TITLE: Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia

Grant Number: 5U01AI151797
Eric Laing, Ph.D., Site Principal Investigator,
Christopher Broder, Ph.D. Co-Investigators,
Spencer Sterling, MS, Scientific Project Coordinator
Prime PI: Peter Daszak, Ph.D.

ATTACHMENT A: Scope of Work

Project Description/Summary: Southeast Asia is one of the world's highest-risk EID hotspots, the origin of the SARS pandemic, Nipah virus, and repeated outbreaks of influenza. This is driven by high diversity of wildlife and rapidly expanding demography that brings human and wildlife populations closer. This proposal will launch the Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH), a collaboration among leaders in emerging disease research in the USA, Thailand, Singapore and the 3 major Malaysian administrative regions. These researchers have networks that span >50 clinics, laboratories and research institutions across almost all SE Asian countries and will use the EID-SEARCH as an early warning system for outbreaks involving exchange of information, reagents, samples and technology, and a collaborative power-house for fundamental and translational research. The EID-SEARCH will also act as a significant asset to scale-up and deploy resources in the case of an outbreak in the region. This EIDRC will conduct research to: 1) Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife, by analyzing previously-archived wildlife samples, conducting targeted wildlife surveillance, and using serology & PCR assays to identify novel viruses. These will be characterized to assess risk of spillover to people, and a series of in vitro (receptor binding, cell culture) and in vivo (humanized mouse and collaborative cross models) assays used to assess their potential to infect people and cause disease; 2) Collect samples and questionnaire data from human communities that live in EID hotspots and have high cultural and behavioral risk of animal exposure (e.g. wildlife hunting, bat guano collection). These will be tested with serological assays to identify evidence of novel virus spillover, and analyzed against metadata to identify key risk pathways for transmission; 3) Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts. We will conduct syndromic surveillance at clinics serving the populations in Aim 2, enroll patients with undiagnosed illness and symptoms consistent with emerging viral pathogens, and test samples with molecular and follow-up serological assays to identify causal links between these syndromes and novel viruses.
This research will advance our understanding of the risk of novel viral emergence in a uniquely important region. It will also strengthen in-country research capacity by linking local infectious disease scientists with an international collaborative network that has proven capacity to conduct this work and produce significant findings. The large body of high impact collaborative research from this EIDRC leadership team provides proof-of-concept that EID-SEARCH has the background, collaborative network, experience, and skillset to act as a unique early warning system for novel EIDs of any etiology threatening to emerge in this hottest of the EID hotspots.

Under the supervision of Co-Investigators Dr. Christopher Broder and Dr. Eric Laing, working closely with the project coordinator Spencer Sterling, The Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc. will conduct epidemiology research and characterization of a series of coronaviruses (CoVs), paramyxoviruses (PMVs – particularly Henipaviruses) and filoviruses (FVs) following Scope of Work:

1. **Continue to develop, validate, and refine serology platforms for laboratory analysis of collected human and animal samples.**
   1.1 Develop and validate the regionally specific CoV/Henipavirus MMIA panel.
   1.2 Refine MMIA tests for Marburg and Tai Forest viruses

1.3 Refine and produce antigens for MPOX assay in support of CREID network-wide activities.
1.4 Support the collaboration with PREMISE for PBMC studies in Thailand.
1.5 Provide on-site training to the project members from Conservation Medicine in Malaysia on serology testing.
1.6 Support data analysis in Thailand and Malaysia to ensure high-quality publications from EID-SEARCH
1.7 Support CREID Pilot Research Project proposals, as applicable.
1.8 Support CREID Pilot Proposal Research Project, "Expanding in-country surveillance capacity for bat-borne henipaviruses in Madagascar," (PI, Dr. Hafaliana Christian Ranaivoson; Co-PI, Dr. Cara Brook), cross-Research Center collaboration with CREID-ESP
1.9 Support pilot study on blood-fed mosquitoes in collaboration with the NIAID vector research program (Dr. Lehmann).

2. Project management and coordination

2.1 Ensure all permits and permissions are in place to conduct project activities.
2.2 Represent EID-SEARCH to present relevant research findings at conferences and other relevant meetings.
2.3 Collaborate with the EID-SEARCH partners for data cleaning, analysis, interpretation, and contributing to scientific publications as agreed.
2.4 The subrecipient will adhere to the applicable elements of resource and data management from the approved project proposal as described in Resource Sharing Plan in **ATTACHMENT D**.
2.5 Submit to the requirements of the Federal Funding Accountability and Transparency Act (FFATA) included as **ATTACHMENT C,** with the signed contract.

3. Project communication and reporting

3.1 Participate in monthly meetings with project partners over video conference.
3.2 Attend CREID Network annual meeting and other in-person project meetings as applicable.
3.3 Present MPOX multiplex serology test development, and Marburg virus and fruit bat sero-surveillance in Madagascar (collaboration with Dr. Cara Brook, Co-PI of Pilot Award, CREID-ESP) at ASTHM 2023, Chicago, IL, USA.
3.4 Participate in calls with the EID-SEARCH partners and global team at EHA as requested.
3.5 Complete the following programmatic and financial reporting by the requested deadlines:
3.5.1 Quarterly reporting to EcoHealth Alliance on programmatic activities due on the following dates:
- 01 August 2023
- 01 November 2023
- 01 February 2024
- 01 May 2024

3.5.2 Semi-annual and annual reports to NIH/NIAID.
3.5.3 Annual reports to the CREID Network and other reports requested by NIAID.
3.5.4 Invoices and financial reports to EcoHealth Alliance every two months, including 1-page or longer programmatic update, copies of receipts and/or other supporting documents, and breakdown of the costs in each item as shown in

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ATTACHMENT B: **Project Budget**

	Year 4 (01 June 2023 – 31 May 2024)	
SALARY		
Christopher Broder	Co-Investigator	$ -
Eric Laing	Site Principal Investigator	$ -
Spencer Sterling	Research Associate/Scientific Project Coordinator	$44,562.48
SALARY		$44,562.48
FRINGE	29.79%	$13,110.28
TOTAL SALARY + FRINGE		**$57,672.76**
TRAVEL		
Domestic	Trip to Chicago: Marana Rekedal	$2,000
International	Trip to Malaysia: Spencer Sterling	$4,000
TOTAL TRAVEL		**$6,000**
OTHER DIRECT COSTS		
Materials & Supplies		$8,000
TOTAL OTHER DIRECT COSTS		**$8,000**
TOTAL DIRECT		**$ 71,672.77**
INDIRECT	36.74%	$ 26,332.58
G&A	16.70%	$ 16,366.89
TOTAL		**$114,372.24**

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ATTACHMENT C: Federal Funding Accountability and Transparency Act (FFATA)

The Federal Funding Accountability and Transparency Act (FFATA) was signed on September 26, 2006 and requires information on federal awards (federal financial assistance and expenditures) be made available to the public via a single, searchable website, which is www.USASpending.gov. All contractors receiving funds from EHA are required to provide the following information as a condition of receiving funds.

Please answer the following questions Yes or No.

a. In the previous tax year, was your company's gross income from all sources above $300,000?

☑ Yes ☐ No

b. In Subrecipient 's business or organization's preceding completed fiscal year, did its business or organization (the legal entity to which the UEI number it provided belongs) receive (1) 80 percent or more of its annual gross revenues in U.S. federal contracts, subcontracts, loans, grants, subgrants, and/or cooperative contracts; **and** (2) $30,000 or more in annual gross revenues from U.S. federal contracts, subcontracts, loans, grants, subgrants, and/or cooperative contracts?

☑ Yes ☐ No

c. Does the public have access to information about the compensation of the executives in Subrecipient 's business or organization (the legal entity to which the UEI number it provided belongs) through periodic reports filed under section 13(a) or 15(d) of the Securities Exchange Act of 1934 (15 U.S.C. 78m(a), 78o(d)) or section 6104 of the Internal Revenue Code of 1986?

☑ Yes ☐ No

d. Does your business or organization maintain an active registration in the System for Award Management (www.SAM.gov)?

☑ Yes ☐ No

ATTACHMENT D: Resource Sharing Plan

The following details are from this award's proposal.

To share resources with the academic research community, we will use the uniform Material Transfer Agreement (MTA), which acknowledges that the materials are proprietary to Institutions of the Cooperative Agreement and permitting their use in a manner that is consistent with the Bayh-Dole Act and NIH funding requirements. NIH research grants require that research be made available to the scientific community and public. The primary method of data sharing is through peer-reviewed publications in scientific journals and by presentation at scientific meetings. In addition, data and results created from NIH supported research will be submitted to NIH in annual progress reports per the terms and conditions of this award. Recombinant viruses, transgenic mouse models and experimental recombinant protein constructs will be made available upon request following a standard procedure (below). Several viruses isolated and studied in this program are select agents so these viruses will not be shipped unless appropriate documentation demonstrates the existence of approved BSL3/4 facilities, select agent licenses, and shipment using approved CDC and Department of Commerce procedures.

We already have established MTAs between most of our EID-SEARCH, consortium partners and will ensure these agreements are up to date and agreed upon by our consortium at the start of our project and then reviewed annually. Having these agreements in place will further reduce the time needed to share reagents and other resources in the event of an outbreak when time-sensitive sharing of biological resources and diagnostic reagents is most critical. **At the start of the project, we will work with the EIDRC – Coordinating Center to ensure these agreements and resource sharing plans are compliant and aligned with plans created for NIH's other EIDRCs.**

Data Sharing Plan

EcoHealth Alliance (EHA) will house the Data Management and Analysis (DMA) team for EIDRC SEARCH. EHA has served as the data and analysis hub for numerous multi-institutional, multi-sectoral, international disease research groups, including acting as the Modeling and Analytics lead institution for the USAID-PREDICT project, the Western Asia Bat Research Network lead by co-I Olival (*1*) and EHA's Rift Valley Fever Consortium (*2*). We will leverage our experience and infrastructure from those projects.

Project Database: We will create a dedicated, centralized EIDRC database to ingest, store, link, and provide for analysis all data associated with the proposed study and other expanded projects associated with the research network. The database will be SQL-based and use encrypted, secure cloud hosting services and enable export to archival and platform-independent formats. It will ensure data and metadata compatibility between project components, track data versioning and annotations. The system will be designed to work with both with both paper- and tablet-based field data entry and with laboratory information management systems in place in individual partner labs. We will design and

engineer the systems to be compatible with other NIAID approved data management systems, including those utilized by the EIDRC-CC, by designing secure APIs, and matching data fields and data standards. The database will use existing metadata standards, including NCBI standards for genetic and molecular data and Ecological Metadata Language (EML) for field and wildlife data, as well as other standards and formats designated by the EIRDC CC. This will enable rapid publication and deposition of data. Granular security and privacy controls will be applied so that specific expansion projects undertaken in the network may be managed while maintaining data confidentiality as needed.

Data Identification and Privacy: For human clinical data and questionnaires, data will be identified by a unique identification code assigned to each individual and only this, de-identified code will be accessible in the project database. All questionnaire data and biological samples will be labeled with a unique alphanumeric identification code, assigned to each enrolled, sampled individual that does not identify the individual from whom data are collected. Participants' names and codes, along with other records with identifying information such as informed consent forms, will be stored in a separately secure system accessible to only essential project staff. If participants agree during the consent process, they may be contacted about having their samples or questionnaire data used for future separate studies about new animal infections discovered in the future, and factors that may affect their chances of getting these animal infections. No data will be released for other purposes without full consent from participants. Upon completion of the project, personal identifying information will be destroyed unless this protocol is extended.

Training: Members of the DMA team will team will develop documentation and provide training for field and laboratory teams at all partner institutions in data management, metadata standards and data hygiene best practices. The DMA team will act as trainers and consultants for partner institutions in experimental design, power analysis, data analysis, and computational and reproducibility issues. DMA trainers will visit each partner institution and/or field team base for training workshops and analysis consultations, and partner institution researchers and students will spend extended time at EHA for collaborative analysis, a model that has been successful in building and maintaining analytical capacity under our NSF EcoHealthNet and PREDICT programs.

Computing Resources: EHA operates a cluster of high-performance servers (System76 20- and 36-core Linux servers with NVIDIA deep-learning GPUs), for data analysis activities, as well as infrastructure to launch cloud-based computing environments of virtual machine with identical software infrastructure. Our servers provide a web-based analysis environment with all necessary software for statistical and bioninformatics work that is available to the DMA and partners anywhere in the world. We use a mixture of cloud services (AWS, Azure, Backblaze, GitHub) to provide redundancy, backup, version control, and rapid post-disaster recovery. The cluster is available to all project partners and can be used use for both high-performance and training-level work (under isolated environments for security and performance).

Data and Code Sharing: Data will be available to the public and researchers without cost or registration, and be released under a CC0 license, as soon as related publications are in press. Data will be deposited for in long-term public scientific repositories. All sequence data will be made publicly available via GenBank. Additional ecological data collected in wildlife sampling will be deposited to the Knowledge Network for Biodiversity, and other data will be deposited in appropriate topic-specific or general repositories. Computer code for modeling and statistical analysis will be made available on a code-hosting site (GitHub), and archived in the Zenodo repository under an open-source, unrestrictive MIT license. Limited human survey and clinical data will be released following anonymization and aggregation per IRB requirements. Publications will be released as open-access via deposition to PubMed commons.

Sharing Model Organisms

Within the program, we will utilize standard laboratory mice as well as different Collaborative Cross mouse strains as well as various transgenic mouse strains, several of which are already available at the NIH-supported Mutant Mouse Regional Resource Center (MMMRC) at UNC. The Collaborative Cross mice are already publicly available from the UNC Systems Genetics Core Facility and the Jackson laboratories, and as such available to the scientific community. All genotyping information generated on these populations will be deposited in the appropriate public repositories (e.g. GEO, ImmPort, ENA). Similarly, all phenotypic data generated within this program from studies with mice will be deposited in the Mouse Phenome Database upon publication, as well as ImmPort to ensure dissemination to the community at large.

In accordance with the NIH/NIAID data sharing and release guidelines, we will coordinate the rapid and unrestricted sharing of all data generated as part of this project.

1. Genotypes generated on the MUGA mouse array, including raw x- and y- intensity data and derived genotype calls will be made available for download from the Mutant Mouse Regional Resource Center at UNC's website (https://www.med.unc.edu/mmrrc/genotypes/publications) and at Zenodo (https://zenodo.org/).

Reagent Sharing

For all other reagents/requests, we have established a consistent process for evaluating requests for samples and reagents from outside scientists. In order of priority, these include: 1) requests for reagents that have been published in peer-reviewed journals; 2) requests which enhance/promote a specific agenda of the program projects and faculty; 3) requests that promote scientifically valid collaborations between project faculty and outside scientists; and 4) overall research and public health needs. The general format involves: a) establishing a working knowledge of the research agenda and credentials of the requestor, b) group discussion and agreement, 3) MTA agreement with the appropriate institution, or license agreement with a commercial entity, and 4) inventory checking and sending out of reagents. We will work closely with the appropriate institutional Technology Transfer Office and individuals involved in

these transactions. The goal will be to provide reagents within a few months of receiving a request for traditional research purposes. In the event of an outbreak or emergency situation, we will communicate with the NIH and EIDRC-CC, and rapidly speed up resource sharing among our EID-SEARCH core partners and our extended network. As documented in the Research Strategy, EHA has successfully provided rapid technical assistance for testing and reagent needs during outbreaks under the USAID-PREDICT project, and has strong existing relationships and existing MTAs with our core EID-SEARCH partners to facilitate this. If needed, we will also acquire appropriate letters from the recipient institutions environmental health and safety officers and help coordinate CDC and/or USDA and Department of Commerce permits. The program faculty will not send reagents to individuals or institutions that do not have appropriate documentation of appropriate containment for the materials, might harbor ill-intentions, or are conducting irresponsible research.

Genomic Data Sharing

We will ensure compliance with NIH's Genomic Data Sharing plans for all viral sequence data generated in this project. We anticipate obtaining genetic sequence data for 100s of novel virus genotypes, including RNA-dependent RNA polymerase (RdRp) sequences for all strains/genotypes and sequences of viral attachment glycoproteins. We will generate full viral genomes for a subset of the viruses and human virus strains that we identify. We will also generate host genetic sequence data for relevant cellular receptor genes of wildlife species. We will deposit all genetic sequences in the NIH data bank, NCBI GenBank as soon as possible after data are generated (including ensurance of quality control), and no later than 6 months, so that they are readily available to the scientific community. We will ensure that all meta-data associated with these sequences, including collection locality lat/long, species-level host identification, date of collection, and sequencing protocols will also submitted. We anticipate sequence generation will occur over the 5 year proposed project period.

All datasets and associated meta-data will be additionally submitted to Virus Pathogen Resource (ViPR, http://www.viprbrc.org). All computational models of biological processes will be made available on the BioModels Database (http://www.ebi.ac.uk/biomodels-main/).

Intellectual Property

Intellectual property agreements, identified during the course of this project, will be accomplished by negotiation in good faith among the institutions and inventors. We will work with the inventors in the production of the necessary documents, working with the particular institutions, legal firms and commercial interests. It is anticipated that companies and institutions will have access to these reagents and viruses by MTA (for research purposes) or by a license agreement to be negotiated in good faith with a company.

Literature Cited

1. K. Phelps *et al.*, Bat Research Networks and Viral Surveillance: Gaps and Opportunities in Western Asia. *Viruses* **11**, (2019).
2. V. Msimang *et al.*, Rift Valley Fever Virus Exposure amongst Farmers, Farm Workers, and Veterinary Professionals in Central South Africa. *Viruses* **11**, (2019).

ATTACHMENT E: **Notice of Award**

(See following pages)

ATTACHMENT F: **NIH Grants Policy Statement**

From: Boxley, Kimberly on behalf of Boxley, Kimberly <kimberly.boxley.ctr@usuhs.edu>
To: Hongying Li
Cc: Laing, Eric; Sterling, Spencer; Christopher Broder
Subject: Re: HJF/USU EID-SEARCH Y4 Scope of Work_draft to edit
Date: Wednesday, August 9, 2023 2:31:37 PM
Attachments: USU HJF EID-SEARCH Y4 Scope of Work_draft v04-SLS-EDL.docx

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Hello,

Attached is the updated SOW and budget.

Let me know if you have any questions. And when you will be able to provide the next modification.

Thanks much .... Kim

On Wed, Aug 9, 2023 at 12:10 PM Hongying Li <li@ecohealthalliance.org> wrote:

> Hi Eric and Kim,
>
> If you want to reallocate funds within your budget for the travel to Malaysia meeting, please feel free to do it (We expect that Spencer could travel to Malaysia for training or troubleshooting in the draft scope of work we shared)
>
> If you're concerned about the available fund for travel within your budget, let me know and we can see how to cover the cost from EHA's budget.
>
> Best,
> Hongying
>
> **Hongying Li, MPH**
> *Senior Program Manager & Senior Research Scientist*
>
> EcoHealth Alliance
> 520 Eighth Avenue, Ste. 1200
> New York, NY 10018
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> 1.917.573.2178 (mobile)
> www.ecohealthalliance.org
>
> ***EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.***
>
> On Wed, Aug 9, 2023 at 11:36 AM Laing, Eric <eric.laing@usuhs.edu> wrote:
>
> > Hi Hongying,
> >
> > I made a couple bullet point changes yesterday. Kim has that version and will be able to provide the update. Also, Spencer was invited to a CCM/EHA conference in Oct, can you use EID-SEARCH travel funds for his airfare/lodging?
> >
> > - Eric

Eric D. Laing, Ph.D.
Assistant Professor
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Uniformed Services University
4301 Jones Bridge Road
Bethesda, MD 20814
cell: (301) 980-8192
office: (301) 295-3419
lab: (301) 295-9618

eric.laing@usuhs.edu

On Fri, Aug 4, 2023 at 4:38 PM Boxley, Kimberly <kimberly.boxley.ctr@usuhs.edu> wrote:

> Here is the updated v3 - it includes revisions to the budget.
>
> Thanks much .... Kim
>
> On Wed, Aug 2, 2023 at 10:03 PM Sterling, Spencer <spencer.sterling.ctr@usuhs.edu> wrote:
>
> > I have two comments for Eric regarding the scope of the work.
> >
> > On Wed, Aug 2, 2023 at 11:54 PM Boxley, Kimberly <kimberly.boxley.ctr@usuhs.edu> wrote:
> >
> > > FYI - your project ... bringing you into the discussion.
> > >
> > > Attached is the current SOW - while Eric is on vacation, will you take a look and see if anything needs to be updated/changed.
> > >
> > > Thanks much .... Kim
> > >
> > > ---------- Forwarded message ---------
> > > From: **Boxley, Kimberly** <kimberly.boxley.ctr@usuhs.edu>
> > > Date: Wed, Aug 2, 2023 at 12:51 PM
> > > Subject: Re: HJF/USU EID-SEARCH Y4 Scope of Work_draft to edit
> > > To: Hongying Li <li@ecohealthalliance.org>
> > > Cc: eric.laing_usuhs <eric.laing@usuhs.edu>, Broder, Christopher <christopher.broder@usuhs.edu>, Yongkang Qiu <yqiu@hjf.org>, Tamera Porter Wilmot <TPorterWilmot@hjf.org>
> > >
> > > Sounds good!
> > >
> > > I will get the updated statement of work back to you as soon as possible.
> > >
> > > And we will be looking for the updating reporting requirements on

the next agreement.

Thanks much .... Kim

On Mon, Jul 31, 2023 at 2:58 PM Hongying Li <li@ecohealthalliance.org> wrote:

> Hi Kim,
>
> Thanks for the quick response and update. No problem, we can wait until Dr. Laing is back from vacation.
>
> More changes will be shown in the **body of the contract** that NIH is reviewing now, we'll share them with you once NIH approves it. They are mostly about specifying everything in detail (e.g., we included general languages in the Y3 contract by referring to relevant policies, now we need to elaborate more in writing), and you can see we'll include the whole "NIH Grants Policy Statement" as Attachment F.
>
> In the file I shared, most changes in Attachment A are on #2 and #3 to describe how we manage and oversee the project. So there will be more regular calls at least every month and more regular reporting, including quarterly reporting with specified deadlines (we'll further discuss with the team the most efficient formats for these reportings).
>
> We also included the "Data and Resroucing Sharing Plan" from the originally submitted proposal as Attachment D to demonstrate what we will do for data sharing following the NIH policies.
>
> Please feel free to email or call if anything I can further clarify. And once we get the approved contract template from NIH, it will be helpful if we can get on a Zoom meeting to go through the changes and explain everything as needed.
>
> Thanks,
> Hongying
>
> **Hongying Li, MPH**
> *Senior Program Manager & Senior Research Scientist*
>
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> 520 Eighth Avenue, Ste. 1200
> New York, NY 10018
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>
>
> On Mon, Jul 31, 2023 at 2:34 PM Boxley, Kimberly

<kimberly.boxley.ctr@usuhs.edu> wrote:

> Hello Hongying,
>
> Dr Laing is out on vacation right now - Can you highlight what changes or specific aims NIH would like us to address?
>
> Thanks much ... Kim
>
> On Mon, Jul 31, 2023 at 1:33 PM Hongying Li <li@ecohealthalliance.org> wrote:
>
> > Dear Eric and Chris,
> >
> > I hope you are doing well.
> >
> > While we are waiting for the Year 4 Notice of Award (U01AI151797), we would like to start discussing with you the scope of work for Year 4.
> >
> > Please take a look at the draft in the attached file, and **feel free to edit and add details to the planned research activities and update the budget accordingly.**
> >
> > Please note that the funder specifically required information related to the reporting after they reviewed our Y3 contracts, so we have to include more details and adhere to them.
> >
> > Please let Peter and me know if you have any questions. We can plan to discuss the work plan at the project all-partner meeting next Wednesday or set up a separate call. Thank you very much!
> >
> > Best Regards,
> > Hongying
> >
> > **Hongying Li, MPH**
> > *Senior Program Manager & Senior Research Scientist*
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> >

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Kim N. Boxley l Program Manager 3 l Henry M Jackson Foundation
Uniformed Services University of the Health Sciences l Microbiology and Immunology

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Email: **KIMBERLY.BOXLEY.CTR@USUHS.EDU**

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ATTACHMENT A: Scope of Work

Project Description/Summary: Southeast Asia is one of the world's highest-risk EID hotspots, the origin of the SARS pandemic, Nipah virus, and repeated outbreaks of influenza. This is driven by high diversity of wildlife and rapidly expanding demography that brings human and wildlife populations closer. This proposal will launch the Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH), a collaboration among leaders in emerging disease research in the USA, Thailand, Singapore and the 3 major Malaysian administrative regions. These researchers have networks that span >50 clinics, laboratories and research institutions across almost all SE Asian countries and will use the EID-SEARCH as an early warning system for outbreaks involving exchange of information, reagents, samples and technology, and a collaborative power-house for fundamental and translational research. The EID-SEARCH will also act as a significant asset to scale-up and deploy resources in the case of an outbreak in the region. This EIDRC will conduct research to: 1) Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife, by analyzing previously-archived wildlife samples, conducting targeted wildlife surveillance, and using serology & PCR assays to identify novel viruses. These will be characterized to assess risk of spillover to people, and a series of in vitro (receptor binding, cell culture) and in vivo (humanized mouse and collaborative cross models) assays used to assess their potential to infect people and cause disease; 2) Collect samples and questionnaire data from human communities that live in EID hotspots and have high cultural and behavioral risk of animal exposure (e.g. wildlife hunting, bat guano collection). These will be tested with serological assays to identify evidence of novel virus spillover, and analyzed against metadata to identify key risk pathways for transmission; 3) Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts. We will conduct syndromic surveillance at clinics serving the populations in Aim 2, enroll patients with undiagnosed illness and symptoms consistent with emerging viral pathogens, and test samples with molecular and follow-up serological assays to identify causal links between these syndromes and novel viruses. This research will advance our understanding of the risk of novel viral emergence in a uniquely important region. It will also strengthen in-country research capacity by linking local infectious disease scientists with an international collaborative network that has proven capacity to conduct this work and produce significant findings. The large body of high impact collaborative research from this EIDRC leadership team provides proof-of-concept that EID-SEARCH has the background, collaborative network, experience, and skillset to act as a unique early warning system for novel EIDs of any etiology threatening to emerge in this hottest of the EID hotspots.

Under the supervision of Co-Investigators Dr. Christopher Broder and Dr. Eric Laing, working closely with the project coordinator Spencer Sterling, The Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc. will conduct epidemiology research and characterization of a series of coronaviruses (CoVs), paramyxoviruses (PMVs – particularly Henipaviruses) and filoviruses (FVs) following Scope of Work:

1. **Continue to develop, validate, and refine serology platforms for laboratory analysis of collected human and animal samples.**
   - 1.1 Develop and validate the regionally specific CoV/Henipavirus MMIA panel.
   - 1.2 Refine MMIA tests for Marburg virus, filoviruses???
   - 1.3 Refine and produce antigens for MPOX assay in support of CREID network-wide activities.
   - 1.4 Support the collaboration with PREMISE for on PBMC study in Thailand.
   - 1.5 Provide on-site training to the project members from Conservation Medicine in Malaysia on serology testing.
   - 1.6 Support data analysis in Thailand and Malaysia to ensure high-quality publications from EID-

SEARCH

1.7 Support CREID Pilot Research Project applications as applicable.

1.8 Support pilot study on blood-fed mosquitoes in collaboration with the NIAID vector research program (Dr. Lehmann).

2. Project management and coordination

2.1 Ensure all permits and permissions are in place to conduct project activities.

2.2 Represent EID-SEARCH to present relevant research findings at conferences and other relevant meetings.

2.3 Collaborate with the EID-SEARCH partners for data cleaning, analysis, interpretation, and contributing to scientific publications as agreed.

2.4 The subrecipient will adhere to the applicable elements of resource and data management from the approved project proposal as described in Resource Sharing Plan in **ATTACHMENT D**.

2.5 Submit to the requirements of the Federal Funding Accountability and Transparency Act (FFATA) included as **ATTACHMENT C,** with the signed contract.

3. Project communication and reporting

3.1 Participate in monthly meetings with project partners over video conference.

3.2 Attend CREID Network annual meeting and other in-person project meetings as applicable.

3.3 Participate in calls with the EID-SEARCH partners and global team at EHA as requested.

3.4 Complete the following programmatic and financial reporting by the requested deadlines:

3.4.1 Quarterly reporting to EcoHealth Alliance on programmatic activities due on the following dates:

- 01 August 2023
- 01 November 2023
- 01 February 2024
- 01 May 2024

3.4.2 Semi-annual and annual reports to NIH/NIAID.

3.4.3 Annual reports to the CREID Network and other reports requested by NIAID.

3.4.4 Invoices and financial reports to EcoHealth Alliance every two months, including 1-page or longer programmatic update, copies of receipts and/or other supporting documents, and breakdown of the costs in each item as shown in **ATTACHMENT B** Project Budget.

ATTACHMENT B: **Project Budget**

Commented [HL1]: Please update.

The total amount should be the same as Y3 $114,372.02

	Year 4 (01 June 2023 – 31 May 2024)	
SALARY		
Christopher Broder	Co-Investigator	$ -
Eric Laing	Site Investigator	$ -
Spencer Sterling	Research Associate/Scientific Project Coordinator	$ 37,500.00
SALARY		$ 37,500.00
FRINGE	29.79%	$ 11,171.25
TOTAL SALARY + FRINGE		$ 48,671.25
TRAVEL		
Domestic		$ 2,476.00
International		$ 5,477.00
TOTAL TRAVEL		$ 7,953.00
OTHER DIRECT COSTS		
Materials & Supplies		$ 18,689.00
TOTAL OTHER DIRECT COSTS		$ 18,689.00
TOTAL DIRECT		$ 75,313.25
INDIRECT	30.03%	$ 22,616.57
G&A	16.79%	$ 16,442.42
TOTAL		$114,372.24

ATTACHMENT C: Federal Funding Accountability and Transparency Act (FFATA)

The Federal Funding Accountability and Transparency Act (FFATA) was signed on September 26, 2006 and requires information on federal awards (federal financial assistance and expenditures) be made available to the public via a single, searchable website, which is www.USASpending.gov. All contractors receiving funds from EHA are required to provide the following information as a condition of receiving funds.

Please answer the following questions Yes or No.

a. In the previous tax year, was your company's gross income from all sources above $300,000?

☑ Yes ☐ No

b. In Subrecipient 's business or organization's preceding completed fiscal year, did its business or organization (the legal entity to which the UEI number it provided belongs) receive (1) 80 percent or more of its annual gross revenues in U.S. federal contracts, subcontracts, loans, grants, subgrants, and/or cooperative contracts; **and** (2) $30,000 or more in annual gross revenues from U.S. federal contracts, subcontracts, loans, grants, subgrants, and/or cooperative contracts?

☑ Yes ☐ No

c. Does the public have access to information about the compensation of the executives in Subrecipient 's business or organization (the legal entity to which the UEI number it provided belongs) through periodic reports filed under section 13(a) or 15(d) of the Securities Exchange Act of 1934 (15 U.S.C. 78m(a), 78o(d)) or section 6104 of the Internal Revenue Code of 1986?

☑ Yes ☐ No

d. Does your business or organization maintain an active registration in the System for Award Management (www.SAM.gov)?

☑ Yes ☐ No

ATTACHMENT D: Resource Sharing Plan

The following details are from this award's proposal.

To share resources with the academic research community, we will use the uniform Material Transfer Agreement (MTA), which acknowledges that the materials are proprietary to Institutions of the Cooperative Agreement and permitting their use in a manner that is consistent with the Bayh-Dole Act and NIH funding requirements. NIH research grants require that research be made available to the scientific community and public. The primary method of data sharing is through peer-reviewed publications in scientific journals and by presentation at scientific meetings. In addition, data and results created from NIH supported research will be submitted to NIH in annual progress reports per the terms and conditions of this award. Recombinant viruses, transgenic mouse models and experimental recombinant protein constructs will be made available upon request following a standard procedure (below). Several viruses isolated and studied in this program are select agents so these viruses will not be shipped unless appropriate documentation demonstrates the existence of approved BSL3/4 facilities, select agent licenses, and shipment using approved CDC and Department of Commerce procedures.

We already have established MTAs between most of our EID-SEARCH, consortium partners and will ensure these agreements are up to date and agreed upon by our consortium at the start of our project and then reviewed annually. Having these agreements in place will further reduce the time needed to share reagents and other resources in the event of an outbreak when time-sensitive sharing of biological resources and diagnostic reagents is most critical. **At the start of the project, we will work with the EIDRC – Coordinating Center to ensure these agreements and resource sharing plans are compliant and aligned with plans created for NIH's other EIDRCs.**

Data Sharing Plan

EcoHealth Alliance (EHA) will house the Data Management and Analysis (DMA) team for EIDRC SEARCH. EHA has served as the data and analysis hub for numerous multi-institutional, multi-sectoral, international disease research groups, including acting as the Modeling and Analytics lead institution for the USAID-PREDICT project, the Western Asia Bat Research Network lead by co-I Olival (*1*) and EHA's Rift Valley Fever Consortium (*2*). We will leverage our experience and infrastructure from those projects.

Project Database: We will create a dedicated, centralized EIDRC database to ingest, store, link, and provide for analysis all data associated with the proposed study and other expanded projects associated with the research network. The database will be SQL-based and use encrypted, secure cloud hosting services and enable export to archival and platform-independent formats. It will ensure data and metadata compatibility between project components, track data versioning and annotations. The system will be designed to work with both with both paper- and tablet-based field data entry and with laboratory information management systems in place in individual partner labs. We will design and engineer the systems to be compatible with other NIAID approved data management systems, including those utilized by the EIDRC-CC, by designing secure APIs, and matching data fields and data standards. The database will use existing metadata standards, including NCBI standards for genetic and molecular data and Ecological Metadata Language (EML) for field and wildlife data, as well as other standards and formats designated by the EIRDC CC. This will enable rapid publication and deposition of data. Granular

security and privacy controls will be applied so that specific expansion projects undertaken in the network may be managed while maintaining data confidentiality as needed.

Data Identification and Privacy: For human clinical data and questionnaires, data will be identified by a unique identification code assigned to each individual and only this, de-identified code will be accessible in the project database. All questionnaire data and biological samples will be labeled with a unique alphanumeric identification code, assigned to each enrolled, sampled individual that does not identify the individual from whom data are collected. Participants' names and codes, along with other records with identifying information such as informed consent forms, will be stored in a separately secure system accessible to only essential project staff. If participants agree during the consent process, they may be contacted about having their samples or questionnaire data used for future separate studies about new animal infections discovered in the future, and factors that may affect their chances of getting these animal infections. No data will be released for other purposes without full consent from participants. Upon completion of the project, personal identifying information will be destroyed unless this protocol is extended.

Training: Members of the DMA team will team will develop documentation and provide training for field and laboratory teams at all partner institutions in data management, metadata standards and data hygiene best practices. The DMA team will act as trainers and consultants for partner institutions in experimental design, power analysis, data analysis, and computational and reproducibility issues. DMA trainers will visit each partner institution and/or field team base for training workshops and analysis consultations, and partner institution researchers and students will spend extended time at EHA for collaborative analysis, a model that has been successful in building and maintaining analytical capacity under our NSF EcoHealthNet and PREDICT programs.

Computing Resources: EHA operates a cluster of high-performance servers (System76 20- and 36-core Linux servers with NVIDIA deep-learning GPUs), for data analysis activities, as well as infrastructure to launch cloud-based computing environments of virtual machine with identical software infrastructure. Our servers provide a web-based analysis environment with all necessary software for statistical and bioninformatics work that is available to the DMA and partners anywhere in the world. We use a mixture of cloud services (AWS, Azure, Backblaze, GitHub) to provide redundancy, backup, version control, and rapid post-disaster recovery. The cluster is available to all project partners and can be used use for both high-performance and training-level work (under isolated environments for security and performance).

Data and Code Sharing: Data will be available to the public and researchers without cost or registration, and be released under a CC0 license, as soon as related publications are in press. Data will be deposited for in long-term public scientific repositories. All sequence data will be made publicly available via GenBank. Additional ecological data collected in wildlife sampling will be deposited to the Knowledge Network for Biodiversity, and other data will be deposited in appropriate topic-specific or general repositories. Computer code for modeling and statistical analysis will be made available on a code-hosting site (GitHub), and archived in the Zenodo repository under an open-source, unrestrictive MIT license. Limited human survey and clinical data will be released following anonymization and aggregation per IRB requirements. Publications will be released as open-access via deposition to PubMed commons.

Sharing Model Organisms

Within the program, we will utilize standard laboratory mice as well as different Collaborative Cross mouse strains as well as various transgenic mouse strains, several of which are already available at the NIH-supported Mutant Mouse Regional Resource Center (MMMRC) at UNC. The Collaborative Cross mice are already publicly available from the UNC Systems Genetics Core Facility and the Jackson laboratories, and as such available to the scientific community. All genotyping information generated on these populations will be deposited in the appropriate public repositories (e.g. GEO, ImmPort, ENA). Similarly, all phenotypic data generated within this program from studies with mice will be deposited in the Mouse Phenome Database upon publication, as well as ImmPort to ensure dissemination to the community at large.

In accordance with the NIH/NIAID data sharing and release guidelines, we will coordinate the rapid and unrestricted sharing of all data generated as part of this project.

1. Genotypes generated on the MUGA mouse array, including raw x- and y- intensity data and derived genotype calls will be made available for download from the Mutant Mouse Regional Resource Center at UNC's website (https://www.med.unc.edu/mmrrc/genotypes/publications) and at Zenodo (https://zenodo.org/).

Reagent Sharing

For all other reagents/requests, we have established a consistent process for evaluating requests for samples and reagents from outside scientists. In order of priority, these include: 1) requests for reagents that have been published in peer-reviewed journals; 2) requests which enhance/promote a specific agenda of the program projects and faculty; 3) requests that promote scientifically valid collaborations between project faculty and outside scientists; and 4) overall research and public health needs. The general format involves: a) establishing a working knowledge of the research agenda and credentials of the requestor, b) group discussion and agreement, 3) MTA agreement with the appropriate institution, or license agreement with a commercial entity, and 4) inventory checking and sending out of reagents. We will work closely with the appropriate institutional Technology Transfer Office and individuals involved in these transactions. The goal will be to provide reagents within a few months of receiving a request for traditional research purposes. In the event of an outbreak or emergency situation, we will communicate with the NIH and EIDRC-CC, and rapidly speed up resource sharing among our EID-SEARCH core partners and our extended network. As documented in the Research Strategy, EHA has successfully provided rapid technical assistance for testing and reagent needs during outbreaks under the USAID-PREDICT project, and has strong existing relationships and existing MTAs with our core EID-SEARCH partners to facilitate this. If needed, we will also acquire appropriate letters from the recipient institutions environmental health and safety officers and help coordinate CDC and/or USDA and Department of Commerce permits. The program faculty will not send reagents to individuals or institutions that do not have appropriate documentation of appropriate containment for the materials, might harbor ill-intentions, or are conducting irresponsible research.

Genomic Data Sharing

We will ensure compliance with NIH's Genomic Data Sharing plans for all viral sequence data generated

in this project. We anticipate obtaining genetic sequence data for 100s of novel virus genotypes, including RNA-dependent RNA polymerase (RdRp) sequences for all strains/genotypes and sequences of viral attachment glycoproteins. We will generate full viral genomes for a subset of the viruses and human virus strains that we identify. We will also generate host genetic sequence data for relevant cellular receptor genes of wildlife species. We will deposit all genetic sequences in the NIH data bank, NCBI GenBank as soon as possible after data are generated (including ensurance of quality control), and no later than 6 months, so that they are readily available to the scientific community. We will ensure that all meta-data associated with these sequences, including collection locality lat/long, species-level host identification, date of collection, and sequencing protocols will also submitted. We anticipate sequence generation will occur over the 5 year proposed project period.

All datasets and associated meta-data will be additionally submitted to Virus Pathogen Resource (ViPR, http://www.viprbrc.org). All computational models of biological processes will be made available on the BioModels Database (http://www.ebi.ac.uk/biomodels-main/).

Intellectual Property

Intellectual property agreements, identified during the course of this project, will be accomplished by negotiation in good faith among the institutions and inventors. We will work with the inventors in the production of the necessary documents, working with the particular institutions, legal firms and commercial interests. It is anticipated that companies and institutions will have access to these reagents and viruses by MTA (for research purposes) or by a license agreement to be negotiated in good faith with a company.

Literature Cited

1. K. Phelps *et al.*, Bat Research Networks and Viral Surveillance: Gaps and Opportunities in Western Asia. *Viruses* **11**, (2019).
2. V. Msimang *et al.*, Rift Valley Fever Virus Exposure amongst Farmers, Farm Workers, and Veterinary Professionals in Central South Africa. *Viruses* **11**, (2019).

ATTACHMENT E: **Notice of Award**

(See following pages)

ATTACHMENT F: **NIH Grants Policy Statement**

From: Hongying Li on behalf of Hongying Li <li@ecohealthalliance.org>
To: eric.laing_usuhs; Broder, Christopher
Cc: Yongkang Qiu; Boxley, Kimberly; Tamera Porter Wilmot
Subject: HJF/USU EID-SEARCH Y4 Scope of Work_draft to edit
Date: Monday, July 31, 2023 1:34:18 PM
Attachments: USU HJF EID-SEARCH Y4 Scope of Work_draft v01.docx

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Dear Eric and Chris,

I hope you are doing well.

While we are waiting for the Year 4 Notice of Award (U01AI151797), we would like to start discussing with you the scope of work for Year 4.

Please take a look at the draft in the attached file, and **feel free to edit and add details to the planned research activities and update the budget accordingly.**

Please note that the funder specifically required information related to the reporting after they reviewed our Y3 contracts, so we have to include more details and adhere to them.

Please let Peter and me know if you have any questions. We can plan to discuss the work plan at the project all-partner meeting next Wednesday or set up a separate call. Thank you very much!

Best Regards,
Hongying

Hongying Li, MPH
Senior Program Manager & Senior Research Scientist

EcoHealth Alliance
520 Eighth Avenue, Ste. 1200
New York, NY 10018

1.917.573.2178 (mobile)
www.ecohealthalliance.org

Department of Health and Human Services
National Institutes of Health
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

Notice of Award
FAIN# U01AI151797
Federal Award Date
09-21-2022

Recipient Information

1. Recipient Name
ECOHEALTH ALLIANCE INC.
520 8TH AVE RM 1200

NEW YORK, 10018

2. Congressional District of Recipient
12

3. Payment System Identifier (ID)
1311726494A1

4. Employer Identification Number (EIN)
311726494

5. Data Universal Numbering System (DUNS)
077090066

6. Recipient's Unique Entity Identifier
TKS7NBB4JDN6

7. Project Director or Principal Investigator
Peter Daszak, PHD
Executive Director
daszak@ecohealthalliance.org
212-380-4460

8. Authorized Official
Dr. Peter Daszak
daszak@ecohealthalliance.org;
chmura@ecohealthalliance.org
212-380-4473

Federal Agency Information

9. Awarding Agency Contact Information
Shaun W Gratton
Grants Management Specialist
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
Shaun.Gratton@nih.gov
240-627-3594

10. Program Official Contact Information
SARA ELAINE Woodson

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
sara.woodson@nih.gov
301-761-6478

Federal Award Information

11. Award Number
5U01AI151797-03

12. Unique Federal Award Identification Number (FAIN)
U01AI151797

13. Statutory Authority
42 USC 241 31 USC 6305 42 CFR 52

14. Federal Award Project Title
Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia

15. Assistance Listing Number
93.855

16. Assistance Listing Program Title
Allergy and Infectious Diseases Research

17. Award Action Type
Non-Competing Continuation

18. Is the Award R&D?
Yes

Summary Federal Award Financial Information

19. Budget Period Start Date 06-01-2022 **– End Date** 05-31-2023	
20. Total Amount of Federal Funds Obligated by this Action	$1,504,400
20 a. Direct Cost Amount	$1,597,725
20 b. Indirect Cost Amount	$156,777
21. Authorized Carryover	$250,102
22. Offset	$0
23. Total Amount of Federal Funds Obligated this budget period	$1,504,400
24. Total Approved Cost Sharing or Matching, where applicable	$0
25. Total Federal and Non-Federal Approved this Budget Period	$1,504,400
26. Project Period Start Date 06-17-2020 **– End Date** 05-31-2025	
27. Total Amount of the Federal Award including Approved Cost Sharing or Matching this Project Period	$4,556,712

28. Authorized Treatment of Program Income
Additional Costs

29. Grants Management Officer - Signature
Vandhana Khurana

30. Remarks

Acceptance of this award, including the "Terms and Conditions," is acknowledged by the recipient when funds are drawn down or otherwise requested from the grant payment system.

SECTION I – AWARD DATA – 5U01AI151797-03

Principal Investigator(s):
Peter Daszak, PHD

Award e-mailed to: chmura@ecohealthalliance.org

Dear Authorized Official:

The National Institutes of Health hereby awards a grant in the amount of $1,504,400 (see "Award Calculation" in Section I and "Terms and Conditions" in Section III) to EcoHealth Alliance in support of the above referenced project. This award is pursuant to the authority of 42 USC 241 31 USC 6305 42 CFR 52 and is subject to the requirements of this statute and regulation and of other referenced, incorporated or attached terms and conditions.

Acceptance of this award, including the "Terms and Conditions," is acknowledged by the recipient when funds are drawn down or otherwise requested from the grant payment system.

Each publication, press release, or other document about research supported by an NIH award must include an acknowledgment of NIH award support and a disclaimer such as "Research reported in this publication was supported by the National Institute Of Allergy And Infectious Diseases of the National Institutes of Health under Award Number U01AI151797. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health." Prior to issuing a press release concerning the outcome of this research, please notify the NIH awarding IC in advance to allow for coordination.

Award recipients must promote objectivity in research by establishing standards that provide a reasonable expectation that the design, conduct and reporting of research funded under NIH awards will be free from bias resulting from an Investigator's Financial Conflict of Interest (FCOI), in accordance with the 2011 revised regulation at 42 CFR Part 50 Subpart F. The Institution shall submit all FCOI reports to the NIH through the eRA Commons FCOI Module. The regulation does not apply to Phase I Small Business Innovative Research (SBIR) and Small Business Technology Transfer (STTR) awards. Consult the NIH website http://grants.nih.gov/grants/policy/coi/ for a link to the regulation and additional important information.

If you have any questions about this award, please direct questions to the Federal Agency contacts.

Sincerely yours,

Vandhana Khurana
Grants Management Officer
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

Additional information follows

Cumulative Award Calculations for this Budget Period (U.S. Dollars)

Salaries and Wages	$253,818
Fringe Benefits	$88,734
Personnel Costs (Subtotal)	$342,552
Consultant Services	$30,000
Materials & Supplies	$12,917
Travel	$72,483
Other	$24,977
Subawards/Consortium/Contractual Costs	$1,107,796
Publication Costs	$7,000
Federal Direct Costs	$1,597,725
Federal F&A Costs	$156,777
Approved Budget	$1,754,502
Total Amount of Federal Funds Authorized (Federal Share)	$1,504,400
Cumulative Authorized Carryover and Offset for this Budget Period	$250,102
TOTAL FEDERAL AWARD AMOUNT	$1,504,400
AMOUNT OF THIS ACTION (FEDERAL SHARE)	$1,504,400

SUMMARY TOTALS FOR ALL YEARS (for this Document Number)		
YR	**THIS AWARD**	**CUMULATIVE TOTALS**
3	$1,504,400	$1,504,400
4	$1,503,220	$1,503,220
5	$1,502,037	$1,502,037

Recommended future year total cost support, subject to the availability of funds and satisfactory progress of the project

Fiscal Information:

Payment System Identifier:	1311726494A1
Document Number:	UAI151797A
PMS Account Type:	P (Subaccount)
Fiscal Year:	2022

IC	CAN	2022	2023	2024
AI	8472315	$1,504,400	$1,503,220	$1,502,037

Recommended future year total cost support, subject to the availability of funds and satisfactory progress of the project

NIH Administrative Data:
PCC: M56A B / **OC**: 41029 / **Released**: Khurana, Vandhana 09-21-2022
Award Processed: 09/22/2022 12:02:43 AM

SECTION II – PAYMENT/HOTLINE INFORMATION – 5U01AI151797-03

For payment and HHS Office of Inspector General Hotline information, see the NIH Home Page at http://grants.nih.gov/grants/policy/awardconditions.htm

SECTION III – STANDARD TERMS AND CONDITIONS – 5U01AI151797-03

This award is based on the application submitted to, and as approved by, NIH on the above-titled project and is subject to the terms and conditions incorporated either directly or by reference in the following:

a. The grant program legislation and program regulation cited in this Notice of Award.
b. Conditions on activities and expenditure of funds in other statutory requirements, such as those included in appropriations acts.
c. 45 CFR Part 75.
d. National Policy Requirements and all other requirements described in the NIH Grants Policy Statement, including addenda in effect as of the beginning date of the budget period.
e. Federal Award Performance Goals: As required by the periodic report in the RPPR or in the final progress report when applicable.
f. This award notice, INCLUDING THE TERMS AND CONDITIONS CITED BELOW.

(See NIH Home Page at http://grants.nih.gov/grants/policy/awardconditions.htm for certain references cited above.)

Research and Development (R&D): All awards issued by the National Institutes of Health (NIH) meet the definition of "Research and Development" at 45 CFR Part§ 75.2**.** As such, auditees should identify NIH awards as part of the R&D cluster on the Schedule of Expenditures of Federal Awards (SEFA). The auditor should test NIH awards for compliance as instructed in Part V, Clusters of Programs. NIH recognizes that some awards may have another classification for purposes of indirect costs. The auditor is not required to report the disconnect (i.e., the award is classified as R&D for Federal Audit Requirement purposes but non-research for indirect cost rate purposes), unless the auditee is charging indirect costs at a rate other than the rate(s) specified in the award document(s).

Carry over of an unobligated balance into the next budget period requires Grants Management Officer prior approval.

This award is subject to the requirements of 2 CFR Part 25 for institutions to obtain a unique entity identifier (UEI) and maintain an active registration in the System for Award Management (SAM). Should a consortium/subaward be issued under this award, a UEI requirement must be included. See http://grants.nih.gov/grants/policy/awardconditions.htm for the full NIH award term implementing this requirement and other additional information.

This award has been assigned the Federal Award Identification Number (FAIN) U01AI151797. Recipients must document the assigned FAIN on each consortium/subaward issued under this award.

Based on the project period start date of this project, this award is likely subject to the Transparency Act subaward and executive compensation reporting requirement of 2 CFR Part 170. There are conditions that may exclude this award; see http://grants.nih.gov/grants/policy/awardconditions.htm for additional award applicability information.

In accordance with P.L. 110-161, compliance with the NIH Public Access Policy is now mandatory. For more information, see NOT-OD-08-033 and the Public Access website: http://publicaccess.nih.gov/.

In accordance with the regulatory requirements provided at 45 CFR 75.113 and Appendix XII to 45 CFR Part 75, recipients that have currently active Federal grants, cooperative agreements, and procurement contracts with cumulative total value greater than $10,000,000 must report and maintain information in the System for Award Management (SAM) about civil, criminal, and administrative proceedings in connection with the award or performance of a Federal award that reached final disposition within the most recent five-year period. The recipient must also make semiannual disclosures regarding such proceedings. Proceedings information will be made publicly available in the designated integrity and performance system (currently the Federal Awardee Performance and Integrity Information System

(FAPIIS)). Full reporting requirements and procedures are found in Appendix XII to 45 CFR Part 75. This term does not apply to NIH fellowships.

Treatment of Program Income:

Additional Costs

SECTION IV – AI SPECIFIC AWARD CONDITIONS – 5U01AI151797-03

Clinical Trial Indicator: No

This award does not support any NIH-defined Clinical Trials. See the NIH Grants Policy Statement Section 1.2 for NIH definition of Clinical Trial.

THIS AWARD CONTAINS GRANT SPECIFIC RESTRICTIONS. THESE RESTRICTIONS MAY ONLY BE LIFTED BY A REVISED NOTICE OF AWARD.

SPECIFIC AWARD CONDITION:

Restriction: This award is made based on the interinstitutional agreement (IIA) between the recipient and Tufts University, which has been approved by the Office of Laboratory Animal Welfare (OLAW) in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals. If there are any changes to this IIA, the recipient must notify OLAW and NIAID within 15 days of the change and no further charges for research involving live vertebrate animals may be incurred on this award without prior approval by OLAW and by NIAID.

****

The recipient must conduct or arrange for the conduct of onsite subrecipient facility inspections every 6 months to ensure that subaward activities are being properly executed. The recipient must provide certification in the Research Performance Progress Report that it is in compliance with this term and condition of award.

****

The recipient must provide NIAID with copies of FSRS reporting of all (existing and newly established) subaward agreements within 30 days of submitting to FSRS.

****

The recipient must provide NIH with copies of updated subaward agreements within 30 days of execution of any initial and subsequent subaward agreement(s). The subaward agreements must demonstrate compliance with the NIH Grants Policy Statement (NIH GPS) 15.2.1 Written Agreement. The subaward agreements must state the correct F&A rate which, for foreign subrecipients is 8% (see NIH GPS 16.6) and include descriptions of the biosafety monitoring plans for each project, where appropriate.

Failure to comply with this special condition can result in withholding of support, audit disallowances, suspension and/or termination of this award, and/or other appropriate enforcement actions. This award is subject to the Transparency Act subaward reporting requirement of 2 CFR Part 170, which must be reported through the Federal Funding Accountability and Transparency Subaward Reporting System (FSRS).

****

The recipient is required to submit semi-annual progress reports, Research Performance Progress Report (RPPR), and Federal Financial Reports (FFR) to NIAID via the Request for Additional Materials module within eRA. The semi-annual reports for this budget period is due on December 01, 2022. NIAID will initiate a RAM request within eRA at least 15 days prior to the due date of the semi-annual progress report, RPPR and FFR. Upon receipt of the RAM request, the recipient is required to upload the requested documents in Commons within RAM no later than the due date listed above.

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Summary of Comments on NIH_NOA_5U01AI151797-03

Page: 6

Number: 1	Author: macdurian	Subject: Highlight	Date: 4/12/2023 8:49:16 PM	
Number: 2	Author: macdurian	Subject: Highlight	Date: 9/26/2022 11:35:35 AM	
Number: 3	Author: macdurian	Subject: Highlight	Date: 9/26/2022 11:35:55 AM	
Number: 4	Author: macdurian	Subject: Highlight	Date: 9/26/2022 11:36:04 AM	
Number: 5	Author: macdurian	Subject: Highlight	Date: 9/26/2022 11:36:48 AM	
Number: 6	Author: macdurian	Subject: Highlight	Date: 9/26/2022 11:37:06 AM	
Number: 7	Author: macdurian	Subject: Highlight	Date: 9/26/2022 11:37:16 AM	

The authority for automatic no-cost extension has been removed for this award. The recipient must request and receive written prior approval from NIAID before any extension of the final budget period.

+++++++

This award includes a carryover of **$250,102**. The carryover is approved for the purpose stated in the request dated **08/31/2021** and will be subject to a downward adjustment should the available unobligated balance from the **01** year FFR be less than the cumulative carryover amount; no adjustments will be made to the "TOTAL FEDERAL AWARD AMOUNT" on the Notice of Award

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This award is issued at the committed level for direct costs and facilities and administrative costs. The awardee must adhere to the applicable policies for rebudgeting costs as outlined in the NIH Grants Policy Statement.

****

This award does not include funds to support research subject to the *Department of Health and Human Services Framework for Guiding Funding Decisions about Proposed Research Involving Enhanced Potential Pandemic Pathogens* (DHHS P3CO Framework) Therefore:

- For Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife, the building of chimeric SARS-like bat coronaviruses will be based on the SHC014 or the pangolin coronavirus molecular clones and the building of chimeric MERS-CoV will be based on the HKU5 strain. Prior to further altering the mutant viruses you must provide NIAID with a detailed description of the proposed alterations and supporting evidence for the anticipated phenotypic characteristics of each virus.
- Alternative approaches to those referenced above, including building chimeras based on SARS-CoV-1, SARS-CoV-2, and MERS-CoV, may be subject to the DHHS P3CO Framework and must be submitted to NIAID for review and approval prior to the work commencing.

If any of the experiments proposed for Aim 1 result in a virus with a phenotype of enhanced pathogenicity and/or transmissibility, enhanced growth by more than 10 fold when compared to wild type strains, or if the mice display significant increases in weight loss, viral titer, or mortality when compared to wild-type strains, the recipient must immediately stop the work and notify the NIAID Program Officer, Grants Management Specialist, and appropriate institutional biosafety committee. Policy changes regarding the classification of these experiments or components used in these experiments may be subject to immediate halting of experimentation. No NIH funding can be used to perform such experiments until these experiments have been approved by NIAID with a revised NOA.

****
Dissemination of study data will be in accord with the Recipient's accepted genomic data sharing plan as stated on page(s) **373** of the application. Failure to adhere to the sharing plan as mutually agreed upon by the Recipient and the NIAID may result in Enforcement Actions as described in the NIH Grants Policy Statement.

****
This award includes human subject research studies and must conform to the DHHS policies for the Protection of Human Subjects research, which are a term and condition of award. Human subjects research is covered by the 2018 Common Rule, and may not be initiated until the associated protocols have received IRB approval as specified in 45 CFR 46. Failure to comply with the terms and conditions of award may result in the disallowance of costs and/or additional enforcement actions as outlined in Section 8.5 of the NIH Grants Policy Statement.

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Number: 1 Author: macdurian Subject: Highlight Date: 9/26/2022 11:37:26 AM

Number: 2 Author: macdurian Subject: Highlight Date: 9/26/2022 11:37:35 AM

Number: 3 Author: macdurian Subject: Sticky Note Date: 9/26/2022 11:38:21 AM

Make sure Joe updates FFR

The Research Performance Progress Report (RPPR), Section G.9 (Foreign component), includes reporting requirements for all research performed outside of the United States. Research conducted at the following site(s) must be reported in your RPPR:

Jeppesen Field Consulting Australia - AUSTRALIA
Conservation Medicine Ltd. - MALAYSIA
Duke-NUS Medical School - SINGAPORE
Chulalongkorn University - THAILAND

****
This Notice of Award (NoA) includes funds for activity with **Conservation Medicine Ltd. - MALAYSIA** in the amount of $299,997 (including carryover)

****
This Notice of Award (NoA) includes funds for activity with **Duke-NUS Medical School - SINGAPORE** in the amount of $174,596 (including carryover)

****
This Notice of Award (NoA) includes funds for activity with **Chulalongkorn University - THAILAND** in the amount of $215,944

****
This Notice of Award (NoA) includes funds for activity with **The University of North Carolina at Chapel Hill** in the amount of $237,923 (including carryover)

****
This Notice of Award (NoA) includes funds for activity **The Henry M. Jackson Fdn. for the Adv'mt. of Mil. Med., Inc**. in the amount of $179,336 (including carryover)

****
This award is issued as a Cooperative Agreement, a financial assistance mechanism in which substantial NIH scientific and/or programmatic involvement is anticipated in the performance of the activity. This award is subject to the Terms and Conditions of Award set forth in Section VI: Award Administrative Information of **RFA AI-19-028**, which are hereby incorporated by reference as special terms and conditions of award.

The RFA may be accessed at: http://grants.nih.gov/grants/guide/index.html

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This award is subject to the Clinical Terms of Award referenced in the NIH Guide for Grants and Contracts, July 8, 2002, NOT AI-02-032. These terms and conditions are hereby incorporated by reference, and can be accessed via the following World Wide Web address: https://www.niaid.nih.gov/grants-contracts/niaid-clinical-terms-award All submissions required by the NIAID Clinical Terms of Award must be forwarded electronically or by mail to the responsible NIAID Program Official identified on this Notice of Award.

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Highly Pathogenic Agents:
NIAID defines a Highly Pathogenic Agent as an infectious Agent or Toxin that may warrant a biocontainment safety level of BSL3 or higher according to the current edition of the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories (BMBL) (https://www.cdc.gov/labs/BMBL.html). Research funded under this grant must adhere to the BMBL, including using the BMBL-recommended biocontainment level at a minimum. If the Institutional Biosafety Committee (IBC) (or equivalent body) or designated institutional biosafety official recommends a higher biocontainment level, the higher recommended containment level must be used.
When submitting future Progress Reports indicate at the beginning of the report:

If no research with a Select Agent (see 42 CFR 73 for the relevant human Select Agents and Toxins; and 7 CFR 331 and 9 CFR 121 for the relevant animal and plant Select Agents and Toxins at https://www.selectagents.gov/regulations/ and https://www.selectagents.gov/sat/list.htm) and/or has been performed or is planned to be performed under this grant.

If the IBC or equivalent body or official has determined, for example, by conducting a risk assessment, that the work being planned or performed under this grant may be conducted at a biocontainment safety level that is lower than BSL3.

If the work involves Select Agents and/or Highly Pathogenic Agents, also address the following points:

Any NIAID pre-approved changes in the use of the Select Agents and/or Highly Pathogenic Agents including its restricted experiments that have resulted in a change in the required biocontainment level, and any resultant change in location, if applicable, as determined by the IBC or equivalent body or official.

If work with a new or additional Select Agents and/or Highly Pathogenic Agents is proposed in the upcoming project period, provide:

- A list of the new and/or additional Agent(s) that will be studied;
- A description of the work that will be done with the Agent(s), and whether or not the work is a restricted experiment;
- The title and location for each biocontainment resource/facility, including the name of the organization that operates the facility, and the biocontainment level at which the work will be conducted, with documentation of approval by the IBC or equivalent body or official. It is important to note if the work is being done in a new location;
- Any biosafety incidents that occurred and were reported to NIH/NIAID.

SPREADSHEET SUMMARY
AWARD NUMBER: 5U01AI151797-03

INSTITUTION: EcoHealth Alliance

Budget	Year 3	Year 4	Year 5
Salaries and Wages	$253,818	$272,938	$272,938
Fringe Benefits	$88,734	$96,628	$96,628
Personnel Costs (Subtotal)	$342,552	$369,566	$369,566
Consultant Services	$30,000	$15,000	$15,000
Materials & Supplies	$12,917	$7,918	$7,918
Travel	$72,483	$72,225	$72,225
Other	$24,977	$27,000	$27,000
Subawards/Consortium/Contractual Costs	$1,107,796	$854,164	$852,981
Publication Costs	$7,000		
TOTAL FEDERAL DC	$1,597,725	$1,345,873	$1,344,690
TOTAL FEDERAL F&A	$156,777	$157,347	$157,347
TOTAL COST	$1,504,400	$1,503,220	$1,502,037

Facilities and Administrative Costs	Year 3	Year 4	Year 5

F&A Cost Rate 1	32%	32%	32%
F&A Cost Base 1	$489,929	$491,709	$491,709
F&A Costs 1	$156,777	$157,347	$157,347

A. COVER PAGE

Project Title: Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia	
Grant Number: 5U01AI151797-04	**Project/Grant Period:** 06/17/2020 - 05/31/2025
Reporting Period: 06/01/2022 - 05/31/2023	**Requested Budget Period:** 06/01/2023 - 05/31/2024
Report Term Frequency: Annual	**Date Submitted:** 03/31/2023
Program Director/Principal Investigator Information: PETER DASZAK , PHD BS **Phone Number:** 212 380 4460 **Email:** daszak@ecohealthalliance.org	**Recipient Organization:** ECOHEALTH ALLIANCE, INC. ECOHEALTH ALLIANCE, INC. 520 EIGHTH AVENUE NEW YORK, NY 100181620 **DUNS:** 077090066 **UEI:** TKS7NBB4JDN6 **EIN:** 1311726494A1 **RECIPIENT ID:**
Change of Contact PD/PI: NA	
Administrative Official: ALEKSEI CHMURA 460 W 34th St., 17th Floor New York, NY 10001 **Phone number:** 1.212.380.4473 **Email:** chmura@ecohealthalliance.org	**Signing Official:** ALEKSEI CHMURA 460 W 34th St., 17th Floor New York, NY 10001 **Phone number:** 1.212.380.4473 **Email:** chmura@ecohealthalliance.org
Human Subjects: Yes **HS Exempt:** NA **Exemption Number:** **Phase III Clinical Trial:** NA	**Vertebrate Animals:** Yes
hESC: No	**Inventions/Patents:** No

B. ACCOMPLISHMENTS

B.1 WHAT ARE THE MAJOR GOALS OF THE PROJECT?

Southeast Asia is one of the world's highest-risk EID hotspots, and the origin of the SARS pandemic, repeated outbreaks of novel influenza strains, and the spillover of dangerous viral pathogens such as the Nipah virus. It is a wildlife 'megadiversity' region, where a rapidly expanding human population is increasing contact with wildlife, and increasing the risk of zoonotic disease outbreaks. The overarching goal of this proposal is to launch the Emerging Infectious Diseases - South East Asia Research Collaboration Hub (EID-SEARCH) to analyze the diversity of key viral pathogens in wildlife, the frequency and causes of their spillover, and to identify viral etiologies of undiagnosed 'cryptic' outbreaks in people. EID-SEARCH includes leaders in the field of emerging viral pathogens at key US institutions, and in Thailand, Singapore, and the 3 major Malaysian administrative regions, whose collaborative networks span >50 clinics, laboratories, and research institutes across almost all SE Asian countries. This hub, and the network, will act as an early warning system for outbreaks - a way to exchange information, reagents, samples, and technology, and a collaborative power-house for translational research. The long-term collaboration among the key personnel and multidisciplinary skillsets from epidemiology, clinical management, lab analysis, through wildlife biology and data analysis will act as significant assets when deployed to help counter outbreaks in the region. The research goals of this EIDRC follow three specific aims:

Specific Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife. We will: 1) analyze some of the tens of thousands of archived wildlife samples at our disposal, conduct geographically- and taxonomically-targeted field surveillance in wild mammals (bats, rodents, primates), and use serological & PCR assays to identify known high-profile zoonotic pathogens, or close relatives with potential to infect people; 2) biologically characterize novel viruses that our analyses suggest have high spillover and pandemic potential; and 3) conduct in vitro receptor binding assays and cell culture experiments, and in vivo animal model infections using humanized mice and the collaborative cross mouse to assess their potential to infect people and cause disease.

Specific Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays. Assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities and approaches that can deal with the low statistical probability of identifying rare events. To achieve this, we will 1) conduct targeted cross-sectional serological surveys of human communities with extremely high geographic and cultural, occupational, and behavioral exposure to wildlife-origin viruses; 2) design and deploy novel serological assays to identify baseline spillover of known or novel CoVs, PMVs, and FVs in these populations; and 3) analyze and test hypotheses on the occupational, cultural and other risk factors for spillover (e.g. hunting wildlife).

Specific Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts. Our prior work provides substantial evidence of spillover leading to undiagnosed illness in people in the region. To test if these represent 'cryptic' outbreaks of novel viruses, we will conduct syndromic surveillance at regional clinics for the communities sampled in SA2. We will: 1) enroll and collect biological samples, and detailed survey data on risk factors, from patients presenting with influenza-like illness, severe respiratory illness, encephalitis, and other specific symptoms; 2) conduct molecular and follow-up serological diagnostic assays to test causal links between their syndromes and known and novel viral agents identified in SA1. Where viruses are identified, we will attempt to isolate and characterize them, then use the survey data, ecological and phylogenetic analyses to identify likely reservoir hosts/spillover pathways and inform intervention programs.

This research will advance our understanding of the risk of novel viral emergence in a uniquely important region. It will strengthen in-country research capacity by linking local infectious disease scientists with an international collaborative network that has proven capacity to conduct this work and produce significant findings. These include testing of tens of thousands of samples from wildlife, humans, and livestock in the region; discovery of hundreds of novel viruses from zoonotic viral families in wildlife; outbreak investigations in rural communities across SE Asia; discovery of the bat-origin of SARSr-CoVs; discovery of a novel bat-origin SADS-CoV killing >25,000 pigs in S. China; and development of novel serological and molecular assays for high-impact viruses, and state-of-the-art in vitro and in vivo assays to characterize viral pathogenic potential. This body of collaborative research provides proof-of-concept that EID-SEARCH has the background, collaborative network, experience, and skillset to act as a unique early warning system for novel EIDs of any etiology threatening to emerge in this hottest of the EID

hotspots.

B.1.a Have the major goals changed since the initial competing award or previous report?

No

B.2 WHAT WAS ACCOMPLISHED UNDER THESE GOALS?

File Uploaded : B.2 Accomplishments final_updated.pdf

B.3 COMPETITIVE REVISIONS/ADMINISTRATIVE SUPPLEMENTS

For this reporting period, is there one or more Revision/Supplement associated with this award for which reporting is required?

No

B.4 WHAT OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT HAS THE PROJECT PROVIDED?

File Uploaded : B.4 Training and professional development_final.pdf

B.5 HOW HAVE THE RESULTS BEEN DISSEMINATED TO COMMUNITIES OF INTEREST?

PI Daszak, and Co-investigators Wacharapluesadee, Hughes, Olival, Wang, Baric, Broder, Laing, and other key personnel from the EID-SEARCH were invited to give lectures, keynote speeches, government and inter-government briefings in Thailand, Malaysia, Southeast Asia, and internationally using EID-SEARCH work to demonstrate how disease ecology, lab virology, and social science approaches can provide a unique platform for a pandemic prediction and prevention, and share research strategies, technologies and results from this project.

Government and inter-government agency briefings
- Meeting with the Disease Control Checkpoint Officers, Field Epidemiology & Management Team (FEMT) program of the Ministry of Public Health, Thailand, for information sharing and capacity building purposes.
- Meeting with multi-sectoral stakeholders in the Philippines to share experience on approaches for One Health surveillance for Coronaviruses, Paramyxoviruses, and Filoviruses in the Philippines.
- Meeting with the Department of Wildlife and National Parks, Peninsular Malaysia
- Meetings with the Ministry of Health (MOH) of Malaysia, including the Zoonosis Sector, the Disease Control Division, the National Public Health Laboratory, infectious disease physicians, State Health Department staff from the States of Pahang, Perak, and Kelantan, Sabah State Health Department, the Kota Kinabalu Public Health Laboratory, and Jabatan Perlindungan Hidupan Liar dan Taman Negara (PERHILITAN) for project updates, results reporting, and research plan discussion.
- Meeting with the US Ambassador to Malaysia, the Economic Counsellor, the Economic Officer, and the Health Officer to discuss EID-SEARCH project progress, results, and plans.
- Meeting with Sabah Wildlife Department's Director, Deputy Director and Veterinarians to discuss EID-SEARCH project progress, molecular and serology testing results and work plans.
- Meeting with Sabah State Health Department (SSHD) Director, Head of Sabah Centre for Disease Control, Director of the Kota Kinabalu Public Health Laboratory (KKPHL), staff from Public Health and Zoonotic Surveillance sections, the Director of the Borneo Medical Health Research Centre (BMHRC) at Universiti Malaysia Sabah (UMS) and staff from Hospital UMS, to discuss EID-SEARCH project progress, molecular and serology testing results, the strategy for syndromic surveillance and work plans.
- Meeting with the Director of the BMHRC and the Director of the KKPHL to discuss how to proceed with Syndromic Surveillance in Sabah, Malaysia.

Panel, workshop, training, webinar
- Workshop to design SBCC campaign to reduce illegal wild meat demand and prevent pandemics in Thailand, USAID Reducing Demand for Wildlife (USAID RDW) and WildAid, Thailand
- 41st Thailand Wildlife Seminar on wildlife surveillance, Kasetsart University, Thailand
- Strengthening surveillance of adverse events following immunization (AEFI) and laboratory identification of pathogenic viruses among network partners, Bureau of Epidemiology, Department of Disease Control, Thailand
- CEPI and NIAID panels for selecting coronaviruses with high pandemic risk.
- G20 webinar on Prevention and Preparedness based on the One Health Approach to Address Threats at The Human-Animal-Environment Interface. Contributed to policy brief via the T20 (Indonesia) in advance of the G20 meeting in Lombok, Indonesia in 2022.
- NSF-funded EcoHealthNet training program for One Health students. "Workshop Session 1: Future of Pandemics"
One Health training for the Thailand One Health University Network (THOHUN) for their field epidemiology One Health training Southeast Asia One Health University Network (SEAOHUN) regional conference and helped moderate partnership meeting, Bangkok, Thailand.
Panel discussion for the Food and Agriculture Organization (FAO) ONE HEALTH Dialogue Series No.6 "Environment and One Health"
Technical Workshop on Bat Surveillance in the Mekong Region" hosted by King Chulalongkorn Memorial Hospital and the World Health Organization in Bangkok, Thailand.

Conference and university lectures
- Prince Mahidol Award Conference Side Meeting on "Addressing Ecosystem Change and Pandemic Emergence through Strengthening One Health Implementation", joint organized by EID-SEARCH and PICREID, Bangkok
- Joint International Tropical Medicine Meeting 2022, Bangkok on "Way Forward to Leverage Pandemic Prevention"
- 3rd International Symposium on Infectious Diseases of Bats, Ft. Collins, CO. on "Bat coronaviruses surveillance in Western Asia" and "A strategy to assess spillover risk of bat SARS-related coronaviruses in Southeast Asia."
- 19th International Bat Research Conference, organizer and chair of Symposium: "Advances in understanding bat health and disease dynamics" and Moderator for Panel discussion during symposium.
- 19th Ecology and Evolution of Infectious Diseases conference in Atlanta, GA on "Ecological correlates of coronavirus infection in wild bats"
- Presentation to the Responding to Epidemics and Crises in Health (REACH) Fellowship, Columbia University. ICAP on "One Health Approaches to Pandemic Prevention, Preparedness, and Response"
- ASTMH meeting Seattle, WA. Symposium on "Pandemic Surveillance and Pathogen Prediction: What's next and what will it take to keep us safe?"
- World One Health Congress, "Secondary health and ecological benefits of bat virus surveillance", "One Health 'Smart Surveillance' – using disease modeling to pinpoint high-risk spillover interfaces."
- Presentation and training on One Health Leadership for the One Health Young Leaders (OHYL) program of the Indonesia One Health University Network (INDOHUN) (virtual)
- Institute of Global Health, University of Geneva in Switzerland on "One Health and Ecological Approaches to Zoonotic Disease Prevention, Preparedness, and Response"
- Keynote panel on the Approaches to pandemic preparedness at the World Antiviral Congress in San Diego, CA.
- Keynote address for iSEE Congress 2023: Confronting Crises of Planetary Scale: Lessons from Pandemics and Climate Change, Urbana, IL
- Kasetsart University, Thailand on "Species distribution models/ecological niche models as a tool for disease risk assessment"
- Thai Red Cross Emerging Infectious Diseases Clinical Center EID Conference on "Next Generation Serology for Pathogen Discovery: Multiplex Immunoassays (MIA)"
- Department of Medicine, Chulalongkorn University on "Antibody durability, vaccine-related symptoms, and omicron neutralization after BNT162b2 mRNA COVID-19 vaccination in the Prospective Assessment of SARS-CoV-2 Seroconversion (PASS) Study"
- Asia Pacific Biorisk Conference on field biosafety
- Asia Society of Conservation Medicine on field biosafety

Public outreach
- Field trip organizing for the SEAOHUN Competence Based Education Workshop led by research staff at TRC EIDCC on bat-human interface research under EID-SEARCH
- J&J on "Analytics to Support Pandemic Prevention".
- Field biosafety standards developed through the CREID/ EID-SEARCH project trained and implemented in various EHA-led projects in Vietnam, Liberia, Guinea
- Griffin Foundation

B.6 WHAT DO YOU PLAN TO DO DURING THE NEXT REPORTING PERIOD TO ACCOMPLISH THE GOALS?

In the next reporting period, we will follow the research plans laid out in our proposal with the specific actions as follows (no modifications of the scope of work):

Specific Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife
1.1 Wildlife (bats, rodents, non-human primates) sampling at selected sites.
1.1.1 Increase sampling efforts for rodents/tree shrews and non-human primates.
1.1.2 Conduct longitudinal sampling at a few selected sites to analyze viral shedding patterns and spillover risk.
1.1.3 Targeted surveillance to identify the animal host of the Mojiang-related virus.
1.2 Continue to develop predictive host-range models and spatial risk models to prioritize sampling sites and species in Years 4-5 of the project.
1.3 Conduct viral family-level molecular screening of collected wildlife samples for coronaviruses and paramyxoviruses. (Filoviruses testing is suspended due to low detection rate in swabs)
1.4 Perform serology testing on the wildlife samples collected in Y1-4.
1.4.1 Perform serological testing for Coronaviruses, Filoviruses, and Henipaviruses.
1.4.2 Perform serological characterization of Sarbecovirus-positive samples using surrogate virus neutralization test (sVNT) RBD-based assay.
1.5 Conduct whole-genome and spike glycoprotein sequencing with newly identified viruses.
1.6 Continue research to further characterize a subset of novel viruses, including virus isolation, human cell infection experiments, and animal modeling for viral pathogenesis studies.
1.6.1 Rescue additional pangolin viruses that are genetically more distant from SARS-CoV-2 and the pangolin GD strain, as well as continue efforts to synthetically reconstruct and characterize the BANAL-52 isolate using in vitro and in vivo approaches.
1.6.2 Review novel bat sarbecovirus whole genomes identified in Y1-4 for synthetic recovery.
1.7 Develop zoonotic risk analyses for relevant viruses.
1.7.1 Analyze Spike and whole-genome sequences to predict host range using a variety of in silico approaches.
1.7.2 Model the host range, geographic distribution, extent of overlap with high-density human populations, etc., using existing apps/models to assess potential zoonotic disease risk.
1.7.3 Improve data workflow to integrate viral characterization results into zoonotic virus prediction models.
1.7.4 Conduct analysis to understand the diversity and evolution of SARS-CoV-2-related viruses.
1.7.5 Develop a risk assessment pipeline that determines the spillover risk, disease severity, and protective levels in individuals with pre-existing SARS-CoV-2 immunity.

Specific Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays
2.1 Increase the number of sites and enrolled participants for community surveillance among at-risk populations to collect biological and behavioral data collection.
2.1.1 Enrolling human participants from additional community sites in Thailand where human-wildlife contact occurs.
2.1.2 Perform whole genome sequencing of identified CoVs from bat guano collectors.
2.1.3 Collect PBMCs from individuals with detectable antibodies against the Mojiang-related viruses for further characterization in Thailand.
2.1.4 Obtain MoA to enroll human participants at community sites in Malaysia.
2.2 Continue identifying community sites concurrent with animal surveillance sites (Specific Aim 1).

2.3 Perform serology testing of collected human samples with optimized multiplex assays for coronaviruses, filoviruses, and Henipaviruses.
2.4 Continue epidemiological analyses of biological and behavioral data.

Specific Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts
3.1 Enrolling participants at selected hospital/clinic sites for syndromic surveillance.
3.1.1 Enroll human participants from additional hospital sites in Thailand.
3.1.2 Obtain MOA to start enrollment at hospital/clinic sites in Malaysia.
3.1.3 Perform PCR and serological tests with newly collected and archived samples if available.
3.2 Continue to identify potential clinical cohorts and sites in the catchment of the community (Specific Aim 2) and animal surveillance (Specific Aim 1) sites.
3.3 Continue serological and molecular testing on collected human samples.
3.4 Perform viral characterization work if any novel viruses are identified.

Training and capacity-building activities to achieve project goals
- Conduct annual field biosafety and animal surveillance training (bats, rodents, non-human primates) with lectures, discussion, and hands-on guidance.
- Conduct training in enriched RNAseq protocols for coronavirus whole-genome sequencing
- Conduct virtual and in-person viral phylogenetic and metagenomics analysis training.
- Continue training and troubleshooting on multiplex microsphere immunoassay (MMIA) data analysis and interpretation.
- Conduct refresher training on human research regarding survey design, interview skills, and data analysis.
- Conduct 2nd-round lab biosafety evaluation and regular research facilities inspection.

Additional activities
- Finalize the standard operating procedures (SOP) manual for emerging infectious disease research field biosafety for peer review, publication, and broad distribution.
- Continue refining and sharing research resources (protocols, SOPs, technologies) and results within the scientific community and local government promptly.
- Organize meetings and workshops with scientists from Southeast Asia to identify common challenges and opportunities for emerging infectious disease research in the region.
- Continue supporting in-country outbreak research and response as requested.

The Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH) is a multidisciplinary research center with **specific objectives** to: 1) characterize diverse coronaviruses, paramyxoviruses, filoviruses, and other viral pathogens in wildlife; 2) assess the frequency and causes of their spillover; & 3) identify viral etiologies of undiagnosed 'cryptic' outbreaks. Here we report the **major activities** and **significant results** for the period of 06/01/2022 – 05/31/2023:

Human and wildlife surveillance in Thailand and Malaysia

	Year 1	Year 2	Year 3
WILDLIFE			
# Individual	100 NHPs[1], 1,648 bats	2454 bats, 106 rodents, 6 NHPs	586 bats, 191 rodents, 168 NHPs, 2 carnivores
# Species	1 (NHP), 20 (bats)	~30 (bats), 4 (rodents), 2 (NHPs)	~20 (bats), ~24 (rodents/treeshrew), ~3 (NHPs), 1(Malayan civet)
# Specimen[2]	12,694	20,747	6,261
# Site	6	12	8
# Individuals for PCR test	805 (NHPs, bats)	1,741 (rodents, bats, NHPs)	2,054 (bats, NHPs, rodents)
# PCR test	3,004 CoVs (two assays)	6,240 CoVs (two assays)	7,822 CoVs (two assays)
	1,502 PMVs	2,248 PMVs	2,878 PMVs
	1,502 FLVs	2,248 FLVs	2,479 FLVs
	200 for IFV-A	814 for IFV-A	306 for IFV-A
# Divergent viral PCR sequence (RdRp) detected[3]	5 novel CoVs from 107 bats (3 species)	7 novel CoV from 189 bats	CoV positive samples in 79 bats (8 species), analysis ongoing
	4 novel PMVs from 11 bats (3 species)	3 known PMVs from 4 rodents 7 novel PMVs from 14 bats	3 known PMVs from 3 bat (1 species) 4 novel PMVs from bats (2 species)
# Individuals for MMIA[4] test	0	1,225 bats (FLVs & Henipaviruses)	Archived samples of 392 bats, 64 rodents, 1 moonrat; 100 NHPs, 160 rodents
# Sero-positive[5]		*Result analysis ongoing*	1% (1/100 NHPs detectable level of antibodies reactive with the MERS-CoV)
			1% (1/100 NHPs detectable level of antibodies reactive with the Mojiang)
HUMAN			
# Enrollment	0	58	92
# Specimen	0	321	465
# Site	0	2	3
# PCR test	0	58 CoVs, 58 PMVs, 58 FVs, 58 IFV-A	146 CoVs, 146 PMVs
# PCR positive	0	0	CoVs positive in 9 participants (Quan) and 2 participants (Watanabe)
# Individuals for MMIA test	0	0	150 from this project and 1,590 archived samples from previous projects
# Specimen tested	0	0	407
# Sero-positive[5]	0	0	45/238 (18.91%) individuals with detected antibodies to FLVs
	0	0	38/214 (17.76%) individuals with detected antibodies to CoVs
	0	0	64/238 (26.89%) individuals with detected antibodies to henipaviruses
	0	0	264/265 (99.6%) individuals with detected antibodies to common human CoVs
DOMESTIC ANIMAL AND LIVESTOCK			
# Individuals for MMIA test	0	0	381 dogs, 15 goats, 7 wild boars, 7 NHPs, 4 rodents, 2 civets, 2 rabbits for CoVs (archived samples collected from at-risk community, wild boars, NHP, rodents and civets all wildlife kept as pet)
# Sero-positive[5]			7 were reactive to CoVs, *result analysis ongoing*

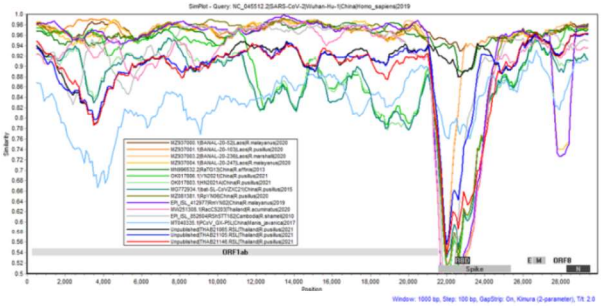
Note: [1]NHP: non-human primate; [2]Specimen include oral swabs, rectal swabs, urogenital swabs, and whole blood or serum, types of specimens may vary among individual animals and human participants; [3]Partial-gene phylogenetic trees suggest that these are potentially "novel viruses", further studies are underway to classify and characterize these divergent viral sequences; [4]MMIA: multiplex microsphere-based immunoassay; [5]Further analysis of serological results is ongoing.

Characterization of bat coronaviruses

Following our finding (Yr2) of SC2r-CoVs in *R. pusillus*, we continued sampling the same bat colony and obtained whole genome sequences (WGS) from 8 individual bats **(Table 1).** Three WGS were further analyzed using a similarity plot against SARS-CoV-2 isolate Wuhan-Hu-1 **(Fig. 1)** and show relatively similar overall genomic sequence except for the spike region. This indicates several SC2r-CoVs are circulating and evolving in the same bat population. Recombination analysis is ongoing. Genomes are being analyzed from 4 other CoVs from 4 different bat species (*R. pusillus, R. amplexicaudatus, C. plicatus, H. larvatus*).

Table 2 (left). Whole genome sequencing of SC2r-CoVs from bat specimen, collected from *R. pusillus* March and October 2021; **Fig. 1 (right).** Similarity plot against SARS-CoV-2 plot showing the relative sequence similarity of each genome against SARS-CoV-2 calculated from a window size of 1000 bp, 100 bp step using Kimura (2-parameter).

Bat ID	Percent identity to SARS-CoV-2 isolate Wuhan-Hu-1 (NC_045512)	Percent identity to bat SARS-CoV-2	Percent genome complete ness
THAB21028	74.30%	74.97% (BANAL20247)	81.26%
THAB21105	90.37%	90.86% (BANAL20236)	100%
THAB21114	88.96%	89.23% (BANAL20236)	97.92%
THAB21122	90.34%	90.96% (BANAL20236)	99.98%
THAB21135	54.84%	55.38% (BANAL20247)	60.24%
THAB21140	86.42%	86.68% (BANAL20236)	96.22%
THAB21146	89.77%	90.3% (BANAL20236)	100%
THAB21149	-	-	20.95%



Meanwhile, further characterization of the previously identified bat CoV (Rp-65) were conducted, the results show this bat CoV binds to human ACE2 **(Fig 2A).** Using human serum samples collected from individuals vaccinated against SARS-CoV-2, we observed significant cross-neutralization activities to the bat CoV Rp-65 using sVNT and pseudovirus neutralization test **(Fig 2B).** Bat CoV Rp-65 is less fusogenic than SARS-CoV-2 and bat CoV BANAL-52 **(Fig 2C).** Considering the cross-reactivity of SARS-CoV-2 neutralizing antibodies and

less fusogenic phenotype, individuals with pre-existing immunity to SARS-CoV-2 are likely protected against Bat CoV Rp-65.

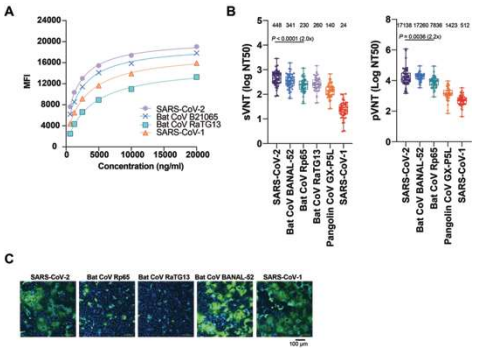


Fig 2. Characterization of the Bat CoV Rp-65 RBD. (A) ACE2 binding SARS-CoV-2, Bat CoV Rp-6, Bat CoV- RaTG13 and SARS-CoV-1 RBD. **(B)** Neutralization titers determined by sVNT and pVNT for SARS-CoV-2 ancestral, Bat CoV BANAL-52, Bat CoV Rp-65, Pangolin CoV GX-P5L, and SARS-CoV-1. **(C)** Fusogenicity of SARS-CoV-2, Bat CoV Rp-65, Bat CoV RaTG13, Bat CoV BANAL-52, and SARS-CoV-1 spike protein

Serological testing of human samples

Serological testing was conducted on samples from rural community participants in Thailand, including bat guano collectors for henipavirus, filovirus, and wildlife and human associated CoV antigens. We found 99.62% (264/265) seroprevalence for HCoV-OC43 in pre- (n=215) and post-COVID-19 (n=50) individuals. All specimens in 2017 and 2018 were negative for SARS-CoV-2. We detected antibodies to both filo-like viruses (18.91%, 45/238) and henipa-like viruses (26.89%, 64/238), including a high seroprevalence to the MojV RBP (21.85%, 52/238) **(Fig 3).** These results suggest possible exposure to novel MojV-related viruses within this population.

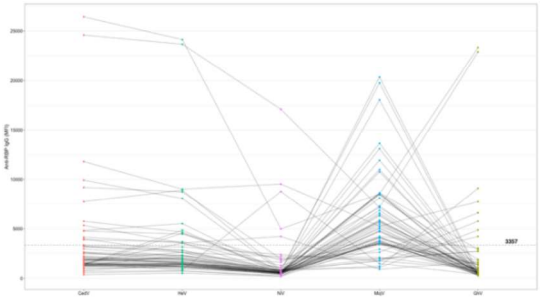


Fig 3. Median fluorescence intensities (MFI) values for henipavirus antigens of sera collected in 2017, 2018, 2021, and 2023 from the community site. Seropositivity is defined by seroreactivity to at least one of the antigens tested within the viral families and does not necessarily indicate the virus of exposure. Further characterization of the functional antibody responses such as neutralization tests would be required.

Surrogate virus neutralization (sVNT) assay development

At Duke NUS, the multiplex sVNT panel to detect neutralizing antibodies targeting multiple human and animal sarbecoviruses has been updated to include recent SARS-CoV-2 Omicron variants (e.g., BA.1, BA.2, BA.5, XBB.1 and XBB.1.5) and the bat CoV Rp-65 detected in Thailand. Apart from the sarbecovirus, the team has expanded the multiplex sVNT to detect neutralizing antibodies targeting members of merbecovirus, henipavirus, and ebolavirus, aming to establish an all-in-one sVNT platform for viral pathogens with pandemic potential.

Host range, transmissibility, and antigenicity of a pangolin coronavirus

At Duke NUS, using a synthetically derived infectious cDNA clone, we recovered the Guangdong wildtype pangolin (Pg) CoV-GD and derivatives encoding indicator genes (nanoluc and GFP introduced into ORF7). We have further characterized it, and assessed its ability to infect human cells. Full details are given in the semi-annual report during Yr 3, and a paper is under 2nd round of review.

Outbreak Research and Response

Time Period	Aims	Funds Pivoted to this Project	Stakeholders/Partners Engaged for Project	Progress/Outcomes to Date
April-June 2022	Monkeypox outbreak preparedness	No direct funds were pivoted however, members from the EID-SEARCH assisted with laboratory testing and provided information to Thai government.	-Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC EIDCC) -Department of Disease Control, Thailand (DDC) -Division of High-Consequence Pathogens and Pathology, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, US	The first 5 cases of Mpox in Thailand were tested and confirmed at the TRC EIDCC laboratory. Whole-genome sequencing of Mpox was conducted and deposited in GISAID.
June 2021	Access to COVID vaccine in Malaysia	No direct funds were pivoted however, members from the EID-SEARCH assisted with facilitating the communication between US Embassy and the Ministry of Health Malaysia	-Conservation Medicine -Ministry of Health Malaysia -US Embassy in Malaysia	1 million Pfizer vaccines donated to Malaysia by the US government in July 2021
September 2020	Access to COVID testing supplies in Malaysia	No direct funds were pivoted however, members from the EID-SEARCH helped organise, source, and coordinate the delivery of supplies for testing 10,000 people	-Conservation Medicine -Ministry of Health Malaysia -US Embassy in Malaysia -Kota Kinabalu Public Health Laboratory	Supplies for testing 10,000 people were delivered to support the Ministry of Health in their impressive response to the COVID-19 outbreak.
August 2020	Wildlife testing for SARS-CoV-2	No direct funds were pivoted however, members from the EID-SEARCH assisted with laboratory testing and provided information to local government.	-Conservation Medicine -EcoHealth Alliance	1,254 archive animal samples in Malaysia from 1,207 animals, including 28 species considered potential reservoir or host species for SARS-CoV-2, were screened using a conventional PCR. SARS-CoV-2 was not detected in any of the samples.

Publications Full details in Section C.

During this reporting period, EID-SEARCH provided training sessions to individuals involved in the activities supported by the project that were focused on biosafety, the application of new technologies, and field and laboratory standard operating procedures to enhance in-country research capacity and improve information sharing. Training was conducted by EID-SEARCH project members and partners among in-country stakeholders in Thailand and Malaysia from local government, universities, research institutes, and NGOs. Some training activities are highlighted as follows:

November-December 2022: The Duke-NUS team provided continuous support for various training development opportunities with the other EID-SEARCH partners, including: Hosting 2 representatives from the Thai team (TRC-EID) during their visit to Singapore from 2 November to 12 November 2022. On-site training and technology transfer of the multiplex sVNT and pseudovirus neutralization assays developed by the Duke-NUS team were conducted for TRC-EID team members. Five research personnel from the Duke-NUS team including the co-Investigator Prof Wang visited TRC-EID lab from 6 December to 11 December 2022, as a follow up and also to assist with the establishment and optimization of the assays. The TRC-EID team also made arrangements to visit the bat caves in Thailand, where members of the Duke-NUS team were able to experience first-hand how the bats were caught in the wild and were taught procedures for processing bat tissues. EID-SEARCH members from Duke-NUS and TRC-EID attended the Joint International Tropical Medicine Meeting 2022 in person, where Prof Wang and Dr Supaporn presented results generated from this project in the session titled "*Way Forward to Leverage Pandemic Prevention"*.

July 2022: USU team provided instructional training to two laboratory members from TRC-EID on the serologic techniques used to detect binding antibodies. The training covers various topics of antigen design, assay techniques and testing strategies, and preliminary data analysis.

24-25 April 2022: Three Conservation Medicine (CM) staff completed a 2 day Outdoor First Aid course. This was not included in the last progress report, as this activity was completed after the report submission.

25 April 2022: Nine Conservation Medicine staff completed an annual 1-day Outdoor Refresher First Aid course. This was not included in the last progress report, as this activity was completed after the report submission.

14 May 2022: Conservation Medicine conducted a Biosafety in Field and Specimen Storage Training at Sabah Wildlife Department's (SWD) - Sepilok Orangutan Rehabilitation Centre (SORC), Sandakan, Sabah. This one-day training included 10 participants from the Bornean Sun Bear Conservation Centre (BSBCC) and 11 participants from SORC – both organizations that have previously provided opportunities for wildlife sampling to EID-SEARCH. PPE donning and doffing, spill clean-up, and sample cold chain maintenance training were given. The objective of this training was to build capacity for those involved with the bio-surveillance of wildlife species at SORC and BSBCC. The training utilized lectures and PowerPoint presentations. After each presentation, post training quizzes were completed by all participants to assess their understanding of each session. Wet lab sessions including Mask Fit Test, PPE donning and doffing, and spill clean-up were conducted. Participants left the training knowing what type of N95 and N100 masks fitted them and the correct size of PPE that they need for lab and field work. This was not included in the last progress report, as this activity was completed after the report submission.

30-31 July 2022: Conservation Medicine Field Coordinator attended a workshop at Kota Kinabalu organized by Sabah Wildlife Department.

04 September – 25 November 2022: Conservation Medicine trained 6 staff from the Ministry of Health - National Public Health Laboratory on EID-SEARCH serology data analysis during the screening of archived human samples using the coronavirus multiplex immunoassay. This training was given over the course of 20 days by CM Laboratory Coordinator on the assay's theory, refresher training on the technique, and results analysis.

16 March 2023: 2 Conservation Medicine contract staff participated in EID-SEARCH lab related training. This training was given by CM Senior Laboratory Technician on Laboratory Safety, Biosafety and PPE, and TRIzol as a part of induction training for EID-SEARCH PCR testing.

20-21 March 2023: Conservation Medicine Field Coordinator and Senior Ranger attended the "Managing People and Macaques in shared spaces Workshop" organized by SWD. The workshop aimed to work towards solving the human-macaque conflict in Sabah, promote conservation for macaques and raise awareness about one health and the role macaques play in zoonosis in Sabah.

Mentorship. Through the CREID pilot research program, Dr. Cadhla Firth from EID-SEARCH has been mentoring pilot project awardees in 2022, Dr. Jurre Siegers and Vireak Heang, on research implementation in Cambodia and relevant data analysis. Dr. Firth continued providing mentorship support to Dr. Nguyen Van Cuong, awardee in 2021, on data analysis and manuscript development, as well as joint grant application. In addition, regular meetings were held among EID-SEARCH members and partners for collaborative laboratory results discussion, data analysis, and manuscript development, to support professional development of graduate students who work on this project.

EID-SEARCH members were able to participate training provided by the **CREID network** including the CREID Network Bioinformatics Training Workshop hosted by IRESSEF and CIGASS-UCAD in October 2022 and the CREID Biorepository Community of Practice (CoP) focuses on knowledge exchange through member presentations, invited seminars, and external stakeholder presentations.

Senior Field Veterinarian, Dr. Marc Valitutto, continued working with EID-SEARCH partners for collaborative advancement of **field biosafety standards** to develop specific field biosafety levels for a risk-based approach to practicing biosafety in the field.

C. PRODUCTS

C.1 PUBLICATIONS

Are there publications or manuscripts accepted for publication in a journal or other publication (e.g., book, one-time publication, monograph) during the reporting period resulting directly from this award?

Yes

Publications Reported for this Reporting Period

Public Access Compliance	Citation
Complete	Jantarabenjakul W, Sodsai P, Chantasrisawad N, Jitsatja A, Ninwattana S, Thippamom N, Ruenjaiman V, Tan CW, Pradit R, Sophonphan J, Wacharapluesadee S, Wang LF, Puthanakit T, Hirankarn N, Putcharoen O. Dynamics of Neutralizing Antibody and T-Cell Responses to SARS-CoV-2 and Variants of Concern after Primary Immunization with CoronaVac and Booster with BNT162b2 or ChAdOx1 in Health Care Workers. Vaccines. 2022 April 19;10(5). PubMed PMID: 35632395; PubMed Central PMCID: PMC9147589; DOI: 10.3390/vaccines10050639.
Complete	Tse LV, Meganck RM, Araba KC, Yount BL, Shaffer KM, Hou YJ, Munt JE, Adams LE, Wykoff JA, Morowitz JM, Dong S, Magness ST, Marzluff WF, Gonzalez LM, Ehre C, Baric RS. Genomewide CRISPR knockout screen identified PLAC8 as an essential factor for SADS-CoVs infection. Proceedings of the National Academy of Sciences of the United States of America. 2022 May 3;119(18):e2118126119. PubMed PMID: 35476513; PubMed Central PMCID: PMC9170153; DOI: 10.1073/pnas.2118126119.
Complete	Yadana S, Cheun-Arom T, Li H, Hagan E, Mendelsohn E, Latinne A, Martinez S, Putcharoen O, Homvijitkul J, Sathaporntheera O, Rattanapreeda N, Chartpituck P, Yamsakul S, Sutham K, Komolsiri S, Pornphatthananikhom S, Petcharat S, Ampoot W, Francisco L, Hemachudha T, Daszak P, Olival KJ, Wacharapluesadee S. Behavioral-biological surveillance of emerging infectious diseases among a dynamic cohort in Thailand. BMC infectious diseases. 2022 May 16;22(1):472. PubMed PMID: 35578171; PubMed Central PMCID: PMC9109443; DOI: 10.1186/s12879-022-07439-7.
Non-Compliant	Chaiyes A, Duengkae P, Suksavate W, Pongpattananurak N, Wacharapluesadee S, Olival KJ, Srikulnath K, Pattanakiat S, Hemachudha T. Mapping Risk of Nipah Virus Transmission from Bats to Humans in Thailand. EcoHealth. 2022 June;19(2):175-189. PubMed PMID: 35657574; DOI: 10.1007/s10393-022-01588-6.
Complete	Wacharapluesadee S, Hirunpatrawong P, Petcharat S, Torvorapanit P, Jitsatja A, Thippamom N, Ninwattana S, Phanlop C, Buathong R, Tangwangvivat R, Klungthong C, Chinnawirotpisan P, Hunsawong T, Suthum K, Komolsiri S, Jones AR, Fernandez S, Putcharoen O. Simultaneous detection of omicron and other SARS-CoV-2 variants by multiplex PCR MassARRAY technology. Scientific reports. 2023 February 6;13(1):2089. PubMed PMID: 36747014; PubMed Central PMCID: PMC9900542; DOI: 10.1038/s41598-023-28715-9.
Complete	Speranskaya AS, Artiushin IV, Samoilov AE, Korneenko EV, Khabudaev KV, Ilina EN, Yusefovich AP, Safonova MV, Dolgova AS, Gladkikh AS, Dedkov VG, Daszak P. Identification and Genetic Characterization of MERS-Related Coronavirus Isolated from Nathusius' Pipistrelle (*Pipistrellus nathusii*) near Zvenigorod (Moscow Region, Russia). International journal of environmental research and public health. 2023 February 19;20(4). PubMed PMID: 36834395; PubMed Central PMCID: PMC9965006; DOI: 10.3390/ijerph20043702.

Non-compliant Publications Previously Reported for this Project

Public Access Compliance	Citation
Non-Compliant	Tse LV, Meganck RM, Yount BL, Hou YJ, Munt JE, Adams LE, Dong S, Baric RS. Genome-wide CRISPR Knockout Screen Identified PLAC8 as an Essential Factor for SADS-CoVs Infection. The Proceedings of the National Academy of Sciences. Forthcoming.

C.2 WEBSITE(S) OR OTHER INTERNET SITE(S)

NOTHING TO REPORT

C.3 TECHNOLOGIES OR TECHNIQUES

Category	Explanation
Protocols	Field biosafety guidance EID-SEARCH is developing a comprehensive field biosafety manual that provides explicit direction on protection measures in the field, accounting for differences across field projects in local contexts. The areas of focus include: - Identification of health risks (disease, routes of exposure, field equipment, medical supplies, climate, trauma, etc.) - Understanding how to mitigate risks (personal health, immunizations, site risk analysis, personal protective equipment [selection, donning, doffing], disinfection, & disposal) - Responding to health threats (pathogen exposure, trauma, envenomation, physical exhaustion) Field biosafety information is being collated for sharing in several forms of documents and media, including 1) A comprehensive standard operating procedure (SOP) manual that is under peer review that is being shared broadly with the CREID network during Years 3-5 of our project; 2) Lecture presentations with recorded voiceovers; 3) Quick reference guides for field use; 4) Video demonstrations, and 5) Web-based photo guides with descriptors. A draft has been prepared and undergoing peer review.

C.4 INVENTIONS, PATENT APPLICATIONS, AND/OR LICENSES

Have inventions, patent applications and/or licenses resulted from the award during the reporting period? No

If yes, has this information been previously provided to the PHS or to the official responsible for patent matters at the grantee organization? No

C.5 OTHER PRODUCTS AND RESOURCE SHARING

NOTHING TO REPORT

D. PARTICIPANTS

D.1 WHAT INDIVIDUALS HAVE WORKED ON THE PROJECT?

Commons ID	S/K	Name	Degree(s)	Role	Cal	Aca	Sum	Foreign Org	Country	SS
DASZAK	Y	Daszak, Peter	BS,PHD	PD/PI	3.0	0.0	0.0			NA
RALPH_BARIC	Y	Baric, Ralph	PhD	Co-Investigator	1.0	0.0	0.0			NA
OLIVAL	Y	Olival, Kevin J.	PHD	Co-Investigator	3.0	0.0	0.0			NA
LINFA66	Y	Wang, Linfa		Co-Investigator	1.0	0.0	0.0	Duke NUS	SINGAPORE	NA
SU_YADANA	N	Yadana, Su	MPH	Epidemiologist	1.0	0.0	0.0			NA
CECILIA_SANCHEZ	Y	Sanchez, Cecilia	PhD	Research Scientist	3.0	0.0	0.0			NA
OPASS.P	Y	Putcharoen, Opass		Co-Investigator	2.0	0.0	0.0	Chulalongkorn University	THAILAND	NA
	Y	Wacharapluesadee, Supaporn	PHD	Co-Investigator	4.0	0.0	0.0	Chulalongkorn University	THAILAND	NA
	Y	Broder, Christopher	PhD	Co-Investigator	1.0	0.0	0.0			NA
	Y	Keusch, Gerald	MD	Co-Investigator	1.0	0.0	0.0			NA
	Y	Corley, Ronald	PhD	Co-Investigator	1.0	0.0	0.0			NA
	Y	Laing, Eric	PhD	Co-Investigator	2.0	0.0	0.0			NA
	Y	Field, Hume	PhD	Consultant	1.0	0.0	0.0	Jeppesen Field Consulting	AUSTRALIA	NA
	Y	Yeo, Tsin Wen	PhD	Consultant	1.0	0.0	0.0	Lee Kong Chian School of Medicine	SINGAPORE	NA
	Y	Hickey, Andrew	PhD	Consultant	1.0	0.0	0.0	Thailand MOPH-CDC	THAILAND	NA
	Y	Rajahram, Giri Shan	MRCP	Infectious Disease Epidemiologist	1.0	0.0	0.0	Queen Elizabeth State Hospital	MALAYSIA	NA
	Y	Hamzah, Nadia Diyana	MD	Medical Officer and Clinician	1.0	0.0	0.0	Bario Clinic, Rural Area Service Ministry of Health Malaysia	MALAYSIA	NA
	Y	Gee Lee, Heng	MRCP	Senior Clinician	1.0	0.0	0.0	Queen Elizabeth State Hospital	MALAYSIA	NA
	Y	Faisal Ali, Anwarali Khan	PhD	Zoologist and Biotechnician	1.0	0.0	0.0	University Malaysia Sarawak	MALAYSIA	NA
	Y	Hemachudha, Pasin	MD	Immunologist and Clinician	1.0	0.0	0.0	Chulalongkorn University Hospital	THAILAND	NA
	Y	Phelps, Kendra	PhD	Field Scientist	1.0	0.0	0.0			NA

	Y	Spencer, Sterling Lee	MPH	Research Project Coordinator	6.0	0.0	0.0			NA
	Y	Li, Hongying	MPH	Epidemiologist	2.0	0.0	0.0			NA
	N	Mendelsohn, Emma	MEM	Data Scientist	1.0	0.0	0.0			NA
	Y	Valitutto, Marc	VMD	Senior Field Veterinarian	3.0	0.0	0.0			NA
	Y	Firth, Cadhla	PhD	Senior Research Scientist	2.0	0.0	0.0			NA
	Y	Sekaran, Jayaseelan		Co-Investigator	1.0	0.0	0.0	Lintang Clinic, Kulala Kangsar District Health Office	MALAYSIA	NA
	Y	Tan, Cheng Siang	PhD	Co-Investigator	1.0	0.0	0.0	Jalan Datuk Mohammad Musa	MALAYSIA	NA
	Y	Kamruddin, Ahmed		Co-Investigator	1.0	0.0	0.0	Universiti Malaysia Sabah	MALAYSIA	NA
	Y	Lin, Ingrid Ting Pao	MD	Co-Investigator	1.0	0.0	0.0	Hospital Miri	MALAYSIA	NA
	Y	Hughes, Tom	Post-Grad Diploma	Co-Investigator	1.0	0.0	0.0	Conservation Medicine	MALAYSIA	NA
	Y	Lasimbang, Helen	MBBS	Co-Investigator	1.0	0.0	0.0	Hospital Universiti Malaysia Sabah	MALAYSIA	NA
	Y	Chmura, Aleksei	PhD	Senior Program Manager	1.0	0.0	0.0			NA
	Y	William, Timothy	FRCP	Co-Investigator	1.0	0.0	0.0	Gleneagles Hospital	MALAYSIA	NA

Glossary of acronyms:
S/K - Senior/Key
Cal - Person Months (Calendar)
Aca - Person Months (Academic)
Sum - Person Months (Summer)
Foreign Org - Foreign Organization Affiliation
SS - Supplement Support
RS - Reentry Supplement
DS - Diversity Supplement
OT - Other
NA - Not Applicable

D.2 PERSONNEL UPDATES

D.2.a Level of Effort

Will there be, in the next budget period, either (1) a reduction of 25% or more in the level of effort from what was approved by the agency for the PD/PI(s) or other senior/key personnel designated in the Notice of Award, or (2) a reduction in the level of effort below the minimum amount of effort required by the Notice of Award?

No

D.2.b New Senior/Key Personnel

Are there, or will there be, new senior/key personnel?

Yes

File Uploaded: New Key Personnel.pdf

D.2.c Changes in Other Support

Has there been a change in the active other support of senior/key personnel since the last reporting period?

No

D.2.d New Other Significant Contributors

Are there, or will there be, new other significant contributors?

No

D.2.e Multi-PI (MPI) Leadership Plan

Will there be a change in the MPI Leadership Plan for the next budget period?

NA

OMB No. 0925-0001 and 0925-0002 (Rev. 10/2021 Approved Through 09/30/2024)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Sterling, Spencer Lee

eRA COMMONS USER NAME (credential, e.g., agency login):

POSITION TITLE: Research Project Coordinator

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of Maryland, College Park (MD, USA)	B.S.	05/2015	Biological Sciences
University of Nebraska Medical Center	MPH	05/2020	Epidemiology

A. Personal Statement

As a current employee with Dr. Eric Laing under his emerging infectious diseases research program. My academic training and research experience have provided me with a background in molecular biology, virology, and epidemiological techniques for zoonosis investigations. As part of my original post-baccalaureate work, I worked in a small team lead by Dr. Chris Border tasked with developing a serological binding assay for Coronaviruses, Filoviruses, and Henipaviruses. During this process, I worked closely with Dr. Laing and scientists from multiple Asian countries in developing and applying the multiplex techniques for rapid, high-throughput serological assays. Throughout this time, I have led technical and data analysis trainings in Cambodia, Bangladesh, and Thailand, and have assisted collaborators in Singapore, Malaysia, Australia, India, South Africa, and the United Kingdom in implementing these novel techniques. Currently, I am a visiting scientist with Dr. Supaporn Wacharapluesadee in the Emerging Infectious Diseases Clinical Center at King Chulalongkorn Medical Hospital as part of the CREID-EID SEARCH program.

1. Laing ED, **Sterling SL**, Richard S., Phogat S., et al. “A betacoronavirus multiplex microsphere immunoassay detects early SARS-CoV-2 seroconversion and antibody cross reactions”. (2021). *Pre-print.*
2. **Sterling, SL**, "Evaluation of the humoral immune response as a potential indicator of disease severity for patients hospitalized with COVID-19" (2020). *Capstone Experience*. 121. https://digitalcommons.unmc.edu/coph_slce/121
3. Yan L, **Sterling SL**, Laing ED, and Broder CC. “Expression System for Recombinant Henipavirus Glycoproteins”. *(submitted, 2020).*
4. “**Sterling SL**, Laing ED, Yan L, Feng Y, Epstein JH, Broder CC. Development of a novel pan-filovirus and henipavirus serological assay for zoonotic surveillance.” Uniformed Services University 2019 Research Days. May 13-17, 2019. Bethesda, Maryland.

B. Positions, Scientific Appointments, and Honors

05/22-present Visiting Scientist, King Chulalongkorn Medical Hospital, Bangkok, Thailand. Advisors: Dr. Supaporn Wacharapluesadee, Dr. Opass Putchaeron, and Dr. Eric D. Laing.

02/22-present Scientific Project Coordinator, Henry M. Jackson Foundation, Department of Microbiology, Uniformed Services University, Bethesda, MD. Advisors: Dr. Christopher C. Broder and Dr. Eric D. Laing

08/20-02/22 Research Associate 2, Henry M. Jackson Foundation, Department of Microbiology, Uniformed Services University, Bethesda, MD. Advisor: Dr. Christopher C. Broder

08/19-08/20 Senior Research Assistant, Henry M. Jackson Foundation, Department of Microbiology, Uniformed Services University, Bethesda, MD. Advisor: Dr. Christopher C. Broder

06/15 – 08/19 Research Assistant, Henry M. Jackson Foundation, Department of Microbiology, Uniformed Services University, Bethesda, MD. Advisor: Dr. Christopher C. Broder

01/14-08/14 Laboratory research internship, Department of Microbiology, Uniformed Services University, Bethesda, MD. Advisor: Dr. Christopher C. Broder

C. Contributions to Science

1. **Post-Baccalaureate:** My post-baccalaureate research works primarily targeted understanding the adaptive immune response to emerging zoonotic viruses as a tool for virus discovery. As part of the assay development team, I designed and expressed soluble, native-like filovirus and henipavirus attachment glycoproteins and developed the monoclonal antibodies and antisera used as standards in the assay. I have led technical training for international projects and assisted in data analysis
    a. Laing ED, Mendenhall IH, Linster M, Low DHW, Chen Y, Yan L, **Sterling SL**, et al. “Serologic evidence of fruit bat exposure to filoviruses, Singapore, 2011–2016”. (2018). Emerg Infect Dis. 24(1):114-117. doi: 10.3201/eid2401.170401. PMID: 29260678.
    b. Schulz JE, Seifert SN, Thompson JT, Avanzato V, **Sterling SL**, et al. “Serological evidence for henipavirus-like and filovirus-like viruses in Trinidad bats.” (2020) J Infect Dis. pii: jiz648. doi: 10.1093/infdis/jiz648. PMID: 32034942.
    c. Yan L, **Sterling SL**, Laing ED, and Broder CC. “Expression System for Recombinant Henipavirus Glycoproteins”. *(submitted, 2020).*
    d. **Sterling SL,** Hip P, Ly P, Ouch P, Mao M, Low DHW, Yan LY, Tso M, Smith GJ, Broder CC, Hertz JC Mendenhall IH, Laing ED. “Serological evidence of exposure to known and unknown henipaviruses in Cambodia” World One Health Congress. November 7-11, 2022. Singapore, SGP.

2. **Professional Studies:** While employed, I continued my studies by pursuing a Master’s in Public Health with a focus on Epidemiology. Immediately preceding my thesis work, COVID-19 was declared a pandemic, and I worked along side Dr. Eric Laing to develop and standardize a human coronavirus serological panel to track the serological response to SARS-CoV-2 infections and vaccinations among a military cohort. For my thesis, I evaluated the humoral immune response to SARS-CoV-2 patients as it related to the severity of the disease, where we observed significantly more robust antibody responses in people with more severe disease.
    a. Laing ED, **Sterling SL**, Richard S., Phogat S., et al. “A betacoronavirus multiplex microsphere immunoassay detects early SARS-CoV-2 seroconversion and antibody cross reactions”. (2021). *Pre-print.*
    b. **Sterling, SL**, "Evaluation of the humoral immune response as a potential indicator of disease severity for patients hospitalized with COVID-19" (2020). *Capstone Experience*. 121. https://digitalcommons.unmc.edu/coph_slce/121
    c. Laing ED, Weiss CD, Samuels EC, Coggins AS, Wang W, Wang R, Vassell R, **Sterling SL**, et al. “Durability of antibody responses and frequency of clinical and subclinical SARS-CoV-2 infection six months after BNT162b2 COVID-19 vaccination in healthcare workers” (2021). *Pre-print*. https://doi.org/10.1101/2021.10.16.21265087
    d. Laing ED, Epsi NJ, Stephanie A. SA, Samuels EC, Wang W, Vassell R, Ewing DF, Herrup R, **Sterling SL**, et al. “SARS-CoV-2 antibodies remain detectable 12 months after infection and antibody magnitude is associated with age and COVID-19 severity”. (2021). *Pre-print*. https://doi.org/10.1101/2021.04.27.21256207

OMB No. 0925-0001 and 0925-0002 (Rev. 10/2021 Approved Through 09/30/2024)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Putcharoen, Opass

eRA COMMONS USER NAME (credential, e.g., agency login): OPASS.P

POSITION TITLE: Assistant Professor, Division of Infectious Diseases, Department of Medicine, King Chulalongkorn Memorial Hospital, Chulalongkorn University

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Kon Kaen University, Thailand	M.D.	1995	Medicine
Chulalongkorn University, Thailand	M.Sc	2007	Medical Sciences

A. Personal Statement

I am the director of the Emerging Infectious Diseases Clinical Center, Thai Red Cross, and a senior lecturer of the division of infectious diseases, Department of Medicine, King Chulalongkorn Memorial Hospital, Chulalongkorn University, Bangkok, Thailand. I am also an assistant professor at the faculty of medicine, Chulalongkorn University. In 1998, I received a medical degree from Kon Kean University, Thailand, and then completed my residency and fellowship (infectious diseases) training at Chulalongkorn University. During 2008-2010, I received training grants from the Fogarty International Center at NIH, USA. From 2008-2011, I was a research fellow in HIV drug resistance at the department of immunology and infectious diseases, Harvard School of Public and a postdoctoral fellow at the section of retroviral therapeutics, division of infectious diseases, Brigham and Women's Hospital, Harvard School of Medicine, Boston, MA, USA. My current research focuses on long-term complications of antiretroviral therapy, HIV drug resistance, co-infections in resource-limited settings, and emerging infectious diseases.

B. Positions, Scientific Appointments, and Honors

Positions

1995-2001 General practitioner Panusnikom District Hospital, Chonburi, Thailand
2001-2005 Internist in Panusnikom District Hospital (200-bed community hospital), Chonburi, Thailand
2006-2008 Clinical fellow, Department of Infectious Diseases, Chulalongkorn Hospital, Bangkok, Thailand
2008-2010 Research fellow, Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA
2010- Instructor in Medicine, Department of Medicine, Chulalongkorn University, Bangkok, Thailand
2017- Director, Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital

Honors

1994 Scholarship from Case Western Reserve University for short course training in "Introduction to Clinical Infectious Diseases"
2007 Award for selected high-quality abstracts in 17th European Congress of Clinical Microbiology and Infectious Diseases and 25th International Congress of Chemotherapy Munich/Germany

D.2.b (New Key Personnel.pdf)

C. Contributions to Science

a. **Putcharoen, O.,** Suankratay, C. (2007). Salmonella gas-forming femoral osteomyelitis and pyomyositis: The first case and review of the literature. Journal of the Medical Association of Thailand, 90 (9): 1943-1947.
b. **Putcharoen, O.** (2008). It is time to change the starting point of initiating antiretroviral therapy and to advocate more active HIV testing. Asian Biomedicine, 2 (3): 245-246.
c. Kerr, S.J., Avihingsanon, A., **Putcharoen, O.**, Chetchotisakd, P., Layton, M., Ubolyam, S., Ruxrungtham, K., Cooper, D.A., Phanuphak, P., Duncombe, C. (2012). Assessing adherence in Thai patients taking combination antiretroviral therapy. International Journal of STD and AIDS, 23 (3): 160-165.
d. **Putcharoen, O**., Lee, S.H., Henrich, T.J., Hu, Z., Vanichanan, J., Coakley, E., Greaves, W., Gulick, R.M., Kuritzkes, D.R., Tsibris, A.M.N. (2012). HIV-1 clinical isolates resistant to CCR5 antagonists exhibit delayed entry kinetics that are corrected in the presence of drug. Journal of Virology, 86 (2): 1119-1128.
e. Rangwala, F., **Putcharoen, O.,** Bowonwatanuwong, C., Edwards-Jackson, N., Kramomthong, S., Kim, J.H., Corey, G.R., Ananworanich, J. (2012). Histoplasmosis and penicilliosis among HIV infected Thai patients: A retrospective review. Southeast Asian Journal of Tropical Medicine and Public Health, 43 (2): 436-441.
f. Tsibris, A.M.N., Hu, Z., Paredes, R., Leopold, K.E., **Putcharoen, O**., Schure, A.L., Mazur, N., Coakley, E., Su, Z., Gulick, R.M., Kuritzkes, D.R. (2012). Vicriviroc resistance decay and relative replicative fitness in HIV-1 clinical isolates under sequential drug selection pressures. Journal of Virology, 86 (12): 6416-6426.
g. Permpalung, N., **Putcharoen, O**., Avihingsanon, A., Ruxrungtham, K. (2012). Treatment of HIV infection with once-daily regimens. Expert Opinion on Pharmacotherapy, 13 (16): 2301-2317.
h. **Putcharoen, O**., Ruxrungtham, K. (2013). Rilpivirine in treatment-naive patients: What did we learn from the THRIVE and ECHO studies? Future Virology, 8 (2): 113-120.
i. **Putcharoen, O.**, Kerr, S.J., Ruxrungtham, K. (2013). An update on clinical utility of rilpivirine in the management of HIV infection in treatment-naïve patients. HIV/AIDS - Research and Palliative Care, 5: 231-241.
j. **Putcharoen, O**., Wattanachanya, L., Sophonphan, J., Siwamogsatham, S., Sapsirisavat, V., Gatechompol, S., Phonphithak, S., Kerr, S.J., Chattranukulchai, P., Avihingsanon, Y., Ruxrungtham, K., Avihingsanon, A. (2017). New-onset diabetes in HIV-treated adults: Predictors, long-term renal and cardiovascular outcomes. AIDS, 31 (11): 1535-1543.
k. **Putcharoen, O**., Pleumkanitkul, S., Chutinet, A., Vongsayan, P., Samajarn, J., Sophonphan, J., Kerr, S.J., Hiransuthikul, A., Siwamogsatham, S., Ruxrungtham, K., Avihingsanon, A. (2019). Comparable carotid intima-media thickness among long-term virologically suppressed individuals with HIV and those without HIV in Thailand. Journal of Virus Eradication, 5 (1).
l. Sarin, S.K., Choudhury, A., Lau, G.K., Zheng, M.-H., Ji, D., Abd-Elsalam, S., Hwang, J., Qi, X., Cua, I.H., Suh, J.I., Park, J.G., **Putcharoen, O**., Kaewdech, A., Piratvisuth, T., Treeprasertsuk, S., Park, S., Wejnaruemarn, S., Payawal, D.A., Baatarkhuu, O., Ahn, S.H., Yeo, C.D., Alonzo, U.R., Chinbayar, T., Loho, I.M., Yokosuka, O., Jafri, W., Tan, S., Soo, L.I., Tanwandee, T., Gani, R., Anand, L., Esmail, E.S., Khalaf, M., Alam, S., Lin, C.-Y., Chuang, W.-L., Soin, A.S., Garg, H.K., Kalista, K., Batsukh, B., Purnomo, H.D., Dara, V.P., Rathi, P., Al Mahtab, M., Shukla, A., Sharma, M.K., Omata, M. (2020). Pre-existing liver disease is associated with poor outcome in patients with SARS CoV2 infection; The APCOLIS Study (APASL COVID-19 Liver Injury Spectrum Study). Hepatology International, 14 (5): 690-700.
m. **Putcharoen, O.**, Wacharapluesadee, S., Chia, W.N., Paitoonpong, L., Tan, C.W., Suwanpimolkul, G., Jantarabenjakul, W., Ruchisrisarod, C., Wanthong, P., Sophonphan, J., Chariyavilaskul, P., Wang, L.-F., Hemachudha, T. (2021). Early detection of neutralizing antibodies against SARS-CoV-2 in COVID-19 patients in Thailand. PLoS ONE, 16: e0246864.
n. Hunsawong T, Fernandez S, Buathong R, Khadthasrima N, Rungrojchareonkit K, Lohachanakul J, Suthangkornkul R, Tayong K, Huang AT, Klungthong C, Chinnawirotpisan P, Poolpanichupatam Y, Jones AR, Lombardini ED, Wacharapluesadee S, **Putcharoen O**. (2021). Limited and Short-Lasting Virus Neutralizing Titers Induced by Inactivated SARS-CoV-2 Vaccine. Emerg Infect Dis, 27(12):3178-3180.
o. Jantarabenjakul W, Chantasrisawad N, Puthanakit T, Wacharapluesadee S, Hirankarn N, Ruenjaiman V, Paitoonpong L, Suwanpimolkul G, Torvorapanit P, Pradit R, Sophonphan J, **Putcharoen O**. (2022). Short-term immune response after inactivated SARS-CoV-2 (CoronaVac®, Sinovac) and ChAdOx1 nCoV-19 (Vaxzevria®, Oxford-AstraZeneca) vaccinations in health care workers. Asian Pac J Allergy Immunol, 40(3):269-277.

p. Jantarabenjakul W, Sodsai P, Chantasrisawad N, Jitsatja A, Ninwattana S, Thippamom N, Ruenjaiman V, Tan CW, Pradit R, Sophonphan J, Wacharapluesadee S, Wang LF, Puthanakit T, Hirankarn N, **Putcharoen O**. (2022). Dynamics of Neutralizing Antibody and T-Cell Responses to SARS-CoV-2 and Variants of Concern after Primary Immunization with CoronaVac and Booster with BNT162b2 or ChAdOx1 in Health Care Workers. Vaccines (Basel), 19;10(5):639.
q. Buathong R, Hunsawong T, Wacharapluesadee S, Guharat S, Jirapipatt R, Ninwattana S, Thippamom N, Jitsatja A, Jones AR, Rungrojchareonkit K, Lohachanakul J, Suthangkornkul R, Tayong K, Klungthong C, Fernandez S, **Putcharoen O**. (2022). Homologous or Heterologous COVID-19 Booster Regimens Significantly Impact Sero-Neutralization of SARS-CoV-2 Virus and Its Variants. Vaccines (Basel), 15;10(8):1321.
r. Tan CW, Chia WN, Zhu F, Young BE, Chantasrisawad N, Hwa SH, Yeoh AY, Lim BL, Yap WC, Pada SKMS, Tan SY, Jantarabenjakul W, Toh LK, Chen S, Zhang J, Mah YY, Chen VC, Chen MI, Wacharapluesadee S, Sigal A, **Putcharoen O**, Lye DC, Wang LF. (2022). SARS-CoV-2 Omicron variant emerged under immune selection. Nat Microbiol. 7(11):1756-1761.
s. Wacharapluesadee S, Hirunpatrawong P, Petcharat S, Torvorapanit P, Jitsatja A, Thippamom N, Ninwattana S, Phanlop C, Buathong R, Tangwangvivat R, Klungthong C, Chinnawirotpisan P, Hunsawong T, Suthum K, Komolsiri S, Jones AR, Fernandez S**, Putcharoen O.** (2023). Simultaneous detection of omicron and other SARS-CoV-2 variants by multiplex PCR MassARRAY technology. Sci Rep, 6;13(1):2089.

E. IMPACT

E.1 WHAT IS THE IMPACT ON THE DEVELOPMENT OF HUMAN RESOURCES?

Not Applicable

E.2 WHAT IS THE IMPACT ON PHYSICAL, INSTITUTIONAL, OR INFORMATION RESOURCES THAT FORM INFRASTRUCTURE?

EID-SEARCH project, in collaboration with Conservation Medicine in Malaysia, is committed to build in-country capacity for emerging infectious surveillance, the long-term collaboration with local government and institutions in Malaysia has led to significant infrastructure improvement as detailed below:

In January 2020, Conservation Medicine started the process of getting the Wildlife Health, Genetic and Forensic Laboratory (WHGFL) in Sabah, a BSL-2 laboratory certified to CDC and NIH laboratory standards, that Conservation Medicine designed and with support from EcoHealth Alliance established with Sabah Wildlife Department (SWD), ISO 17025 accredited. This accreditation will allow the laboratory to conduct forensic investigations for the Sabah Wildlife Department, an important development in combatting the illegal wildlife trade and its associated risks with the spread of zoonotic disease. The accreditation will also include the Disease Unit coordinated by Conservation Medicine and our CoV, PMV, and FLV testing. From 1st April 2020, we started the ISO 17025 training (16 people trained to date) and document preparations with guidance from our consultant. The document audit with Standards Malaysia was conducted on 28th July 2022, and we were found to be adequate. The pre-assessment on-site audit with Standards Malaysia was conducted on 12-13 January 2023. Addressing the few non-conformities identified during this audit is ongoing, and we are working closely with SWD with the hope of having the lab accredited before the end of 2023.

On 18 July 2022, the WHGFL in Sabah was certified for the ninth year in a row as a BSL- 2 laboratory, in accordance with the Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition (December 2009), which is the US standard for laboratory specifications.

On 3 October 2022, the Molecular Zoonosis Laboratory at PERHILITAN's National Wildlife Forensic Laboratory was certified for the sixth year in a row as a BSL-2 laboratory in accordance with the Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition (December 2009).

On 27 February 2023, with approved funding, EID-SEARCH/Conservation Medicine purchased the MAGPIX Multiplex System for serology work in Sabah. This will be located at the Borneo Medical and Health Research Centre (BMHRC) and Universiti Malaysia Sabah (UMS). It will be used to screen human and wildlife samples for EID-SEARCH as agreed with Sabah State Health Department, SWD, and UMS. Conservation Medicine are working with BMHRC to get the lab ready, so that the wildlife serology screening can start in June 2023, followed by human serology once all approvals are in place. The laboratory and work will be managed according to internationally recognized BioSafety standards.

Additional resources were kindly provided by the NIAID Office of Global Research (OGR) via the CREID network, which helped to improve the molecular viral screening and characterization capacity in Thailand and Malaysia.

E.3 WHAT IS THE IMPACT ON TECHNOLOGY TRANSFER?

Not Applicable

E.4 WHAT DOLLAR AMOUNT OF THE AWARD'S BUDGET IS BEING SPENT IN FOREIGN COUNTRY(IES)?

Dollar Amount	Country

$431,551	MALAYSIA
$108,006	SINGAPORE
$215,944	THAILAND

F. CHANGES

F.1 CHANGES IN APPROACH AND REASONS FOR CHANGE

Not Applicable

F.2 ACTUAL OR ANTICIPATED CHALLENGES OR DELAYS AND ACTIONS OR PLANS TO RESOLVE THEM

The biggest challenge we have encountered during this reporting period goes back to the large number of hospitalized patients in local hospitals due to COVID-19. Patient influx delayed human participant enrollment at targeted hospital sites and the subsequent laboratory analysis we anticipated in Year 2. During Year 3, we have worked hard to address this challenge and will increase human enrollment at both hospital and community sites in Malaysia and Thailand as we move into Year 4. To address these issues, we have 1) added additional hospitals, including both larger hospitals and community clinics; 2) refined the study tools and provided refresher training to hospital staff to improve the enrollment and data collection procedures to allow faster processing. Meanwhile, we have included 3) archived samples with the undiagnosed causes of infection or death cases of encephalitis from the local surveillance program into this program.

In Malaysia, while waiting for government approval for human surveillance, we have continued working on archived human samples and are already getting exciting results, which will be reported to NIAID after they are validated and approved in the coming weeks.

F.3 SIGNIFICANT CHANGES TO HUMAN SUBJECTS, VERTEBRATE ANIMALS, BIOHAZARDS, AND/OR SELECT AGENTS

F.3.a Human Subject

No Change

F.3.b Vertebrate Animals

No Change

F.3.c Biohazards

No Change

F.3.d Select Agents

No Change

G. SPECIAL REPORTING REQUIREMENTS SPECIAL REPORTING REQUIREMENTS

G.1 SPECIAL NOTICE OF AWARD TERMS AND FUNDING OPPORTUNITIES ANNOUNCEMENT REPORTING REQUIREMENTS

File(s) uploaded:
G.1 Special reporting requirement_final.pdf

G.2 RESPONSIBLE CONDUCT OF RESEARCH

Not Applicable

G.3 MENTOR'S REPORT OR SPONSOR COMMENTS

Not Applicable

G.4 HUMAN SUBJECTS

Sub-Project ID	Study ID	Study Title	Delayed Onset	Clinical Trial	NCT	NIH-Defined Phase 3	ACT
	293221	Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia	NO	NO			
	380835	Monitoring and characterizing SARS-CoV-2 variants in Thailand	NO	NO			

G.5 HUMAN SUBJECTS EDUCATION REQUIREMENT

Are there personnel on this project who are newly involved in the design or conduct of human subjects research?

No

G.6 HUMAN EMBRYONIC STEM CELLS (HESCS)

Does this project involve human embryonic stem cells (only hESC lines listed as approved in the NIH Registry may be used in NIH funded research)?

No

G.7 VERTEBRATE ANIMALS

Does this project involve vertebrate animals?

Yes

G.8 PROJECT/PERFORMANCE SITES

Organization Name	UEI	Congressional District	Address
Primary: ECOHEALTH ALLIANCE, INC.	TKS7NBB4JDN6	NY-010	520 Eighth Avenue SUITE 1200 NEW YORK, NY 100184183
Chulalongkorn University	L237QX57L1K7		254 Phayathai Road Pathumwan, Bangkok, 10330
Conservation Medicine	XKAJBKBNJLV7		13H Villamas Condo Villamas Jalan Villamas Sungai Buloh, NONE 47000
National University of Singapore	KZGWU4FU9UK5		21 Lower Kent Ridge Road Singapore, NONE 119077
Uniformed Services University	UYLKBRENAPG5	MD-008	4301 Jones Bridge Rd Bethesda, MD 208144799
University of North Carolina, Chapel Hill	D3LHU66KBLD5	NC-004	135 Dauer Drive Chapel Hill, NC 275997400
National Emerging Infectious Diseases Laboratories	FBYMGMHW4X95	MA-007	620 Albany St. Boston, MA 021182516

G.9 FOREIGN COMPONENT

Organization Name: Chulalongkorn University
Country: THAILAND
Description of Foreign Component:
The site will 1) collect biological specimens from target animal and human populations; 2) collect questionnaire survey data from target human populations; 3) conduct laboratory RT-PCR testing, sequencing, and serological testing with collected specimens; 4) conduct viral isolation, full genome sequencing and initial data analysis; and 5) share data and samples with US collaborator.

Organization Name: Conservation Medicine
Country: MALAYSIA
Description of Foreign Component:
The site will 1) collect biological specimens from target animal and human populations; 2) collect questionnaire survey data from target human populations; 3) conduct laboratory RT-PCR testing, sequencing, and serological testing with collected specimens; 4) conduct viral isolation, full genome sequencing, and initial data analysis; and 5) share data and samples with US collaborators.

Organization Name: Duke-NUS Medical School
Country: SINGAPORE

Description of Foreign Component:
The site will 1) develop serological platforms for the human biological sample testing; 2) provide the serological assays to project collaborators in Thailand and Malaysia; and 3) provide lab technical support and consultation regarding any specific pathogens that are identified as high interest to the CREID and NIH.

G.10 ESTIMATED UNOBLIGATED BALANCE

G.10.a Is it anticipated that an estimated unobligated balance (including prior year carryover) will be greater than 25% of the current year's total approved budget?

No

G.11 PROGRAM INCOME

Is program income anticipated during the next budget period? No

G.12 F&A COSTS

Is there a change in performance sites that will affect F&A costs?

No

G.1 (G.1 Special reporting requirement_final.pdf)

All special reporting requirements specified in the award terms and conditions in the Notice of Award (NoA) have been addressed and reported to the Grants Management Specialist and Program Office.

EcoHealth Alliance certifies that during the current reporting period, onsite subrecipient facility inspections were conducted as required in our NoA to ensure that subaward activities are being properly executed.

Section 1 - Basic Information (Study 293221)

1.1. Study Title *

Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia

1.2. Is this study exempt from Federal Regulations * ○ Yes ● No

1.3. Exemption Number ❑ 1 ❑ 2 ❑ 3 ❑ 4 ❑ 5 ❑ 6 ❑ 7 ❑ 8

1.4. Clinical Trial Questionnaire *

1.4.a. Does the study involve human participants?	● Yes	○ No
1.4.b. Are the participants prospectively assigned to an intervention?	○ Yes	● No
1.4.c. Is the study designed to evaluate the effect of the intervention on the participants?	○ Yes	● No
1.4.d. Is the effect that will be evaluated a health-related biomedical or behavioral outcome?	○ Yes	● No

1.5. Provide the ClinicalTrials.gov Identifier (e.g. NCT87654321) for this trial, if applicable

Section 2 - Study Population Characteristics (Study 293221)

2.1. Conditions or Focus of Study

- Humans living in geographic hotspot areas/close contact with wild animals

2.2. Eligibility Criteria

ELIGIBILITY CRITERIA

Participants to be enrolled in this study will be individuals from Thailand (Ratchaburi and Chonburi provinces), Peninsular Malaysia, Sabah Malaysia, or Sarawak # 12 years old living or working around wildlife habitats (e.g. bat caves/roosts), those who hunt wildlife, work with wildlife or livestock farming, transportation, selling, or slaughtering wildlife or visiting or working in high-risk sites (e.g. wildlife markets) who meet the inclusion criteria outlined below. Study sites are prioritized based on the hotspot geographic areas described in Aim 1, according to ecological and epidemiological conditions associated with a high risk for the coronaviruses, henipaviruses, filoviruses spillover.

Research participants will be enrolled in two settings:

1. Community – We aim to enroll and collect biological samples and survey responses individuals' living, working, or visiting targeted high-risk communities (as defined above) who have close contact with wildlife, specifically bats, rodents, non-human primates, with a range of exposures to these animals. Enrolled research participants will be asked to provide biological samples and complete a questionnaire that is designed to obtain detailed information into wildlife contact frequency and exposures related to: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house and other livestock animals (e.g. farming, butchering, slaughtering); 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported symptoms relating to a) severe/acute respiratory illness (SARI/ARI); b) Influenza-like illness (ILI); c) fever of unknown origin (FUO); d) encephalitis; or e) hemorrhagic fever; or f) diarrhea in combination with any of the previously mentioned illnesses within the 12 months and lifetime.

Additional inclusion criteria:

- Adults (18 years of age or older) who provide informed consent
- Children aged 12-17 years of age who provide assent along with an accompanying parent or guardian who is able to provide informed consent and
- Pregnant women will be considered eligible for inclusion

Exclusion criteria:

- Adults (18 years of age or older) who are unable to provide informed consent, including individuals with physiologically or medically induced cognitive impairments
- Individuals under 12 years of age
- Children without an accompanying parent or guardian who is able to provide informed consent, or a child 12-17 years old unable or unwilling to provide assent or children who are wards of the state
- Prisoners

2. Hospital - Both out-patients and in-patients at clinics or hospitals presenting with clinically defined symptoms of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever; or 6) diarrhea in combination with any of the previously mentioned illnesses of unknown etiology. Biological samples will be collected from the patients and the patient, will complete a questionnaire. We will follow up with these participants 35 days after enrollment to collect another biological sample to assess the development of IgG/IgM and collect additional data on the course of symptoms in the interim period.

Additional inclusion criteria:

- Adults (18 years of age or greater) who provide informed consent
- Children aged 12-17 years of age who provide assent along with an accompanying parent or guardian who is able to provide informed consent and
- Pregnant women will be considered eligible for inclusion

Exclusion criteria:

- Individuals over the age of 12 years who refuse to provide informed consent
- Adults unable to provide informed consent, including individuals with physiologically or medically induced cognitive impairments
- Children, aged 12-17 years, without an accompanying parent or guardian who is able to provide informed consent, or a child aged 12 to 17 who is unable or unwilling to provide assent
- Children < 12 years of age or children who are wards of the state
- Prisoners

2.3. Age Limits Min Age: 12 Years Max Age: N/A (No limit)

2.3.a. Inclusion of Individuals Across the Lifespan

2.4. Inclusion of Women and Minorities	Inclusion_of_Women_Min_Children_FINAL.pdf
2.5. Recruitment and Retention Plan	Section_2_Attm_Recruitment_Retention_Plan_FINAL.pdf
2.6. Recruitment Status	Not yet recruiting
2.7. Study Timeline	Section_2_Attm_Study_Timeline_FINAL.pdf
2.8. Enrollment of First Participant (SEE SECTION 6.3)	

INCLUSION OF WOMEN AND MINORITIES:

This study will enroll men and women, including pregnant women, as study participants. Subjects will be enrolled in this study without regard to ethnicity.

Women who volunteer to participate are not at an increased risk based on pregnancy status and are at the same exposure risk as non-pregnant women. Every effort will be made to protect the privacy, dignity, and well-being of all study participants especially special populations who participate in this study.

Individuals in sub-sites in selected geographic hotspot regions will be the primary mechanism for identifying subjects. We will make every effort to have men and women equally represented in this study and no individuals will be excluded based on ethnicity.

- **At community sites,** living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock farming, transportation, selling, or slaughtering wildlife in the surveyed areas will be the primary criteria for identifying participants in community.

- **At clinic sites**, only patients who present at the healthcare facility who meet the clinical case definition of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever; or 6) diarrhea in combination with any of the previously mentioned illnesses of unknown etiology will be recruited for this study, and no patients will be excluded based on ethnicity or gender.

INCLUSION OF CHILDREN:

Children aged 12–17 years will be included in this study, and there will be no maximum age restriction for adults, at both community and clinical sites

- Previous clinic-based studies have shown that children are one of the major populations who present to healthcare facilities with severe/acute respiratory illness (SARI/ARI), Influenza-like illness (ILI), or fever of unknown origin (FUO). Our behavioral study in Thailand and Malaysia also suggested the close contact with wild animals among children in the study regions via activities of animal hunting, trade, or butchering.

- Children aged 12 years or older are post-primary school in Thailand and Malaysia and are able to comprehend and respond to the questionnaire autonomously which increases the reliability of responses. We will not enroll children aged 12-17 years without an accompanying parent or guardian who is able to provide informed consent, or a child aged 12-17 who is unable to or unwilling to provide assent.

- Children under age 12 in target communities are mainly school children who have very limited exposures to wild animals under the scenarios of interest to the study, and ethically we do not want to collect or enroll participants without strong scientific need for inclusion. We will not enroll children who are wars of the state

- Every effort will be made to protect the privacy, dignity, and well-being of children who participate in this study. Our in-country human research team are well-trained medical doctors and researchers who have extensive experience working with children, as well as their parents, at both community and clinical settings. Prior to the start of human subject research activities, all research staff will be CITI-trained and further trained on conducting ethical human subject research training including a module on the special considerations for working with children

regarding risk and coercion. Enrollment of children will be monitored and annually reported to the IRB.

RECRUITMENT AND RETENTION PLAN

In order to improve recruitment within target communities, introductory visits will be made by project staff to each of the selected sub-sites. These visits will be advertised through word of mouth and a project description letter to village/town/city leaders and letters that can be posted or shared in a central community location. The letter will inform the community that a team will be coming on a particular day(s) to enroll voluntary participants and after discuss health issues related to animal contact. This letter will be for informational sharing not be used for recruitment purposes. It will only be used to inform the community of the research visits. The project description letter will be written in the local language with a Flesch–Kincaid readability score equivalent to a 7th grade reading level or below (primary school in Thailand and Malaysia), to assure that community leaders and potential community participants understand the study purpose, eligibility, and inclusion guidelines.

Community visits will begin with discussions and meetings with local authorities and community leaders to introduce ourselves and our project, and when appropriate following approval from local authorities, the study team will post flyers to inform the community when the team will be speaking about enrollment and later coming back to enroll interested individuals. Attending this “town hall” style meeting will be completely voluntary and based on our experience, those interested are likely to attend. Although local authorities may be present to introduce the study team members, they will not be involved in the recruitment and/or consent of the participants for the study. Individuals will be clearly informed during the recruitment process that their participation in the study is voluntary. If research visits or recruitment events are held at a workplace individuals choice of involvement will not impact their employment, nor will information discussed be shared with employers. With local permissions and accompanied by local community leaders, district health officers, or authorities the study team members will engage in community town halls and ‘walkabouts’ during which they will discuss study details, dates, times, and locations for enrollment and participation in the study.

Participation in the study will be strictly voluntary and will require signed informed consent for all participants and signed assent for participants aged 12-17 along with parent or guardian consent. During the enrollment process interested individuals will be given a consent form and research staff will read the consent form to potential participants. Together they will review the consent form and study staff will explain details of the study including: why they were selected, what the study procedures are and what will be expected from them, potential risks and benefits of their participation, that their participation is completely voluntary, and that they can withdraw their participation at any time. After reviewing the consent form individuals will be given as much time as needed to ask questions. At that time if individuals wish to participate they will sign two copies of the consent form and it will be countersigned by the research staff, with a copy given to the participant for their records. Included in the consent form is the contact information for local research staff and a local IRB contact for participants if they have questions in the future. Responses will be kept strictly confidential. Measures will be taken to assure the privacy, dignity, and respect of each participant. Ethical human subjects research methods will be a focus in all training of research staff, we will emphasize the importance of avoiding coercion during enrollment and protecting the privacy of participants.

Community-based recruitment: Participants from the community will be recruited through town hall meetings and community ‘walkabouts’ as described above. Meeting dates, times, and locations for enrollment and participation will be shared during these activities, and individuals who wish to enroll can volunteer to participate at these times and locations.

Clinic-based recruitment: Patients eligible for enrollment will be identified during standard intake procedures or from overnight intake logs, or in the emergency room, ward, or intensive care unit of each participating clinic or hospital by collaborating clinic staff. Employed staff at each location will identify potential participants meeting the clinical case definition of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever; or 6) diarrhea in combination with any of the previously mentioned illnesses of unknown etiology. Patients will be screened for eligibility according to the inclusion/exclusion criteria based on available clinical information and clinical presentation.

We have set a minimum target enrollment sample size to detect live virus in patients at each hospital assuming a population prevalence of 1% with a 95% probability. We will work with the local institution review board to determine the maximum enrollment of patients without undue burden on the population. However, in larger

tertiary healthcare centers where many cases fitting study inclusion are expected or are being enrolled we will regularly evaluate enrollment logs to be sure we are prepared to collect samples throughout the length of the sample collection timeline as to not miss a change in circulating virus. If we need to control the number of patients being enrolled at a hospital interval sampling will be implemented by selecting every Nth case of those individuals who meet enrollment criteria. The interval will be determined in collaboration with the local research staff and implementing partners based on an evaluation of the enrolled participants to date and expected number of cases presenting at the site within a given year in order to best meet study design and sample size criteria and stay IRB compliant. In terms of retention, we will express our gratitude to subjects for their participation and discuss the research importance of the follow-up data collection. Nonetheless, we expect to have an approximate 40% loss to follow up and have included this in our sample size calculations.

STUDY TIMELINE

At each sampling time point, patients/participants will be asked to volunteer approximately 1 hour of their time for participation in the study, including providing biological samples and completing the questionnaire.

This will be an ongoing five-year project from the time of award.

- We anticipate obtaining all required IRB approvals and local permissions in the first 6 months of projects;
- We will start human subject enrollment at community and clinical sites in Year 0.5 at the earliest, and enrollment will continue through Year 5, to be completed by the conclusion of the project;
- Human sample testing will start in Year 0.5 at the earliest, with completion of analyses by the end of the award.

2.9. Inclusion Enrollment Reports

IER ID#	Enrollment Location Type	Enrollment Location
IER 291622	Foreign	Ratchaburi and Chonburi provinces in Thailand; Peninsular Malaysia, Sabah Malaysia, and Sarawak in Malaysia

Inclusion Enrollment Report 291622

1. Inclusion Enrollment Report Title* : Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia

2. Using an Existing Dataset or Resource* : ● Yes ○ No

3. Enrollment Location Type* : ○ Domestic ● Foreign

4. Enrollment Country(ies): MYS: MALAYSIA, THA: THAILAND

5. Enrollment Location(s): Ratchaburi and Chonburi provinces in Thailand; Peninsular Malaysia, Sabah Malaysia, and Sarawak in Malaysia

6. Comments:

Planned

Racial Categories	Ethnic Categories				Total
	Not Hispanic or Latino		Hispanic or Latino		
	Female	Male	Female	Male	
American Indian/ Alaska Native	0	0	0	0	0
Asian	4150	4150	0	0	8300
Native Hawaiian or Other Pacific Islander	0	0	0	0	0
Black or African American	0	0	0	0	0
White	0	0	0	0	0
More than One Race	0	0	0	0	0
Total	4150	4150	0	0	8300

Cumulative (Actual)

Racial Categories	Ethnic Categories									Total
	Not Hispanic or Latino			Hispanic or Latino			Unknown/Not Reported Ethnicity			
	Female	Male	Unknown/ Not Reported	Female	Male	Unknown/ Not Reported	Female	Male	Unknown/ Not Reported	
American Indian/ Alaska Native	0	0	0	0	0	0	0	0	0	0
Asian	53	64	0	0	0	0	0	0	0	117
Native Hawaiian or Other Pacific Islander	0	0	0	0	0	0	0	0	0	0
Black or African American	0	0	0	0	0	0	0	0	0	0
White	0	0	0	0	0	0	0	0	0	0
More than One Race	0	0	0	0	0	0	0	0	0	0
Unknown or Not Reported	0	0	0	0	0	0	0	0	0	0
Total	53	64	0	0	0	0	0	0	0	117

Section 3 - Protection and Monitoring Plans (Study 293221)

3.1. Protection of Human Subjects	Section_3_Protection_Human_Subjects_FINAL.pdf
3.2. Is this a multi-site study that will use the same protocol to conduct non-exempt human subjects research at more than one domestic site?	● Yes ○ No ○ N/A
Single IRB plan attachment	Section_3_sIRB_plan_FINAL.pdf
3.3. Data and Safety Monitoring Plan	
3.4. Will a Data and Safety Monitoring Board be appointed for this study?	○ Yes ● No
3.5. Overall structure of the study team	

PROTECTION OF HUMAN SUBJECTS:

1. Risks to Human Subjects

1.1 Human Subjects Involvement, Characteristics, and Design

This project is a study of human spillover and exposure to animal coronaviruses, henipaviruses, and filoviruses in Southeast Asia, with active sample collection in Thailand and Malaysia and testing of archived samples in Singapore. As there is substantial evidence that these viruses likely spillover regularly to people, are often unreported or misdiagnosed and thus underestimated; and this targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence Subjects will be enrolled on a voluntary basis and informed consent will be obtained from all participants and assent from all participants aged 12-17. Consenting participants will provide biological samples for PCR or serological testing and complete a questionnaire to collect information on wildlife exposures and frequency. Subjects will be individuals: 1) who are highly exposed to wildlife, specifically bats, rodents, and non-human primates, in community settings, through hunting, butchering, or general handling within the context of their living or working environments (≥ 18 years old); and 2) patients admitted to hospitals and clinics presenting with disease symptoms of clinically-defined 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever; or 6) diarrhea in combination with any of the previously mentioned illnesses of unknown etiology.

The study population will be selected from the subnational geographic hotspot regions listed in Aim 1. We will enroll participants from: 1) communities at 4 sub-sites from each of the regions of interest in Ratchaburi (Thailand), Chonburi (Thailand), Peninsular Malaysia, Sabah Malaysia, and Sarawak, 175 individuals from each of sub-site in the 5 regions will be collected and pooled across the region for a total of 700 participants per region, allowing us to make region-level comparisons of differing effects, enrolling a total of 3,500 participants; and 2) patients from the selected 2 town-level level clinics and 2 provincial-level hospitals in each of the geographic regions of Thailand, Peninsular Malaysia, Sabah and Sarawak, we will enroll a minimum of 300 participants per clinic or hospital, for a total of 16 healthcare facilities and 4,800 total clinical participants. This will yield 2,880 participants that will be available for follow-up blood sampling assuming for an estimated 40% loss from follow-up. The community and clinical sites are further defined in Specific Aims 2 and 3.

There are no data to suggest a gender or ethnic bias for coronaviruses, henipaviruses, and filoviruses exposure or infection, therefore individuals will be enrolled based on exposure criteria alone and individuals will not be excluded based on ethnicity or gender. We will also monitor sampling enrollment to ensure equal representation of sex, demographic, and socio-economic factors in each community site.

1.2 Sources of Materials

Biological samples to be collected and tested for coronaviruses, henipaviruses, and filoviruses include whole blood, serum, and nasal/oropharyngeal swabs. Samples will be collected by locally trained medical personnel and a questionnaire will be administered by research or collaborating staff from the local hospitals and clinics.

In community sites, whole blood samples will be collected from participants one time during the data collection period during Years 2-5 of the study. The whole blood samples will be aliquoted into at least one max. 500 µL whole blood and two 500 µL serum samples. Samples will be tested for coronaviruses, henipaviruses, and filoviruses using developed ELISA by consortium partners. For participants who report the symptoms relating to 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever; or 6) diarrhea in combination with any of the previously mentioned illnesses of unknown etiology within the last 10 days, an additional sample type will be collected, nasal or oropharyngeal swabs (2x). These samples will be marked for additional PCR-based assays to identify presence of known and novel coronaviruses, henipaviruses and

filoviruses, and for isolation and biological characterization of potential pathogens if PCR results are positive.

In clinic sites, both whole blood samples and nasal/oropharyngeal swabs will be collected at enrollment, whole blood samples will be aliquoted into at least one max. 500 µL whole blood and two 500 µL serum samples. Samples will be tested for coronaviruses, henipaviruses, and filoviruses using consensus PCR (cPCR). We will follow up 35 days after enrollment to collect an additional blood sample of 5mL to be separated and aliquoted into a minimum of two 500 µL serum samples that will be serological tested with the developed ELISA assay.

All blood samples will be kept frozen for future harvesting of Peripheral Blood Mononuclear Cells (PBMCs) for those study participants whose serum tests are positive for emerging viral pathogens. These will be used for harvesting polyclonal and monoclonal antibodies as potential therapeutics.

During data collection a standardized questionnaire will be administered to both community and clinic participants. This survey will collect data on exposure type and frequency with wildlife focusing on: 1) occupation and occupational exposures; 2) observed or reported interactions with wildlife, especially bats, rodents, and non-human primates, in/around house; 3) proximity of residence or workplace to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12 months. During the follow-up with clinic participants a standardized questionnaire supplement will be administered to collect additional data on the course of symptoms in the interim period. All electronic data will be password protected, and all hardcopy files and biological samples will be stored in secure storage facilities. All consent forms and participant logs will be stored separately from research data in locked filing cabinets.

1.3 Potential Risks

The potential risks to study participants as a result of study participation are minimal. The biological specimen will be collected by locally certified healthcare professionals proficient in phlebotomy techniques, the volume of blood being collected is within normal safety limits and the swab sample is not overly invasive. The questionnaire will be designed to assess exposure risk and may ask personal questions, however, administration will be conducted privately and confidentially to protect individuals' personal health and exposure information. There may be some stress or discomfort for participants who are informed that they have been exposed to a zoonotic virus, to reduce this counseling will be available and options for future medical care will be included in the discussion with the health official or physician reporting results back to individuals.

2. Adequacy of Protection against Risks

2.1 Recruitment and Informed Consent

Potential study participants at each site will be recruited after obtaining local permissions and support from local community leaders, district health officers, and/or authorities the study team members will engage in community town hall meetings and 'walkabouts' during which they will discuss study details, dates, times, and locations for enrollment and participation in the study for individuals who wish volunteer to participate. The team will be trained on conducting ethical human subjects research before the commencement of data collection and enrollment of participants. This will include the importance of avoiding coercion during enrollment, protecting the privacy of participants, how to effectively communicate the research objectives, what is being asked of participants, any risks or benefits to participation, with sufficient support to be able to address any questions that potential participants may have. Training will also include a module on special populations for advanced training on working with minors during human subjects research. During the enrollment process interested individuals will be given a consent form in the local language and research staff will read the consent form to potential participants, via an interpreter in local dialects if necessary. Together they will review the consent form and study staff will explain details of the study including: why they were selected, what the study procedures are and what will be expected from them, potential risks and benefits of their participation, that their participation is completely voluntary, and that they can withdraw their participation at any time. After reviewing the consent form individuals will be given as much time as needed to

ask questions. At that time if individuals wish to participate they will sign two copies of the consent form and it will be countersigned by the research staff, with a copy given to the participant for their records. Contact details for local research staff member, a local IRB contact, and project PI will be provided to all subjects in the consent form to answer any future questions or requests for withdrawal participants may have.

2.2 Protection against Risks

Biological sample collection: collection of whole blood samples and nasal or oropharyngeal swab samples pose minimal risk to subjects. The potential complications associated with whole blood draw include pain and/or hematoma at the site of venipuncture. Nasal or oropharyngeal swab sample collection may cause minor irritation at the time of collection. To protect against and minimize potential complications, all biological sampling will be done by a locally trained and certified healthcare professional and/or clinic staff, and the sample collection sites will be monitored according to existing health facility protocols.

Risk factor questionnaire survey: potential risks associated with the administration of the questionnaire may be discomfort or concern providing responses related to wild animal contact or consumption if practices are taboo or prohibited by local laws. To minimize this risk, questionnaire data will be collected in a strictly confidential manner. The questionnaire will be conducted in private, ensuring that others cannot overhear participant responses and a barrier will be used or created so that no other individuals can view the participants. Depending on the location, this could be a private room, behind a building or fence, or behind a line of trees, obstructing view so that confidentiality may be maintained. The interview team will take care to pair interviewers and participants by sex to the best of their ability to increase the level of comfort of the participant and the team will ensure the privacy and confidentiality of response data. Children aged 12-17 will not be interviewed in the absence of a parent or guardian. Every effort will be made to ensure the privacy, dignity, and well-being of children and adults who participate in this study. In addition, identifying information will not be linked to responses, and data will be stored in secure, password protected files or locked secure storage facilities.

Participants may feel some stress or discomfort if informed that they have been exposed to a known or novel zoonotic virus, to reduce this counseling will be available and options for future medical care will be included in the discussion with the health official or physician reporting results back to individuals. Additionally, we will provide participating hospitals, clinicians, and community leaders with information and background data on relevant zoonotic viruses.

3. Potential Benefits to Subjects and Others

There are no measurable benefits to the individual study participants enrolled in this study. There may be secondary benefits including receiving a physical exam/health check from a medical officer at the time of enrollment or advanced non-diagnostic testing assays that add clarity medical history for clinic participants. There are also benefits to the community and regional healthcare providers understand the risk of zoonotic infections among high-risk populations. At the conclusion of the study, we will deliver an educational workshop reporting study findings that will be open to both study and non-study participants, describing the health benefits of using PPE and hand-washing during animal handling activities throughout the day, as well as to share other prevention interventions that emerge from the research data.

4. The Importance of Knowledge to be Gained

There are valuable potential benefits to the general public from the knowledge to be gained from this study. One key benefit of this study to the community an understanding the risk of zoonotic spillover events among high-risk populations. This strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an ‘early warning system’ to conduct public health interventions and disrupt disease emergence. As well as share information with communities on practices that could reduce exposure and related health risks such as the avoidance of particular animals or the need for PPE and extra care when handling wildlife may substantially reduce the risk zoonotic pathogen transmission.

Knowledge gained will also increase understanding of the conditions and human activities associated with the introduction of zoonotic infections into human populations, which may have implications for disease control more broadly.

SINGLE INSTITUIONAL REVIEW BOARD (sIRB)

In compliance with the NIH Policy on the use of a single IRB of record for multi-site research EcoHealth Alliance will prepare, submit, and work the institutional review board that follows the ethical standards set forth by the HHS regulations at 45 CFR 46. Once this single IRB is approved in the US it will functions as the IRB of record and will be relied on at all planned sites and any future sites.

We are currently anticipating working with HummingbirdIRB to serve as the IRB of record for all study sites. All of our local research partners, partner institutions, and study staff will rely on the IRB protocol that is approved at the IRB of record for all planned and future sites where data collection will occur. All data collection (biological and questionnaire) procedures and protocols and consent processes will be conducted using the same protocols outlined in the approved IRB of record and consistent for all location sites. The approved protocol at IRB of record will serve as the foundation for all locally submitted IRB packages in all partner countries, Thailand, Malaysia, and potential Singapore for inclusion of archived samples for testing.

EcoHealth Alliance will submit for IRB approval and maintain all records, and annually manage and submit for continuing review approvals at the IRB of record. Additionally, EcoHealth Alliance will manage the authorization and reliance agreements between partners and implementation the communication plan. Each partner implementing human subjects research in Thailand, Malaysia, and Singapore will maintain regular communication with scheduled updates to the EcoHealth Alliance point of contact on enrollment and recruitment numbers, breakdown of enrollment of special populations and report any adverse events within 8 hours if not sooner.

Prior to commencing study enrollment or sample testing, the partner organization that is managing human subjects enrollment in Thailand, Malaysia, and Singapore will sign a reliance agreement that will acknowledge the role of the IRB of record and responsibilities of the participating institutional partners.

Section 4 - Protocol Synopsis (Study 293221)

4.1. Study Design

4.1.a. Detailed Description

4.1.b. Primary Purpose

4.1.c. Interventions

Type	Name	Description

4.1.d. Study Phase

Is this an NIH-defined Phase III Clinical Trial? ❍ Yes ❍ No

4.1.e. Intervention Model

4.1.f. Masking ❍ Yes ❍ No

❑ Participant ❑ Care Provider ❑ Investigator ❑ Outcomes Assessor

4.1.g. Allocation

4.2. Outcome Measures

Type	Name	Time Frame	Brief Description

4.3. Statistical Design and Power

4.4. Subject Participation Duration

4.5. Will the study use an FDA-regulated intervention? ❍ Yes ❍ No

4.5.a. If yes, describe the availability of Investigational Product (IP) and Investigational New Drug (IND)/ Investigational Device Exemption (IDE) status

4.6. Is this an applicable clinical trial under FDAAA? (SEE SECTION 6.6)

4.7. Dissemination Plan

From:	Hongying Li on behalf of Hongying Li <li@ecohealthalliance.org>
To:	Supaporn Wacharapluesadee; Chris Broder; Baric, Ralph S; Baric, Toni C; Tom Hughes; Jimmy Lee; Wang Linfa; Mei-Ho Lee; Sasiprapa Ninwattana; Ananporn Jenny supataragul; Spencer Sterling; Tan Chee Wah; Opass ID; Zhu Feng; Gralinski, Lisa E; Ong Xin Mei; eric.laing_usuhs; Edwards, Caitlin E; Khwankamon Rattanatumhi
Cc:	Kevin Olival, PhD; Marc Valitutto; Cadhla Firth; Cecilia Sanchez; Alix Villaneuva; Aleksei Chmura; Alison Andre; Peter Daszak
Subject:	EID-SEARCH NoA and Year 3 Report as submitted
Date:	Thursday, April 13, 2023 9:42:25 AM
Attachments:	NIH_NOA_1U01AI151797-03 with highlights.pdf 5U01AI151797 Y3 Annual Report As Submitted_without budget.pdf

Dear All,

Thank you for the call yesterday!

As discussed, I am sharing the Year 3 Notice of Award (NoA) of the EID-SEARCH project and the Year 3 Research Performance Progress Report (RPPR) report as submitted.

The NoA is issued annually, and this Y3 NoA has been attached to the Y3 contracts we signed. You can see these special requirements as highlighted on Pages 6-7.

In addition to the annual RPPR report (dues April 1 every year), we are also required to submit a semi-annual report. The semi-annual report is less structured compared to the RPPR, focusing on research activities mainly without needing to fill in different sections.

In the attached RPPR report, Page 27 G.1 Section is where we report about the special reporting requirements as described in the NoA.

Please feel free to let Peter, Aleksei, and me know if you have any questions about these documents. And thank you very much for your understanding to help the project comply with these requirements.

Sincerely,
Hongying

Hongying Li, MPH
Senior Program Manager & Senior Research Scientist

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On Tue, Apr 11, 2023 at 3:07 PM Peter Daszak <daszak@ecohealthalliance.org> wrote:

> Dear All,

Reminder of our EID-SEARCH all partner meeting coming up tomorrow (Wednesday night US time, Thursday morning SE Asian time).

We're going to be doing these every month, with the key goals of 1) identifying important issues from EID-SEARCH HQ, e.g. requests from NIAID etc.; 2) to help each partner to find out what the other partners are doing; 3) brainstorm solutions to problems, and ways to increase efficiency of sampling, testing, characterization, surveys and paper publishing.

At this first meeting, I'll start off with a quick round up of overall EID-SEARCH updates, then we'll just go round the table and get an update from each partner.

Please make sure that at least one or two people from your team will be joining, and get ready to be put on the spot to give a quick update…

Look forward to talking and to having these regularly!

Cheers,

Peter

Peter Daszak

President

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USA

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Website: www.ecohealthalliance.org

Twitter: @PeterDaszak

EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation

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From: Peter Daszak <daszak@ecohealthalliance.org>
Sent: Tuesday, March 21, 2023 11:58 AM
To: 'Supaporn Wacharapluesadee' <spwa@hotmail.com>; 'Tom Hughes' <tom@conservationmedicine.org>; 'Chris Broder' <cbroder@usuhs.mil>; 'Wang Linfa' <linfa.wang@duke-nus.edu.sg>; 'rbaric@email.unc.edu' <rbaric@email.unc.edu>
Cc: Mei-Ho Lee <mei-ho@conservationmedicine.org>; Jimmy Lee <jimmy@conservationmedicine.org>; Sasiprapa Ninwattana <sasiprapa.n@outlook.com>; Ananporn Jenny supataragul <ananporn.su@gmail.com>; Khwankamon Rattanatumhi <khwankamon.r@gmail.com>; Opass ID <opassid@gmail.com>; eric.laing_usuhs <eric.laing@usuhs.edu>; Spencer Sterling <spencer.sterling.ctr@usuhs.edu>; 'siana.coggins.ctr@usuhs.edu' <siana.coggins.ctr@usuhs.edu>; 'lianying.yan.ctr@usuhs.edu' <lianying.yan.ctr@usuhs.edu>; 'Tan Chee Wah' <cheewah.tan@duke-nus.edu.sg>; Zhu Feng <feng.zhu@duke-nus.edu.sg>; Ong Xin Mei <xinmei_ong@duke-nus.edu.sg>; Gralinski, Lisa E <lgralins@email.unc.edu>; Edwards, Caitlin E <caitedw@unc.edu>; 'Baric, Toni C' <antoinette_baric@med.unc.edu>; Hongying Li <li@ecohealthalliance.org>; 'Kevin Olival, PhD (olival@ecohealthalliance.org)' <olival@ecohealthalliance.org>; Marc Valitutto <valitutto@ecohealthalliance.org>; 'Cadhla Firth' <firth@ecohealthalliance.org>; Cecilia Sanchez <sanchez@ecohealthalliance.org>; Alix Villaneuva <armero@ecohealthalliance.org>; 'Aleksei Chmura (chmura@ecohealthalliance.org)' <chmura@ecohealthalliance.org>; 'Alison Andre (andre@ecohealthalliance.org)' <andre@ecohealthalliance.org>
Subject: EID-SEARCH All-partner calls every month, starting April 12th (April 13th in

Asia)
Importance: High

Hello EID-SEARCH team!

Now that we're near the end of Year 3, and with work really cranking up now in our project, and multiple groups doing all forms of testing and analyses, I'm canceling the 'lab meetings' and setting up a monthly ALL-PARTNER meeting. The first will be on April 12th at 8:30pm Eastern time which is April 13th in the morning in Asia. These will be on the second Wednesday/Thursday of each month and you will receive an automatic calendar invite with Zoom information from Alison Andre.

It's very important for each partner to have staff on this call every month. The goal of the meeting is to keep everyone updated on news from NIAID and EcoHealth Alliance re. the project, get updates from each of the partners and brainstorm together to maximize our outputs (science, papers, talks, outreach) towards the grant renewal. So please take one hour per month to join the meeting, and feel free to send me and Hongying any discussion items you have.

I won't be sending out agendas, these meetings are intended to be updates from each of us so that all partners can hear what we're all up to.

Also, please note that this all-partner meeting is <u>in addition</u> to the monthly calls we have with each of the partners individually.

Look forward to seeing you on the 12th…

Cheers,

Peter

Peter Daszak

President

EcoHealth Alliance

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USA

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Twitter: @PeterDaszak

EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation

Christopher Broder, Ph.D., Site Investigator
Eric Laing, Ph.D., Co-Investigator
Spencer Sterling, MS, Scientific Project Coordinator
Uniformed Services University of the Health Sciences
The Henry M. Jackson Foundation

Reporting Period: **06/01/2022 – 05/31/2023**

B.2 What was accomplished under these goals (by Aims)? *required

Aim 1: Identify, characterize, and rank spillover risk of high zoonotic potential viruses from wildlife.

In Year 3, we provided henipavirus, filovirus, and coronavirus reagents for testing 92 rodent, 100 non-human primate (NHP), and 700 bat serum samples in Thailand and coronavirus reagents for testing over 3,000 bat serum samples in Malaysia.

In Thailand, there was no seroprevalence of henipavirus, filovirus, or coronavirus antibodies in the rodents that were tested. One NHP (1/100, 1%) had detectable antibodies against the MERS-CoV spike glycoprotein, and three additional NHPs (3/100, 3%) had elevated (but not positive) levels of MERS-CoV reactive antibodies. Additionally, one NHP (1/100, 1%) had positive levels of antibodies reactive with the Mojiang (MojV) receptor binding protein (RBP) and six additional NHPs (6/100, 6%) had elevated (but not positive) levels of anti-MojV antibodies. There were no filovirus antibodies detected within this cohort of NHPs. The proposed bat samples have not been tested during this study period due to troubleshooting optimization of the binding and neutralization assays for the coronavirus proteins.

Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays.

The testing of samples from humans was prioritized in Thailand. We supplied reagents for the serological testing of 457 community samples from 288 individual participants, including 84 samples from a bat guano collection operation, and 100 samples from a human cohort without wildlife contact residing within Bangkok against protein antigens from henipavirus, filovirus, and wildlife and human-associated coronaviruses. We identified a 99.62% (264/265) seroprevalence for HCoV-OC43 in pre- and post-COVID-19 samples. Interestingly, there was minimal wildlife-associated coronavirus seroprevalence in samples collected prior to the COVID-19 pandemic. We observed in this SARS-CoV-2 seroprevalence after the start of the COVID-19 pandemic, likely induced by SARS-CoV-2 infections and COVID-19 vaccinations. Within the community surveillance cohort, we detected antibodies to both filoviruses (18.91%, 45/238) and henipaviruses (26.89%, 64/238), including a high seroprevalence to the MojV RBP (21.85%, 52/238). These results suggest there is infection by novel MojV-like viruses within this population.

Aim 3: Identify and characterize viral etiology of ‘cryptic’ outbreaks in clinical cohorts.

Network Collaboration

Our group at USU has been setting up a material transfer agreement with WAC-EID via UTMB investigator, Dr. Robert Cross. Materials and protocols for henipavirus and filovirus serological testing will be transferred to Dr. Cross and assays will be established at partner labs within WAC-EID.

We also continue to develop a multiplex serology test for MPOX and are awaiting a transfer of convalescent sera from the CDC. In Year 4 we aim to further qualify and optimize a multiplex serology

test, and cross-verify assay performance with colleagues at the Applied Diagnostics Branch at USAMRIID.

Outbreak Research and Response Activities

	Thailand	Malaysia
Wildlife		
# Samples	3,862	29,099
# Individuals	454 bats, 106 rodents, 100 NHPs	3,648 bats, 6 carnivores, 13 NHPs, 9 elephants
# Species	6 (bats), 5 (rodents) 1 (NHP)	37 (bats), 1 (carnivore), 2 (NHPs), 1 (elephant)
# Sites	5	13
# Individuals tested	506	2,732
# PCR tests (CoVs, PMVs, FLVs)	5,063	18,218
Divergent viral PCR sequences (RdRp) detected[3]	3 novel CoVs from 11 bats (in 1 species) 3 known PMVs from 4 rodents (in 2 species) 4 novel PMVs from 11 bats (in 1 species)	5 novel CoVs from 112 bats (in 3 species) 4 novel PMVs from 10 bats (in 2 species) 2 known PMVs from 2 bats (in 1 species)
# Specimens for MMIA testing (FLVs, CoV, Henipaviruses)	100 (macaques), 1 CoV positive, 1 HNV positive 92 (rodents), 0 positive	1,225 (bats)
Human		
# Enrollment	93 enrollment	
# Samples		
# Sites		
# PCR tests (for CoVs, PMVs, FLVs)		
# PCR positive (for CoVs, PMVs, FLVs, respectively)		
# MMIA tests (FLVs, CoV, Henipaviruses)	357 samples in duplicate	
# Sero-positrive (for FLVs, CoV, Henipaviruses, respectively)	36 FLV positive, 77 HNV positive	

[2]Specimens include oral swabs, rectal swabs, urogenital swabs, and whole blood, types of specimens may vary among individual animals.

B.4 What opportunities for training and professional development has the project provided? *required

For each relevant activity, please include information about the topic, time (month/month), number of attendees (gender if available), organizations, if possible.

In July 2022, Mr. Spencer Sterling (USU) provided instructional training on the serologic techniques used to detect binding antibodies. Participants of the training included Dr. Krongkan Srimuang (KCMH-EIDcc) and Ms. Sasiprapa Ninwattana (KCMH-EIDcc). Included in the training were the topics of antigen design, assay techniques and testing strategies, and preliminary data analysis.

Christopher Broder, Ph.D., Site Investigator
Eric Laing, Ph.D., Co-Investigator
Spencer Sterling, MS, Scientific Project Coordinator
Uniformed Services University of the Health Sciences
The Henry M. Jackson Foundation

From October 20-21, 2022, Mr. Sterling participated in the "Technical Workshop – Bat Surveillance in the Mekong Region" hosted by King Chulalongkorn Memorial Hospital and the World Health Organization in Bangkok, Thailand. Mr. Sterling presented research work and participated as a rapporteur.

From November 7-10, 2022, Mr. Sterling attended the World One Health Congress in Singapore. He presented a poster and participated in project meetings.

On November 15, 2022, Dr. Laing was invited to present a seminar, "*Antibody durability, vaccine-related symptoms, and omicron neutralization after BNT162b2 mRNA COVID-19 vaccination in the Prospective Assessment of SARS-CoV-2 Seroconversion (PASS) Study*" to the Department of Medicine, Chulalongkorn University, Bangkok, Thailand.

On January 24, 2023, Mr. Sterling participated as a rapporteur in the Prince Mahidol Award Ceremony – School of Global Health Side Meeting on "Addressing Ecosystem Change and Pandemic Emergence through Strengthening One Health Implementation" in Bangkok, Thailand.

On March 23, 2023, Mr. Sterling presented "Next Generation Serology for Pathogen Discovery: Multiplex Immunoassays (MIA)" at the Thai Red Cross Emerging Infectious Diseases Clinical Center EID Conference 2023 in Bangkok, Thailand

B.5 How have the results been disseminated to communities of interest ? *required

- Government and inter-government agency briefings
- Panels, workshop, webinars
- Conference and university lectures
- Public outreach

B.6 What do you plan to do during the next reporting period to accomplish the goals (by Aims)? *required

Aim 1: Identify, characterize, and rank spillover risk of high zoonotic potential viruses from wildlife.

Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays.

Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts.

C.1 Publications *required

None

C.2 Website(s) or other Internet site(s)
None

C.3 Technologies or techniques *required to check and confirm
Identify technologies or techniques that have resulted from the research activities. Describe the technologies or techniques and how they are being shared. Please select the appropriate category(ies) from the following list and provide a brief description. If the product(s) has been reported or shared through a publication, please include the full reference and/or PubMed ID in the product description. Limit the response to this reporting period. **None**

C.4 Inventions, patent applications, and/or licenses *required to confirm
- Have inventions, patent applications and/or licenses resulted from the award during this reporting period? **None**
- If yes, has this information been previously provided to the PHS or to the official responsible for patent matters at the grantee organization? **N/A**

D. Participants
If you would like to add any key/senior personnel to this project, please send us this person's
None

E.2 What is the impact on physical, institutional, or information resources that form infrastructure?
No

F.2 Actual or anticipated challenges or delays and actions or plans to resolve them
No

F.3.d Select Agents *required to confirm
If the possession, use, or transfer of Select Agents is or will be different from that proposed in the previous submission, including any change in the select agent research location and/or the required level of biocontainment, provide a description and explanation of the differences. If the use of Select Agents was proposed in the previous submission but has not been approved by regulatory authorities, provide an explanation. If studies involving Select Agents are planned and were not part of the originally proposed research design, provide a description of the proposed use, possession, transfer, and research location as described in the competing application instructions.

U.S. Select Agent Registry information: http://www.selectagents.gov/SelectAgentsandToxins.html
No Change

Human Subjects *required
No Change

From: Tamera Porter Wilmot on behalf of Tamera Porter Wilmot <TPorterWilmot@hjf.org>
To: Hongying Li
Cc: Yongkang Qiu; Kimberly Boxley; Christopher Broder; Laing, Eric; Spencer Sterling
Subject: RE: [EXTERNAL] NIH EID-SEARCH Year 3 Progress Report_by March 24, 2023
Date: Friday, March 24, 2023 3:31:29 PM
Attachments: NIH_EID-SEARCH Y3 Annual Report.pdf
image001.png

Good Afternoon,

Please find the annual progress report attached for the project titled: "Understanding Risk of Zoonotic Virus Emergence in Eid Hotspots of Southeast Asia"
Please let us know if you have any questions.
Thank you,
Tammy

Tamera Porter Wilmot - *Currently teleworking*
Grants Specialist - Office of Sponsored Programs
Research Administration and Innovation Management (RAIM)
Phone: 240-694-2008
Email: tporterwilmot@hjf.org

Henry M. Jackson Foundation for the
Advancement of Military Medicine
6720A Rockledge Drive, Suite 100
Bethesda, MD 20817

From: Hongying Li <li@ecohealthalliance.org>
Sent: Wednesday, March 1, 2023 7:41 PM
To: Tom Hughes <tom@conservationmedicine.org>; Supaporn Wacharapluesadee <spwa@hotmail.com>; wang linfa <linfa.wang@duke-nus.edu.sg>; Tan Chee Wah <cheewah.tan@duke-nus.edu.sg>; Ong Xin Mei <xinmei_ong@duke-nus.edu.sg>; eric.laing_usuhs <eric.laing@usuhs.edu>; Spencer Sterling <spencer.sterling.ctr@usuhs.edu>; Broder, Christopher <christopher.broder@usuhs.edu>; Baric, Ralph S <rbaric@email.unc.edu>; Gralinski, Lisa E <lgralins@email.unc.edu>; Marc Valitutto <valitutto@ecohealthalliance.org>; Cadhla Firth <firth@ecohealthalliance.org>; Cecilia Sanchez <sanchez@ecohealthalliance.org>; Sasiprapa Ninwattana <sasiprapa.n@outlook.com>
Cc: Peter Daszak <daszak@ecohealthalliance.org>; Kevin Olival <olival@ecohealthalliance.org>; Siyeun Kim <kim@ecohealthalliance.org>; Samantha Lee <samantha.lee@duke-nus.edu.sg>; Baric, Toni C <antoinette_baric@med.unc.edu>; Joanne Pang <joannepang@duke-nus.edu.sg>; Loh Jian Yun <jianyun.loh@duke-nus.edu.sg>; Mei-Ho Lee <mei-ho@conservationmedicine.org>; Jimmy Lee <jimmy@conservationmedicine.org>; Luke Hamel <hamel@ecohealthalliance.org>; Alison Andre

<andre@ecohealthalliance.org>; Aleksei Chmura <chmura@ecohealthalliance.org>; Vanessa Chuo Kher Sing <vanessa.chuo@duke-nus.edu.sg>; McGlaughon, Ben <benmcg@email.unc.edu>; Garcia, Martha <garcim22@unc.edu>; Williams, Colleen Anne <colleen_williams@unc.edu>; Yongkang Qiu <yqiu@hjf.org>; Kimberly Boxley <kimberly.boxley.ctr@usuhs.edu>
Subject: [EXTERNAL] NIH EID-SEARCH Year 3 Progress Report_by March 24, 2023

Dear EID-SEARCH Members,

The NIH Research Performance Progress Report (reporting period **06/01/2022-05/31/2023**) for EID-SEARCH (U01AI151797) is due on **April 1, 2023**.

I've attached a template with the different sections we would like to get updates from you as part of the report. All relevant work for any section is welcomed (the more detailed, the better, don't worry about the languages or word limits); I also labeled some sections as **required* where are the most important to concentrate.

And as part of the CREID Network, we also need to highlight the cross-Research Center activities, which are now KPIs to evaluate each Research Center's performance by the funder. So if you have anything relevant to report, please provide information on the Word document and fill in the *Excel file* I've attached.

The information required for this report is majorly focused on the research findings and plans and very minimal on administration/finance unless you have any significant changes on project key/senior personnel or budget.

We would greatly appreciate it if you could send us updates by **Friday, March 24, 2023,** so we can have a few days to consolidate everything to submit.

Please do not hesitate to let me know if you have any questions. Thank you very much!

Sincerely,
Hongying

Hongying Li, MPH
Senior Program Manager & Senior Research Scientist

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EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.

Co-circulation dynamics of henipaviruses, filoviruses and rubulaviruses in a bat population

Noam Ross[1,*], Ariful Islam[1], Sarah Hayes[2], A. Marm Kilpatrick[3], Kevin J. Olival[1], Emily S Gurley[4], M. Jahangir Hossain[6,11], Hume. E. Field[2], Gary Crameri[7], Lin-Fa Wang[7,8], Stephen P. Luby[9] Christopher C. Broder[10], Peter Daszak[1] and Jonathan H. Epstein[1]

1. EcoHealth Alliance, New York, New York, USA
2. Imperial College, London, UK
3. Department of Ecology and Evolutionary Biology, University of California, Santa Cruz, California, USA
4. Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland, USA
5. [check Jahangir affiliation]
6. International Centre for Diarrheal Diseases Research, Bangladesh, Dhaka, Bangladesh
7. CSIRO Australian Animal Health Laboratory, Geelong, VIC, Australia
8. Programme in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore
9. Division of Infectious Diseases and Geographic Medicine, Stanford University, Stanford California, USA
10. Department of Microbiology and Immunology, Uniformed Services University, Bethesda, Maryland, USA
11. Medical Research Council Unit The Gambia at the London School of Hygiene & Tropical Medicine, Banjul, The Gambia

* Correspondence: N.R.: ross@ecohealthalliance.org; J.H.E.: epstein@ecohealthalliance.org

Abstract: Bats are hosts for a variety of viruses, some with potential to cause human disease. *Pteropus medius* are fruit bats that live in close association with humans in Bangladesh and are a known source of Nipah virus and other viruses of unknown pathogenic risk. Here we report on dynamics of seroprevalence against multiple viruses in a population of *P. medius* in Bangladesh over five years. We found evidence of regular circulation of Nipah virus, a rubulavirus, and a filovirus. Nipah virus circulated primarily among adult bats, with no seasonal patterns. The rubulavirus exhibited annual cycles in juveniles and near universal seroprevalence in adults. The filovirus exhibited annual cycles and antibody waning in juveniles and little circulation but lower seroprevalence amongst adults. We found no evidence for spatial differences in Nipah virus dynamics, but weak evidence for differences with other viruses. Host-viral interactions result in very different viral circulation patterns in the same individual bats and environments.

Keywords: bats; Nipah virus; filovirus; Rubulavirus; *Pteropus medius*; Bangladesh; serology; disease dynamics; generalized additive models

Introduction

Bats have been associated with a large diversity of viruses, several of which have been linked to human epidemics [1-3]. The discovery of Severe Acute Respiratory Syndrome (SARS)-related coronaviruses (CoVs) in Chinese horseshoe bats (*Rhinolophus spp.*) in 2004 and subsequent discoveries of related viruses[4,5] including close relatives of SARS-CoV-2[6], along with studies of other high consequence pathogens such as Ebola virus, Marburg virus, Nipah virus, Hendra virus have led to questions about whether and why bats were disproportionately represented among mammals as reservoirs for zoonotic viruses[1,2,7].

Bat species can carry diverse viruses which circulate simultaneously within single populations [8-11]. *Rousettus aegyptiacus* is a natural reservoir of Marburg virus, and IgG antibodies against Ebola Zaire virus have also been detected in this bat in Central Africa, suggesting that it may play a role in

Summary of Comments on Email 5 - Attachment 1 - Ross-et-al_bangladesh-bats-cocirculation-serology_2022-08-11_LW_HF_sl_eg_JH (002).pdf

Page: 1

Number: 1 Author: Emily Gurley Date: 9/9/2022 7:21:00 PM
Surprised not to see other icddr,b co-authors from the first paper - Rajib? Salah Uddin?

Number: 2 Author: Hume Field Date: 8/24/2022 9:20:00 AM
Pls add my UQ affiliation Noam.
'School of Veterinary Science, The University of Queensland, Gatton 4343 Australia'

Number: 3 Author: Wang Linfa Date: 8/21/2022 10:57:00 AM
It's better to include my CSIRO affiliation here as the work started when I was still associated with AAHL

Number: 4 Author: Noam Ross Date: 8/10/2022 12:38:00 PM
Please check that your affiliation is as it should be!

Number: 5 Author: Emily Gurley Date: 9/9/2022 7:25:00 PM
Consider providing years of the study

Number: 6 Author: Emily Gurley Date: 9/9/2022 7:23:00 PM
Or, viral families?

Number: 7 Author: Emily Gurley Date: 9/9/2022 7:24:00 PM
Evidence that this is just one virus?

Number: 8 Author: Steve Luby Date: 9/2/2022 9:33:00 AM
This seems an odd sentence to close with. The last sentence is usually a conclusion, but I can't see how the data presented in the abstract supports this conclusion.

Number: 9 Author: Steve Luby Date: 9/2/2022 9:36:00 AM
True, but since the new data presented here don't address this issue, I don't see this as central to the introduction, i.e. Error 3.12

the circulation of multiple filoviruses[12,13]. In addition to these filoviruses, a novel zoonotic and pathogenic paramyxovirus, Sosuga virus, was also found in Ugandan *R. aegyptiacus* populations[14]. *Eidolon helvum*, another pteropodid bat with a broad geographic range in Africa, has been found to carry a lyssavirus, a henipavirus, and several other viruses of unknown pathogenicity[15]. Various surveillance efforts have found diverse viruses from with the same viral family in various bat species[5,16,17], and other studies have used metagenomic approaches to look broadly at viral diversity within individual bat species[9,11,18-21].

Studies of viral diversity in a single taxonomic host group can inform evolutionary history of viruses and their relationships to specific hosts[6,22] and inform public health strategies[23]. However, detection of a zoonotic virus in an animal host is insufficient to determine whether the associated host species is a natural reservoir for the virus, or to characterize the demographic, physiological, or abiotic factors that may influence the timing of viral shedding and therefore risk of spillover to other species, including domestic animals and humans[24]. For these, more detailed demographic and longitudinal studies are required, especially in cases of multiple viruses, as direct or indirect interactions between multiple pathogens may affect disease presence, prevalence, and dynamics[25-27]. Some longitudinal studies of zoonotic viruses in bats have provided insight into the timing of viral infection and shedding, generally of single viruses. Nipah virus in *Pteropus lylei* in Thailand has been observed to have annual shedding patterns[28], whereas Hendra virus, which is closely related to Nipah virus and is carried by multiple pteropid bat species in Australia, has asynchronous and non-periodic cycles which appear to be influenced by localized factors such as specific bat species abundance and climatic factors[29]. Bi-annual pulses of Marburg virus shedding were observed in Uganda, coinciding with synchronous birth pulses in *R. aegyptiacus*[30]. Serological data is often valuable in understanding disease dynamics and transmission[31-33], especially in cases where direct detection and incidence rates of viruses are low[34].

Pteropus sp. are reservoirs of henipaviruses, a group of mostly zoonotic, lethal neurotropic viruses that include Nipah, Hendra, Cedar, Ghana and Mojiang virus[35-37]. In Bangladesh and India, *P. medius* is a reservoir of Nipah virus, which has spilled over to human populations repeatedly[38]. Viruses from eight other viral families have been detected in *P. medius* in Bangladesh[9]. The epidemiology of these other viruses is far less characterized, and little is known about their interactions or zoonotic potential.

Here, we report on the dynamics of seroprevalence against three types of viruses - Nipah virus, a filovirus, and a rubulavirus - circulating simultaneously in *P. medius* populations in three sites in Bangladesh. We conducted a longitudinal study over a period of five years, and two shorter, one-year studies for comparative seasonal data. These studies enable us to develop a detailed picture of the seasonal and demographic serological patterns, and infer the extent and periodicity of viral circulation. We find that Nipah virus circulates primarily among adults without distinct seasonality, while the rubulavirus exhibits strong annual dynamics characteristic of juvenile epidemics driven by waning maternal antibodies. The filovirus, the first detected in *P. medius* in Bangladesh, also circulated in juveniles but exhibited distinct patterns antibody waning and limited maternal inheritance.

2. Results

Bat Dynamics and Demographics

We conducted a five-year longitudinal study sampling *P. medius* bats quarterly from a roost complex in the vicinity of Faridpur, Bangladesh, and also two parallel one-year studies sampling bats monthly from roosts in Chakoria and Ramnagar of Faridpur district, Bangladesh. In the five-year study, we sampled and tested serology of 1,886 bats: 1,224 adults, 623 free-flying juveniles, and 39 weaning juveniles (captured attached to adult females) over 19 sampling events (Figure 1). During the-one-year studies, 919 bats were sampled: 435 in Chakoria (251 adults, 144 free-flying juveniles and 40 weaning juveniles) and 484 in Ramnagar (277 adults, 164 free-flying juveniles and 43 weaning juveniles (Figure S1). Nearly all juveniles (as determined by examination of maturation of sex organs)

Page: 2

Number: 1 Author: Emily Gurley Date: 9/9/2022 7:28:00 PM
Sentence seems much broader than public health concerns

Number: 2 Author: Emily Gurley Date: 9/9/2022 7:29:00 PM
Meaning unclear - are there any species that don't host multiple viruses?

Number: 3 Author: Emily Gurley Date: 9/9/2022 7:31:00 PM
Suggest adding in what we know about seasonal shedding of Nipah already

Number: 4 Author: Emily Gurley Date: 9/9/2022 7:30:00 PM
And perhaps not Mojiang

Number: 5 Author: Steve Luby Date: 9/2/2022 9:41:00 AM
I don't believe this adjective applies to Cedar virus.

Number: 6 Author: Hume Field Date: 8/24/2022 9:29:00 AM
Don’t think this should be here.

Number: 7 Author: Wang Linfa Date: 8/21/2022 10:37:00 AM
To be consistent with other section headings in Results

Number: 8 Author: Jahangir Hossain Date: 9/10/2022 12:28:00 PM
Give time period in years as Emily indicated belwo

Number: 9 Author: Emily Gurley Date: 9/9/2022 7:35:00 PM
Some indication of the frequency of sampling would also be useful to add here

Number: 10 Author: Emily Gurley Date: 9/9/2022 7:33:00 PM
Please include the years of data collection

Number: 11 Author: Jahangir Hossain Date: 9/10/2022 12:27:00 PM
I think both Chakhoria and Ramnagar are in Faridpur district

Number: 12 Author: Steve Luby Date: 9/2/2022 10:29:00 AM
it would be helpful to clarify how far apart these places are, as you have a considerable section exploring spatial comparisons.

were 14 months old or less and could be assigned to a birth cohort based on size. Pregnant and juvenile bats were captured more frequently during the late spring and summer months. Mother-pup pairs were all captured during April and May in the five-year study whilst in the one-year-studies a small number were also captured in June and July. Pregnant females were captured between November and April/May. Lactating females were found between April and July.

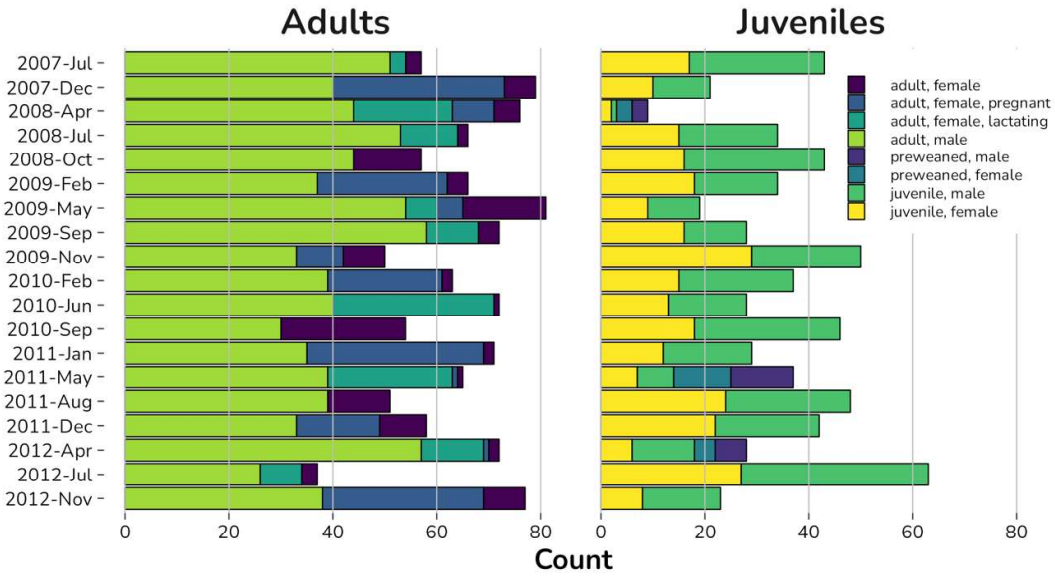

Figure 1. Demographics of bats captured in longitudinal sampling. Pregnant females were captured in months from November to June, lactating females were captured in months from April to July. Females with pre-weaning juveniles attached were found from April to May.

Patterns of Immunity and Co-immunity

We found bats were seropositive for antibodies against the henipavirus (HenipVsero+), a filovirus (EbVsero+), and a rubulavirus (MenVsero+). Among adult bats, antibodies against the rubulavirus were common; 1,314 of 1752 adult bats were MenVsero+. Antibodies against Nipah virus or filovirus were less common – 1,031 were NiVsero+ and 921 were EbVsero+. Among the 921 juveniles, 192 were MenVsero+, 311 were NiVsero+ and 348 were EbVsero+. Of the 122 pre-weaned juveniles, 43 were MenVsero+, 52 were NiVsero+, and 32 were EbVsero+.

Co-exposure to multiple viruses was common (Figure 2). Over half (1,173) of the 1752 adults had antibodies against more than one of the three viruses, and 493 had antibodies against all three. Among the 921 juveniles, 210 had antibodies against more than one, and 39 had antibodies all three. 38 of 122 pre-weaned juveniles had more than one of the three antibodies, with seven having all three.

We found correlations between serostatus between all three pairs of viruses in a model accounting for bat age, sex, and study location, with all viruses tending to co-occur with each other more than would be expected than if they were distributed independently among bats. Nipah virus and filovirus antibodies had a standardized covariance of 0.13 (95% posterior interval 0.08-0.20). Nipah virus and rubulavirus antibodies were also more likely to occur together, with a covariance of 0.15 (0.09-0.21). Filovirus and rubulavirus antibodies had a covariance of 0.23 (0.17-0.29).

Page: 3

Number: 1 Author: Wang Linfa Date: 8/21/2022 10:48:00 AM

I think it is better to treat the three group of viruses equally as you have done for the title. It makes no difference whether we use NiV or Henipavirus in the context of the current study. Luminex-based assays targeting binding antibodies will in theory detect cross-RX antibodies in all three cases. See also comments in Methods

Number: 2 Author: Steve Luby Date: 9/2/2022 10:01:00 AM

Include percentages for all of these comparisons.

Number: 3 Author: Emily Gurley Date: 9/9/2022 7:36:00 PM

Agree with Linfa - unclear how many viruses, better to treat as groups

Number: 4 Author: Steve Luby Date: 9/2/2022 10:03:00 AM

Again, including percentages for all of these comparisons would make this much easier to understand.

Number: 5 Author: Emily Gurley Date: 9/9/2022 7:37:00 PM

Some kind of general cross-reactivity, non-specificity of the assay?

Number: 6 Author: Steve Luby Date: 9/2/2022 10:03:00 AM

I did not see this described in the methods section.

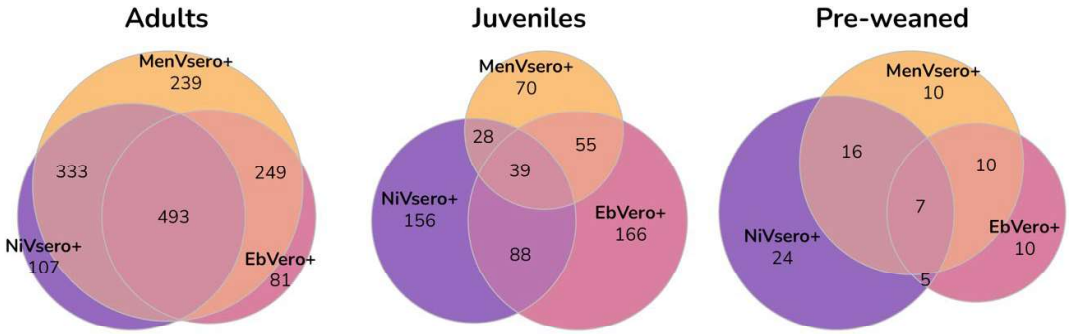

Figure 2. Detection of IgG antibodies against multiple viruses in individual *Pteropus medius*: Venn diagrams for adult, juvenile, and pre-weaned bats testing positive for antibodies against Nipah virus, filovirus, and Rubulavirus. Numbers under labels are counts of bats with only those viruses, numbers in overlapping areas represent number of bats detected with multiple viruses.

Serodynamics

Dynamics of population seroprevalence were different across the viral types. In adults, population seroprevalence against Nipah virus decreased over the study period from 78% (66%-88%) to 50% (40-62%) (Figure 3). Three periods of distinct increase in seroprevalence stood out in the inter-annual signal: early 2008, early 2009, and the second half of 2010. Nipah seroprevalence showed very weak seasonal patterns (Figure 4). (Nipah dynamics were previously reported in Epstein, et al. [34]).

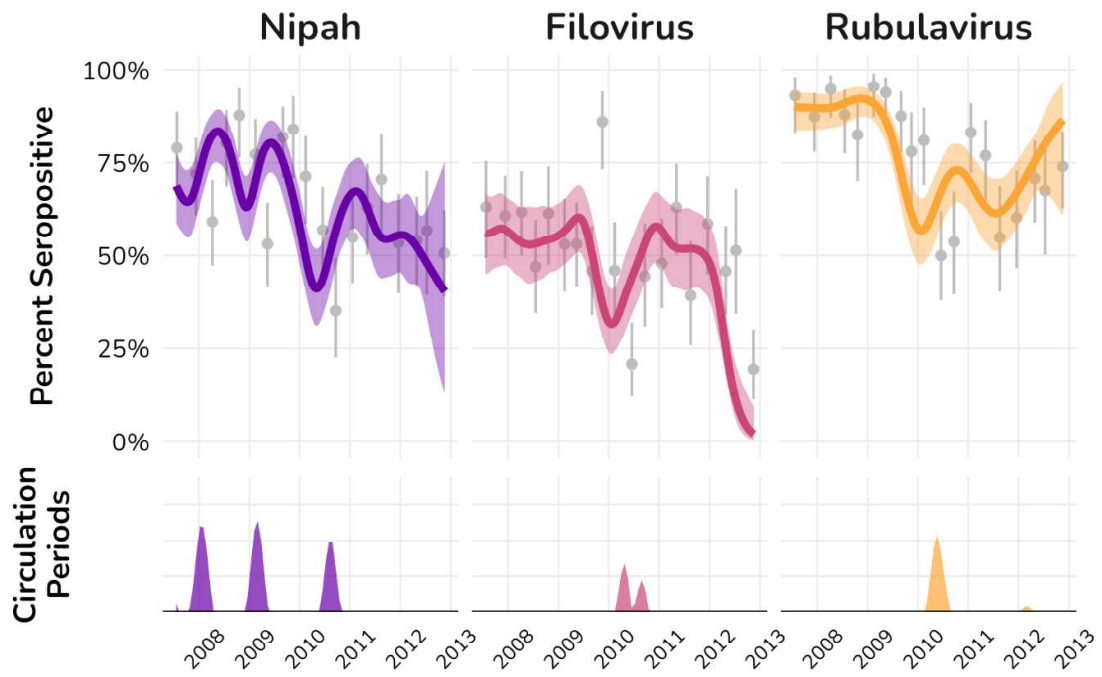

Figure 3. Inter-annual serodynamics in adult bats. Top: Measured and modeled population seroprevalence in adults over the term of the study. X-axis ticks mark the first day of the year. Grey points and bars represent measured population seroprevalence from individual sampling events on and 95% exact binomial confidence intervals. Thick lines and shaded areas represent the inter-annual (non-seasonal) spline component of a generalized additive mixed model (GAMM) of serodynamics and 95% confidence intervals for the mean pattern over time. Bottom: Circulation periods, when modeled slopes of the GAMM splines are positive for >95% of 1000 draws from the model posterior.

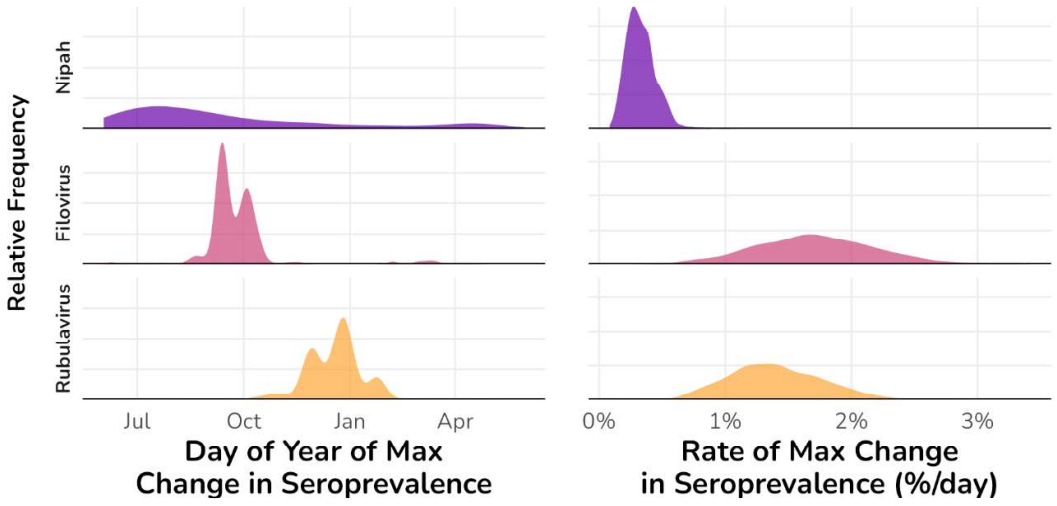

Figure 4. Timing and strength of seasonality of viral circulation in adults. Plots show posterior densities of the date for which increase in seroprevalence is greatest (left), and the rate of increase (right), for adult bats, drawn from GAMM models of seasonality. Densities are of these quantities derived from 1000 samples of model posteriors.

Among juveniles, we examined the dynamics of each cohort over their first year. For Nipah virus, the average cohort had 44% (32%-58%) seroprevalence in June, which consistently decreased over the course of the year to 20% (10%-36%) (Fig. 5). Year-to-year dynamics varied little, with overall declining seroprevalence in all years. No part of the year exhibited regular increases in seroprevalence for juveniles; the maximum rate of increase was indistinguishable from zero (Fig. 6).

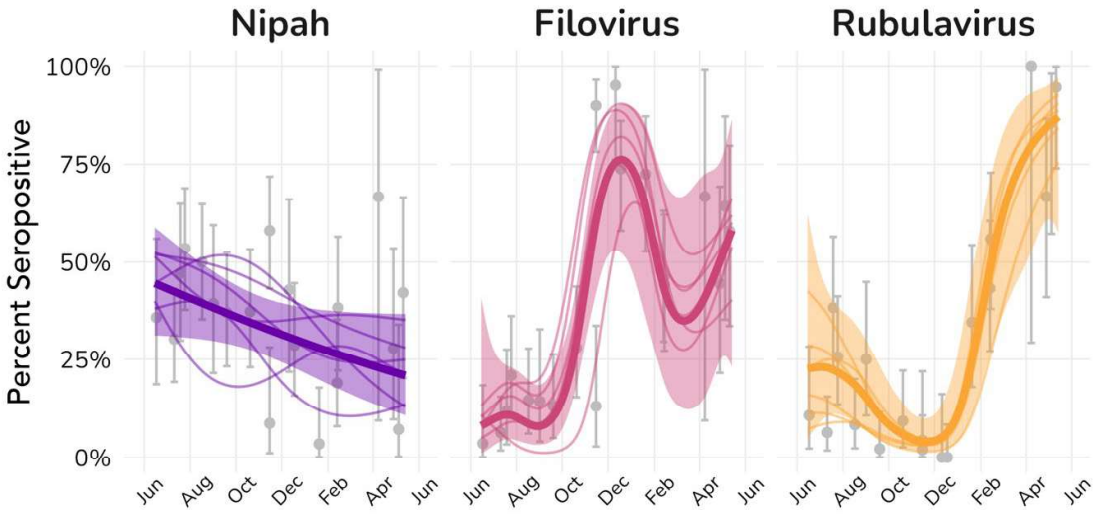

Figure 5. Serodynamics in juvenile bats in five-year study. Measured and modeled population seroprevalence in juvenile bats caught over their first year of life. Sampling events from multiple years are transposed onto a single year starting June (the earliest month free-flying young-of-the-year juveniles were captured). X-axis ticks mark the first day of each month. Grey points and bars represent measured population seroprevalence from individual sampling events on and 95% exact binomial confidence intervals. Thick lines and shaded areas represent GAMM-modeled average serodynamics across all years and 95% confidence intervals for the mean effect. Thin lines represent individual-year deviations from the average year pattern.

Page: 5

Number: 1 Author: Steve Luby Date: 9/2/2022 10:18:00 AM
What is the y axis for these curves?

Number: 2 Author: Emily Gurley Date: 9/9/2022 8:45:00 PM
Also from the last paper, right? Or are these new data?

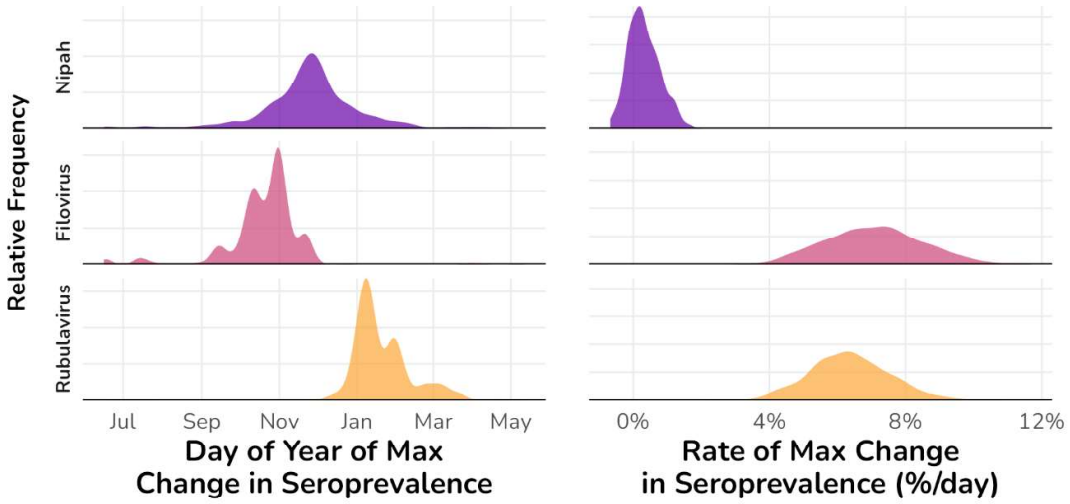

Figure 6. Timing and strength of seasonality of viral circulation in juveniles over the five-year study. Plots show posterior densities of the date for which increase in seroprevalence is greatest (left), and the rate of increase (right), for juvenile bats. Densities are of these quantities derived from 1000 samples of model posteriors.

Filovirus seroprevalence exhibited different patterns. Among adults, seroprevalence against filovirus was lower than for Nipah virus or Rubulavirus. As with Nipah virus, filovirus seroprevalence decreased over the study period, from 64% (50%-76%) to 20% (12%-30%), and had periods of distinct increases in spring/summer 2010 (concurrently with a circulation period for Nipah virus) (Fig. 3). filovirus seroprevalence did not exhibit cyclic annual patterns in adults (Fig. 4).

Among juveniles within the average year, filovirus seroprevalence started very low in June at 8% (2%-40%), but increased to a peak of 44% (32%-58%) in juveniles approximately six months of age before declining. (Fig 5). This pattern was consistent across years; though the peak varied in size, it consistently occurred in December or January. The average date with the greatest rate of seroprevalence increase was Oct 31 (Jul 14-Nov 25), and the maximum rate of increase was 7.1%/day (4.5%/day-9.9%/day).

Rubulavirus seroprevalence had a distinct dynamic pattern. Among adults, rubulavirus seroprevalence was high at 92% (82%-98%), and remained high through the end of the period at 74% (62%-84%), though there were some temporary periods of decline. There were distinct periods of increasing circulation in early 2010 and early 2012. Only for the rubulavirus did adult seroprevalence exhibit patterns of seasonality. Periods of increasing seroprevalence in adults exhibited strong seasonality of 1.6%/day (1.0%/day-2.4%/day) increase on peak days, with the mean peak date occurring on Dec 15 (Oct 15-Feb 1)

In juveniles, seroprevalence against the rubulavirus in the average year was 22% (4%-62%) in the youngest bats in June, though in individual years this could range from 7%-43%. In all years juvenile seroprevalence declined to a low of 1.7% (0.6%, 8.3%) that occurred between Oct 9 and Dec 11, then increased starting in winter, reaching high values in yearling juveniles of 88% (56%-99%) The peak rate of increase for juvenile rubulavirus seroprevalence was 6.4%/day (4.2%-8.8%) and occurred Jan 9 (Dec 21-Mar 16).

Spatial Comparisons

Seroprevalence trends in both adult and juvenile bats within the one-year studies in Chakhoria and Ramnagar broadly mirrored those in the five-year study in Faridpur (Supplementary Figure 2). In adults, Nipah virus seroprevalence increased in both Ramnagar and Chakhoria over the course of April 2010-May 2011, consistent with the period of increased seroprevalence observed in late 2010 in

Page: 6

Number: 1 Author: Steve Luby Date: 9/2/2022 10:19:00 AM
As above. What is the y axis?

Number: 2 Author: Steve Luby Date: 9/2/2022 10:22:00 AM
Is this appropriate to call out when it is not identified by the GAMM splines?

Number: 3 Author: Steve Luby Date: 9/2/2022 10:24:00 AM
Consider including a figure in the appendix that illustrates this.

the Faridpur. In Ramnagar, the seroprevalence in adults was 41% (24%-61%) in April 2010, rising to 60% (36%-81%) in May 2011 whilst in Chakhoria it rose from 26% (10%-48%) to 60% (36%-81%) over the same period. The trend in juveniles was as described for the five-year study.

For the filovirus, the seroprevalence in the one-year studies was higher than the reported average in the five-year study, with seroprevalence ranging between 41% (21%-64%) and 75% (51%-91%) in Ramnagar and between 48% (26%-70%) and 100% (83%-100%) in Chakhoria. A period of distinct increase in spring/summer 2010 was described in the five-year study which overlaps the timing of the one-year studies and may explain this difference in seroprevalence. In Ramnagar the serodynamics in both adults and juveniles were as described for the five-year study. In Chakhoria, there were two periods of increasing seroprevalence in adults over the course of the year. The first of these occurred in late 2010 when the adult seroprevalence increased from 48% (28%-69%) in August 2010 to 100% (83%-100%) in October 2010. The second period of increase occurred at the end of the study period in Spring 2011 with seroprevalence rising from 48% (26%-70%) to 75% (51%-91%) between early March 2011 to late April 2011.

Rubulavirus seroprevalence was high in adults throughout the one-year studies, ranging from 50% (27%-73%) to 95% (76%-100%) in Ramnagar and 29% (11%-52%) to 100% (83%-100%) in Chakhoria, with the lowest values observed in September 2010 in Ramnagar and December 2010/January 2011 in Chakhoria. These decreases were followed by rapid increases back to high seroprevalence, coincident with increasing levels in juveniles. The pattern in juveniles was as described in the five-year study, with seroprevalence estimates in yearlings in April 2011 of 80% (28%-99%) in both locations.

Comparison of the seroprevalence trends within each age group and virus between the two one-year study locations did not support spatial differences in the timing of changes in seroprevalence (Figure 7). Only for the filovirus in adults was there some evidence of a difference in trend between locations coinciding with the period of increased seroprevalence observed in adults in Chakhoria at the end of 2010.

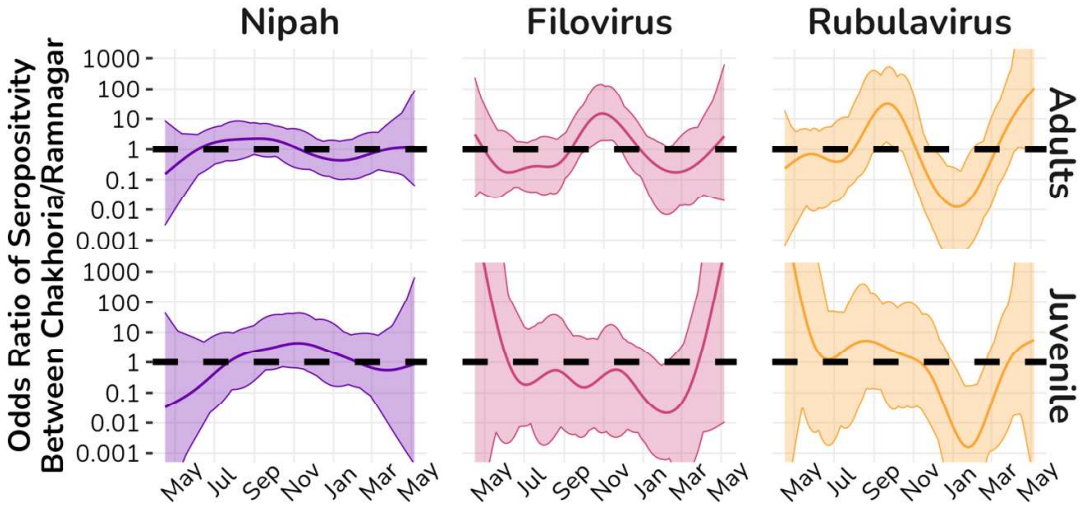

Figure 7. Comparison of splines for seroprevalence trends between locations in adults and juveniles over one-year study. Thick lines and shaded errors represent the estimated difference in trends and their associated 95% confidence intervals. The horizontal dashed line is set at zero. Where the confidence interval excludes zero, there is evidence for a significant difference in the serodynamics between the Ramnagar and Chakhoria populations over that period.

Maternal Inheritance of antibodies

We examined the relationship between mother and pup antibody titers, as measured by our Luminex assays (see Methods). This relationship varied between viruses (Figure 7). For Nipah virus,

Page: 7

Number: 1 Author: Emily Gurley Date: 9/9/2022 8:50:00 PM
Are these ranges or credible intervals around estimates?

the relationship was nonlinear due to clustering at high and low values, but largely followed a 1:1 relationship between mother and pup antibody titers. For the rubulavirus the relationship was near-linear and near 1:1. For the filovirus, though, the relationship between MFI values measured in mothers and their pups fell well below the 1:1 line, indicating pups having low inheritance of antibodies against the filovirus relative to the other two viruses.

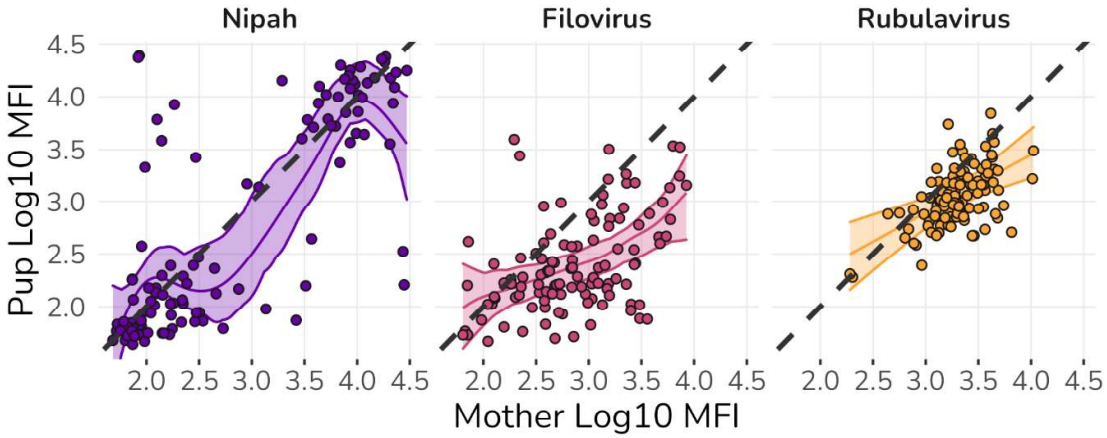

Figure 8. Maternal inheritance of antibodies. In each plot, points are measured log-MFI values for adults and juveniles in mother-pup pairs for each of the three antibody tests. Lines represent the predicted mean relationship between the two and their associated 95% confidence intervals.

3. Discussion

We found serological evidence for regular circulation of multiple viruses in *P. medius* populations in Bangladesh. These included Nipah virus, which spills over from bat populations to humans in the region, as well as a filovirus and a rubulavirus. It was common for bats to have antibodies against multiple viruses, and more common in adults than juveniles. Co-immunity patterns among pre-weaned juveniles mirrored those in adults, reflecting maternal inheritance patterns. Patterns of co-immunity were not random, with antibodies against all three virus types being positively correlated within bats.

The dynamic patterns in population seroprevalence were distinct for the three viral groups. The rubulavirus exhibited the most regular and consistent patterns. Some juveniles in these populations inherit maternal antibodies against the rubulavirus, which wane over the first six months of their life, after which a regular annual epidemic occurs in the juvenile population, causing a steep rise in juvenile seroprevalence by the end of their first year. High adult seroprevalence is likely the consequence of most bats being exposed to the disease as juveniles. When adult seroprevalence drops, as it did in 2009 to 2011, it rapidly rebounds during the same season as juveniles, indicating the same regular epidemics affect both adults and juveniles. We expect circulation and shedding of the rubulavirus to be strongest in the late winter and early spring months in these populations. Mortlock at al.[39] found irregular patterns of rubulavirus shedding in *Rousettus* bats over one year (via PCR detection in pooled urine samples), including a peak during a period of presumed antibody waning, though only within a one-year study.

An outstanding question is how the rubulavirus is maintained in the population despite high seroprevalence in inter-epidemic periods. Recrudescence is one possibility. Evidence for latent infections and recrudescence has been found for Nipah virus in *Pteropus vampyrus*[40], Hendra virus in *P. alecto and P. poliocephalus*[41], and Lagos bat lyssavirus and African henipavirus in *Eidolon helvum*[42]. Another possibility is re-importation. In concurrent work with this study, we found that bat home

Page: 8

Number: 1 Author: Emily Gurley Date: 9/9/2022 8:59:00 PM
I thought maybe not with the filos?

Number: 2 Author: Emily Gurley Date: 9/9/2022 9:00:00 PM
Something about individual bat risk or strange bat immune responses?

ranges overlapped with nearby colonies so as to form a meta-population[34], allowing occasional infection from outside bats, as has been shown to maintain Hendra virus in *Pteropus* populations[43,44].

The identity of the rubulavirus in this population is unclear. Our test was designed for Menangle virus, which has been found in multiple *Pteropus* species in Australia[45,46]. At least 11 distinct Paramyxoviruses have been found in *P. medius* in Bangladesh alone: Nipah virus and ten uncharacterized species, including six Rubulaviruses closely related to Menangle virus and the Tioman virus[9,47]. It is possible that the serological patterns observed represent antibodies against a complex of multiple Rubulaviruses, though the regular interannual patterns in seroprevalence would indicate that they are operating similarly.

Nipah virus serodynamics exhibited a different pattern. We found serodynamics consistent with little to no circulation amongst juveniles. Instead, each cohort of juveniles appeared to inherit some maternal antibodies - maternal inheritance was most consistent for Nipah virus in our mother-pup comparisons - and population seroprevalence declined over their first year for all cohorts. Adults experienced several outbreaks, but Nipah virus serodynamics did not exhibit any seasonal patterns, rather, circulation events occurred in irregular intervals one to two years apart. In Thailand, Nipah virus shedding in *Pteropus lylei* was found to vary in time over two years and this pattern varied by strain; Bangladesh-strain Nipah virus shedding clustered in spring months, but only over two years[28]. Human Nipah virus outbreaks in Bangladesh, on the other hand, exhibit seasonality associated with the palm-sap consumption, the most frequently implicated spillover pathway[48].

We had repeated positive detections of filovirus antibodies, using a test designed for Ebola-Zaire virus. This is the first detection of filovirus antibodies in *P. medius* in Bangladesh, though they have been found in *R. leschenaultii* in the country[49]. This could be one of several known filoviruses or an unknown species. There have been several recent findings of new Ebola-like filoviruses in bats extending across Africa and Asia. These include Bombali virus in Sierra Leone[50], and Měnglà virus in *Rousettus* bats in China[51]. Ebola-Reston virus was found in multiple bat species (*M. australis*, *C. brachyotis* and *Ch. plicata*) in the Philippines[52]. Marburg virus and Ebola-Zaire virus may have been detected in Sierra Leone and Liberia (unpublished,[53,54]). Serological evidence of filoviruses in bats has been found in multiple bat species in Central[12,55] and Western[56] African countries, Singapore [57], China[58], as well as Trinidad[59]. Our finding here adds to the evidence of broad host and geographic host ranges for filoviruses.

Several components of the serological patterns of filovirus antibodies are of interest. Young juveniles were almost all seronegative, and seroprevalence rose over the course of the first six months all years, then declined in the latter half of the year. Adult seroprevalence remained steady despite these regular juvenile outbreaks near 50% over the course of the five-year study. Adult seroprevalence against the filovirus also did not exhibit regular annual cycles, though the one period of detectable increase, in mid-2010, coincided with one of the regular seasonal periods of increase among juveniles. One possible hypothesis explaining this pattern is that the filovirus may exhibit low transmissibility in this system, only circulating when seroprevalence is near zero in young juveniles. It is also possible that bats may exhibit weak or waning filovirus antibody response but remain immune. In *R. aegyptiacus*, infected with the Marburg virus, antibody waning post-infection was found to be rapid, but bats retained protective immunity even with very low antibody titers[60,61]. If a similar mechanism occurs in this population, this could explain low seroprevalence in adults despite regular apparent periods of circulation in juveniles. The regular drop of seroprevalence in juveniles following the peak is consistent with this explanation.

However, if adults remain immune to the filovirus despite low antibody titers, this immunity does not appear to transfer to juveniles, as seroprevalence is near-zero among newborns and circulation in juveniles begins soon after birth. The relationship of pup/dam MFI found for the filovirus antibodies suggests that there may be differential inheritance of antibodies and possibly differential maternally derived immunity. If pups derive weaker immunity from dams than for other viruses, this may provide another mechanism for maintenance of the virus. It possible that patterns in filovirus antibody detection are an artifact of our test, specific to Ebola-Zaire virus, and that temporal trends are in part reflective of differential cross-reaction with viruses in this bat population.

Number: 1 Author: Hume Field Date: 8/24/2022 9:59:00 AM
And recently in Australia.
Barr et al. J Gen Virol. 2022 Aug;103(8).
doi: 10.1099/jgv.0.001785.

However, while this may modify estimates of overall seroprevalence, the differential pup/dam MFI relationship, and consistent rise in seroprevalence among young juveniles, would require the test to exhibit differential sensitivity by age.

Serodynamics in our two one-year studies were broadly similar to those found in the five-year study. We found weak evidence for differences in serodynamics between sites for the filovirus in adults, and the rubulavirus for adults and juveniles. However, limited sample numbers and the shorter study period limit our ability to generalize about seasonal patterns. In a concurrent study[34], we found that flying foxes home ranges in Bangladesh extended an average of 50 km from their roosts. The roosts at Chakoria and Ramnagar, the sites of the one-year studies, are approximately 225 km apart. Thus, we would not expect the dynamics of these sites to be strongly coupled. It is plausible that inter- and even intra-annual dynamics of these viruses could be similar if weak coupling and/or environmental factors drive some of the dynamics. More detailed and longer-term studies at multiple sites in parallel are needed to better understand spatiotemporal variation in viral circulation.

Regular co-circulation of these viruses indicates the potential for viral interactions to affect host-viral dynamics. Viral interactions in hosts – whether direct during co-infection or indirect from infection in different periods – can enhance or suppress pathogen mortality, fecundity, or transmission, via competition for host nutritional or cellular resources, or via immune-mediation involving cross-immunity or antibody-dependent enhancement, and these interactions structure viral communities[26]. These interactions can have complex, even opposite effects when scaled to the population[27]. Here, we found positive correlations between serostatus against different viruses. External factors may also affect these relationships. For instance, all three viruses appear to have circulated in adults in mid-2010. It is possible that common factors like population density and/or nutrition availability[62] affected transmission of multiple viruses.

While rich observational serological data reveal these patterns, greater study is required to characterize these viruses and their effects on the host population and potential for spillover, as well as the degree and mechanisms of interactions. Our inferred periods of viral circulation point to optimal sub-populations and times to sample this population to detect viral shedding and isolate these viruses, and potentially capture co-infected hosts.

Extended longitudinal sampling also sheds light on viral dynamics not found in short-term studies. One- to two-year studies can identify temporal patterns in serology or viral prevalence and shedding, but establishing patterns or variation in seasonality requires extended, multi-year sampling. In our case, while Nipah virus outbreaks occurred in the same season in two years, it was apparent from the longer time series that there was no distinct seasonal pattern in dynamics. The consistency of the filovirus and rubulavirus patterns required repeated years of sampling to establish. Similarly, we were able to understand these patterns far better by separating juvenile and adult patterns, which we would be unable to distinguish in pooled samples.

While such extended individual-capture longitudinal studies are resource-intense, understanding the joint circulation of multiple viruses can be accomplished via multiplex immunoassays such as those used here. The continuous measures from these assays also have the potential to identify key patterns such as the differential inheritance of filovirus antibodies we identified here. Interpretation of these values is challenging and the relationship between immune status, antibody titer, and measured fluorescence is complex[32], but they have much potential to shed light on mechanistic drivers of disease circulation.

Methods

Field collection

We conducted a five-year longitudinal study sampling *P. medius* bats quarterly and two one-year studies in different locations, sampling monthly. All capture and sampling methods were approved by Tufts University IACUC protocol #G929-07 and icddr,b animal ethical review (protocol 2006-012). For the five-year study, we sampled from a roost complex near Faridpur, Bangladesh, as previously described in Epstein, et al. [34] The area of the roost complex consists of patchy forest

Page: 10

Number: 1 Author: Steve Luby Date: 9/2/2022 10:41:00 AM
As noted above, this important detail needs to be mentioned sooner.

Number: 2 Author: Steve Luby Date: 9/2/2022 10:44:00 AM
In addition to uncertainty over which specific viruses these assays are detecting antibodies against, which is addressed earlier in the discussion, other limitations to scientific inference that seem to me important to discuss include:
there were only two sites to assess spatial heterogeneity. A more robust assessment would require more sites.
Although five years is longer than usually studied, it is too short of a time for measuring temporal dynamics that might play out over longer time periods.

Number: 3 Author: Emily Gurley Date: 9/9/2022 9:07:00 PM
Agree

Number: 4 Author: Steve Luby Date: 9/2/2022 10:43:00 AM
Seems a weak concluding statement. I recommend a stronger statement of implications and way forward. To my mind, identification of the specific viruses that are driving these antibody responses would be the highest priority.

Number: 5 Author: Steve Luby Date: 9/2/2022 9:47:00 AM
I recommend listing the local IRB first.

interspersed with agrarian human settlements and large rice paddies. Sampling occurred from July 2007 to November 2012 approximately every three months. The bat colony regularly shifted amongst roosts within the 80km^2 roost complex over the period of the study. Sampling occurred at the largest occupied roost found each quarterly sampling trip, sampling at different roosts within the complex across consecutive sampling nights if required to capture a sufficient number of individuals.

Approximately 100 bats were captured at each sampling event, over 7-10 days. We captured bats with a 10x15m mist net between 11pm and 5am each night as bats returned from foraging until the count of 100 was reached.

In the one-year longitudinal studies, sampling was undertaken in two roost complexes in Ramnagar and Chakhoria, Bangladesh between April 2010 and May 2011. Monthly sampling of approximately 40 bats in each location was performed to obtain data at a finer temporal scale. Details of collection are otherwise as described for the five-year study.

For each study, we recorded each bat's age class, reproductive status, weight, size, and body condition. We collected up to 3.0 mL of blood from each bat's brachial vein into serum tubes with serum clot activator (Vaccutainer, USA). Blood tubes were stored vertically on ice packs in a cold box and serum was allowed to separate overnight. Serum was drawn from the tube after 24 hours and placed in a screw-top cryovial (Corning, USA) and stored in a liquid nitrogen dewar (Princeton Cryogenics, NJ, USA).

As part of a larger study to detect viral RNA, we also collected urine, rectal, and oropharyngeal swabs and pooled urine samples from under colonies. Further details can be found in Epstein, et al. [34]

Serological Assays

Sera from the longitudinal studies were sent to the Australian Animal Health Laboratory and gamma irradiated upon receipt. We used a bead-based microsphere assay that specifically detects antibodies to the soluble attachment glycoproteins[57,63,64] for a panel of viruses. Beads coated with each protein were mixed with sera at a dilution of 1:100. Biotinylated Protein A/G and Streptavidin-PE were then used to detect bound antibody. Beads were interrogated by lasers in a BioRad BioPlex machine and the results recorded as the Median Fluorescent Intensity (MFI) of 100 beads. We report here results for Nipah, Ebola-Zaire, and Menangle, the only three for which we established regular positive results.

While the Nipah virus has been detected in this population[65] and the specificity of the Nipah test is well-established [66], the Ebola-Zaire and Menangle virus tests are cross-reactive with other Ebola and rubulavirus species[67,68]. Thus we refer to these as tests for filovirus and Rubulavirus.

Data Analysis

We determined individual bat serostatus using Bayesian mixture models[69] fit on pooled data across all three longitudinal studies, calculating a cutoff of log-MFI as the point of equal probability between the smallest and second-smallest cluster of equal distributions for each assay.

To determine correlations of serostatus in bats across the three viruses, we fit a Bayesian multivariate probit model[70,71] which allows estimation of the joint outcomes (serostatus against each virus) and the correlation between the outcomes. We included age and sex variables to account for these effects on serostatus.

To examine time-varying changes in population seroprevalence, we fit binomial generalized additive mixed models (GAMMs)[72,73] to the time-series of serostatus measurements. For the five-year study, we fit separate models for adults and juveniles and for each immunoassay. For adults, we fit a model for seroprevalence dynamics over the course of the whole study. This treats the adult population as a single unit, though individuals within the population may turn over via migration, death and recruitment. We included both long-term and annual cyclic components for the multi-year time series of measurements. For juveniles, we treated each year's cohort of new recruits as separate populations with commonalities in overall first-year dynamics. The dynamics of each year's juvenile cohort were modeled as random-effect splines, deviating from an overall mean splines for juveniles

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Number: 1 Author: Steve Luby Date: 9/2/2022 9:49:00 AM
Error 5.4

Number: 2 Author: Emily Gurley Date: 9/9/2022 8:54:00 PM
If data from this aren't included here, does it need to be in the methods?

Number: 3 Author: Wang Linfa Date: 8/21/2022 10:50:00 AM
I am not 100% sure about this. All serological tests (the NiV test included) will detect cross-RX antibodies from related viruses. For NiV, you can argue that longitudinal surveillance has NOT detected any related viruses in the study populations, but we can't say that the Ab test is specific to NiV.

Number: 4 Author: Emily Gurley Date: 9/9/2022 8:56:00 PM
How has it been done in other similar studies? Using the beads from USU now, we see more than one cluster of MFI values below what we're considering positive.

Number: 5 Author: Steve Luby Date: 9/2/2022 9:53:00 AM
Why should we assume that the second smallest cluster is associated with the genuine presence or absence of antibody?

over their first year. To estimate periods of peak viral circulation within the population, we calculated the derivatives of model-derived seroprevalence over time. We sampled these from GAMM posterior distributions and classified periods with >95% of samples with positive derivatives - that is, increasing population seroprevalence - as periods of viral circulation. We also calculated strength and timing seasonality for each virus as the maximum rate of seroprevalence increase and the date at which this maximum occurred, again sampling these values from the model posterior, and calculating mean and high-density posterior interval (HDPI) values.

For the one-year studies, we fit models primarily to detect differences in time-varying changes between sites. We fit binomial generalized additive models (GAMs) to the monthly serostatus measurements with separate models for each immunoassay. We included a separate, fixed-term spline in each model for age (adult or juvenile) and location (Chakhoria or Ramnagar). The trends in serodynamics for each virus in each age group were compared between locations to test for spatial differences[74]. Juveniles identified as being from the previous year's cohort were excluded from the analysis.

To examine patterns of maternal inheritance, we used GAMs to fit a relationship of log-MFI between adult lactating females and their attached pups for each viral assay. We limited these to data from the five-year longitudinal study.

Data Availability: Raw data from this study and reproducible code used to perform analyses is available at the Zenodo Scientific Archive [Zenodo DOI to be generated] and also on GitHub at https://github.com/ecohealthalliance/bangladesh-bat-serodynamics

Author Contributions: Conceptualization: N.R. and J.H.E..; methodology, N.R., L-FW and J.H.E.; software, N.R. and S.H.; formal analysis, N.R. and S.H.; investigation, J.H.E., A.I. and L-FW….; resources, X.X.; data curation, N.R and J.H.E; writing—original draft preparation, N.R. J.H.E., and S.H.; writing—review and editing, HEF, X.X.; visualization, N.R. supervision, N.R. and J.H.E.; project administration, J.H.E.; funding acquisition, P.D., L-FW and J.H.E

Funding: This study was funded by an NIH NIAID awards to JHE (AI153420) and CCB (AI054715), an NIH Fogarty International Center Ecology and Evolution of Infectious Diseases (EEID) award to PD (TW005869) a NSF-NIH EEID award to AMK (EF-0914866), the US Agency for International Development Emerging Pandemic Threats: PREDICT program (NR, PD, JHE , AI), and grants from CSIRO (OCE Science Leader Award) the Singapore National Research Foundation (NRF2012NRF-CRP001-056) to L-FW.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Page: 12

Number: 1 Author: Steve Luby Date: 9/2/2022 9:57:00 AM
How did you assess model fit?

Number: 2 Author: Noam Ross Date: 8/10/2022 12:19:00 PM
All co-authors please insert yourselves as appropriate here. A summary of contribution types can be found here: https://img.mdpi.org/data/contributor-role-instruction.pdf

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Co-circulation dynamics of henipaviruses, filoviruses and rubulaviruses in a bat population

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Abstract: Bats are hosts for a variety of viruses, some with potential to cause human disease. *Pteropus medius* are fruit bats that live in close association with humans in Bangladesh and are a known source of Nipah virus and other viruses of unknown pathogenic risk. Here we report on dynamics of seroprevalence against multiple viruses in a population of *P. medius* in Bangladesh over five years. We found evidence of regular circulation of Nipah virus, a rubulavirus, and a filovirus. Nipah virus circulated primarily among adult bats, with no seasonal patterns. The rubulavirus exhibited annual cycles in juveniles and near universal seroprevalence in adults. The filovirus exhibited annual cycles and antibody waning in juveniles and little circulation but lower seroprevalence amongst adults. We found no evidence for spatial differences in Nipah virus dynamics, but weak evidence for differences with other viruses. Host-viral interactions result in very different viral circulation patterns in the same individual bats and environments.

Keywords: bats; Nipah virus; filovirus; Rubulavirus; *Pteropus medius*; Bangladesh; serology; disease dynamics; generalized additive models

Introduction

Bats have been associated with a large diversity of viruses, several of which have been linked to human epidemics [1-3]. The discovery of Severe Acute Respiratory Syndrome (SARS)-related coronaviruses (CoVs) in Chinese horseshoe bats (*Rhinolophus spp.*) in 2004 and subsequent discoveries of related viruses[4,5] including close relatives of SARS-CoV-2[6], along with studies of other high consequence pathogens such as Ebola virus, Marburg virus, Nipah virus, Hendra virus have led to questions about whether and why bats were disproportionately represented among mammals as reservoirs for zoonotic viruses[1,2,7].

Bat species can carry diverse viruses which circulate simultaneously within single populations [8-11]. *Rousettus aegyptiacus* is a natural reservoir of Marburg virus, and IgG antibodies against Ebola Zaire virus have also been detected in this bat in Central Africa, suggesting that it may play a role in the circulation of multiple filoviruses[12,13]. In addition to these filoviruses, a novel zoonotic and pathogenic paramyxovirus, Sosuga virus, was also found in Ugandan *R. aegyptiacus* populations[14].

Summary of Comments on Email 5 - Attachment 2 - Ross-et-al_bangladesh-bats-cocirculation-serology_2022-08-11_LW_HF_sl_eg.pdf

Page: 1

Number: 1 Author: Emily Gurley Date: 9/9/2022 7:21:00 PM
Surprised not to see other icddr,b co-authors from the first paper - Rajib? Salah Uddin?

Number: 2 Author: Hume Field Date: 8/24/2022 9:20:00 AM
Pls add my UQ affiliation Noam.
'School of Veterinary Science, The University of Queensland, Gatton 4343 Australia'

Number: 3 Author: Wang Linfa Date: 8/21/2022 10:57:00 AM
It's better to include my CSIRO affiliation here as the work started when I was still associated with AAHL

Number: 4 Author: Noam Ross Date: 8/10/2022 12:38:00 PM
Please check that your affiliation is as it should be!

Number: 5 Author: Emily Gurley Date: 9/9/2022 7:25:00 PM
Consider providing years of the study

Number: 6 Author: Emily Gurley Date: 9/9/2022 7:23:00 PM
Or, viral families?

Number: 7 Author: Emily Gurley Date: 9/9/2022 7:24:00 PM
Evidence that this is just one virus?

Number: 8 Author: Steve Luby Date: 9/2/2022 9:33:00 AM
This seems an odd sentence to close with. The last sentence is usually a conclusion, but I can't see how the data presented in the abstract supports this conclusion.

Number: 9 Author: Steve Luby Date: 9/2/2022 9:36:00 AM
True, but since the new data presented here don't address this issue, I don't see this as central to the introduction, i.e. Error 3.12

Eidolon helvum, another pteropodid bat with a broad geographic range in Africa, has been found to carry a lyssavirus, a henipavirus, and several other viruses of unknown pathogenicity[15]. Various surveillance efforts have found diverse viruses from with the same viral family in various bat species[5,16,17], and other studies have used metagenomic approaches to look broadly at viral diversity within individual bat species[9,11,18-21].

Studies of viral diversity in a single taxonomic host group can inform evolutionary history of viruses and their relationships to specific hosts[6,22] and inform public health strategies[23]. However, detection of a zoonotic virus in an animal host is insufficient to determine whether the associated host species is a natural reservoir for the virus, or to characterize the demographic, physiological, or abiotic factors that may influence the timing of viral shedding and therefore risk of spillover to other species, including domestic animals and humans[24]. For these, more detailed demographic and longitudinal studies are required, especially in cases of multiple viruses, as direct or indirect interactions between multiple pathogens may affect disease presence, prevalence, and dynamics[25-27]. Some longitudinal studies of zoonotic viruses in bats have provided insight into the timing of viral infection and shedding, generally of single viruses. Nipah virus in *Pteropus lylei* in Thailand has been observed to have annual shedding patterns[28], whereas Hendra virus, which is closely related to Nipah virus and is carried by multiple pteropid bat species in Australia, has asynchronous and non-periodic cycles which appear to be influenced by localized factors such as specific bat species abundance and climatic factors [29]. Bi-annual pulses of Marburg virus shedding were observed in Uganda, coinciding with synchronous birth pulses in *R. aegyptiacus*[30]. Serological data is often valuable in understanding disease dynamics and transmission[31-33], especially in cases where direct detection and incidence rates of viruses are low[34].

Pteropus sp. are reservoirs of henipaviruses, a group of mostly zoonotic, lethal neurotropic viruses that include Nipah, Hendra, Cedar, Ghana and Mojiang virus[35-37]. In Bangladesh and India, *P. medius* is a reservoir of Nipah virus, which has spilled over to human populations repeatedly[38]. Viruses from eight other viral families have been detected in *P. medius* in Bangladesh[9]. The epidemiology of these other viruses is far less characterized, and little is known about their interactions or zoonotic potential.

Here, we report on the dynamics of seroprevalence against three types of viruses - Nipah virus, a filovirus, and a rubulavirus - circulating simultaneously in *P. medius* populations in three sites in Bangladesh. We conducted a longitudinal study over a period of five years, and two shorter, one-year studies for comparative seasonal data. These studies enable us to develop a detailed picture of the seasonal and demographic serological patterns, and infer the extent and periodicity of viral circulation. We find that Nipah virus circulates primarily among adults without distinct seasonality, while the rubulavirus exhibits strong annual dynamics characteristic of juvenile epidemics driven by waning maternal antibodies. The filovirus, the first detected in *P. medius* in Bangladesh, also circulated in juveniles but exhibited distinct patterns antibody waning and limited maternal inheritance.

2. Results

Bat Dynamics and Demographics

We conducted a five-year longitudinal study sampling *P. medius* bats quarterly from a roost complex in the vicinity of Faridpur, Bangladesh, and also two parallel one-year studies sampling bats monthly from roosts in Chakhoria and Ramnagar, Bangladesh. In the five-year study, we sampled and tested serology of 1,886 bats: 1,224 adults, 623 free-flying juveniles, and 39 weaning juveniles (captured attached to adult females) over 19 sampling events (Figure 1). During the-one-year studies, 919 bats were sampled: 435 in Chakhoria (251 adults, 144 free-flying juveniles and 40 weaning juveniles) and 484 in Ramnagar (277 adults, 164 free-flying juveniles and 43 weaning juveniles (Figure S1). Nearly all juveniles (as determined by examination of maturation of sex organs) were 14 months old or less and could be assigned to a birth cohort based on size. Pregnant and juvenile bats were captured more frequently during the late spring and summer months. Mother-pup pairs were all

Number: 1 Author: Emily Gurley Date: 9/9/2022 7:28:00 PM
Sentence seems much broader than public health concerns

Number: 2 Author: Emily Gurley Date: 9/9/2022 7:29:00 PM
Meaning unclear - are there any species that don't host multiple viruses?

Number: 3 Author: Emily Gurley Date: 9/9/2022 7:31:00 PM
Suggest adding in what we know about seasonal shedding of Nipah already

Number: 4 Author: Emily Gurley Date: 9/9/2022 7:30:00 PM
And perhaps not Mojiang

Number: 5 Author: Steve Luby Date: 9/2/2022 9:41:00 AM
I don't believe this adjective applies to Cedar virus.

Number: 6 Author: Hume Field Date: 8/24/2022 9:29:00 AM
Don't think this should be here.

Number: 7 Author: Wang Linfa Date: 8/21/2022 10:37:00 AM
To be consistent with other section headings in Results

Number: 8 Author: Emily Gurley Date: 9/9/2022 7:35:00 PM
Some indication of the frequency of sampling would also be useful to add here

Number: 9 Author: Emily Gurley Date: 9/9/2022 7:33:00 PM
Please include the years of data collection

Number: 10 Author: Steve Luby Date: 9/2/2022 10:29:00 AM
it would be helpful to clarify how far apart these places are, as you have a considerable section exploring spatial comparisons.

captured during April and May in the five-year study whilst in the one-year-studies a small number were also captured in June and July. Pregnant females were captured between November and April/May. Lactating females were found between April and July.

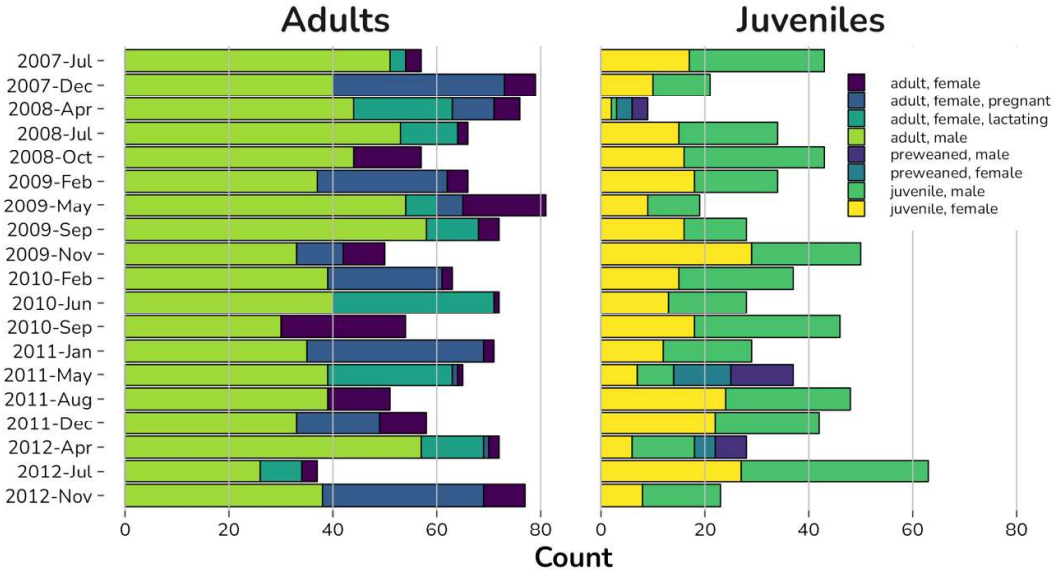

Figure 1. Demographics of bats captured in longitudinal sampling. Pregnant females were captured in months from November to June, lactating females were captured in months from April to July. Females with pre-weaning juveniles attached were found from April to May.

Patterns of Immunity and Co-immunity

We found bats were seropositive for antibodies against the henipavirus (HenipVsero+), a filovirus (EbVsero+), and a rubulavirus (MenVsero+).. Among adult bats, antibodies against the rubulavirus were common; 1,314 of 1752 adult bats were MenVsero+. Antibodies against Nipah virus or filovirus were less common – 1,031 were NiVsero+ and 921 were EbVsero+. Among the 921 juveniles, 192 were MenVsero+, 311 were NiVsero+ and 348 were EbVsero+. Of the 122 pre-weaned juveniles, 43 were MenVsero+, 52 were NiVsero+, and 32 were EbVsero+.

Co-exposure to multiple viruses was common (Figure 2). Over half (1,173) of the 1752 adults had antibodies against more than one of the three viruses, and 493 had antibodies against all three. Among the 921 juveniles, 210 had antibodies against more than one, and 39 had antibodies all three. 38 of 122 pre-weaned juveniles had more than one of the three antibodies, with seven having all three.

We found correlations between serostatus between all three pairs of viruses in a model accounting for bat age, sex, and study location, with all viruses tending to co-occur with each other more than would be expected than if they were distributed independently among bats. Nipah virus and filovirus antibodies had a standardized covariance of 0.13 (95% posterior interval 0.08-0.20). Nipah virus and rubulavirus antibodies were also more likely to occur together, with a covariance of 0.15 (0.09-0.21). Filovirus and rubulavirus antibodies had a covariance of 0.23 (0.17-0.29).

Number: 1 Author: Wang Linfa Date: 8/21/2022 10:48:00 AM

I think it is better to treat the three group of viruses equally as you have done for the title. It makes no difference whether we use NiV or Henipavirus in the context of the current study. Luminex-based assays targeting binding antibodies will in theory detect cross-RX antibodies in all three cases. See also comments in Methods

Number: 2 Author: Steve Luby Date: 9/2/2022 10:01:00 AM

Include percentages for all of these comparisons.

Number: 3 Author: Emily Gurley Date: 9/9/2022 7:36:00 PM

Agree with Linfa - unclear how many viruses, better to treat as groups

Number: 4 Author: Steve Luby Date: 9/2/2022 10:03:00 AM

Again, including percentages for all of these comparisons would make this much easier to understand.

Number: 5 Author: Emily Gurley Date: 9/9/2022 7:37:00 PM

Some kind of general cross-reactivity, non-specificity of the assay?

Number: 6 Author: Steve Luby Date: 9/2/2022 10:03:00 AM

I did not see this described in the methods section.

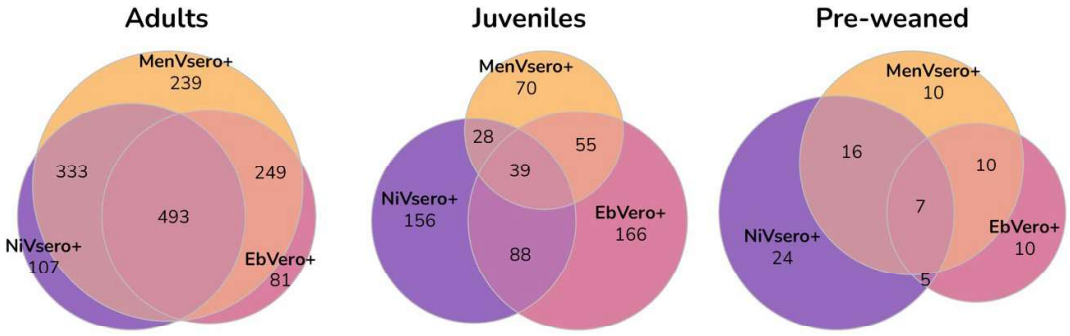

Figure 2. Detection of IgG antibodies against multiple viruses in individual *Pteropus medius*: Venn diagrams for adult, juvenile, and pre-weaned bats testing positive for antibodies against Nipah virus, filovirus, and Rubulavirus. Numbers under labels are counts of bats with only those viruses, numbers in overlapping areas represent number of bats detected with multiple viruses.

Serodynamics

Dynamics of population seroprevalence were different across the viral types. In adults, population seroprevalence against Nipah virus decreased over the study period from 78% (66%-88%) to 50% (40-62%) (Figure 3). Three periods of distinct increase in seroprevalence stood out in the inter-annual signal: early 2008, early 2009, and the second half of 2010. Nipah seroprevalence showed very weak seasonal patterns (Figure 4). (Nipah dynamics were previously reported in Epstein, et al. [34]).

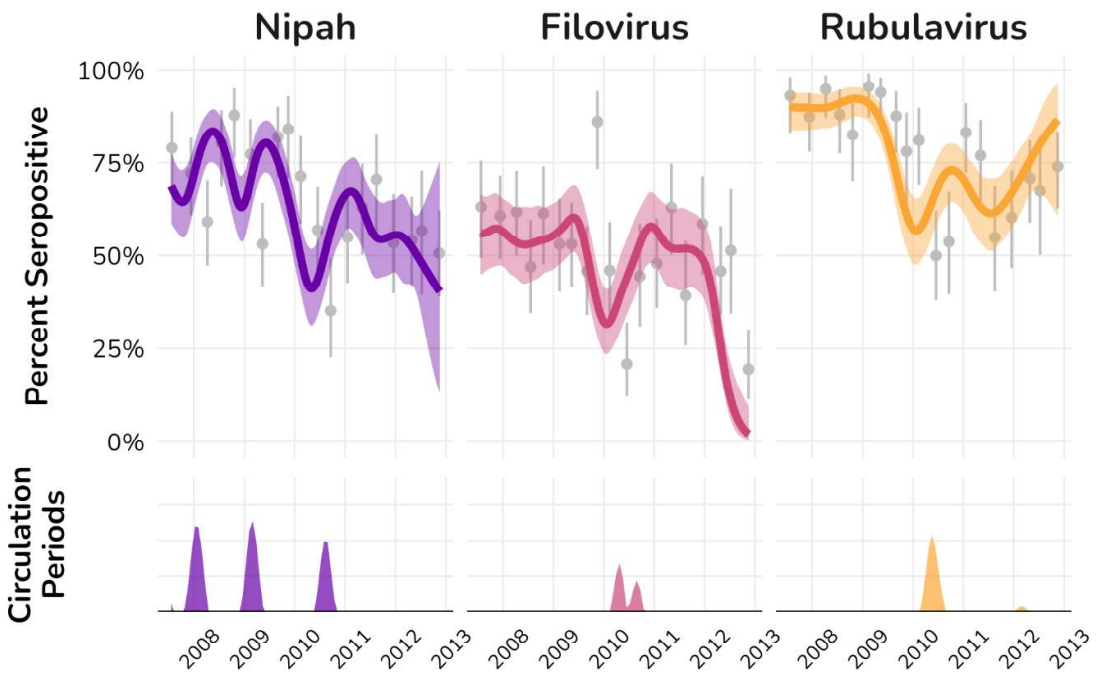

Figure 3. Inter-annual serodynamics in adult bats. Top: Measured and modeled population seroprevalence in adults over the term of the study. X-axis ticks mark the first day of the year. Grey points and bars represent measured population seroprevalence from individual sampling events on and 95% exact binomial confidence intervals. Thick lines and shaded areas represent the inter-annual (non-seasonal) spline component of a generalized additive mixed model (GAMM) of serodynamics and 95% confidence intervals for the mean pattern over time. Bottom: Circulation periods, when modeled slopes of the GAMM splines are positive for >95% of 1000 draws from the model posterior.

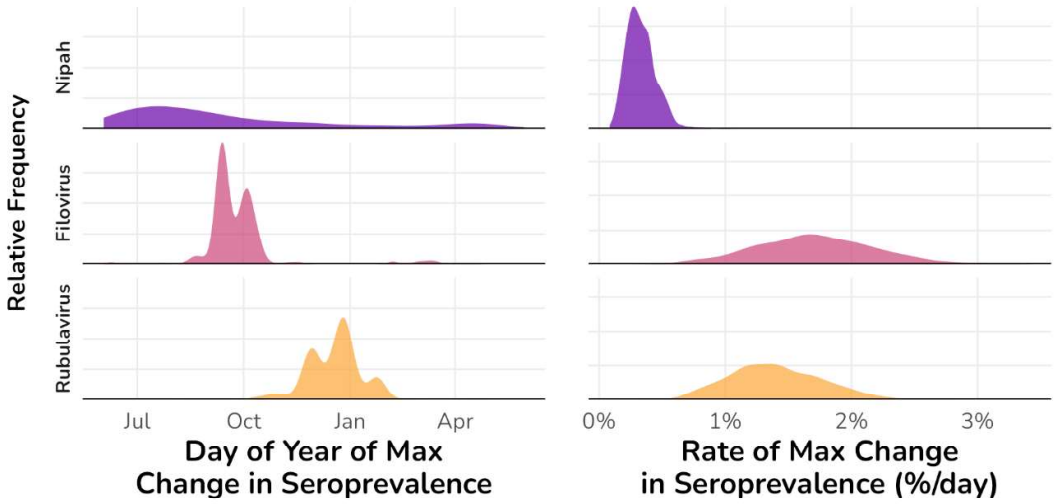

Figure 4. Timing and strength of seasonality of viral circulation in adults. Plots show posterior densities of the date for which increase in seroprevalence is greatest (left), and the rate of increase (right), for adult bats, drawn from GAMM models of seasonality. Densities are of these quantities derived from 1000 samples of model posteriors.

Among juveniles, we examined the dynamics of each cohort over their first year. For Nipah virus, the average cohort had 44% (32%-58%) seroprevalence in June, which consistently decreased over the course of the year to 20% (10%-36%) (Fig. 5). Year-to-year dynamics varied little, with overall declining seroprevalence in all years. No part of the year exhibited regular increases in seroprevalence for juveniles; the maximum rate of increase was indistinguishable from zero (Fig. 6).

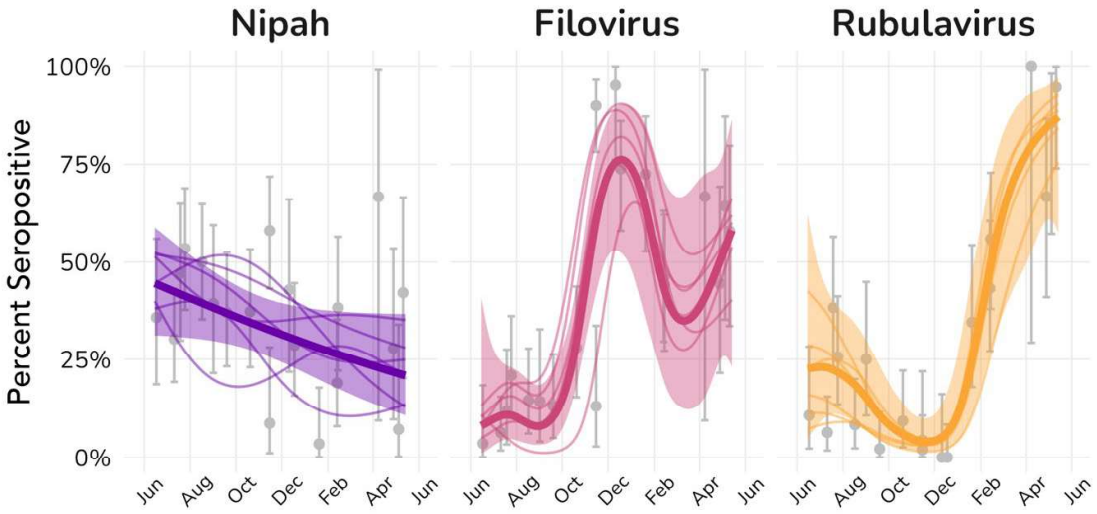

Figure 5. Serodynamics in juvenile bats in five-year study. Measured and modeled population seroprevalence in juvenile bats caught over their first year of life. Sampling events from multiple years are transposed onto a single year starting June (the earliest month free-flying young-of-the-year juveniles were captured). X-axis ticks mark the first day of each month. Grey points and bars represent measured population seroprevalence from individual sampling events on and 95% exact binomial confidence intervals. Thick lines and shaded areas represent GAMM-modeled average serodynamics across all years and 95% confidence intervals for the mean effect. Thin lines represent individual-year deviations from the average year pattern.

Number: 1 Author: Steve Luby Date: 9/2/2022 10:18:00 AM
What is the y axis for these curves?

Number: 2 Author: Emily Gurley Date: 9/9/2022 8:45:00 PM
Also from the last paper, right? Or are these new data?

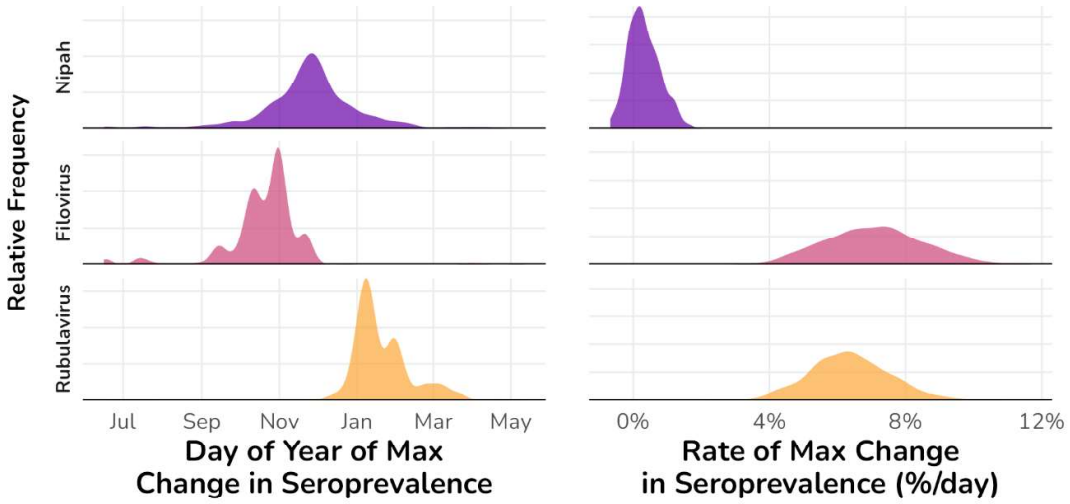

Figure 6. Timing and strength of seasonality of viral circulation in juveniles over the five-year study. Plots show posterior densities of the date for which increase in seroprevalence is greatest (left), and the rate of increase (right), for juvenile bats. Densities are of these quantities derived from 1000 samples of model posteriors.

Filovirus seroprevalence exhibited different patterns. Among adults, seroprevalence against filovirus was lower than for Nipah virus or Rubulavirus. As with Nipah virus, filovirus seroprevalence decreased over the study period, from 64% (50%-76%) to 20% (12%-30%), and had periods of distinct increases in spring/summer 2010 (concurrently with a circulation period for Nipah virus) (Fig. 3). filovirus seroprevalence did not exhibit cyclic annual patterns in adults (Fig. 4).

Among juveniles within the average year, filovirus seroprevalence started very low in June at 8% (2%-40%), but increased to a peak of 44% (32%-58%) in juveniles approximately six months of age before declining. (Fig 5). This pattern was consistent across years; though the peak varied in size, it consistently occurred in December or January. The average date with the greatest rate of seroprevalence increase was Oct 31 (Jul 14-Nov 25), and the maximum rate of increase was 7.1%/day (4.5%/day-9.9%/day).

Rubulavirus seroprevalence had a distinct dynamic pattern. Among adults, rubulavirus seroprevalence was high at 92% (82%-98%), and remained high through the end of the period at 74% (62%-84%), though there were some temporary periods of decline. There were distinct periods of increasing circulation in early 2010 and early 2012. Only for the rubulavirus did adult seroprevalence exhibit patterns of seasonality. Periods of increasing seroprevalence in adults exhibited strong seasonality of 1.6%/day (1.0%/day-2.4%/day) increase on peak days, with the mean peak date occurring on Dec 15 (Oct 15-Feb 1)

In juveniles, seroprevalence against the rubulavirus in the average year was 22% (4%-62%) in the youngest bats in June, though in individual years this could range from 7%-43%. In all years juvenile seroprevalence declined to a low of 1.7% (0.6%, 8.3%) that occurred between Oct 9 and Dec 11, then increased starting in winter, reaching high values in yearling juveniles of 88% (56%-99%) The peak rate of increase for juvenile rubulavirus seroprevalence was 6.4%/day (4.2%-8.8%) and occurred Jan 9 (Dec 21-Mar 16).

Spatial Comparisons

Seroprevalence trends in both adult and juvenile bats within the one-year studies in Chakhoria and Ramnagar broadly mirrored those in the five-year study in Faridpur (Supplementary Figure 2). In adults, Nipah virus seroprevalence increased in both Ramnagar and Chakhoria over the course of April 2010-May 2011, consistent with the period of increased seroprevalence observed in late 2010 in

Number: 1 Author: Steve Luby Date: 9/2/2022 10:19:00 AM
As above. What is the y axis?

Number: 2 Author: Steve Luby Date: 9/2/2022 10:22:00 AM
Is this appropriate to call out when it is not identified by the GAMM splines?

Number: 3 Author: Steve Luby Date: 9/2/2022 10:24:00 AM
Consider including a figure in the appendix that illustrates this.

the Faridpur. In Ramnagar, the seroprevalence in adults was 41% (24%-61%) in April 2010, rising to 60% (36%-81%) in May 2011 whilst in Chakhoria it rose from 26% (10%-48%) to 60% (36%-81%) over the same period. The trend in juveniles was as described for the five-year study.

For the filovirus, the seroprevalence in the one-year studies was higher than the reported average in the five-year study, with seroprevalence ranging between 41% (21%-64%) and 75% (51%-91%) in Ramnagar and between 48% (26%-70%) and 100% (83%-100%) in Chakhoria. A period of distinct increase in spring/summer 2010 was described in the five-year study which overlaps the timing of the one-year studies and may explain this difference in seroprevalence. In Ramnagar the serodynamics in both adults and juveniles were as described for the five-year study. In Chakhoria, there were two periods of increasing seroprevalence in adults over the course of the year. The first of these occurred in late 2010 when the adult seroprevalence increased from 48% (28%-69%) in August 2010 to 100% (83%-100%) in October 2010. The second period of increase occurred at the end of the study period in Spring 2011 with seroprevalence rising from 48% (26%-70%) to 75% (51%-91%) between early March 2011 to late April 2011.

Rubulavirus seroprevalence was high in adults throughout the one-year studies, ranging from 50% (27%-73%) to 95% (76%-100%) in Ramnagar and 29% (11%-52%) to 100% (83%-100%) in Chakhoria, with the lowest values observed in September 2010 in Ramnagar and December 2010/January 2011 in Chakhoria. These decreases were followed by rapid increases back to high seroprevalence, coincident with increasing levels in juveniles. The pattern in juveniles was as described in the five-year study, with seroprevalence estimates in yearlings in April 2011 of 80% (28%-99%) in both locations.

Comparison of the seroprevalence trends within each age group and virus between the two one-year study locations did not support spatial differences in the timing of changes in seroprevalence (Figure 7). Only for the filovirus in adults was there some evidence of a difference in trend between locations coinciding with the period of increased seroprevalence observed in adults in Chakhoria at the end of 2010.

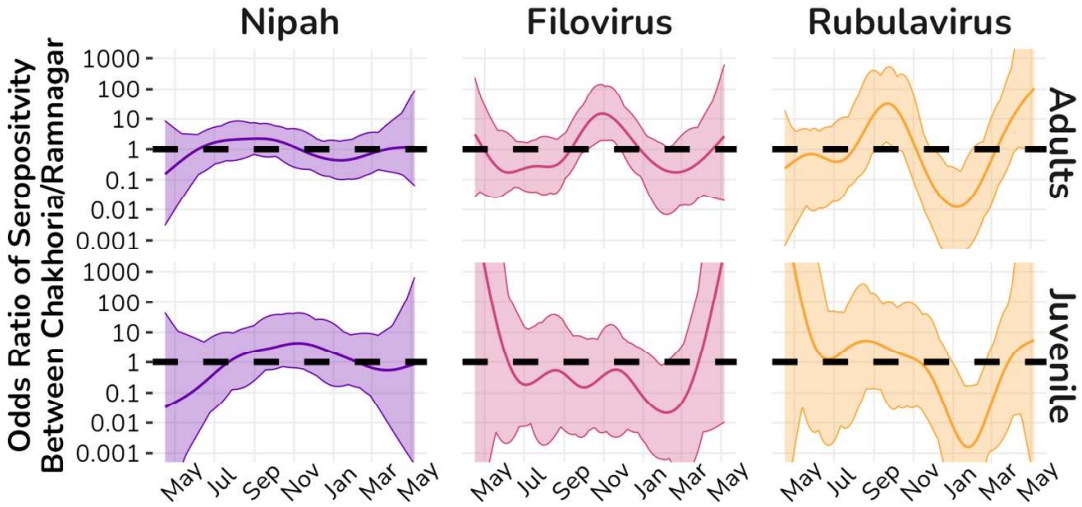

Figure 7. Comparison of splines for seroprevalence trends between locations in adults and juveniles over one-year study. Thick lines and shaded errors represent the estimated difference in trends and their associated 95% confidence intervals. The horizontal dashed line is set at zero. Where the confidence interval excludes zero, there is evidence for a significant difference in the serodynamics between the Ramnagar and Chakhoria populations over that period.

Maternal Inheritance of antibodies

We examined the relationship between mother and pup antibody titers, as measured by our Luminex assays (see Methods). This relationship varied between viruses (Figure 7). For Nipah virus,

Number: 1 Author: Emily Gurley Date: 9/9/2022 8:50:00 PM
Are these ranges or credible intervals around estimates?

the relationship was nonlinear due to clustering at high and low values, but largely followed a 1:1 relationship between mother and pup antibody titers. For the rubulavirus the relationship was near-linear and near 1:1. For the filovirus, though, the relationship between MFI values measured in mothers and their pups fell well below the 1:1 line, indicating pups having low inheritance of antibodies against the filovirus relative to the other two viruses.

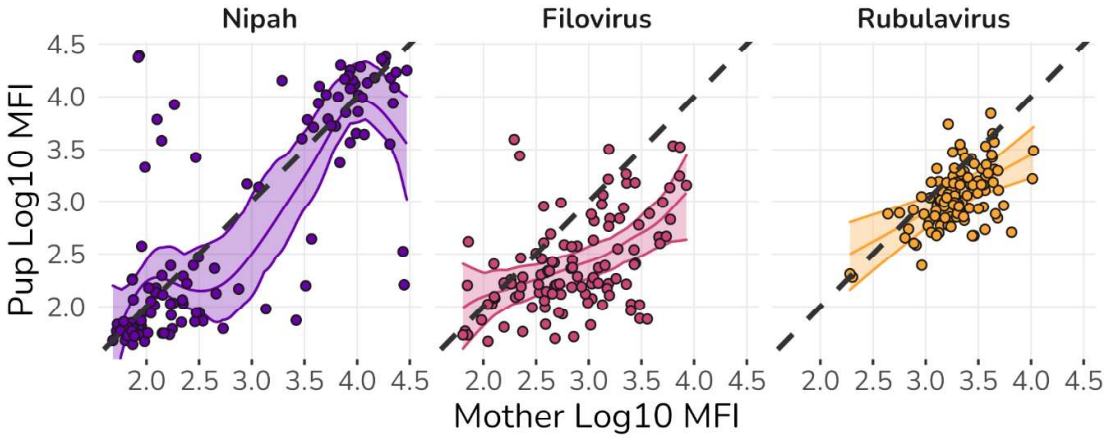

Figure 8. Maternal inheritance of antibodies. In each plot, points are measured log-MFI values for adults and juveniles in mother-pup pairs for each of the three antibody tests. Lines represent the predicted mean relationship between the two and their associated 95% confidence intervals.

3. Discussion

We found serological evidence for regular circulation of multiple viruses in *P. medius* populations in Bangladesh. These included Nipah virus, which spills over from bat populations to humans in the region, as well as a filovirus and a rubulavirus. It was common for bats to have antibodies against multiple viruses, and more common in adults than juveniles. Co-immunity patterns among pre-weaned juveniles mirrored those in adults, reflecting maternal inheritance patterns. Patterns of co-immunity were not random, with antibodies against all three virus types being positively correlated within bats.

The dynamic patterns in population seroprevalence were distinct for the three viral groups. The rubulavirus exhibited the most regular and consistent patterns. Some juveniles in these populations inherit maternal antibodies against the rubulavirus, which wane over the first six months of their life, after which a regular annual epidemic occurs in the juvenile population, causing a steep rise in juvenile seroprevalence by the end of their first year. High adult seroprevalence is likely the consequence of most bats being exposed to the disease as juveniles. When adult seroprevalence drops, as it did in 2009 to 2011, it rapidly rebounds during the same season as juveniles, indicating the same regular epidemics affect both adults and juveniles. We expect circulation and shedding of the rubulavirus to be strongest in the late winter and early spring months in these populations. Mortlock at al.[39] found irregular patterns of rubulavirus shedding in *Rousettus* bats over one year (via PCR detection in pooled urine samples), including a peak during a period of presumed antibody waning, though only within a one-year study.

An outstanding question is how the rubulavirus is maintained in the population despite high seroprevalence in inter-epidemic periods. Recrudescence is one possibility. Evidence for latent infections and recrudescence has been found for Nipah virus in *Pteropus vampyrus*[40], Hendra virus in *P. alecto and P. poliocephalus*[41], and Lagos bat lyssavirus and African henipavirus in *Eidolon helvum*[42]. Another possibility is re-importation. In concurrent work with this study, we found that bat home

Number: 1 Author: Emily Gurley Date: 9/9/2022 8:59:00 PM
I thought maybe not with the filos?

Number: 2 Author: Emily Gurley Date: 9/9/2022 9:00:00 PM
Something about individual bat risk or strange bat immune responses?

ranges overlapped with nearby colonies so as to form a meta-population[34], allowing occasional infection from outside bats, as has been shown to maintain Hendra virus in *Pteropus* populations[43,44].

The identity of the rubulavirus in this population is unclear. Our test was designed for Menangle virus, which has been found in multiple *Pteropus* species in Australia[45,46]. At least 11 distinct Paramyxoviruses have been found in *P. medius* in Bangladesh alone: Nipah virus and ten uncharacterized species, including six Rubulaviruses closely related to Menangle virus and the Tioman virus[9,47]. It is possible that the serological patterns observed represent antibodies against a complex of multiple Rubulaviruses, though the regular interannual patterns in seroprevalence would indicate that they are operating similarly.

Nipah virus serodynamics exhibited a different pattern. We found serodynamics consistent with little to no circulation amongst juveniles. Instead, each cohort of juveniles appeared to inherit some maternal antibodies - maternal inheritance was most consistent for Nipah virus in our mother-pup comparisons - and population seroprevalence declined over their first year for all cohorts. Adults experienced several outbreaks, but Nipah virus serodynamics did not exhibit any seasonal patterns, rather, circulation events occurred in irregular intervals one to two years apart. In Thailand, Nipah virus shedding in *Pteropus lylei* was found to vary in time over two years and this pattern varied by strain; Bangladesh-strain Nipah virus shedding clustered in spring months, but only over two years[28]. Human Nipah virus outbreaks in Bangladesh, on the other hand, exhibit seasonality associated with the palm-sap consumption, the most frequently implicated spillover pathway[48].

We had repeated positive detections of filovirus antibodies, using a test designed for Ebola-Zaire virus. This is the first detection of filovirus antibodies in *P. medius* in Bangladesh, though they have been found in *R. leschenaultii* in the country[49]. This could be one of several known filoviruses or an unknown species. There have been several recent findings of new Ebola-like filoviruses in bats extending across Africa and Asia. These include Bombali virus in Sierra Leone[50], and Měnglà virus in *Rousettus* bats in China[51]. Ebola-Reston virus was found in multiple bat species (*M. australis*, *C. brachyotis* and *Ch. plicata*) in the Philippines[52]. Marburg virus and Ebola-Zaire virus may have been detected in Sierra Leone and Liberia (unpublished,[53,54]). Serological evidence of filoviruses in bats has been found in multiple bat species in Central[12,55] and Western[56] African countries, Singapore [57], China[58], as well as Trinidad[59]. Our finding here adds to the evidence of broad host and geographic host ranges for filoviruses.

Several components of the serological patterns of filovirus antibodies are of interest. Young juveniles were almost all seronegative, and seroprevalence rose over the course of the first six months all years, then declined in the latter half of the year. Adult seroprevalence remained steady despite these regular juvenile outbreaks near 50% over the course of the five-year study. Adult seroprevalence against the filovirus also did not exhibit regular annual cycles, though the one period of detectable increase, in mid-2010, coincided with one of the regular seasonal periods of increase among juveniles. One possible hypothesis explaining this pattern is that the filovirus may exhibit low transmissibility in this system, only circulating when seroprevalence is near zero in young juveniles. It is also possible that bats may exhibit weak or waning filovirus antibody response but remain immune. In *R. aegyptiacus*, infected with the Marburg virus, antibody waning post-infection was found to be rapid, but bats retained protective immunity even with very low antibody titers[60,61]. If a similar mechanism occurs in this population, this could explain low seroprevalence in adults despite regular apparent periods of circulation in juveniles. The regular drop of seroprevalence in juveniles following the peak is consistent with this explanation.

However, if adults remain immune to the filovirus despite low antibody titers, this immunity does not appear to transfer to juveniles, as seroprevalence is near-zero among newborns and circulation in juveniles begins soon after birth. The relationship of pup/dam MFI found for the filovirus antibodies suggests that there may be differential inheritance of antibodies and possibly differential maternally derived immunity. If pups derive weaker immunity from dams than for other viruses, this may provide another mechanism for maintenance of the virus. It possible that patterns in filovirus antibody detection are an artifact of our test, specific to Ebola-Zaire virus, and that temporal trends are in part reflective of differential cross-reaction with viruses in this bat population.

Number: 1 Author: Hume Field Date: 8/24/2022 9:59:00 AM
And recently in Australia.
Barr et al. J Gen Virol. 2022 Aug;103(8).
doi: 10.1099/jgv.0.001785.

However, while this may modify estimates of overall seroprevalence, the differential pup/dam MFI relationship, and consistent rise in seroprevalence among young juveniles, would require the test to exhibit differential sensitivity by age.

Serodynamics in our two one-year studies were broadly similar to those found in the five-year study. We found weak evidence for differences in serodynamics between sites for the filovirus in adults, and the rubulavirus for adults and juveniles. However, limited sample numbers and the shorter study period limit our ability to generalize about seasonal patterns. In a concurrent study[34], we found that flying foxes home ranges in Bangladesh extended an average of 50 km from their roosts. The roosts at Chakoria and Ramnagar, the sites of the one-year studies, are approximately 225 km apart. Thus, we would not expect the dynamics of these sites to be strongly coupled. It is plausible that inter- and even intra-annual dynamics of these viruses could be similar if weak coupling and/or environmental factors drive some of the dynamics. More detailed and longer-term studies at multiple sites in parallel are needed to better understand spatiotemporal variation in viral circulation.

Regular co-circulation of these viruses indicates the potential for viral interactions to affect host-viral dynamics. Viral interactions in hosts – whether direct during co-infection or indirect from infection in different periods – can enhance or suppress pathogen mortality, fecundity, or transmission, via competition for host nutritional or cellular resources, or via immune-mediation involving cross-immunity or antibody-dependent enhancement, and these interactions structure viral communities[26]. These interactions can have complex, even opposite effects when scaled to the population[27]. Here, we found positive correlations between serostatus against different viruses. External factors may also affect these relationships. For instance, all three viruses appear to have circulated in adults in mid-2010. It is possible that common factors like population density and/or nutrition availability[62] affected transmission of multiple viruses.

While rich observational serological data reveal these patterns, greater study is required to characterize these viruses and their effects on the host population and potential for spillover, as well as the degree and mechanisms of interactions. Our inferred periods of viral circulation point to optimal sub-populations and times to sample this population to detect viral shedding and isolate these viruses, and potentially capture co-infected hosts.

Extended longitudinal sampling also sheds light on viral dynamics not found in short-term studies. One- to two-year studies can identify temporal patterns in serology or viral prevalence and shedding, but establishing patterns or variation in seasonality requires extended, multi-year sampling. In our case, while Nipah virus outbreaks occurred in the same season in two years, it was apparent from the longer time series that there was no distinct seasonal pattern in dynamics. The consistency of the filovirus and rubulavirus patterns required repeated years of sampling to establish. Similarly, we were able to understand these patterns far better by separating juvenile and adult patterns, which we would be unable to distinguish in pooled samples.

While such extended individual-capture longitudinal studies are resource-intense, understanding the joint circulation of multiple viruses can be accomplished via multiplex immunoassays such as those used here. The continuous measures from these assays also have the potential to identify key patterns such as the differential inheritance of filovirus antibodies we identified here. Interpretation of these values is challenging and the relationship between immune status, antibody titer, and measured fluorescence is complex[32], but they have much potential to shed light on mechanistic drivers of disease circulation.

Methods

Field collection

We conducted a five-year longitudinal study sampling *P. medius* bats quarterly and two one-year studies in different locations, sampling monthly. All capture and sampling methods were approved by Tufts University IACUC protocol #G929-07 and icddr,b animal ethical review (protocol 2006-012). For the five-year study, we sampled from a roost complex near Faridpur, Bangladesh, as previously described in Epstein, et al. [34] The area of the roost complex consists of patchy forest

Number: 1 Author: Steve Luby Date: 9/2/2022 10:41:00 AM
As noted above, this important detail needs to be mentioned sooner.

Number: 2 Author: Steve Luby Date: 9/2/2022 10:44:00 AM
In addition to uncertainty over which specific viruses these assays are detecting antibodies against, which is addressed earlier in the discussion, other limitations to scientific inference that seem to me important to discuss include:
there were only two sites to assess spatial heterogeneity. A more robust assessment would require more sites.
Although five years is longer than usually studied, it is too short of a time for measuring temporal dynamics that might play out over longer time periods.

Number: 3 Author: Emily Gurley Date: 9/9/2022 9:07:00 PM
Agree

Number: 4 Author: Steve Luby Date: 9/2/2022 10:43:00 AM
Seems a weak concluding statement. I recommend a stronger statement of implications and way forward. To my mind, identification of the specific viruses that are driving these antibody responses would be the highest priority.

Number: 5 Author: Steve Luby Date: 9/2/2022 9:47:00 AM
I recommend listing the local IRB first.

interspersed with agrarian human settlements and large rice paddies. Sampling occurred from July 2007 to November 2012 approximately every three months. The bat colony regularly shifted amongst roosts within the 80km^2 roost complex over the period of the study. Sampling occurred at the largest occupied roost found each quarterly sampling trip, sampling at different roosts within the complex across consecutive sampling nights if required to capture a sufficient number of individuals.

Approximately 100 bats were captured at each sampling event, over 7-10 days. We captured bats with a 10x15m mist net between 11pm and 5am each night as bats returned from foraging until the count of 100 was reached.

In the one-year longitudinal studies, sampling was undertaken in two roost complexes in Ramnagar and Chakhoria, Bangladesh between April 2010 and May 2011. Monthly sampling of approximately 40 bats in each location was performed to obtain data at a finer temporal scale. Details of collection are otherwise as described for the five-year study.

For each study, we recorded each bat's age class, reproductive status, weight, size, and body condition. We collected up to 3.0 mL of blood from each bat's brachial vein into serum tubes with serum clot activator (Vaccutainer, USA). Blood tubes were stored vertically on ice packs in a cold box and serum was allowed to separate overnight. Serum was drawn from the tube after 24 hours and placed in a screw-top cryovial (Corning, USA) and stored in a liquid nitrogen dewar (Princeton Cryogenics, NJ, USA).

As part of a larger study to detect viral RNA, we also collected urine, rectal, and oropharyngeal swabs and pooled urine samples from under colonies. Further details can be found in Epstein, et al. [34]

Serological Assays

Sera from the longitudinal studies were sent to the Australian Animal Health Laboratory and gamma irradiated upon receipt. We used a bead-based microsphere assay that specifically detects antibodies to the soluble attachment glycoproteins[57,63,64] for a panel of viruses. Beads coated with each protein were mixed with sera at a dilution of 1:100. Biotinylated Protein A/G and Streptavidin-PE were then used to detect bound antibody. Beads were interrogated by lasers in a BioRad BioPlex machine and the results recorded as the Median Fluorescent Intensity (MFI) of 100 beads. We report here results for Nipah, Ebola-Zaire, and Menangle, the only three for which we established regular positive results.

While the Nipah virus has been detected in this population[65] and the specificity of the Nipah test is well-established [66], the Ebola-Zaire and Menangle virus tests are cross-reactive with other Ebola and rubulavirus species[67,68]. Thus we refer to these as tests for filovirus and Rubulavirus.

Data Analysis

We determined individual bat serostatus using Bayesian mixture models[69] fit on pooled data across all three longitudinal studies, calculating a cutoff of log-MFI as the point of equal probability between the smallest and second-smallest cluster of equal distributions for each assay.

To determine correlations of serostatus in bats across the three viruses, we fit a Bayesian multivariate probit model[70,71] which allows estimation of the joint outcomes (serostatus against each virus) and the correlation between the outcomes. We included age and sex variables to account for these effects on serostatus.

To examine time-varying changes in population seroprevalence, we fit binomial generalized additive mixed models (GAMMs)[72,73] to the time-series of serostatus measurements. For the five-year study, we fit separate models for adults and juveniles and for each immunoassay. For adults, we fit a model for seroprevalence dynamics over the course of the whole study. This treats the adult population as a single unit, though individuals within the population may turn over via migration, death and recruitment. We included both long-term and annual cyclic components for the multi-year time series of measurements. For juveniles, we treated each year's cohort of new recruits as separate populations with commonalities in overall first-year dynamics. The dynamics of each year's juvenile cohort were modeled as random-effect splines, deviating from an overall mean splines for juveniles

Number: 1 Author: Steve Luby Date: 9/2/2022 9:49:00 AM
Error 5.4

Number: 2 Author: Emily Gurley Date: 9/9/2022 8:54:00 PM
If data from this aren't included here, does it need to be in the methods?

Number: 3 Author: Wang Linfa Date: 8/21/2022 10:50:00 AM
I am not 100% sure about this. All serological tests (the NiV test included) will detect cross-RX antibodies from related viruses. For NiV, you can argue that longitudinal surveillance has NOT detected any related viruses in the study populations, but we can't say that the Ab test is specific to NiV.

Number: 4 Author: Emily Gurley Date: 9/9/2022 8:56:00 PM
How has it been done in other similar studies? Using the beads from USU now, we see more than one cluster of MFI values below what we're considering positive.

Number: 5 Author: Steve Luby Date: 9/2/2022 9:53:00 AM
Why should we assume that the second smallest cluster is associated with the genuine presence or absence of antibody?

over their first year. To estimate periods of peak viral circulation within the population, we calculated the derivatives of model-derived seroprevalence over time. We sampled these from GAMM posterior distributions and classified periods with >95% of samples with positive derivatives - that is, increasing population seroprevalence - as periods of viral circulation. We also calculated strength and timing seasonality for each virus as the maximum rate of seroprevalence increase and the date at which this maximum occurred, again sampling these values from the model posterior, and calculating mean and high-density posterior interval (HDPI) values.

For the one-year studies, we fit models primarily to detect differences in time-varying changes between sites. We fit binomial generalized additive models (GAMs) to the monthly serostatus measurements with separate models for each immunoassay. We included a separate, fixed-term spline in each model for age (adult or juvenile) and location (Chakhoria or Ramnagar). The trends in serodynamics for each virus in each age group were compared between locations to test for spatial differences[74]. Juveniles identified as being from the previous year's cohort were excluded from the analysis.

To examine patterns of maternal inheritance, we used GAMs to fit a relationship of log-MFI between adult lactating females and their attached pups for each viral assay. We limited these to data from the five-year longitudinal study.

Data Availability: Raw data from this study and reproducible code used to perform analyses is available at the Zenodo Scientific Archive [Zenodo DOI to be generated] and also on GitHub at https://github.com/ecohealthalliance/bangladesh-bat-serodynamics

Author Contributions: Conceptualization: N.R. and J.H.E..; methodology, N.R., L-FW and J.H.E.; software, N.R. and S.H.; formal analysis, N.R. and S.H.; investigation, J.H.E., A.I. and L-FW….; resources, X.X.; data curation, N.R and J.H.E; writing—original draft preparation, N.R. J.H.E., and S.H.; writing—review and editing, HEF, X.X.; visualization, N.R. supervision, N.R. and J.H.E.; project administration, J.H.E.; funding acquisition, P.D., L-FW and J.H.E

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Number: 1 Author: Steve Luby Date: 9/2/2022 9:57:00 AM
How did you assess model fit?

Number: 2 Author: Noam Ross Date: 8/10/2022 12:19:00 PM
All co-authors please insert yourselves as appropriate here. A summary of contribution types can be found here: https://img.mdpi.org/data/contributor-role-instruction.pdf

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Co-circulation dynamics of henipaviruses, filoviruses and rubulaviruses in a bat population

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Abstract: Bats are hosts for a variety of viruses, some with potential to cause human disease. *Pteropus medius* are fruit bats that live in close association with humans in Bangladesh and are a known source of Nipah virus and other viruses of unknown pathogenic risk. Here we report on dynamics of seroprevalence against multiple viruses in a population of *P. medius* in Bangladesh over five years. We found evidence of regular circulation of Nipah virus, a rubulavirus, and a filovirus. Nipah virus circulated primarily among adult bats, with no seasonal patterns. The rubulavirus exhibited annual cycles in juveniles and near universal seroprevalence in adults. The filovirus exhibited annual cycles and antibody waning in juveniles with a lower seroprevalence amongst adults suggesting limited circulation or immunity despite low antibody titers. We found no evidence for spatial differences in Nipah virus dynamics, but weak evidence for differences with other viruses. Host-viral interactions result in very different viral circulation patterns in the same individual bats and environments.

Keywords: Chiroptera; Nipah virus; Ebola Zaire; Menangle virus; flying fox; Bangladesh; serology; longitudinal surveillance; disease dynamics; generalized additive models

Introduction

Bats have been associated with a large diversity of viruses, several of which have been linked to human epidemics [1-3]. The discovery of Severe Acute Respiratory Syndrome (SARS)-related coronaviruses (CoVs) in Chinese horseshoe bats (*Rhinolophus spp.*) in 2004 and subsequent discoveries of related viruses[4,5] including close relatives of SARS-CoV-2[6], along with studies of other high consequence pathogens such as Ebola virus, Marburg virus, Nipah virus, Hendra virus have led to questions about whether and why bats were disproportionately represented among mammals as reservoirs for zoonotic viruses[1,2,7].

Bat species can carry diverse viruses which circulate simultaneously within single populations [8-11]. *Rousettus aegyptiacus* is a natural reservoir of Marburg virus, and IgG antibodies against Ebola Zaire virus have also been detected in this bat in Central Africa, suggesting that it may play a role in the circulation of multiple filoviruses[12,13]. In addition to these filoviruses, a novel zoonotic and

Summary of Comments on Email 5 - Attachment 3 - Ross-et-al_bangladesh-bats-cocirculation-serology_2022-08-11_LW_HF_KJO.pdf

Page: 1

Number: 1 Author: Hume Field Date: 8/24/2022 9:20:00 AM

Pls add my UQ affiliation Noam.
'School of Veterinary Science, The University of Queensland, Gatton 4343 Australia'

Number: 2 Author: Wang Linfa Date: 8/21/2022 10:57:00 AM

It's better to include my CSIRO affiliation here as the work started when I was still associated with AAHL

Number: 3 Author: Noam Ross Date: 8/10/2022 12:38:00 PM

Please check that your affiliation is as it should be!

Number: 4 Author: Kevin Olival Date: 9/6/2022 12:02:00 AM

Per comments from Linfa and Hume, I think we may want to change the way we describe these throughout given the potential for cross-reactivity with all assays (in fact most serological assays!). At the moment, it's really only when you get to the methods or later in the discussion that it's clear the assays are for Menangle virus and Ebola Zaire viruses specifically. Also, weren't there other Filos in the panel? If so, curious about cross reactivity with any other Filos, e.g. Reston?

How about instead of "a rubulavirus" or "a filovirus" we just go with: Nipah-like, Meangle-like and Ebola-Zaire-like? OR Nipah-related, Menangle-related, and Ebola-Zaire-related viruses? Or we just use the nomenclature you have below, e.g. MenVsero+, EbVsero+… and then later in the discussion have a section about the implications of cross reactivity, i.e. that these may be novel viruses or even multiple related viruses, and not exactly EboV or MenV.

Number: 5 Author: Kevin Olival Date: 9/6/2022 12:10:00 AM

Always good to go with keywords not in the title already, or even abstract, to maximize search optimization.

Number: 6 Author: Kevin Olival Date: 9/6/2022 12:15:00 AM

The Letko et al. 2020 Nature Review Microb. is a good one to cite too. Also check out the section on adaptive immunity for some relevant discussion about filos vs henipas, etc. https://doi.org/10.1038/s41579-020-0394-z

pathogenic paramyxovirus, Sosuga virus, was also found in Ugandan *R. aegyptiacus* populations[14]. *Eidolon helvum,* another pteropodid bat with a broad geographic range in Africa, has been found to carry a lyssavirus, a henipavirus, and several other viruses of unknown pathogenicity[15]. Various surveillance efforts have found diverse viruses from with the same viral family in various bat species[5,16,17], and other studies have used metagenomic approaches to look broadly at viral diversity within individual bat species[9,11,18-21].

Studies of viral diversity in a single taxonomic host group can inform evolutionary history of viruses and their relationships to specific hosts[6,22] and inform public health strategies[23]. However, detection of a zoonotic virus in an animal host is insufficient to determine whether the associated host species is a natural reservoir for the virus, or to characterize the demographic, physiological, or abiotic factors that may influence the timing of viral shedding and therefore risk of spillover to other species, including domestic animals and humans[24]. For these, more detailed demographic and longitudinal studies are required, especially in cases of multiple viruses, as direct or indirect interactions between multiple pathogens may affect disease presence, prevalence, and dynamics[25-27]. Some longitudinal studies of zoonotic viruses in bats have provided insight into the timing of viral infection and shedding, generally of single viruses. Nipah virus in *Pteropus lylei* in Thailand has been observed to have annual shedding patterns [28], whereas Hendra virus, which is closely related to Nipah virus and is carried by multiple pteropid bat species in Australia, has asynchronous and non-periodic cycles which appear to be influenced by localized factors such as specific bat species abundance and climatic factors [29]. Bi-annual pulses of Marburg virus shedding were observed in Uganda, coinciding with synchronous birth pulses in *R. aegyptiacus*[30]. Serological data is often valuable in understanding disease dynamics and transmission[31-33], especially in cases where direct detection and incidence rates of viruses are low[34].

Pteropus sp. are reservoirs of henipaviruses, a group of mostly zoonotic, lethal neurotropic viruses that include Nipah, Hendra, Cedar, Ghana and Mojiang virus[35-37]. In Bangladesh and India, *P. medius* is a reservoir of Nipah virus, which has spilled over to human populations repeatedly[38]. Viruses from eight other viral families have been detected in *P. medius* in Bangladesh[9]. The epidemiology of these other viruses is far less characterized, and little is known about their interactions or zoonotic potential.

Here, we report on the dynamics of seroprevalence against three types of viruses - Nipah virus, a filovirus, and a rubulavirus - circulating simultaneously in *P. medius* populations in three sites in Bangladesh. We conducted a longitudinal study over a period of five years, and two shorter, one-year studies for comparative seasonal data. These studies enable us to develop a detailed picture of the seasonal and demographic serological patterns, and infer the extent and periodicity of viral circulation. We find that Nipah virus circulates primarily among adults without distinct seasonality, while the rubulavirus exhibits strong annual dynamics characteristic of juvenile epidemics driven by waning maternal antibodies. The filovirus, the first detected in *P. medius* in Bangladesh, also circulated in juveniles but exhibited distinct patterns antibody waning and limited maternal inheritance.

2. Results

Bat Dynamics and Demographics

We conducted a five-year longitudinal study (2007-2012) sampling *P. medius* bats quarterly from a roost complex in the vicinity of Faridpur, Bangladesh, and also two parallel one-year studies (2010-2011) sampling bats monthly from roosts in Chakhoria and Ramnagar, Bangladesh. In the five-year study, we sampled and tested serology of 1,886 bats: 1,224 adults, 623 free-flying juveniles, and 39 weaning juveniles (captured attached to adult females) over 19 sampling events (Figure 1). During the-one-year studies, 919 bats were sampled: 435 in Chakhoria (251 adults, 144 free-flying juveniles and 40 weaning juveniles) and 484 in Ramnagar (277 adults, 164 free-flying juveniles and 43 weaning juveniles (Figure S1). Nearly all juveniles (as determined by examination of maturation of sex organs) were 14 months old or less and could be assigned to a birth cohort based on size. Pregnant and

Page: 2

Number: 1 Author: Hume Field Date: 8/24/2022 9:29:00 AM
Don’t think this should be here.

Number: 2 Author: Kevin Olival Date: 9/6/2022 12:21:00 AM
Agree. Delete or move to results or discussion if not repetitive.

Number: 3 Author: Wang Linfa Date: 8/21/2022 10:37:00 AM
To be consistent with other section headings in Results

juvenile bats were captured more frequently during the late spring and summer months. Mother-pup pairs were all captured during April and May in the five-year study whilst in the one-year-studies a small number were also captured in June and July. Pregnant females were captured between November and April/May. Lactating females were found between April and July.

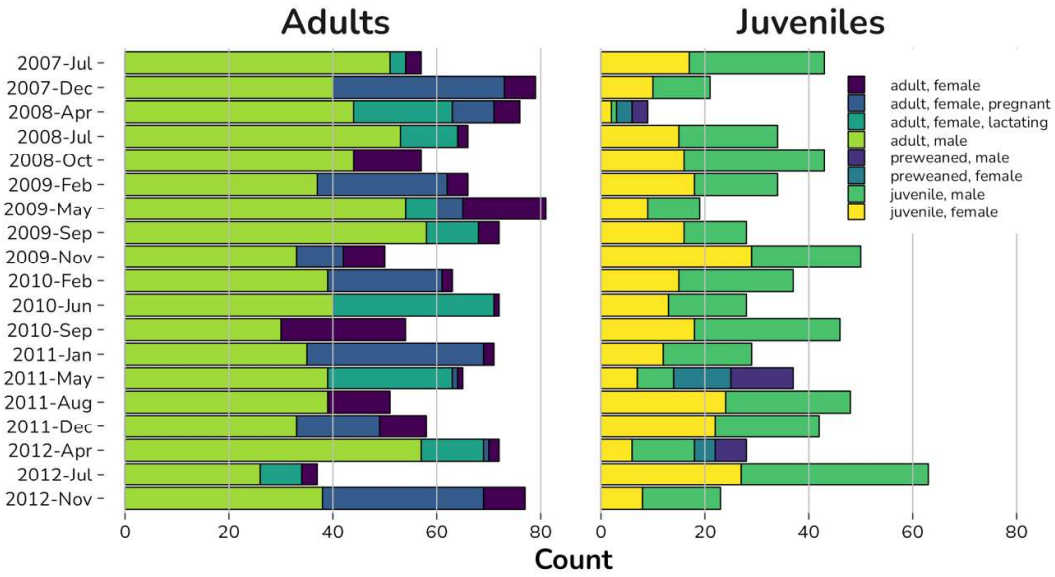

Figure 1. Demographics of bats captured in longitudinal sampling. Pregnant females were captured in months from November to June, lactating females were captured in months from April to July. Females with pre-weaning juveniles attached were found from April to May.

Patterns of Immunity and Co-immunity

We found bats were seropositive for antibodies against the henipavirus (HenipVsero+), a filovirus (EbVsero+), and a rubulavirus (MenVsero+).. Among adult bats, antibodies against the rubulavirus were common; 1,314 of 1752 adult bats were MenVsero+. Antibodies against Nipah virus or filovirus were less common – 1,031 were NiVsero+ and 921 were EbVsero+. Among the 921 juveniles, 192 were MenVsero+, 311 were NiVsero+ and 348 were EbVsero+. Of the 122 pre-weaned juveniles, 43 were MenVsero+, 52 were NiVsero+, and 32 were EbVsero+.

Co-exposure to multiple viruses was common (Figure 2). Two-thirds (1,173) of the 1752 adults had antibodies against more than one of the three viruses, and 493 (28%) had antibodies against all three. Among the 921 juveniles, 210 (23%) had antibodies against more than one, and 39 had antibodies all three. Almost a third, 38 of 122 pre-weaned juveniles, had more than one of the three antibodies, with seven having all three.

We found correlations between serostatus between all three pairs of viruses in a model accounting for bat age, sex, and study location, with all viruses tending to co-occur with each other more than would be expected than if they were distributed independently among bats. Nipah virus and filovirus antibodies had a standardized covariance of 0.13 (95% posterior interval 0.08-0.20). Nipah virus and rubulavirus antibodies were also more likely to occur together, with a covariance of 0.15 (0.09-0.21). Filovirus and rubulavirus antibodies had the highests covariance of 0.23 (0.17-0.29).

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Number: 1 Author: Kevin Olival Date: 9/6/2022 12:24:00 AM

Minor: but consider separating out figure legend, one for adult panel and one key for the juv panel, as colors repeat and a little confusing.

Number: 2 Author: Kevin Olival Date: 9/6/2022 12:05:00 AM

Just flagging as we aren't consistent with using these "sero+" nomenclatures on seropositivity later, as we keep referring generically to rubula and filo throughout.

Number: 3 Author: Wang Linfa Date: 8/21/2022 10:48:00 AM

I think it is better to treat the three group of viruses equally as you have done for the title. It makes no difference whether we use NiV or Henipavirus in the context of the current study. Luminex-based assays targeting binding antibodies will in theory detect cross-RX antibodies in all three cases. See also comments in Methods

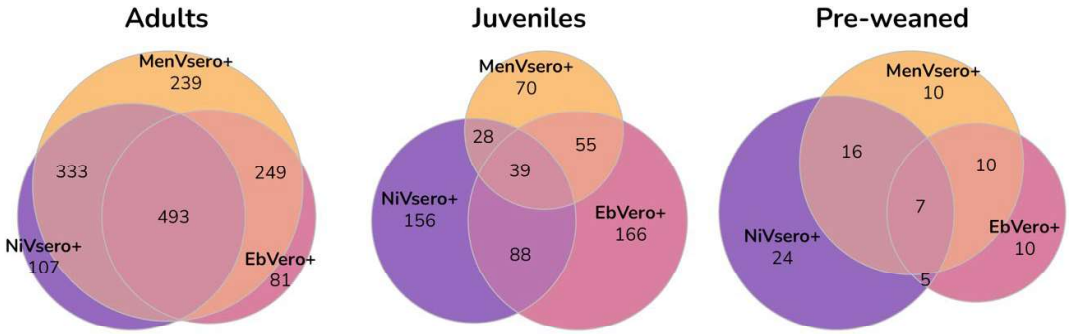

Figure 2. Detection of IgG antibodies against multiple viruses in individual *Pteropus medius*: Venn diagrams for adult, juvenile, and pre-weaned bats testing positive for antibodies against Nipah virus, filovirus, and Rubulavirus. Numbers under labels are counts of bats with only those viruses, numbers in overlapping areas represent number of bats detected with multiple viruses.

Serodynamics

Dynamics of population seroprevalence were different across the viral types. In adults, population seroprevalence against Nipah virus decreased over the study period from 78% (66%-88%) to 50% (40-62%) (Figure 3). Three periods of distinct increase in seroprevalence stood out in the inter-annual signal: early 2008, early 2009, and the second half of 2010. Nipah seroprevalence showed very weak seasonal patterns (Figure 4). (Nipah dynamics were previously reported in Epstein, et al. [34]).

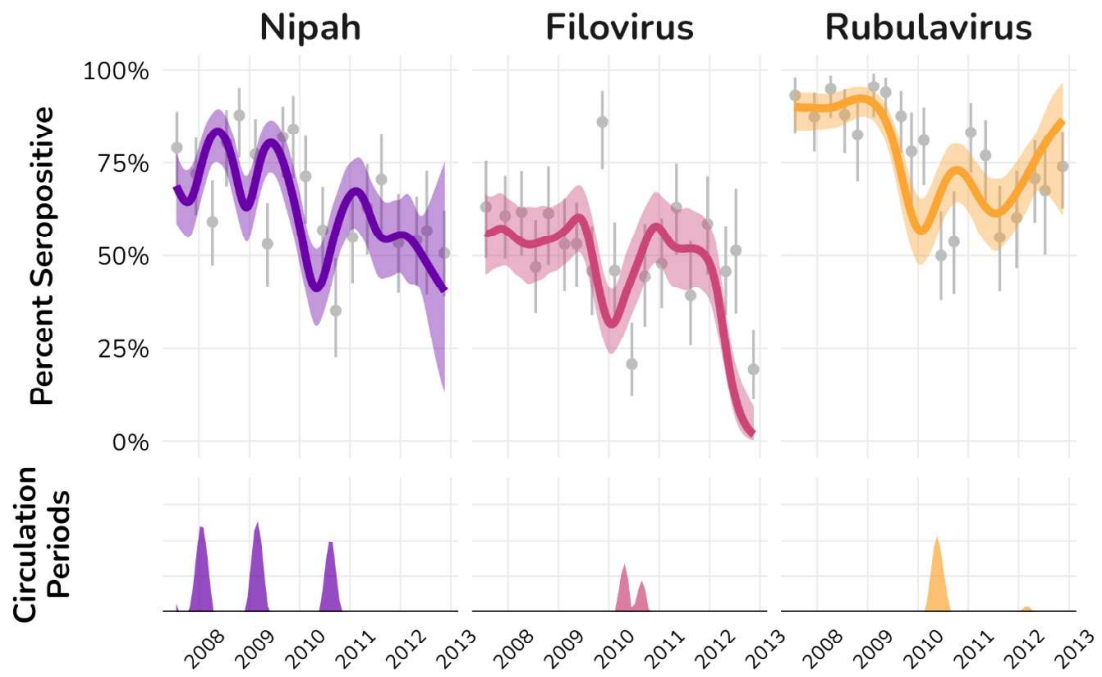

Figure 3. Inter-annual serodynamics in adult bats. Top: Measured and modeled population seroprevalence in adults over the term of the study. X-axis ticks mark the first day of the year. Grey points and bars represent measured population seroprevalence from individual sampling events on and 95% exact binomial confidence intervals. Thick lines and shaded areas represent the inter-annual (non-seasonal) spline component of a generalized additive mixed model (GAMM) of serodynamics and 95% confidence intervals for the mean pattern over time. Bottom: Circulation periods, when modeled slopes of the GAMM splines are positive for >95% of 1000 draws from the model posterior.

Page: 4

Number: 1 Author: Kevin Olival Date: 9/10/2022 4:42:00 AM

Don’t see tick marks on the axis itself, but do see the gridlines which are nice to guide the interpretation, just show up super faint and didn’t even show on my print version.

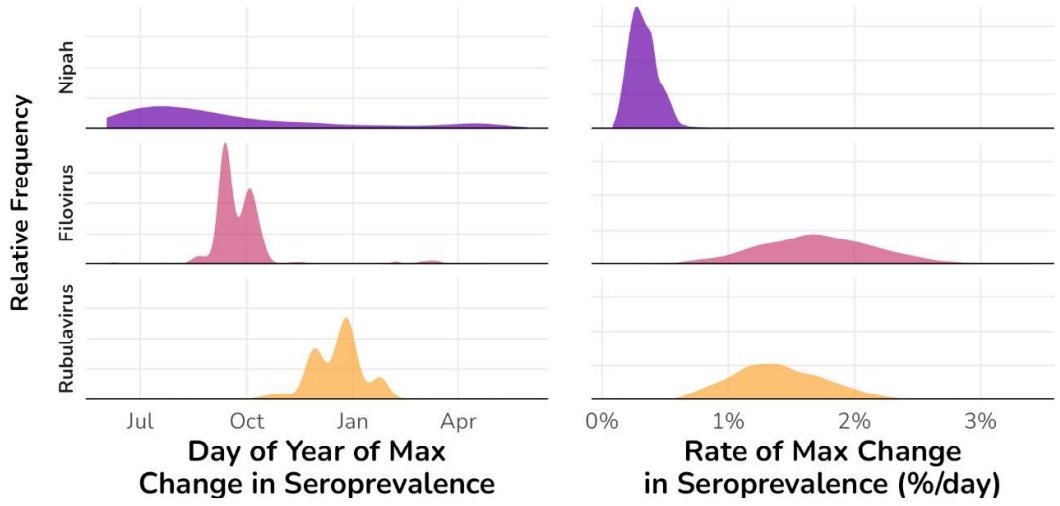

Figure 4. Timing and strength of seasonality of viral circulation in adults over five-year study. Plots show posterior densities of the date for which increase in seroprevalence is greatest (left), and the rate of increase (right), for adult bats, drawn from GAMM models of seasonality. Densities are of these quantities derived from 1000 samples of model posteriors.

Among juveniles, we examined the dynamics of each cohort over their first year. For Nipah virus, the average cohort had 44% (32%-58%) seroprevalence in June, which consistently decreased over the course of the year to 20% (10%-36%) (Fig. 5). Year-to-year dynamics varied little, with overall declining seroprevalence in all years. No part of the year exhibited regular increases in seroprevalence for juveniles; the maximum rate of increase was indistinguishable from zero (Fig. 6).

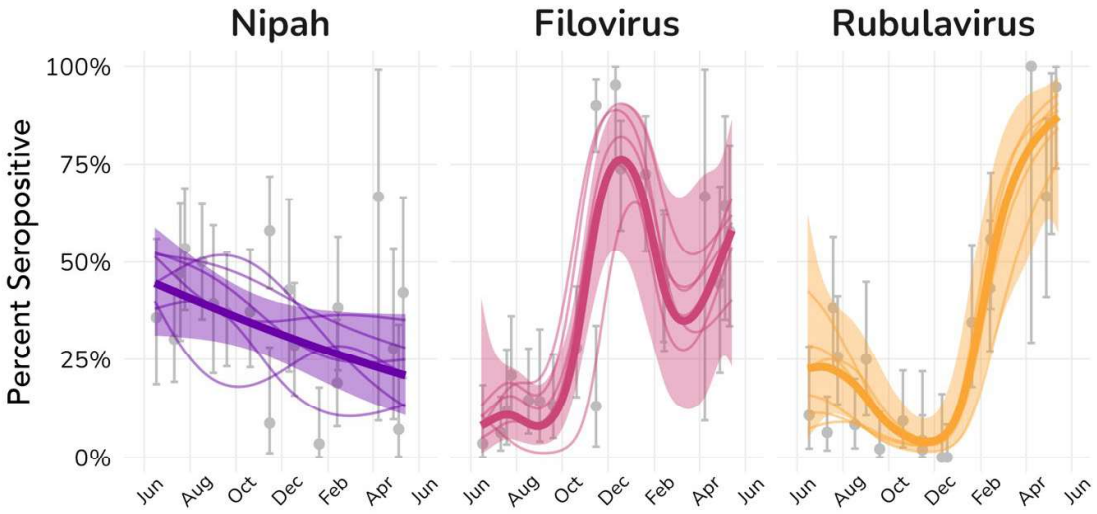

Figure 5. Serodynamics in juvenile bats in five-year study. Measured and modeled population seroprevalence in juvenile bats caught over their first year of life. Sampling events from multiple years are transposed onto a single year starting June (the earliest month free-flying young-of-the-year juveniles were captured). X-axis ticks mark the first day of each month. Grey points and bars represent measured population seroprevalence from individual sampling events on and 95% exact binomial confidence intervals. Thick lines and shaded areas represent GAMM-modeled average serodynamics across all years and 95% confidence intervals for the mean effect. Thin lines represent individual-year deviations from the average year pattern.

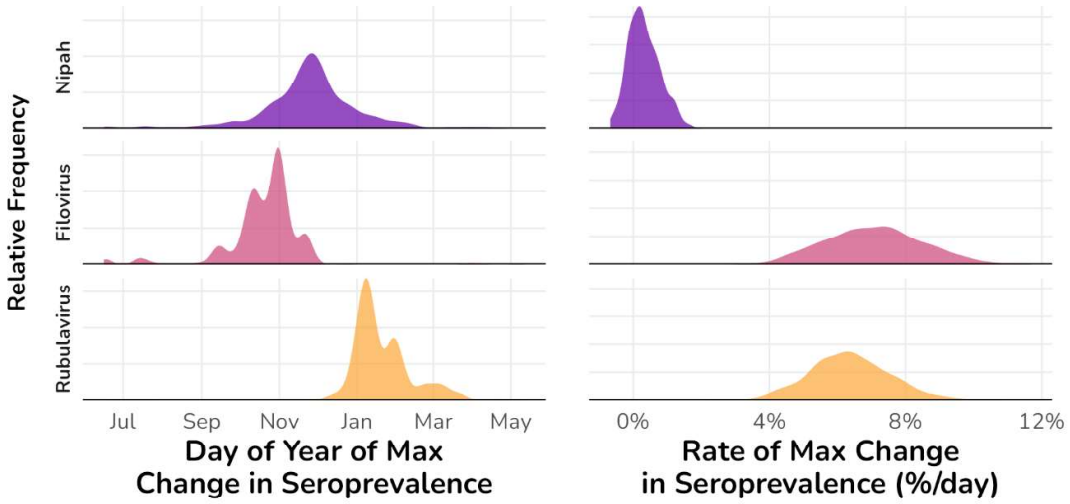

Figure 6. Timing and strength of seasonality of viral circulation in juveniles over the five-year study. Plots show posterior densities of the date for which increase in seroprevalence is greatest (left), and the rate of increase (right), for juvenile bats. Densities are of these quantities derived from 1000 samples of model posteriors.

Filovirus seroprevalence exhibited different patterns. Among adults, seroprevalence against filovirus was lower than for Nipah virus or Rubulavirus. As with Nipah virus, filovirus seroprevalence decreased over the study period, from 64% (50%-76%) to 20% (12%-30%), and had periods of distinct increases in spring/summer 2010 (concurrently with a circulation period for Nipah virus) (Fig. 3). Filovirus seroprevalence did not exhibit cyclic annual patterns in adults (Fig. 4).

Among juveniles within the average year, filovirus seroprevalence started very low in June at 8% (2%-40%), but increased to a peak of 44% (32%-58%) in juveniles approximately six months of age before declining. (Fig 5). This pattern was consistent across years; though the peak varied in size, it consistently occurred in December or January. The average date with the greatest rate of seroprevalence increase was Oct 31 (Jul 14-Nov 25), and the maximum rate of increase was 7.1%/day (4.5%/day-9.9%/day).

Rubulavirus seroprevalence had a distinct dynamic pattern. Among adults, rubulavirus seroprevalence was high at 92% (82%-98%), and remained high through the end of the period at 74% (62%-84%), though there were some temporary periods of decline (Fig 3). There were distinct periods of increasing circulation in early 2010 and early 2012 (Fig 3). Only for the rubulavirus did adult seroprevalence exhibit patterns of seasonality. Periods of increasing seroprevalence in adults exhibited strong seasonality of 1.6%/day (1.0%/day-2.4%/day) increase on peak days, with the mean peak date occurring on Dec 15 (Oct 15-Feb 1) (Fig 4).

In juveniles, seroprevalence against the rubulavirus in the average year was 22% (4%-62%) in the youngest bats in June, though in individual years this could range from 7%-43%. In all years juvenile seroprevalence declined to a low of 1.7% (0.6%, 8.3%) that occurred between Oct 9 and Dec 11, then increased starting in winter, reaching high values in yearling juveniles of 88% (56%-99%). The peak rate of increase for juvenile rubulavirus seroprevalence was 6.4%/day (4.2%-8.8%) and occurred Jan 9 (Dec 21-Mar 16) (Fig 5).

Spatial Comparisons

Seroprevalence trends in both adult and juvenile bats within the one-year studies in Chakhoria and Ramnagar broadly mirrored those in the five-year study in Faridpur (Supplementary Figure 2). In adults, Nipah virus seroprevalence increased in both Ramnagar and Chakhoria over the course of April 2010-May 2011, consistent with the period of increased seroprevalence observed in late 2010 in

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Number: 1 Author: Kevin Olival Date: 9/10/2022 4:48:00 AM

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the Faridpur. In Ramnagar, the seroprevalence in adults was 41% (24%-61%) in April 2010, rising to 60% (36%-81%) in May 2011 whilst in Chakhoria it rose from 26% (10%-48%) to 60% (36%-81%) over the same period. The trend in juveniles was as described for the five-year study.

For the filovirus, the seroprevalence in the one-year studies was higher than the reported average in the five-year study, with seroprevalence ranging between 41% (21%-64%) and 75% (51%-91%) in Ramnagar and between 48% (26%-70%) and 100% (83%-100%) in Chakhoria. A period of distinct increase in spring/summer 2010 was described in the five-year study which overlaps the timing of the one-year studies and may explain this difference in seroprevalence. In Ramnagar the serodynamics in both adults and juveniles were as described for the five-year study. In Chakhoria, there were two periods of increasing seroprevalence in adults over the course of the year. The first of these occurred in late 2010 when the adult seroprevalence increased from 48% (28%-69%) in August 2010 to 100% (83%-100%) in October 2010. The second period of increase occurred at the end of the study period in Spring 2011 with seroprevalence rising from 48% (26%-70%) to 75% (51%-91%) between early March 2011 to late April 2011.

Rubulavirus seroprevalence was high in adults throughout the one-year studies, ranging from 50% (27%-73%) to 95% (76%-100%) in Ramnagar and 29% (11%-52%) to 100% (83%-100%) in Chakhoria, with the lowest values observed in September 2010 in Ramnagar and December 2010/January 2011 in Chakhoria. These decreases were followed by rapid increases back to high seroprevalence, coincident with increasing levels in juveniles. The pattern in juveniles was as described in the five-year study, with seroprevalence estimates in yearlings in April 2011 of 80% (28%-99%) in both locations.

Comparison of the seroprevalence trends within each age group and virus between the two one-year study locations did not support spatial differences in the timing of changes in seroprevalence (Figure 7). Only for the filovirus in adults was there some evidence of a difference in trend between locations coinciding with the period of increased seroprevalence observed in adults in Chakhoria at the end of 2010.

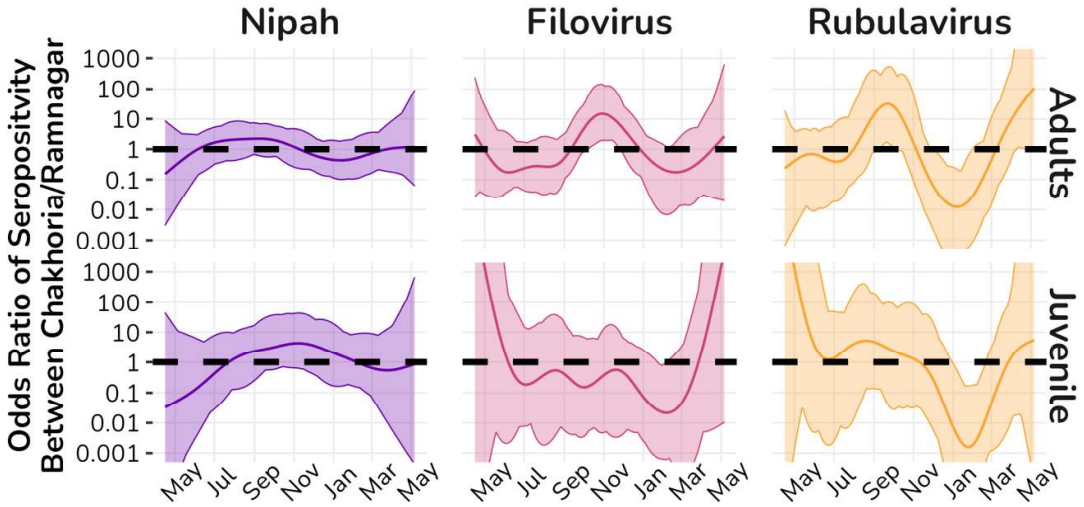

Figure 7. Comparison of splines for seroprevalence trends between locations in adults and juveniles over one-year study. Thick lines and shaded errors represent the estimated difference in trends and their associated 95% confidence intervals. The horizontal dashed line is set at zero. Where the confidence interval excludes zero, there is evidence for a significant difference in the serodynamics between the Ramnagar and Chakhoria populations over that period.

Maternal Inheritance of antibodies

We examined the relationship between mother and pup antibody titers, as measured by our Luminex assays (see Methods). This relationship varied between viruses (Figure 7). For Nipah virus,

the relationship was nonlinear due to clustering at high and low values, but largely followed a 1:1 relationship between mother and pup antibody titers. For the rubulavirus the relationship was near-linear and near 1:1. For the filovirus, though, the relationship between MFI values measured in mothers and their pups fell well below the 1:1 line, indicating pups having low inheritance of antibodies against the filovirus relative to the other two viruses.

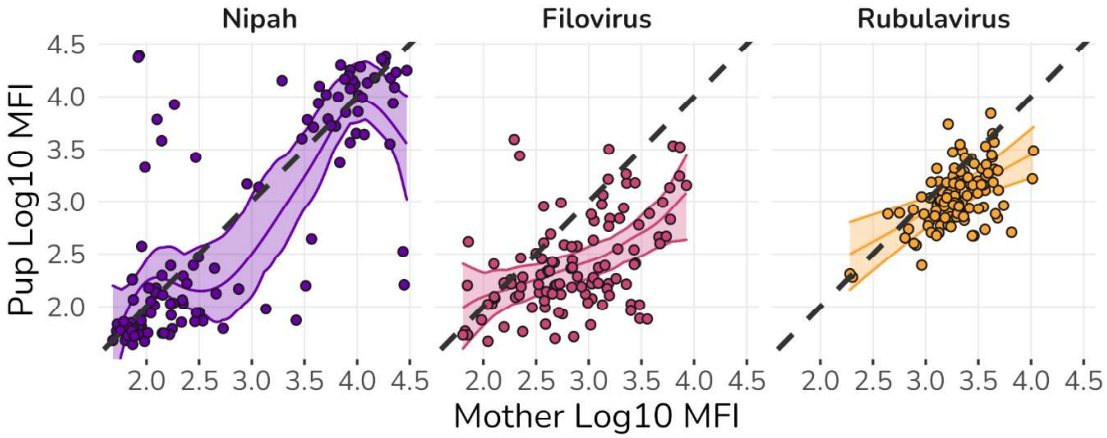

Figure 8. Maternal inheritance of antibodies. In each plot, points are measured log-MFI values for adults and juveniles in mother-pup pairs for each of the three antibody tests. Lines represent the predicted mean relationship between the two and their associated 95% confidence intervals.

3. Discussion

We found serological evidence for regular circulation of multiple viruses in *P. medius* populations in Bangladesh. These included Nipah virus, which spills over from bat populations to humans in the region, as well as a filovirus and a rubulavirus. It was common for bats to have antibodies against multiple viruses, and more common in adults than juveniles. Co-immunity patterns among pre-weaned juveniles mirrored those in adults, reflecting maternal inheritance patterns. Patterns of co-immunity were not random, with antibodies against all three virus types being positively correlated within bats.

The dynamic patterns in population seroprevalence were distinct for the three viral groups. The rubulavirus exhibited the most regular and consistent patterns. Some juveniles in these populations inherit maternal antibodies against the rubulavirus, which wane over the first six months of their life, after which a regular annual epidemic occurs in the juvenile population, causing a steep rise in juvenile seroprevalence by the end of their first year. High adult seroprevalence is likely the consequence of most bats being exposed to the disease as juveniles. When adult seroprevalence drops, as it did in 2009 to 2011, it rapidly rebounds during the same season as juveniles, indicating the same regular epidemics affect both adults and juveniles. We expect circulation and shedding of the rubulavirus to be strongest in the late winter and early spring months in these populations. Mortlock at al.[39] found irregular patterns of rubulavirus shedding in *Rousettus* bats over one year (via PCR detection in pooled urine samples), including a peak during a period of presumed antibody waning, though only within a one-year study.

An outstanding question is how the rubulavirus is maintained in the population despite high seroprevalence in inter-epidemic periods. Recrudescence is one possibility. Evidence for latent infections and recrudescence has been found for Nipah virus in *Pteropus vampyrus*[40], Hendra virus in *P. alecto and P. poliocephalus*[41], and Lagos bat lyssavirus and African henipavirus in *Eidolon helvum*[42]. Another possibility is the re-introduction of virus, or distinct viral strains, from outside bat populations. We have previously shown that bat home ranges overlapped with nearby colonies so as

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Number: 1 Author: Kevin Olival Date: 9/10/2022 4:52:00 AM

Just as example if we're changing nomenclature, this would be "how Menangle-related viruses are maintained"…

Number: 2 Author: Kevin Olival Date: 9/10/2022 4:51:00 AM

Not clear what this means?

to form a meta-population[34], findings further supported by population genetic studies of *P. medius* in Bangladesh [REF]. These metapopulation dynamics may allow for occasional infection of viruses or novel viral strains from outside bats, as has been shown to maintain Hendra virus in *Pteropus* populations[43,44].

The identity of the rubulavirus in this population is unclear. Our test was designed for Menangle virus, which has been found in multiple *Pteropus* species in Australia[45,46]. At least 11 distinct Paramyxoviruses have been found in *P. medius* in Bangladesh alone: Nipah virus and ten uncharacterized species, including six Rubulaviruses closely related to Menangle virus and the Tioman virus[9,47]. It is possible that the serological patterns observed represent antibodies against a complex of multiple Rubulaviruses, though the regular interannual patterns in seroprevalence would indicate that they are operating similarly. The development of more specific serological assays for the six known rubulaviruses found in this species would help to further disentangle these patterns.

Nipah virus serodynamics exhibited a different pattern. We found serodynamics consistent with little to no circulation amongst juveniles. Instead, each cohort of juveniles appeared to inherit some maternal antibodies - maternal inheritance was most consistent for Nipah virus in our mother-pup comparisons - and population seroprevalence declined over their first year for all cohorts. Adults experienced several outbreaks, but Nipah virus serodynamics did not exhibit any seasonal patterns, rather, circulation events occurred in irregular intervals one to two years apart. In Thailand, Nipah virus shedding in *Pteropus lylei* was found to vary in time over two years and this pattern varied by strain; Bangladesh-strain Nipah virus shedding clustered in spring months, but only over two years[28]. Human Nipah virus outbreaks in Bangladesh, on the other hand, exhibit seasonality associated with the palm-sap consumption, the most likely spillover mechanism[48].

We had repeated positive detections of filovirus antibodies, using a test designed for Ebola-Zaire virus. This is the first detection of filovirus antibodies in *P. medius* in Bangladesh, though they have been found in *R. leschenaultii* in the country[49]. Interestingly, *P. medius* and *R. leschenaultii* have been observed sharing a common food resources, i.e. data palm sap [REF], which represents a pathway for cross-species EboV transmission and also a potential route of exposure to humans. There have been several recent findings of new Ebola-like filoviruses in bats extending across Africa and Asia, and it's likely the virus we are detecting is not Ebola-Zaire but rather a novel and related virus. New bat filovirus discoveries include Bombali virus in Sierra Leone[50], and Měnglà virus in *Rousettus* bats in China[51]. Ebola-Reston virus was found in multiple bat species (*M. australis*, *C. brachyotis* and *Ch. plicata*) in the Philippines[52]. Marburg virus and Ebola-Zaire virus may have been detected in Sierra Leone and Liberia (unpublished,[53,54]). Serological evidence of filoviruses in bats has been found in multiple bat species in Central[12,55] and Western[56] African countries, Singapore [57], China[58], as well as Trinidad[59]. Our finding here adds to the evidence of broad host and geographic host ranges for filoviruses.

Several components of the serological patterns of filovirus antibodies are of interest. Young juveniles were almost all seronegative, and seroprevalence rose over the course of the first six months all years, then declined in the latter half of the year. Adult seroprevalence remained steady despite these regular juvenile outbreaks near 50% over the course of the five-year study. Adult seroprevalence against the filovirus also did not exhibit regular annual cycles, though the one period of detectable increase, in mid-2010, coincided with one of the regular seasonal periods of increase among juveniles. One possible hypothesis explaining this pattern is that the filovirus may exhibit low transmissibility in this system, only circulating when seroprevalence is near zero in young juveniles. It is also possible that bats may exhibit weak or waning filovirus antibody response but remain immune. In *R. aegyptiacus*, infected with the Marburg virus, antibody waning post-infection was found to be rapid, but bats retained protective immunity even with very low antibody titers[60,61]. If a similar mechanism occurs in this population, this could explain low seroprevalence in adults despite regular apparent periods of circulation in juveniles. The regular drop of seroprevalence in juveniles following the peak is consistent with this explanation.

However, if adults remain immune to the filovirus despite low antibody titers, this immunity does not appear to transfer to juveniles, as seroprevalence is near-zero among newborns and

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Number: 1 Author: Kevin Olival Date: 9/10/2022 4:57:00 AM
Olival et al 2019. DOI: 10.1111/mec.15288

Number: 2 Author: Kevin Olival Date: 9/10/2022 5:10:00 AM
Khan SU, Gurley ES, Hossain MJ, Nahar N, Sharker MAY, et al. (2012) A Randomized Controlled Trial of Interventions to Impede Date Palm Sap Contamination by Bats to Prevent Nipah Virus Transmission in Bangladesh. PLoS ONE 7(8): e42689. doi:10.1371/journal.pone.0042689

Number: 3 Author: Kevin Olival Date: 9/10/2022 5:15:00 AM
I wouldn't site news reports and also say "may have been detected" perhaps delete this?

Number: 4 Author: Hume Field Date: 8/24/2022 9:59:00 AM
And recently in Australia.
Barr et al. J Gen Virol. 2022 Aug;103(8).
doi: 10.1099/jgv.0.001785.

Number: 5 Author: Kevin Olival Date: 9/10/2022 6:15:00 AM
Random thought, and pretty speculative, but could expression of some endogenous viral elements cause a pattern like this (or any of the juvenile patterns we observed, as opposed to a strictly maternal antibody inheritance model)? I guess if so, all individuals would show antibodies.

Number: 6 Author: Kevin Olival Date: 9/10/2022 5:16:00 AM
I think this 2010 finding is an interesting and understated finding of ours… namely that all 3 viruses showed significant viral circulation during this period. What inter-annual dyanmics could have led to this? Food shortage (ala some of Raina's work (a new paper coming out soon on this), other environmental or physiological stressors?

Number: 7 Author: Kevin Olival Date: 9/10/2022 5:18:00 AM
We don't know much about this in bats I guess (maybe for Marburg) but Filos in people aren't spread very easily, so some intrinsically lower Rt for filos over other bat viruses?

circulation in juveniles begins soon after birth. The relationship of pup/dam MFI found for the filovirus antibodies suggests that there may be differential inheritance of antibodies and possibly differential maternally derived immunity. If pups derive weaker immunity from dams than for other viruses, this may provide another mechanism for maintenance of the virus. It's also possible that patterns in filovirus antibody detection are an artifact of our test, specific to Ebola-Zaire virus, and that temporal trends are in part reflective of differential cross-reaction with viruses in this bat population. However, while this may modify estimates of overall seroprevalence, the differential pup/dam MFI relationship, and consistent rise in seroprevalence among young juveniles, would require the test to exhibit differential sensitivity by age which we believe is unlikely.

Serodynamics in our two one-year studies were broadly similar to those found in the five-year study. We found weak evidence for differences in serodynamics between sites for the filovirus in adults, and the rubulavirus for adults and juveniles. However, limited sample numbers and the shorter study period limit our ability to generalize about seasonal patterns. In a concurrent study[34], we found that flying foxes home ranges in Bangladesh extended an average of 50 km from their roosts. The roosts at Chakoria and Ramnagar, the sites of the one-year studies, are approximately 225 km apart. Thus, we would not expect the dynamics of these sites to be strongly coupled. It is plausible that inter- and even intra-annual dynamics of these viruses could be similar if weak coupling and/or environmental factors drive some of the dynamics. More detailed and longer-term studies at multiple sites in parallel are needed to better understand spatiotemporal variation in viral circulation.

Regular co-circulation of these viruses indicates the potential for viral interactions to affect host-viral dynamics. Viral interactions in hosts – whether direct during co-infection or indirect from infection in different periods – can enhance or suppress pathogen mortality, fecundity, or transmission, via competition for host nutritional or cellular resources, or via immune-mediation involving cross-immunity or antibody-dependent enhancement, and these interactions structure viral communities[26]. These interactions can have complex, even opposite effects when scaled to the population[27]. Here, we found positive correlations between serostatus against three different viruses. Notably, all three viruses had a significant increase in viral circulation in adults in mid-2010. External factors may explain these inter-annual viral circulation patterns, for example changes in population density and/or physiological stress due to climate or nutrition availability[62] could simultaneously affected transmission of multiple viruses.

While rich observational serological data reveal these patterns, further studies are required to characterize these viruses and their effects on the host population and potential for spillover, as well as the degree and mechanisms of interactions. Our model of longitudinal, serological data was able to infer periods of increased viral circulation – providing a tool to more precisely target optimal sub-populations and times to sample bats or other wildlife reservoirs to detect viral shedding, isolate viruses, and potentially capture co-infected hosts.

Extended longitudinal sampling also sheds light on viral dynamics not found in short-term studies. One- to two-year studies can identify temporal patterns in serology or viral prevalence and shedding, but establishing patterns or variation in seasonality requires extended, multi-year sampling. In our case, while Nipah virus outbreaks occurred in the same season in two years, it was apparent from the longer time series that there was no distinct seasonal pattern in dynamics. The consistency of the filovirus and rubulavirus patterns required repeated years of sampling to establish. Similarly, we were able to understand these patterns far better by separating juvenile and adult patterns, which we would be unable to distinguish in pooled samples. Future work should examine these inter-annual patterns in viral dynamics with ecological correlates and intrinsic health parameters for bats in the population

While such extended individual-capture longitudinal studies are resource-intense, understanding the joint circulation of multiple viruses can be accomplished via multiplex immunoassays such as those used here. The continuous measures from these assays also have the potential to identify key patterns such as the differential inheritance of filovirus antibodies we identified here. Interpretation of these values is challenging and the relationship between immune

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Number: 1 Author: Kevin Olival Date: 9/10/2022 6:18:00 AM
Do we have other Filos on the Luminex panel we can compare the EBOV-Z data with? If so, maybe for next paper?

Number: 2 Author: Kevin Olival Date: 9/10/2022 6:19:00 AM
Seems to be what we're implying here, but feel free to modify.

Number: 3 Author: Kevin Olival Date: 9/6/2022 12:19:00 AM
This struck me as a really interesting finding I think we should make a bigger deal about. As per Fig 3, for two of our viruses this period in 2010 was the only with significant virus circulation. Made some edits here.

Number: 4 Author: Kevin Olival Date: 9/6/2022 12:19:00 AM
This struck me as a really interesting finding I think we should make a bigger deal about. As per Fig 3, for two of our viruses this period in 2010 was the only with significant virus circulation.

Number: 5 Author: Kevin Olival Date: 9/6/2022 12:18:00 AM
Also. Plowright, R. K. et al. Reproduction and nutritional
stress are risk factors for Hendra virus infection in little
red flying foxes (Pteropus scapulatus). Proc. Biol. Sci.
275, 861–869 (2008).
Raina has a new paper coming out around now that measures food availability and links w HeV shedding (as shown at BatID meeting)

status, antibody titer, and measured fluorescence is complex[32], but they have much potential to shed light on mechanistic drivers of disease circulation.

Methods

Field collection

We conducted a five-year longitudinal study sampling *P. medius* bats quarterly and two one-year studies in different locations, sampling monthly. All capture and sampling methods were approved by Tufts University IACUC protocol #G929-07 and icddr,b animal ethical review (protocol 2006-012). For the five-year study, we sampled from a roost complex near Faridpur, Bangladesh, as previously described in Epstein, et al. [34] The area of the roost complex consists of patchy forest interspersed with agrarian human settlements and large rice paddies. Sampling occurred from July 2007 to November 2012 approximately every three months. The bat colony regularly shifted amongst roosts within the 80km^2 roost complex over the period of the study. Sampling occurred at the largest occupied roost found each quarterly sampling trip, sampling at different roosts within the complex across consecutive sampling nights if required to capture a sufficient number of individuals.

Approximately 100 bats were captured at each sampling event, which lasted 7-10 days. We captured bats with a 10x15m mist net between 11pm and 5am each night as bats returned from foraging until the count of 100 was reached.

In the one-year longitudinal studies, sampling was undertaken in two roost complexes approximately 225km apart in Ramnagar and Chakhoria, Bangladesh between April 2010 and May 2011. Monthly sampling of approximately 40 bats in each location was performed to obtain data at a finer temporal scale. Details of collection are otherwise as described for the five-year study.

For each study, we recorded each bat's age class, reproductive status, weight, size, and body condition. We collected up to 3.0 mL of blood from each bat's brachial vein into serum tubes with serum clot activator (Vaccutainer, USA). Blood tubes were stored vertically on ice packs in a cold box and serum was allowed to separate overnight. Serum was drawn from the tube after 24 hours and placed in a screw-top cryovial (Corning, USA) and stored in a liquid nitrogen dewar (Princeton Cryogenics, NJ, USA).

As part of a larger study to detect viral RNA, we also collected urine, rectal, and oropharyngeal swabs and pooled urine samples from under colonies. Further details can be found in Epstein, et al. [34]

Serological Assays

Sera from the longitudinal studies were sent to the Australian Animal Health Laboratory and gamma irradiated upon receipt. We used a bead-based microsphere assay that specifically detects antibodies to the soluble attachment glycoproteins[57,63,64] for a panel of viruses. Beads coated with each protein were mixed with sera at a dilution of 1:100. Biotinylated Protein A/G and Streptavidin-PE were then used to detect bound antibody. Beads were interrogated by lasers in a BioRad BioPlex machine and the results recorded as the Median Fluorescent Intensity (MFI) of 100 beads. We report here results for Nipah, Ebola-Zaire, and Menangle, the only three for which we established regular positive results.

While the Nipah virus has been detected in this population[65] and the specificity of the Nipah test is well-established [66], the Ebola-Zaire and Menangle virus tests are cross-reactive with other Ebola and rubulavirus species[67,68]. Thus we refer to these as tests for filovirus and Rubulavirus.

Data Analysis

We determined individual bat serostatus using Bayesian mixture models[69] fit on pooled data across all three longitudinal studies, calculating a cutoff of log-MFI as the point of equal probability between the smallest and second-smallest cluster of equal distributions for each assay.

To determine correlations of serostatus in bats across the three viruses, we fit a Bayesian multivariate probit model[70,71] which allows estimation of the joint outcomes (serostatus against each

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Do we give GPS lat/long anywhere, even if rounded up some decimal points?

Number: 2 Author: Wang Linfa Date: 8/21/2022 10:50:00 AM

I am not 100% sure about this. All serological tests (the NiV test included) will detect cross-RX antibodies from related viruses. For NiV, you can argue that longitudinal surveillance has NOT detected any related viruses in the study populations, but we can't say that the Ab test is specific to NiV.

virus) and the correlation between the outcomes. We included age and sex variables to account for these effects on serostatus.

To examine time-varying changes in population seroprevalence, we fit binomial generalized additive mixed models (GAMMs)[72,73] to the time-series of serostatus measurements. For the five-year study, we fit separate models for adults and juveniles and for each immunoassay. For adults, we fit a model for seroprevalence dynamics over the course of the whole study. This treats the adult population as a single unit, though individuals within the population may turn over via migration, death and recruitment. We included both long-term and annual cyclic components for the multi-year time series of measurements. For juveniles, we treated each year's cohort of new recruits as separate populations with commonalities in overall first-year dynamics. The dynamics of each year's juvenile cohort were modeled as random-effect splines, deviating from an overall mean splines for juveniles over their first year. To estimate periods of peak viral circulation within the population, we calculated the derivatives of model-derived seroprevalence over time. We sampled these from GAMM posterior distributions and classified periods with >95% of samples with positive derivatives - that is, increasing population seroprevalence - as periods of viral circulation. We also calculated strength and timing seasonality for each virus as the maximum rate of seroprevalence increase and the date at which this maximum occurred, again sampling these values from the model posterior, and calculating mean and high-density posterior interval (HDPI) values.

For the one-year studies, we fit models primarily to detect differences in time-varying changes between sites. We fit binomial generalized additive models (GAMs) to the monthly serostatus measurements with separate models for each immunoassay. We included a separate, fixed-term spline in each model for age (adult or juvenile) and location (Chakhoria or Ramnagar). The trends in serodynamics for each virus in each age group were compared between locations to test for spatial differences[74]. Juveniles identified as being from the previous year's cohort were excluded from the analysis.

To examine patterns of maternal inheritance, we used GAMs to fit a relationship of log-MFI between adult lactating females and their attached pups for each viral assay. We limited these to data from the five-year longitudinal study.

Data Availability: Raw data from this study and reproducible code used to perform analyses is available at the Zenodo Scientific Archive [Zenodo DOI to be generated] and also on GitHub at https://github.com/ecohealthalliance/bangladesh-bat-serodynamics

Author Contributions: Conceptualization: N.R. and J.H.E..; methodology, N.R., L-F.W and J.H.E.; software, N.R. and S.H.; formal analysis, N.R. and S.H.; investigation, J.H.E., A.I. and L-F.W….; resources, X.X.; data curation, N.R and J.H.E; writing—original draft preparation, N.R. J.H.E., and S.H.; writing—review and editing, H.E.F., K.J.O., X.X.; visualization, N.R. supervision, N.R. and J.H.E.; project administration, J.H.E.; funding acquisition, P.D., L-F.W and J.H.E

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All co-authors please insert yourselves as appropriate here. A summary of contribution types can be found here: https://img.mdpi.org/data/contributor-role-instruction.pdf

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Co-circulation dynamics of henipaviruses, filoviruses and rubulaviruses in a bat population

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Abstract: Bats are hosts for a variety of viruses, some with potential to cause human disease. *Pteropus medius* are fruit bats that live in close association with humans in Bangladesh and are a known source of Nipah virus and other viruses of unknown pathogenic risk. Here we report on dynamics of seroprevalence against multiple viruses in a population of *P. medius* in Bangladesh over five years. We found evidence of regular circulation of Nipah virus, a rubulavirus, and a filovirus. Nipah virus circulated primarily among adult bats, with no seasonal patterns. The rubulavirus exhibited annual cycles in juveniles and near universal seroprevalence in adults. The filovirus exhibited annual cycles and antibody waning in juveniles and little circulation but lower seroprevalence amongst adults. We found no evidence for spatial differences in Nipah virus dynamics, but weak evidence for differences with other viruses. Host-viral interactions result in very different viral circulation patterns in the same individual bats and environments.

Keywords: bats; Nipah virus; filovirus; Rubulavirus; *Pteropus medius*; Bangladesh; serology; disease dynamics; generalized additive models

Introduction

Bats have been associated with a large diversity of viruses, several of which have been linked to human epidemics [1-3]. The discovery of Severe Acute Respiratory Syndrome (SARS)-related coronaviruses (CoVs) in Chinese horseshoe bats (*Rhinolophus spp.*) in 2004 and subsequent discoveries of related viruses[4,5] including close relatives of SARS-CoV-2[6], along with studies of other high consequence pathogens such as Ebola virus, Marburg virus, Nipah virus, Hendra virus have led to questions about whether and why bats were disproportionately represented among mammals as reservoirs for zoonotic viruses[1,2,7].

Bat species can carry diverse viruses which circulate simultaneously within single populations [8-11]. *Rousettus aegyptiacus* is a natural reservoir of Marburg virus, and IgG antibodies against Ebola Zaire virus have also been detected in this bat in Central Africa, suggesting that it may play a role in the circulation of multiple filoviruses[12,13]. In addition to these filoviruses, a novel zoonotic and pathogenic paramyxovirus, Sosuga virus, was also found in Ugandan *R. aegyptiacus* populations[14].

Summary of Comments on Email 5 - Attachment 4 - Ross-et-al_bangladesh-bats-cocirculation-serology_2022-08-11.pdf

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Eidolon helvum, another pteropodid bat with a broad geographic range in Africa, has been found to carry a lyssavirus, a henipavirus, and several other viruses of unknown pathogenicity[15]. Various surveillance efforts have found diverse viruses from with the same viral family in various bat species[5,16,17], and other studies have used metagenomic approaches to look broadly at viral diversity within individual bat species[9,11,18-21].

Studies of viral diversity in a single taxonomic host group can inform evolutionary history of viruses and their relationships to specific hosts[6,22] and inform public health strategies[23]. However, detection of a zoonotic virus in an animal host is insufficient to determine whether the associated host species is a natural reservoir for the virus, or to characterize the demographic, physiological, or abiotic factors that may influence the timing of viral shedding and therefore risk of spillover to other species, including domestic animals and humans[24]. For these, more detailed demographic and longitudinal studies are required, especially in cases of multiple viruses, as direct or indirect interactions between multiple pathogens may affect disease presence, prevalence, and dynamics[25-27]. Some longitudinal studies of zoonotic viruses in bats have provided insight into the timing of viral infection and shedding, generally of single viruses. Nipah virus in *Pteropus lylei* in Thailand has been observed to have annual shedding patterns [28], whereas Hendra virus, which is closely related to Nipah virus and is carried by multiple pteropid bat species in Australia, has asynchronous and non-periodic cycles which appear to be influenced by localized factors such as specific bat species abundance and climatic factors [29]. Bi-annual pulses of Marburg virus shedding were observed in Uganda, coinciding with synchronous birth pulses in *R. aegyptiacus*[30]. Serological data is often valuable in understanding disease dynamics and transmission[31-33], especially in cases where direct detection and incidence rates of viruses are low[34].

Pteropus sp. are reservoirs of henipaviruses, a group of mostly zoonotic, lethal neurotropic viruses that include Nipah, Hendra, Cedar, Ghana and Mojiang virus[35-37]. In Bangladesh and India, *P. medius* is a reservoir of Nipah virus, which has spilled over to human populations repeatedly[38]. Viruses from eight other viral families have been detected in *P. medius* in Bangladesh[9]. The epidemiology of these other viruses is far less characterized, and little is known about their interactions or zoonotic potential.

Here, we report on the dynamics of seroprevalence against three types of viruses - Nipah virus, a filovirus, and a rubulavirus - circulating simultaneously in *P. medius* populations in three sites in Bangladesh. We conducted a longitudinal study over a period of five years, and two shorter, one-year studies for comparative seasonal data. These studies enable us to develop a detailed picture of the seasonal and demographic serological patterns, and infer the extent and periodicity of viral circulation. We find that Nipah virus circulates primarily among adults without distinct seasonality, while the rubulavirus exhibits strong annual dynamics characteristic of juvenile epidemics driven by waning maternal antibodies. The filovirus, the first detected in *P. medius* in Bangladesh, also circulated in juveniles but exhibited distinct patterns antibody waning and limited maternal inheritance.

2. Results

Bat dynamics and demographics

We conducted a five-year longitudinal study sampling *P. medius* bats quarterly from a roost complex in the vicinity of Faridpur, Bangladesh, and also two parallel one-year studies sampling bats monthly from roosts in Chakhoria and Ramnagar, Bangladesh. In the five-year study, we sampled and tested serology of 1,886 bats: 1,224 adults, 623 free-flying juveniles, and 39 weaning juveniles (captured attached to adult females) over 19 sampling events (Figure 1). During the-one-year studies, 919 bats were sampled: 435 in Chakhoria (251 adults, 144 free-flying juveniles and 40 weaning juveniles) and 484 in Ramnagar (277 adults, 164 free-flying juveniles and 43 weaning juveniles (Figure S1). Nearly all juveniles (as determined by examination of maturation of sex organs) were 14 months old or less and could be assigned to a birth cohort based on size. Pregnant and juvenile bats were captured more frequently during the late spring and summer months. Mother-pup pairs were all

captured during April and May in the five-year study whilst in the one-year-studies a small number were also captured in June and July. Pregnant females were captured between November and April/May. Lactating females were found between April and July.

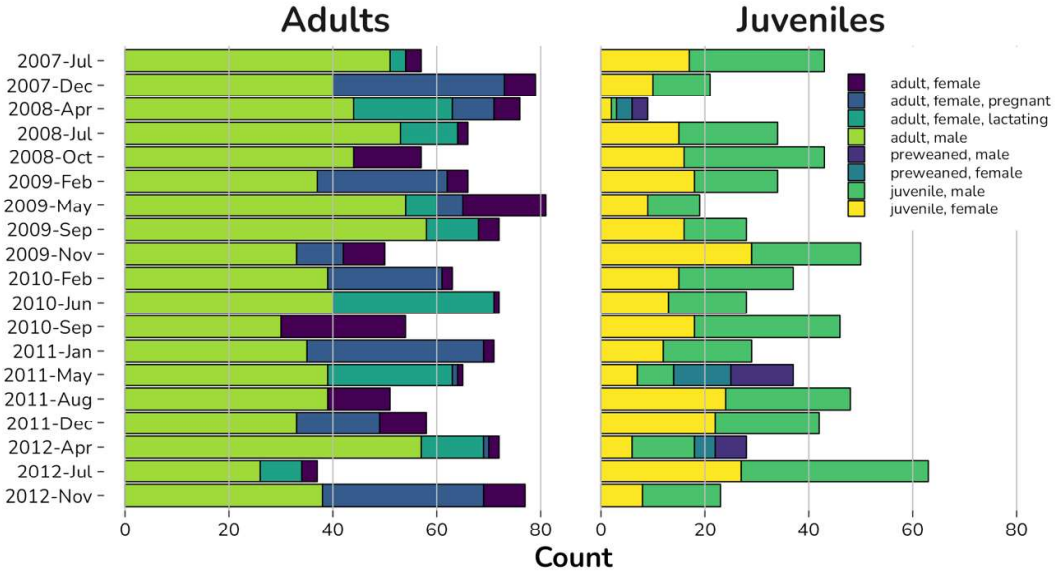

Figure 1. Demographics of bats captured in longitudinal sampling. Pregnant females were captured in months from November to June, lactating females were captured in months from April to July. Females with pre-weaning juveniles attached were found from April to May.

Patterns of Immunity and Co-immunity

We found bats were seropositive for antibodies against the Nipah virus (NiVsero+), a filovirus (EbVsero+), and a rubulavirus (MenVsero+).. Among adult bats, antibodies against the rubulavirus were common; 1,314 of 1752 adult bats were MeNsero+. Antibodies against Nipah virus or filovirus were less common – 1,031 were NiVsero+ and 921 were EbVsero+. Among the 921 juveniles, 192 were MenVsero+, 311 were NiVsero+ and 348 were EbVsero+. Of the 122 pre-weaned juveniles, 43 were MenVsero+, 52 were NiVsero+, and 32 were EbVsero+.

Co-exposure to multiple viruses was common (Figure 2). Over half (1,173) of the 1752 adults had antibodies against more than one of the three viruses, and 493 had antibodies against all three. Among the 921 juveniles, 210 had antibodies against more than one, and 39 had antibodies all three. 38 of 122 pre-weaned juveniles had more than one of the three antibodies, with seven having all three.

We found correlations between serostatus between all three pairs of viruses in a model accounting for bat age, sex, and study location, with all viruses tending to co-occur with each other more than would be expected than if they were distributed independently among bats. Nipah virus and filovirus antibodies had a standardized covariance of 0.13 (95% posterior interval 0.08-0.20). Nipah virus and rubulavirus antibodies were also more likely to occur together, with a covariance of 0.15 (0.09-0.21). Filovirus and rubulavirus antibodies had a covariance of 0.23 (0.17-0.29).

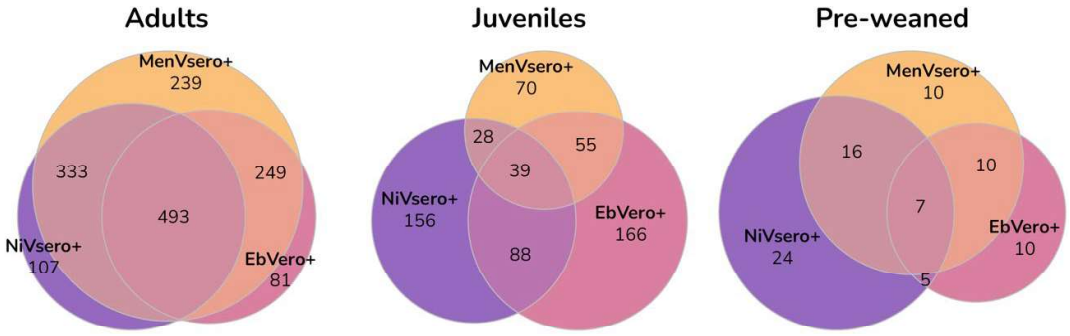

Figure 2. Detection of IgG antibodies against multiple viruses in individual *Pteropus medius*: Venn diagrams for adult, juvenile, and pre-weaned bats testing positive for antibodies against Nipah virus, filovirus, and Rubulavirus. Numbers under labels are counts of bats with only those viruses, numbers in overlapping areas represent number of bats detected with multiple viruses.

Serodynamics

Dynamics of population seroprevalence were different across the viral types. In adults, population seroprevalence against Nipah virus decreased over the study period from 78% (66%-88%) to 50% (40-62%) (Figure 3). Three periods of distinct increase in seroprevalence stood out in the inter-annual signal: early 2008, early 2009, and the second half of 2010. Nipah seroprevalence showed very weak seasonal patterns (Figure 4). (Nipah dynamics were previously reported in in Epstein, et al. [34]).

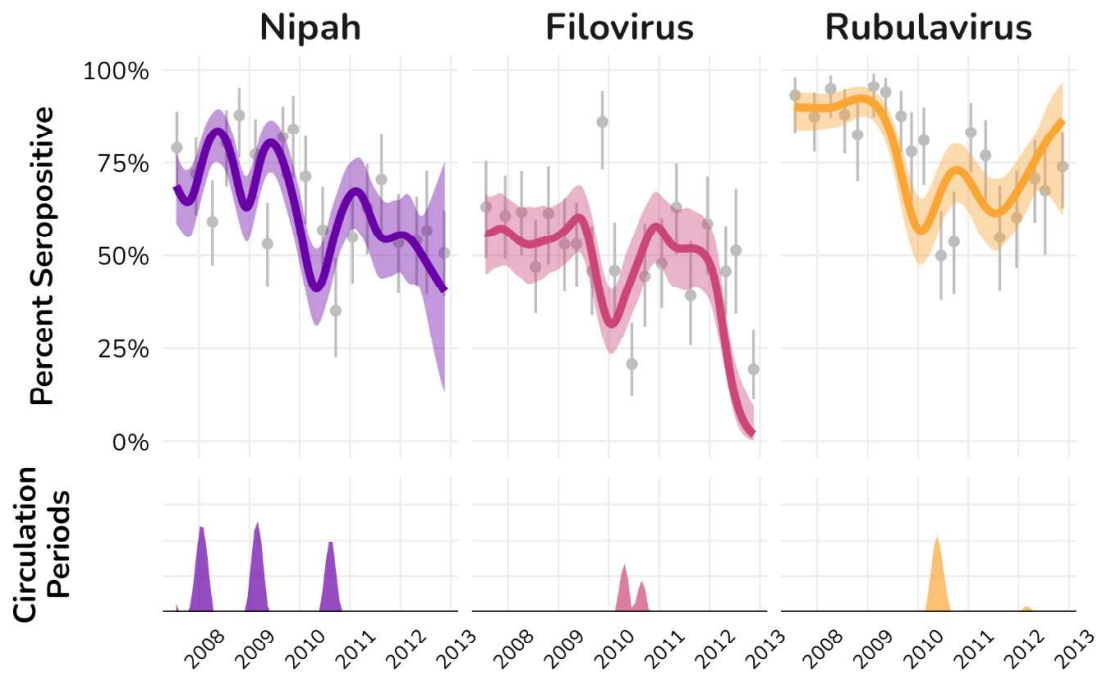

Figure 3. Inter-annual serodynamics in adult bats. Top: Measured and modeled population seroprevalence in adults over the term of the study. X-axis ticks mark the first day of the year. Grey points and bars represent measured population seroprevalence from individual sampling events on and 95% exact binomial confidence intervals. Thick lines and shaded areas represent the inter-annual (non-seasonal) spline component of a generalized additive mixed model (GAMM) of serodynamics and 95% confidence intervals for the mean pattern over time. Bottom: Circulation periods, when modeled slopes of the GAMM splines are positive for >95% of 1000 draws from the model posterior.

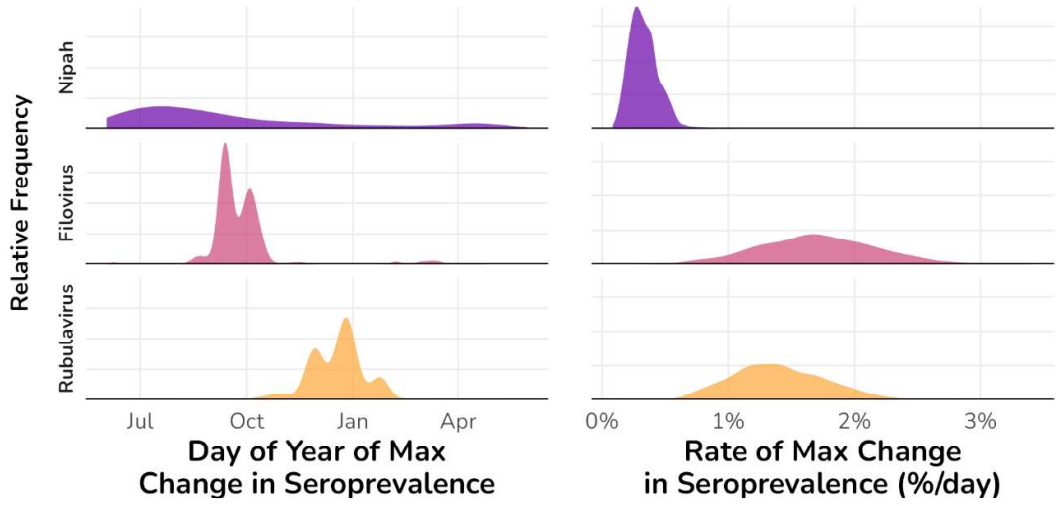

Figure 4. Timing and strength of seasonality of viral circulation in adults. Plots show posterior densities of the date for which increase in seroprevalence is greatest (left), and the rate of increase (right), for adult bats, drawn from GAMM models of seasonality. Densities are of these quantities derived from 1000 samples of model posteriors.

Among juveniles, we examined the dynamics of each cohort over their first year. For Nipah virus, the average cohort had 44% (32%-58%) seroprevalence in June, which consistently decreased over the course of the year to 20% (10%-36%) (Fig. 5). Year-to-year dynamics varied little, with overall declining seroprevalence in all years. No part of the year exhibited regular increases in seroprevalence for juveniles; the maximum rate of increase was indistinguishable from zero (Fig. 6).

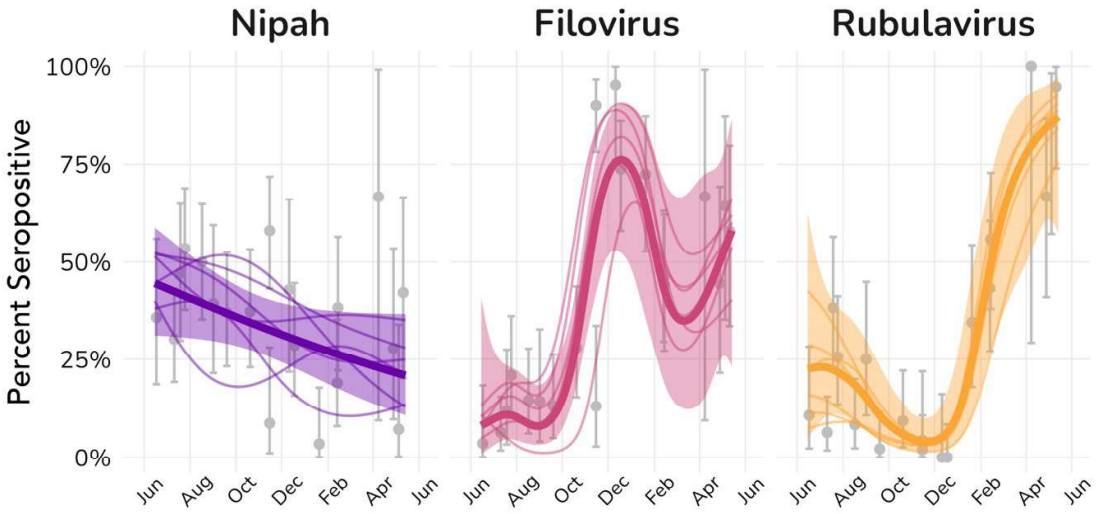

Figure 5. Serodynamics in juvenile bats in five-year study. Measured and modeled population seroprevalence in juvenile bats caught over their first year of life. Sampling events from multiple years are transposed onto a single year starting June (the earliest month free-flying young-of-the-year juveniles were captured). X-axis ticks mark the first day of each month. Grey points and bars represent measured population seroprevalence from individual sampling events on and 95% exact binomial confidence intervals. Thick lines and shaded areas represent GAMM-modeled average serodynamics across all years and 95% confidence intervals for the mean effect. Thin lines represent individual-year deviations from the average year pattern.

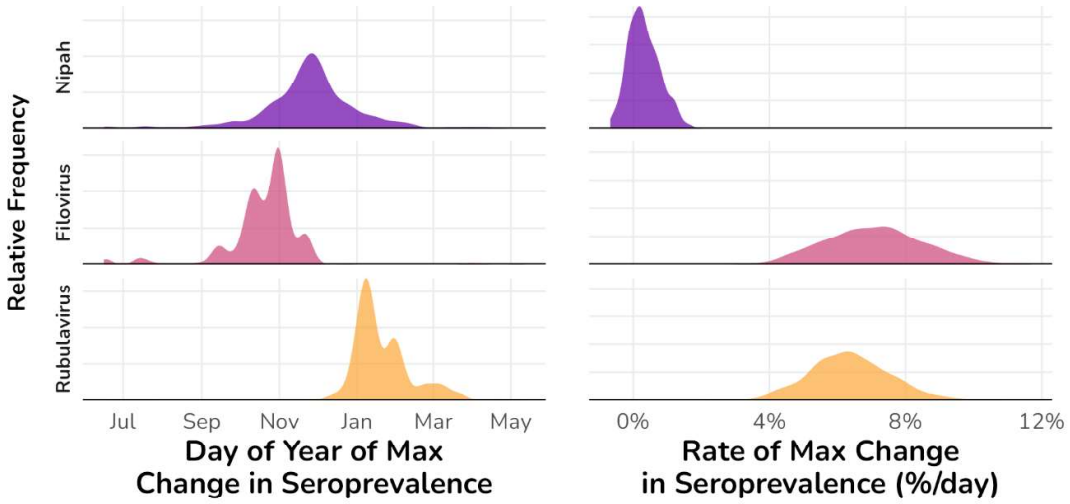

Figure 6. Timing and strength of seasonality of viral circulation in juveniles over the five-year study. Plots show posterior densities of the date for which increase in seroprevalence is greatest (left), and the rate of increase (right), for juvenile bats. Densities are of these quantities derived from 1000 samples of model posteriors.

Filovirus seroprevalence exhibited different patterns. Among adults, seroprevalence against filovirus was lower than for Nipah virus or Rubulavirus. As with Nipah virus, filovirus seroprevalence decreased over the study period, from 64% (50%-76%) to 20% (12%-30%), and had periods of distinct increases in spring/summer 2010 (concurrently with a circulation period for Nipah virus) (Fig. 3). filovirus seroprevalence did not exhibit cyclic annual patterns in adults (Fig. 4).

Among juveniles within the average year, filovirus seroprevalence started very low in June at 8% (2%-40%), but increased to a peak of 44% (32%-58%) in juveniles approximately six months of age before declining. (Fig 5). This pattern was consistent across years; though the peak varied in size, it consistently occurred in December or January. The average date with the greatest rate of seroprevalence increase was Oct 31 (Jul 14-Nov 25), and the maximum rate of increase was 7.1%/day (4.5%/day-9.9%/day).

Rubulavirus seroprevalence had a distinct dynamic pattern. Among adults, rubulavirus seroprevalence was high at 92% (82%-98%), and remained high through the end of the period at 74% (62%-84%), though there were some temporary periods of decline. There were distinct periods of increasing circulation in early 2010 and early 2012. Only for the rubulavirus did adult seroprevalence exhibit patterns of seasonality. Periods of increasing seroprevalence in adults exhibited strong seasonality of 1.6%/day (1.0%/day-2.4%/day) increase on peak days, with the mean peak date occurring on Dec 15 (Oct 15-Feb 1)

In juveniles, seroprevalence against the rubulavirus in the average year was 22% (4%-62%) in the youngest bats in June, though in individual years this could range from 7%-43%. In all years juvenile seroprevalence declined to a low of 1.7% (0.6%, 8.3%) that occurred between Oct 9 and Dec 11, then increased starting in winter, reaching high values in yearling juveniles of 88% (56%-99%) The peak rate of increase for juvenile rubulavirus seroprevalence was 6.4%/day (4.2%-8.8%) and occurred Jan 9 (Dec 21-Mar 16).

Spatial Comparisons

Seroprevalence trends in both adult and juvenile bats within the one-year studies in Chakhoria and Ramnagar broadly mirrored those in the five-year study in Faridpur (Supplementary Figure 2). In adults, Nipah virus seroprevalence increased in both Ramnagar and Chakhoria over the course of April 2010-May 2011, consistent with the period of increased seroprevalence observed in late 2010 in

the Faridpur. In Ramnagar, the seroprevalence in adults was 41% (24%-61%) in April 2010, rising to 60% (36%-81%) in May 2011 whilst in Chakhoria it rose from 26% (10%-48%) to 60% (36%-81%) over the same period. The trend in juveniles was as described for the five-year study.

For the filovirus, the seroprevalence in the one-year studies was higher than the reported average in the five-year study, with seroprevalence ranging between 41% (21%-64%) and 75% (51%-91%) in Ramnagar and between 48% (26%-70%) and 100% (83%-100%) in Chakhoria. A period of distinct increase in spring/summer 2010 was described in the five-year study which overlaps the timing of the one-year studies and may explain this difference in seroprevalence. In Ramnagar the serodynamics in both adults and juveniles were as described for the five-year study. In Chakhoria, there were two periods of increasing seroprevalence in adults over the course of the year. The first of these occurred in late 2010 when the adult seroprevalence increased from 48% (28%-69%) in August 2010 to 100% (83%-100%) in October 2010. The second period of increase occurred at the end of the study period in Spring 2011 with seroprevalence rising from 48% (26%-70%) to 75% (51%-91%) between early March 2011 to late April 2011.

Rubulavirus seroprevalence was high in adults throughout the one-year studies, ranging from 50% (27%-73%) to 95% (76%-100%) in Ramnagar and 29% (11%-52%) to 100% (83%-100%) in Chakhoria, with the lowest values observed in September 2010 in Ramnagar and December 2010/January 2011 in Chakhoria. These decreases were followed by rapid increases back to high seroprevalence, coincident with increasing levels in juveniles. The pattern in juveniles was as described in the five-year study, with seroprevalence estimates in yearlings in April 2011 of 80% (28%-99%) in both locations.

Comparison of the seroprevalence trends within each age group and virus between the two one-year study locations did not support spatial differences in the timing of changes in seroprevalence (Figure 7). Only for the filovirus in adults was there some evidence of a difference in trend between locations coinciding with the period of increased seroprevalence observed in adults in Chakhoria at the end of 2010.

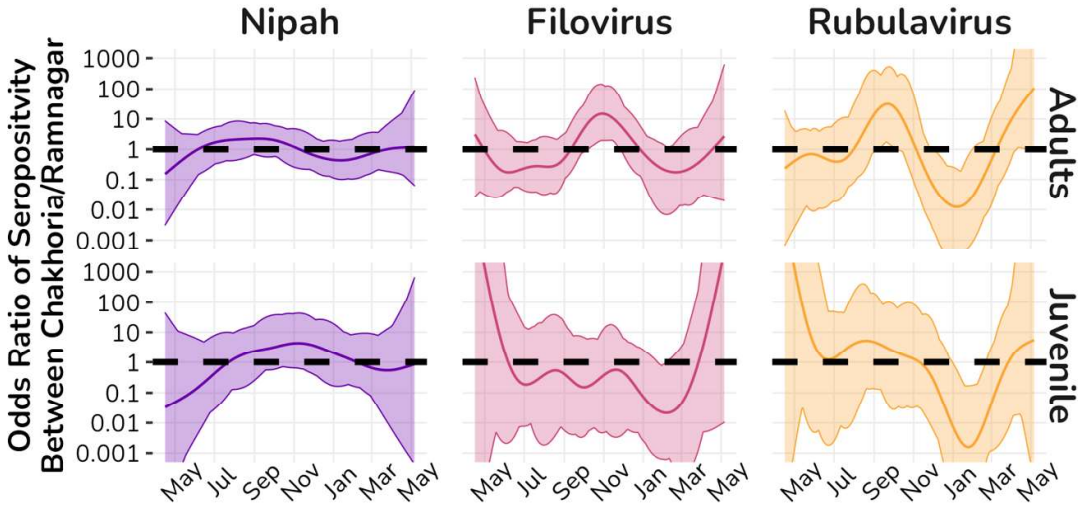

Figure 7. Comparison of splines for seroprevalence trends between locations in adults and juveniles over one-year study. Thick lines and shaded errors represent the estimated difference in trends and their associated 95% confidence intervals. The horizontal dashed line is set at zero. Where the confidence interval excludes zero, there is evidence for a significant difference in the serodynamics between the Ramnagar and Chakhoria populations over that period.

Maternal Inheritance of antibodies

We examined the relationship between mother and pup antibody titers, as measured by our Luminex assays (see Methods). This relationship varied between viruses (Figure 7). For Nipah virus,

the relationship was nonlinear due to clustering at high and low values, but largely followed a 1:1 relationship between mother and pup antibody titers. For the rubulavirus the relationship was near-linear and near 1:1. For the filovirus, though, the relationship between MFI values measured in mothers and their pups fell well below the 1:1 line, indicating pups having low inheritance of antibodies against the filovirus relative to the other two viruses.

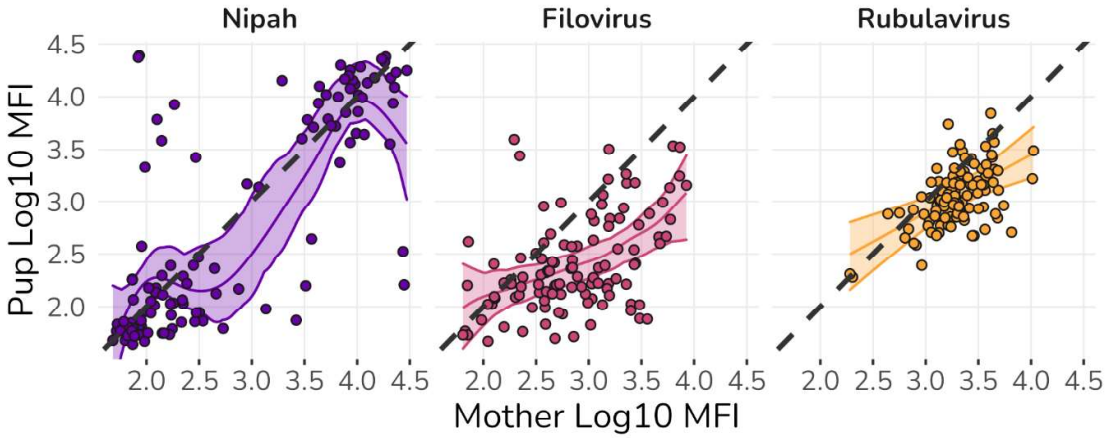

Figure 8. Maternal inheritance of antibodies. In each plot, points are measured log-MFI values for adults and juveniles in mother-pup pairs for each of the three antibody tests. Lines represent the predicted mean relationship between the two and their associated 95% confidence intervals.

3. Discussion

We found serological evidence for regular circulation of multiple viruses in *P. medius* populations in Bangladesh. These included Nipah virus, which spills over from bat populations to humans in the region, as well as a filovirus and a Rubulavirus. It was common for bats to have antibodies against multiple viruses, and more common in adults than juveniles. Co-immunity patterns among pre-weaned juveniles mirrored those in adults, reflecting maternal inheritance patterns. Patterns of co-immunity were not random, with antibodies against all three virus types being positively correlated within bats.

The dynamic patterns in population seroprevalence were distinct for the three viral groups. The rubulavirus exhibited the most regular and consistent patterns. Some juveniles in these populations inherit maternal antibodies against the Rubulavirus, which wane over the first six months of their life, after which a regular annual epidemic occurs in the juvenile population, causing a steep rise in juvenile seroprevalence by the end of their first year. High adult seroprevalence is likely the consequence of most bats being exposed to the disease as juveniles. When adult seroprevalence drops, as it did in 2009 to 2011, it rapidly rebounds during the same season as juveniles, indicating the same regular epidemics affect both adults and juveniles. We expect circulation and shedding of the rubulavirus to be strongest in the late winter and early spring months in these populations. Mortlock at al.[39] found irregular patterns of rubulavirus shedding in *Rousettus* bats over one year (via PCR detection in pooled urine samples), including a peak during a period of presumed antibody waning, though only within a one-year study.

An outstanding question is how the rubulavirus is maintained in the population despite high seroprevalence in inter-epidemic periods. Recrudescence is one possibility. Evidence for latent infections and recrudescence has been found for Nipah virus in *Pteropus vampyrus*[40], Hendra virus in *P. Medius*[41], and Lagos bat lyssavirus and African henipavirus in *Eidolon helvum*[42]. Another possibility is re-importation. In concurrent work with this study, we found that bat home ranges overlapped

with nearby colonies so as to form a meta-population[34], allowing occasional infection from outside bats, as has been shown to maintain Hendra virus in *Pteropus* populations[43,44].

The identity of the rubulavirus in this population is unclear. Our test was designed for Menangle virus, which has been found in *P. medius* in Australia[45,46]. At least 11 distinct Paramyxoviruses have been found in *P. medius* in Bangladesh alone: Nipah virus and ten uncharacterized species, including six Rubulaviruses closely related to Menangle virus and the Tioman virus[9,47]. It is possible that the serological patterns observed represent antibodies against a complex of multiple Rubulaviruses, though the regular interannual patterns in seroprevalence would indicate that they are operating similarly.

Nipah virus serodynamics exhibited a different pattern. We found serodynamics consistent with little to no circulation amongst juveniles. Instead, each cohort of juveniles appeared to inherit some maternal antibodies - maternal inheritance was most consistent for Nipah virus in our mother-pup comparisons - and population seroprevalence declined over their first year for all cohorts. Adults experienced several outbreaks, but Nipah virus serodynamics did not exhibit any seasonal patterns, rather, circulation events occurred in irregular intervals one to two years apart. In Thailand, Nipah virus shedding in *Pteropus lylei* was found to vary in time over two years and this pattern varied by strain; Bangladesh-strain Nipah virus shedding clustered in spring months, but only over two years[28]. Human Nipah virus outbreaks in Bangladesh, on the other hand, exhibit seasonality associated with the palm-sap consumption, the most likely spillover mechanism[48].

We had repeated positive detections of filovirus antibodies, using a test designed for Ebola-Zaire virus. This is the first detection of filovirus antibodies in *P. medius* in Bangladesh, though they have been found in *R. leschenaultii* in the country[49]. This could be one of several known filoviruses or an unknown species. There have been several recent findings of new Ebola-like filoviruses in bats extending across Africa and Asia. These include Bombali virus in Sierra Leone[50], and Měnglà virus in *Rousettus* bats in China[51]. Ebola-Reston virus was found in multiple bat species (*M. australis*, *C. brachyotis* and *Ch. plicata*) in the Philippines[52]. Marburg virus and Ebola-Zaire virus may have been detected in Sierra Leone and Liberia (unpublished,[53,54]). Serological evidence of filoviruses in bats has been found in multiple bat species in Central[12,55] and Western[56] African countries, Singapore [57], China[58], as well as Trinidad[59]. Our finding here adds to the evidence of broad host and geographic host ranges for filoviruses.

Several components of the serological patterns of filovirus antibodies are of interest. Young juveniles were almost all seronegative, and seroprevalence rose over the course of the first six months all years, then declined in the latter half of the year. Adult seroprevalence remained steady despite these regular juvenile outbreaks near 50% over the course of the five-year study. Adult seroprevalence against the filovirus also did not exhibit regular annual cycles, though the one period of detectable increase, in mid-2010, coincided with one of the regular seasonal periods of increase among juveniles. One possible hypothesis explaining this pattern is that the filovirus may exhibit low transmissibility in this system, only circulating when seroprevalence is near zero in young juveniles. It is also possible that bats may exhibit weak or waning filovirus antibody response but remain immune. In *R. aegyptiacus*, infected with the Marburg virus, antibody waning post-infection was found to be rapid, but bats retained protective immunity even with very low antibody titers[60,61]. If a similar mechanism occurs in this population, this could explain low seroprevalence in adults despite regular apparent periods of circulation in juveniles. The regular drop of seroprevalence in juveniles following the peak is consistent with this explanation.

However, if adults remain immune to the filovirus despite low antibody titers, this immunity does not appear to transfer to juveniles, as seroprevalence is near-zero among newborns and circulation in juveniles begins soon after birth. The relationship of pup/dam MFI found for the filovirus antibodies suggests that there may be differential inheritance of antibodies and possibly differential maternally derived immunity. If pups derive weaker immunity from dams than for other viruses, this may provide another mechanism for maintenance of the virus. It possible that patterns in filovirus antibody detection are an artifact of our test, specific to Ebola-Zaire virus, and that temporal trends are in part reflective of differential cross-reaction with viruses in this bat population.

However, while this may modify estimates of overall seroprevalence, the differential pup/dam MFI relationship, and consistent rise in seroprevalence among young juveniles, would require the test to exhibit differential sensitivity by age.

Serodynamics in our two one-year studies were broadly similar to those found in the five-year study. We found weak evidence for differences in serodynamics between sites for the filovirus in adults, and the rubulavirus for adults and juveniles. However, limited sample numbers and the shorter study period limit our ability to generalize about seasonal patterns. In a concurrent study[34], we found that flying foxes home ranges in Bangladesh extended an average of 50 km from their roosts. The roosts at Chakoria and Ramnagar, the sites of the one-year studies, are approximately 225 km apart. Thus, we would not expect the dynamics of these sites to be strongly coupled. It is plausible that inter- and even intra-annual dynamics of these viruses could be similar if weak coupling and/or environmental factors drive some of the dynamics. More detailed and longer-term studies at multiple sites in parallel are needed to better understand spatiotemporal variation in viral circulation.

Regular co-circulation of these viruses indicates the potential for viral interactions to affect host-viral dynamics. Viral interactions in hosts – whether direct during co-infection or indirect from infection in different periods – can enhance or suppress pathogen mortality, fecundity, or transmission, via competition for host nutritional or cellular resources, or via immune-mediation involving cross-immunity or antibody-dependent enhancement, and these interactions structure viral communities[26]. These interactions can have complex, even opposite effects when scaled to the population[27]. Here, we found positive correlations between serostatus against different viruses. External factors may also affect these relationships. For instance, all three viruses appear to have circulated in adults in mid-2010. It is possible that common factors like population density and/or nutrition availability[62] affected transmission of multiple viruses.

While rich observational serological data reveal these patterns, greater study is required to characterize these viruses and their effects on the host population and potential for spillover, as well as the degree and mechanisms of interactions. Our inferred periods of viral circulation point to optimal sub-populations and times to sample this population to detect viral shedding and isolate these viruses, and potentially capture co-infected hosts.

Extended longitudinal sampling also sheds light on viral dynamics not found in short-term studies. One- to two-year studies can identify temporal patterns in serology or viral prevalence and shedding, but establishing patterns or variation in seasonality requires extended, multi-year sampling. In our case, while Nipah virus outbreaks occurred in the same season in two years, it was apparent from the longer time series that there was no distinct seasonal pattern in dynamics. The consistency of the filovirus and rubulavirus patterns required repeated years of sampling to establish. Similarly, we were able to understand these patterns far better by separating juvenile and adult patterns, which we would be unable to distinguish in pooled samples.

While such extended individual-capture longitudinal studies are resource-intense, understanding the joint circulation of multiple viruses can be accomplished via multiplex immunoassays such as those used here. The continuous measures from these assays also have the potential to identify key patterns such as the differential inheritance of filovirus antibodies we identified here. Interpretation of these values is challenging and the relationship between immune status, antibody titer, and measured fluorescence is complex[32], but they have much potential to shed light on mechanistic drivers of disease circulation.

Methods

Field collection

We conducted a five-year longitudinal study sampling *P. medius* bats quarterly and two one-year studies in different locations, sampling monthly. All capture and sampling methods were approved by Tufts University IACUC protocol #G929-07 and icddr,b animal ethical review (protocol 2006-012). For the five-year study, we sampled from a roost complex near Faridpur, Bangladesh, as previously described in Epstein, et al. [34] The area of the roost complex consists of patchy forest

interspersed with agrarian human settlements and large rice paddies. Sampling occurred from July 2007 to November 2012 approximately every three months. The bat colony regularly shifted amongst roosts within the 80km^2 roost complex over the period of the study. Sampling occurred at the largest occupied roost found each quarterly sampling trip, sampling at different roosts within the complex across consecutive sampling nights if required to capture a sufficient number of individuals.

Approximately 100 bats were captured at each sampling event, which lasted 7-10 days. We captured bats with a 10x15m mist net between 11pm and 5am each night as bats returned from foraging until the count of 100 was reached.

In the one-year longitudinal studies, sampling was undertaken in two roost complexes in Ramnagar and Chakhoria, Bangladesh between April 2010 and May 2011. Monthly sampling of approximately 40 bats in each location was performed to obtain data at a finer temporal scale. Details of collection are otherwise as described for the five-year study.

For each study, we recorded each bat's age class, reproductive status, weight, size, and body condition. We collected up to 3.0 mL of blood from each bat's brachial vein into serum tubes with serum clot activator (Vaccutainer, USA). Blood tubes were stored vertically on ice packs in a cold box and serum was allowed to separate overnight. Serum was drawn from the tube after 24 hours and placed in a screw-top cryovial (Corning, USA) and stored in a liquid nitrogen dewar (Princeton Cryogenics, NJ, USA).

As part of a larger study to detect viral RNA, we also collected urine, rectal, and oropharyngeal swabs and pooled urine samples from under colonies. Further details can be found in Epstein, et al. [34]

Serological Assays

Sera from the longitudinal studies were sent to the Australian Animal Health Laboratory and gamma irradiated upon receipt. We used a bead-based microsphere assay that specifically detects antibodies to the soluble attachment glycoproteins[57,63,64] for a panel of viruses. Beads coated with each protein were mixed with sera at a dilution of 1:100. Biotinylated Protein A/G and Streptavidin-PE were then used to detect bound antibody. Beads were interrogated by lasers in a BioRad BioPlex machine and the results recorded as the Median Fluorescent Intensity (MFI) of 100 beads. We report here results for Nipah, Ebola-Zaire, and Menangle, the only three for which we established regular positive results.

While the Nipah virus has been detected in this population[65] and the specificity of the Nipah test is well-established [66], the Ebola-Zaire and Menangle virus tests are cross-reactive with other Ebola and rubulavirus species[67,68]. Thus we refer to these as tests for filovirus and Rubulavirus.

Data Analysis

We determined individual bat serostatus using Bayesian mixture models[69] fit on pooled data across all three longitudinal studies, calculating a cutoff of log-MFI as the point of equal probability between the smallest and second-smallest cluster of equal distributions for each assay.

To determine correlations of serostatus in bats across the three viruses, we fit a Bayesian multivariate probit model[70,71] which allows estimation of the joint outcomes (serostatus against each virus) and the correlation between the outcomes. We included age and sex variables to account for these effects on serostatus.

To examine time-varying changes in population seroprevalence, we fit binomial generalized additive mixed models (GAMMs)[72,73] to the time-series of serostatus measurements. For the five-year study, we fit separate models for adults and juveniles and for each immunoassay. For adults, we fit a model for seroprevalence dynamics over the course of the whole study. This treats the adult population as a single unit, though individuals within the population may turn over via migration, death and recruitment. We included both long-term and annual cyclic components for the multi-year time series of measurements. For juveniles, we treated each year's cohort of new recruits as separate populations with commonalities in overall first-year dynamics. The dynamics of each year's juvenile cohort were modeled as random-effect splines, deviating from an overall mean splines for juveniles

over their first year. To estimate periods of peak viral circulation within the population, we calculated the derivatives of model-derived seroprevalence over time. We sampled these from GAMM posterior distributions and classified periods with >95% of samples with positive derivatives - that is, increasing population seroprevalence - as periods of viral circulation. We also calculated strength and timing seasonality for each virus as the maximum rate of seroprevalence increase and the date at which this maximum occurred, again sampling these values from the model posterior, and calculating mean and high-density posterior interval (HDPI) values.

For the one-year studies, we fit models primarily to detect differences in time-varying changes between sites. We fit binomial generalized additive models (GAMs) to the monthly serostatus measurements with separate models for each immunoassay. We included a separate, fixed-term spline in each model for age (adult or juvenile) and location (Chakhoria or Ramnagar). The trends in serodynamics for each virus in each age group were compared between locations to test for spatial differences[74]. Juveniles identified as being from the previous year's cohort were excluded from the analysis.

To examine patterns of maternal inheritance, we used GAMs to fit a relationship of log-MFI between adult lactating females and their attached pups for each viral assay. We limited these to data from the five-year longitudinal study.

Data Availability: Raw data from this study and reproducible code used to perform analyses is available at the Zenodo Scientific Archive [Zenodo DOI to be generated] and also on GitHub at https://github.com/ecohealthalliance/bangladesh-bat-serodynamics

Author Contributions: Conceptualization: N.R. and J.H.E..; methodology, N.R. and J.H.E.; software, N.R. and S.H.; formal analysis, N.R. and S.H.; investigation, J.H.E., A.I. and ….; resources, X.X.; data curation, N.R and J.H.E; writing—original draft preparation, N.R. J.H.E., and S.H.; writing—review and editing, X.X.; visualization, N.R. supervision, N.R. and J.H.E.; project administration, J.H.E.; funding acquisition, P.D. and J.H.E

Funding: This study was funded by an NIH NIAID awards to JHE (AI153420) and CCB (AI054715), an NIH Fogarty International Center Ecology and Evolution of Infectious Diseases (EEID) award to PD (TW005869) a NSF-NIH EEID award to AMK (EF-0914866), and the US Agency for International Development Emerging Pandemic Threats: PREDICT program (NR, PD, JHE , AI).

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Number: 1 Author: Noam Ross Date: 8/10/2022 12:19:00 PM
All co-authors please insert yourselves as appropriate here. A summary of contribution types can be found here: https://img.mdpi.org/data/contributor-role-instruction.pdf

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From:	Jahangir Hossain on behalf of Jahangir Hossain <Jahangir.Hossain@lshtm.ac.uk>
To:	Emily Gurley; Kevin Olival; Noam Ross; Jon Epstein
Cc:	Steve Luby; Ariful Islam; Hayes, Sarah; A. Marm Kilpatrick; Dr. Jahangir Hossain; Gary Crameri; Linfa Wang; Chris Broder; Peter Daszak; Madeline Salino; Hume Field
Subject:	RE: Draft manuscript: Co-circulation dynamics of viruses in a bat population
Date:	Saturday, September 10, 2022 9:07:32 AM
Attachments:	Ross-et-al_bangladesh-bats-cocirculation-serology_2022-08-11_LW_HF_sl_eg_JH.docx

Hi Jon,
I have a few minor editions and comments with others.

Thank you,
Jahangir Hossain

From: Emily Gurley <egurley1@jhu.edu>
Sent: 10 September 2022 01:08
To: Kevin Olival olival@ecohealthalliance.org; Noam Ross ross@ecohealthalliance.org; Jon Epstein <epstein@ecohealthalliance.org>
Cc: Steve Luby <sluby@stanford.edu>; Ariful Islam <arif@ecohealthalliance.org>; Hayes, Sarah <sarah.hayes16@imperial.ac.uk>; A. Marm Kilpatrick <akilpatr@ucsc.edu>; Dr. Jahangir Hossain <jhossain@mrc.gm>; Gary Crameri <garycrameri1@gmail.com>; Linfa Wang <linfa.wang@duke-nus.edu.sg>; Chris Broder <christopher.broder@usuhs.edu>; Peter Daszak <daszak@ecohealthalliance.org>; Madeline Salino <salino@ecohealthalliance.org>; Hume Field <hume.field@ecohealthalliance.org>
Subject: RE: Draft manuscript: Co-circulation dynamics of viruses in a bat population

*** This message originated outside MRCG @ LSHTM ***
Sorry to be late to the party – a few more comments attached to add to the insightful suggestions you've already received.

Jon – still think it would be great to link up so we can describe antibody dynamics over time in our recaptures!

Have a great weekend,
Emily

From: Kevin Olival <olival@ecohealthalliance.org>
Sent: Friday, September 9, 2022 5:39 PM
To: Noam Ross <ross@ecohealthalliance.org>; Jon Epstein <epstein@ecohealthalliance.org>
Cc: Steve Luby <sluby@stanford.edu>; Ariful Islam <arif@ecohealthalliance.org>; Hayes, Sarah <sarah.hayes16@imperial.ac.uk>; A. Marm Kilpatrick <akilpatr@ucsc.edu>; Emily Gurley <egurley1@jhu.edu>; Dr. Jahangir Hossain <jhossain@mrc.gm>; Gary Crameri <garycrameri1@gmail.com>; Linfa Wang <linfa.wang@duke-nus.edu.sg>; Chris Broder <christopher.broder@usuhs.edu>; Peter Daszak <daszak@ecohealthalliance.org>; Madeline Salino <salino@ecohealthalliance.org>; Hume Field <hume.field@ecohealthalliance.org>
Subject: Re: Draft manuscript: Co-circulation dynamics of viruses in a bat population

Noam and Jon,

Attached are my edits and comments. I started working on this using the version from Hume and Linfa, so my version here does not include Steve's valuable comments.

Great paper.

Cheers,
Kevin

Kevin J. Olival, PhD
Vice President for Research

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EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation

> On Sep 3, 2022, at 2:54 AM, Steve Luby <sluby@stanford.edu> wrote:
>
> Hi Jon,
>
> Thanks for sharing this interesting work. Kudos to Noam and the team for pulling all of this together.
>
> Attached are my comments.
>
> To reduce the amount of my time and improve the thoroughness of my review, I use error codes when I identify common errors within a scientific document. A full description of the error and strategies for addressing it can be found in a scientific writing guide written by Dorothy Southern and I (Pathway to Publishing-A Guide to Quantitative Writing in the Health Sciences) published as an Open Access ebook through Springer. It can be downloaded from here: https://link.springer.com/book/10.1007/978-3-030-98175-4
>
> Steve
>
> hume.field@ecohealthalliance.org wrote on 8/23/2022 6:40 PM:
>
> > Great to see Jon, Noam. Reads well, nice graphics. Thanks for co-

authorship.. my edits added to Linfa's version.

Regards all
Hume

Hume Field PhD MSc BVSc MACVS
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Cc: Noam Ross <ross@ecohealthalliance.org>; Madeline Salino <salino@ecohealthalliance.org>
Subject: Draft manuscript: Co-circulation dynamics of viruses in a bat population

Colleagues,
I hope you're all doing well. I'm excited to share a draft manuscript, led by Noam, that details the serodynamics of henipaviruses, filoviruses and rubulaviruses in a population of *Pteropus medius.* We plan to submit this to *Nature Communications* in September. Please send Noam and I your comments by **September 10th**.

Cheers,
Jon

--
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--

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<Ross-et-al_bangladesh-bats-cocirculation-serology_2022-08-11_LW_HF_sl.docx>

Co-circulation dynamics of henipaviruses, filoviruses and rubulaviruses in a bat population

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Abstract: Bats are hosts for a variety of viruses, some with potential to cause human disease. *Pteropus medius* are fruit bats that live in close association with humans in Bangladesh and are a known source of Nipah virus and other viruses of unknown pathogenic risk. Here we report on dynamics of seroprevalence against multiple viruses in a population of *P. medius* in Bangladesh over five years. We found evidence of regular circulation of Nipah virus, a rubulavirus, and a filovirus. Nipah virus circulated primarily among adult bats, with no seasonal patterns. The rubulavirus exhibited annual cycles in juveniles and near universal seroprevalence in adults. The filovirus exhibited annual cycles and antibody waning in juveniles and little circulation but lower seroprevalence amongst adults. We found no evidence for spatial differences in Nipah virus dynamics, but weak evidence for differences with other viruses. Host-viral interactions result in very different viral circulation patterns in the same individual bats and environments.

Keywords: bats; Nipah virus; filovirus; Rubulavirus; *Pteropus medius*; Bangladesh; serology; disease dynamics; generalized additive models

Introduction

Bats have been associated with a large diversity of viruses, several of which have been linked to human epidemics [1-3]. The discovery of Severe Acute Respiratory Syndrome (SARS)-related coronaviruses (CoVs) in Chinese horseshoe bats (*Rhinolophus spp.*) in 2004 and subsequent discoveries of related viruses[4,5] including close relatives of SARS-CoV-2[6], along with studies of other high consequence pathogens such as Ebola virus, Marburg virus, Nipah virus, Hendra virus have led to questions about whether and why bats were disproportionately represented among mammals as reservoirs for zoonotic viruses[1,2,7].

Bat species can carry diverse viruses which circulate simultaneously within single populations [8-11]. *Rousettus aegyptiacus* is a natural reservoir of Marburg virus, and IgG antibodies against Ebola Zaire virus have also been detected in this bat in Central Africa, suggesting that it may play a role in the circulation of multiple filoviruses[12,13]. In addition to these filoviruses, a novel zoonotic and pathogenic paramyxovirus, Sosuga virus, was also found in Ugandan *R. aegyptiacus* populations[14].

Summary of Comments on Email 6 - Attachment 1 - Ross-et-al_bangladesh-bats-cocirculation-serology_2022-08-11_LW_HF_sl (002).pdf

Page: 1

Number: 1 Author: Hume Field Date: 8/24/2022 9:20:00 AM

Pls add my UQ affiliation Noam.
'School of Veterinary Science, The University of Queensland, Gatton 4343 Australia'

Number: 2 Author: Wang Linfa Date: 8/21/2022 10:57:00 AM

It's better to include my CSIRO affiliation here as the work started when I was still associated with AAHL

Number: 3 Author: Noam Ross Date: 8/10/2022 12:38:00 PM

Please check that your affiliation is as it should be!

Number: 4 Author: Steve Luby Date: 9/2/2022 9:33:00 AM

This seems an odd sentence to close with. The last sentence is usually a conclusion, but I can't see how the data presented in the abstract supports this conclusion.

Number: 5 Author: Steve Luby Date: 9/2/2022 9:36:00 AM

True, but since the new data presented here don't address this issue, I don't see this as central to the introduction, i.e. Error 3.12

Eidolon helvum, another pteropodid bat with a broad geographic range in Africa, has been found to carry a lyssavirus, a henipavirus, and several other viruses of unknown pathogenicity[15]. Various surveillance efforts have found diverse viruses from with the same viral family in various bat species[5,16,17], and other studies have used metagenomic approaches to look broadly at viral diversity within individual bat species[9,11,18-21].

Studies of viral diversity in a single taxonomic host group can inform evolutionary history of viruses and their relationships to specific hosts[6,22] and inform public health strategies[23]. However, detection of a zoonotic virus in an animal host is insufficient to determine whether the associated host species is a natural reservoir for the virus, or to characterize the demographic, physiological, or abiotic factors that may influence the timing of viral shedding and therefore risk of spillover to other species, including domestic animals and humans[24]. For these, more detailed demographic and longitudinal studies are required, especially in cases of multiple viruses, as direct or indirect interactions between multiple pathogens may affect disease presence, prevalence, and dynamics[25-27]. Some longitudinal studies of zoonotic viruses in bats have provided insight into the timing of viral infection and shedding, generally of single viruses. Nipah virus in *Pteropus lylei* in Thailand has been observed to have annual shedding patterns[28], whereas Hendra virus, which is closely related to Nipah virus and is carried by multiple pteropid bat species in Australia, has asynchronous and non-periodic cycles which appear to be influenced by localized factors such as specific bat species abundance and climatic factors [29]. Bi-annual pulses of Marburg virus shedding were observed in Uganda, coinciding with synchronous birth pulses in *R. aegyptiacus*[30]. Serological data is often valuable in understanding disease dynamics and transmission[31-33], especially in cases where direct detection and incidence rates of viruses are low[34].

Pteropus sp. are reservoirs of henipaviruses, a group of mostly zoonotic, lethal neurotropic viruses that include Nipah, Hendra, Cedar, Ghana and Mojiang virus[35-37]. In Bangladesh and India, *P. medius* is a reservoir of Nipah virus, which has spilled over to human populations repeatedly[38]. Viruses from eight other viral families have been detected in *P. medius* in Bangladesh[9]. The epidemiology of these other viruses is far less characterized, and little is known about their interactions or zoonotic potential.

Here, we report on the dynamics of seroprevalence against three types of viruses - Nipah virus, a filovirus, and a rubulavirus - circulating simultaneously in *P. medius* populations in three sites in Bangladesh. We conducted a longitudinal study over a period of five years, and two shorter, one-year studies for comparative seasonal data. These studies enable us to develop a detailed picture of the seasonal and demographic serological patterns, and infer the extent and periodicity of viral circulation. We find that Nipah virus circulates primarily among adults without distinct seasonality, while the rubulavirus exhibits strong annual dynamics characteristic of juvenile epidemics driven by waning maternal antibodies. The filovirus, the first detected in *P. medius* in Bangladesh, also circulated in juveniles but exhibited distinct patterns antibody waning and limited maternal inheritance.

2. Results

Bat Dynamics and Demographics

We conducted a five-year longitudinal study sampling *P. medius* bats quarterly from a roost complex in the vicinity of Faridpur, Bangladesh, and also two parallel one-year studies sampling bats monthly from roosts in Chakhoria and Ramnagar, Bangladesh. In the five-year study, we sampled and tested serology of 1,886 bats: 1,224 adults, 623 free-flying juveniles, and 39 weaning juveniles (captured attached to adult females) over 19 sampling events (Figure 1). During the-one-year studies, 919 bats were sampled: 435 in Chakhoria (251 adults, 144 free-flying juveniles and 40 weaning juveniles) and 484 in Ramnagar (277 adults, 164 free-flying juveniles and 43 weaning juveniles (Figure S1). Nearly all juveniles (as determined by examination of maturation of sex organs) were 14 months old or less and could be assigned to a birth cohort based on size. Pregnant and juvenile bats were captured more frequently during the late spring and summer months. Mother-pup pairs were all

Page: 2

Number: 1 Author: Steve Luby Date: 9/2/2022 9:41:00 AM
I don't believe this adjective applies to Cedar virus.

Number: 2 Author: Hume Field Date: 8/24/2022 9:29:00 AM
Don’t think this should be here.

Number: 3 Author: Wang Linfa Date: 8/21/2022 10:37:00 AM
To be consistent with other section headings in Results

Number: 4 Author: Steve Luby Date: 9/2/2022 10:29:00 AM
it would be helpful to clarify how far apart these places are, as you have a considerable section exploring spatial comparisons.

captured during April and May in the five-year study whilst in the one-year-studies a small number were also captured in June and July. Pregnant females were captured between November and April/May. Lactating females were found between April and July.

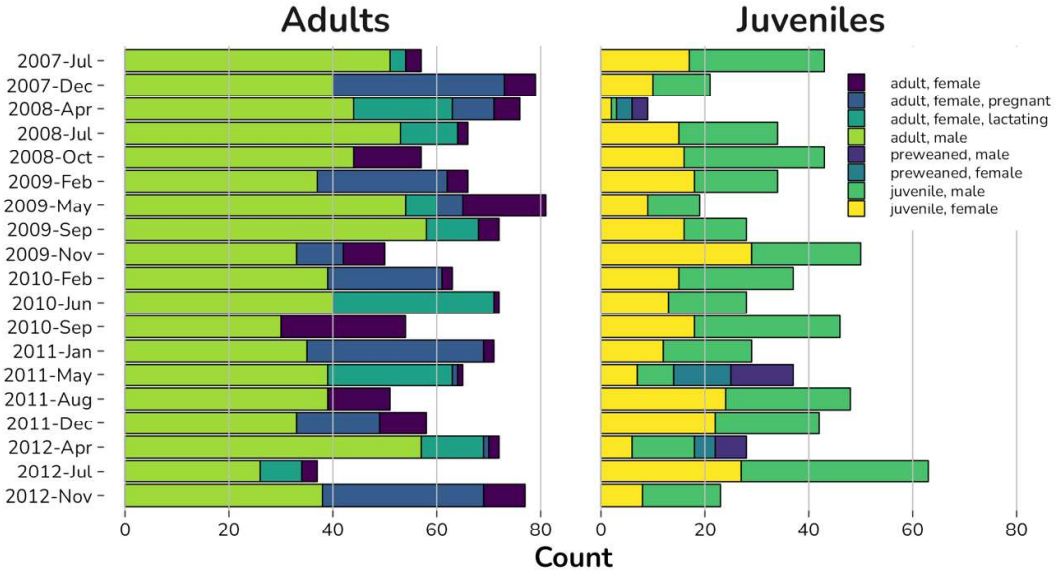

Figure 1. Demographics of bats captured in longitudinal sampling. Pregnant females were captured in months from November to June, lactating females were captured in months from April to July. Females with pre-weaning juveniles attached were found from April to May.

Patterns of Immunity and Co-immunity

We found bats were seropositive for antibodies against the henipavirus (HenipVsero+), a filovirus (EbVsero+), and a rubulavirus (MenVsero+).. Among adult bats, antibodies against the rubulavirus were common; 1,314 of 1752 adult bats were MenVsero+. Antibodies against Nipah virus or filovirus were less common – 1,031 were NiVsero+ and 921 were EbVsero+. Among the 921 juveniles, 192 were MenVsero+, 311 were NiVsero+ and 348 were EbVsero+. Of the 122 pre-weaned juveniles, 43 were MenVsero+, 52 were NiVsero+, and 32 were EbVsero+.

Co-exposure to multiple viruses was common (Figure 2). Over half (1,173) of the 1752 adults had antibodies against more than one of the three viruses, and 493 had antibodies against all three. Among the 921 juveniles, 210 had antibodies against more than one, and 39 had antibodies all three. 38 of 122 pre-weaned juveniles had more than one of the three antibodies, with seven having all three.

We found correlations between serostatus between all three pairs of viruses in a model accounting for bat age, sex, and study location, with all viruses tending to co-occur with each other more than would be expected than if they were distributed independently among bats. Nipah virus and filovirus antibodies had a standardized covariance of 0.13 (95% posterior interval 0.08-0.20). Nipah virus and rubulavirus antibodies were also more likely to occur together, with a covariance of 0.15 (0.09-0.21). Filovirus and rubulavirus antibodies had a covariance of 0.23 (0.17-0.29).

Page: 3

Number: 1 Author: Wang Linfa Date: 8/21/2022 10:48:00 AM

I think it is better to treat the three group of viruses equally as you have done for the title. It makes no difference whether we use NiV or Henipavirus in the context of the current study. Luminex-based assays targeting binding antibodies will in theory detect cross-RX antibodies in all three cases. See also comments in Methods

Number: 2 Author: Steve Luby Date: 9/2/2022 10:01:00 AM

Include percentages for all of these comparisons.

Number: 3 Author: Steve Luby Date: 9/2/2022 10:03:00 AM

Again, including percentages for all of these comparisons would make this much easier to understand.

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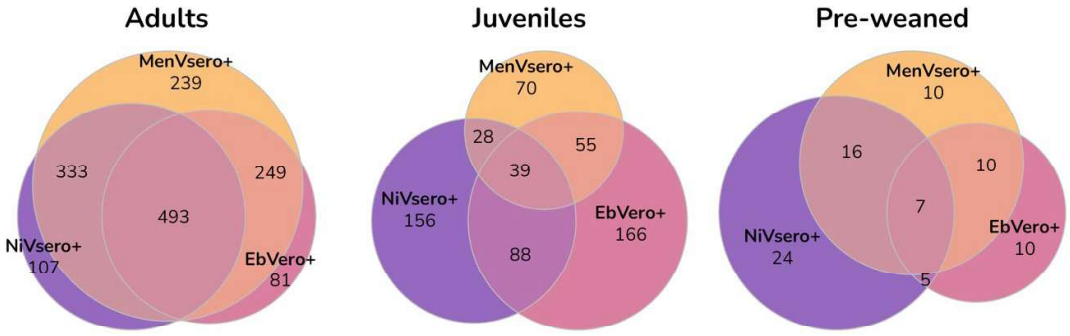

Figure 2. Detection of IgG antibodies against multiple viruses in individual *Pteropus medius*: Venn diagrams for adult, juvenile, and pre-weaned bats testing positive for antibodies against Nipah virus, filovirus, and Rubulavirus. Numbers under labels are counts of bats with only those viruses, numbers in overlapping areas represent number of bats detected with multiple viruses.

Serodynamics

Dynamics of population seroprevalence were different across the viral types. In adults, population seroprevalence against Nipah virus decreased over the study period from 78% (66%-88%) to 50% (40-62%) (Figure 3). Three periods of distinct increase in seroprevalence stood out in the inter-annual signal: early 2008, early 2009, and the second half of 2010. Nipah seroprevalence showed very weak seasonal patterns (Figure 4). (Nipah dynamics were previously reported in Epstein, et al. [34]).

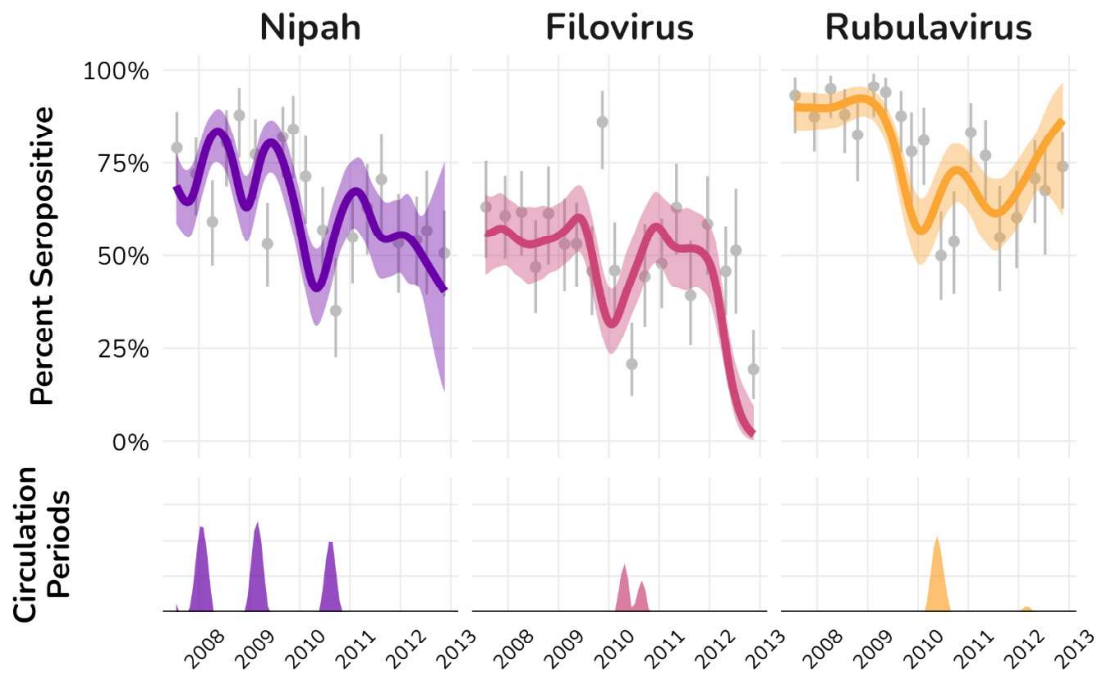

Figure 3. Inter-annual serodynamics in adult bats. Top: Measured and modeled population seroprevalence in adults over the term of the study. X-axis ticks mark the first day of the year. Grey points and bars represent measured population seroprevalence from individual sampling events on and 95% exact binomial confidence intervals. Thick lines and shaded areas represent the inter-annual (non-seasonal) spline component of a generalized additive mixed model (GAMM) of serodynamics and 95% confidence intervals for the mean pattern over time. Bottom: Circulation periods, when modeled slopes of the GAMM splines are positive for >95% of 1000 draws from the model posterior.

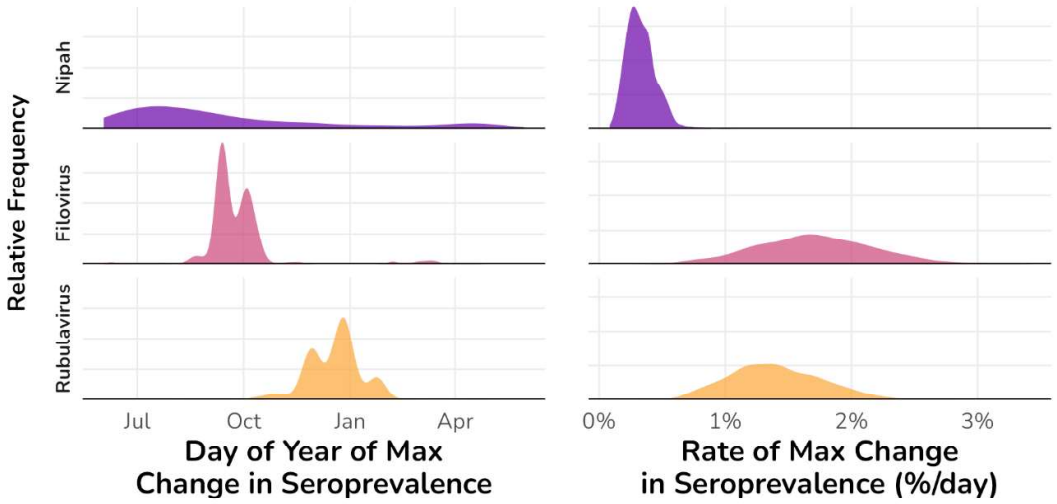

Figure 4. Timing and strength of seasonality of viral circulation in adults. Plots show posterior densities of the date for which increase in seroprevalence is greatest (left), and the rate of increase (right), for adult bats, drawn from GAMM models of seasonality. Densities are of these quantities derived from 1000 samples of model posteriors.

Among juveniles, we examined the dynamics of each cohort over their first year. For Nipah virus, the average cohort had 44% (32%-58%) seroprevalence in June, which consistently decreased over the course of the year to 20% (10%-36%) (Fig. 5). Year-to-year dynamics varied little, with overall declining seroprevalence in all years. No part of the year exhibited regular increases in seroprevalence for juveniles; the maximum rate of increase was indistinguishable from zero (Fig. 6).

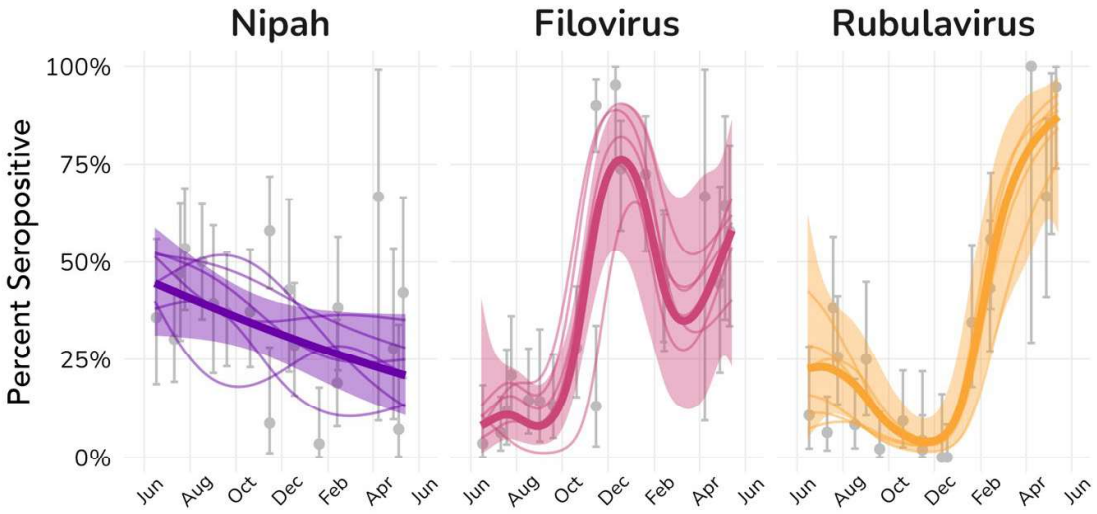

Figure 5. Serodynamics in juvenile bats in five-year study. Measured and modeled population seroprevalence in juvenile bats caught over their first year of life. Sampling events from multiple years are transposed onto a single year starting June (the earliest month free-flying young-of-the-year juveniles were captured). X-axis ticks mark the first day of each month. Grey points and bars represent measured population seroprevalence from individual sampling events on and 95% exact binomial confidence intervals. Thick lines and shaded areas represent GAMM-modeled average serodynamics across all years and 95% confidence intervals for the mean effect. Thin lines represent individual-year deviations from the average year pattern.

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Number: 1 Author: Steve Luby Date: 9/2/2022 10:18:00 AM
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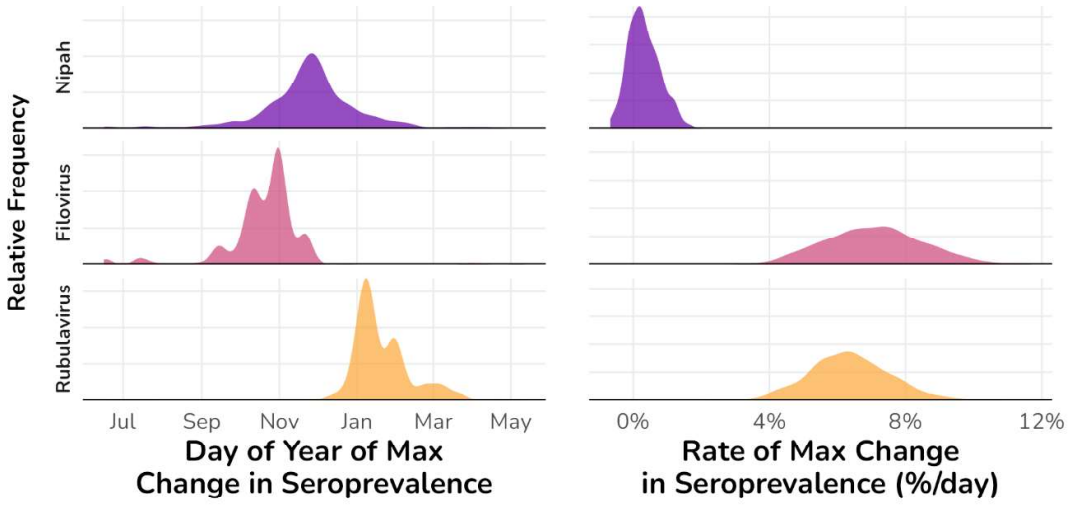

Figure 6. Timing and strength of seasonality of viral circulation in juveniles over the five-year study. Plots show posterior densities of the date for which increase in seroprevalence is greatest (left), and the rate of increase (right), for juvenile bats. Densities are of these quantities derived from 1000 samples of model posteriors.

Filovirus seroprevalence exhibited different patterns. Among adults, seroprevalence against filovirus was lower than for Nipah virus or Rubulavirus. As with Nipah virus, filovirus seroprevalence decreased over the study period, from 64% (50%-76%) to 20% (12%-30%), and had periods of distinct increases in spring/summer 2010 (concurrently with a circulation period for Nipah virus) (Fig. 3). filovirus seroprevalence did not exhibit cyclic annual patterns in adults (Fig. 4).

Among juveniles within the average year, filovirus seroprevalence started very low in June at 8% (2%-40%), but increased to a peak of 44% (32%-58%) in juveniles approximately six months of age before declining. (Fig 5). This pattern was consistent across years; though the peak varied in size, it consistently occurred in December or January. The average date with the greatest rate of seroprevalence increase was Oct 31 (Jul 14-Nov 25), and the maximum rate of increase was 7.1%/day (4.5%/day-9.9%/day).

Rubulavirus seroprevalence had a distinct dynamic pattern. Among adults, rubulavirus seroprevalence was high at 92% (82%-98%), and remained high through the end of the period at 74% (62%-84%), though there were some temporary periods of decline. There were distinct periods of increasing circulation in early 2010 and early 2012. Only for the rubulavirus did adult seroprevalence exhibit patterns of seasonality. Periods of increasing seroprevalence in adults exhibited strong seasonality of 1.6%/day (1.0%/day-2.4%/day) increase on peak days, with the mean peak date occurring on Dec 15 (Oct 15-Feb 1)

In juveniles, seroprevalence against the rubulavirus in the average year was 22% (4%-62%) in the youngest bats in June, though in individual years this could range from 7%-43%. In all years juvenile seroprevalence declined to a low of 1.7% (0.6%, 8.3%) that occurred between Oct 9 and Dec 11, then increased starting in winter, reaching high values in yearling juveniles of 88% (56%-99%) The peak rate of increase for juvenile rubulavirus seroprevalence was 6.4%/day (4.2%-8.8%) and occurred Jan 9 (Dec 21-Mar 16).

Spatial Comparisons

Seroprevalence trends in both adult and juvenile bats within the one-year studies in Chakhoria and Ramnagar broadly mirrored those in the five-year study in Faridpur (Supplementary Figure 2). In adults, Nipah virus seroprevalence increased in both Ramnagar and Chakhoria over the course of April 2010-May 2011, consistent with the period of increased seroprevalence observed in late 2010 in

Page: 6

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As above. What is the y axis?

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Is this appropriate to call out when it is not identified by the GAMM splines?

Number: 3 Author: Steve Luby Date: 9/2/2022 10:24:00 AM
Consider including a figure in the appendix that illustrates this.

the Faridpur. In Ramnagar, the seroprevalence in adults was 41% (24%-61%) in April 2010, rising to 60% (36%-81%) in May 2011 whilst in Chakhoria it rose from 26% (10%-48%) to 60% (36%-81%) over the same period. The trend in juveniles was as described for the five-year study.

For the filovirus, the seroprevalence in the one-year studies was higher than the reported average in the five-year study, with seroprevalence ranging between 41% (21%-64%) and 75% (51%-91%) in Ramnagar and between 48% (26%-70%) and 100% (83%-100%) in Chakhoria. A period of distinct increase in spring/summer 2010 was described in the five-year study which overlaps the timing of the one-year studies and may explain this difference in seroprevalence. In Ramnagar the serodynamics in both adults and juveniles were as described for the five-year study. In Chakhoria, there were two periods of increasing seroprevalence in adults over the course of the year. The first of these occurred in late 2010 when the adult seroprevalence increased from 48% (28%-69%) in August 2010 to 100% (83%-100%) in October 2010. The second period of increase occurred at the end of the study period in Spring 2011 with seroprevalence rising from 48% (26%-70%) to 75% (51%-91%) between early March 2011 to late April 2011.

Rubulavirus seroprevalence was high in adults throughout the one-year studies, ranging from 50% (27%-73%) to 95% (76%-100%) in Ramnagar and 29% (11%-52%) to 100% (83%-100%) in Chakhoria, with the lowest values observed in September 2010 in Ramnagar and December 2010/January 2011 in Chakhoria. These decreases were followed by rapid increases back to high seroprevalence, coincident with increasing levels in juveniles. The pattern in juveniles was as described in the five-year study, with seroprevalence estimates in yearlings in April 2011 of 80% (28%-99%) in both locations.

Comparison of the seroprevalence trends within each age group and virus between the two one-year study locations did not support spatial differences in the timing of changes in seroprevalence (Figure 7). Only for the filovirus in adults was there some evidence of a difference in trend between locations coinciding with the period of increased seroprevalence observed in adults in Chakhoria at the end of 2010.

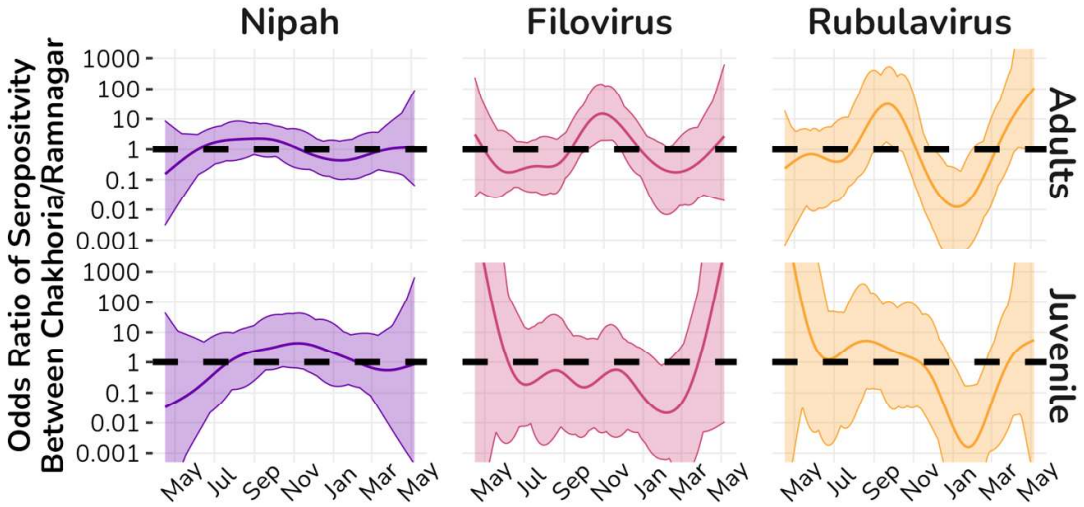

Figure 7. Comparison of splines for seroprevalence trends between locations in adults and juveniles over one-year study. Thick lines and shaded errors represent the estimated difference in trends and their associated 95% confidence intervals. The horizontal dashed line is set at zero. Where the confidence interval excludes zero, there is evidence for a significant difference in the serodynamics between the Ramnagar and Chakhoria populations over that period.

Maternal Inheritance of antibodies

We examined the relationship between mother and pup antibody titers, as measured by our Luminex assays (see Methods). This relationship varied between viruses (Figure 7). For Nipah virus,

the relationship was nonlinear due to clustering at high and low values, but largely followed a 1:1 relationship between mother and pup antibody titers. For the rubulavirus the relationship was near-linear and near 1:1. For the filovirus, though, the relationship between MFI values measured in mothers and their pups fell well below the 1:1 line, indicating pups having low inheritance of antibodies against the filovirus relative to the other two viruses.

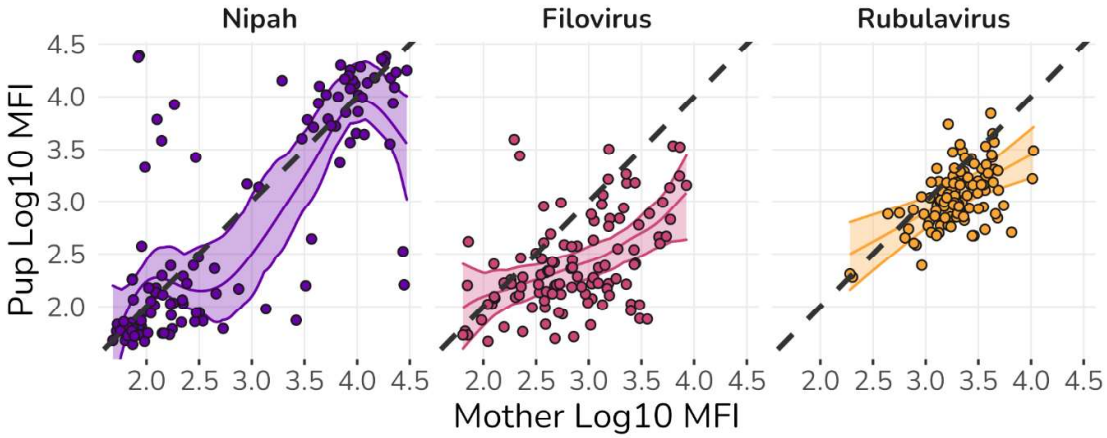

Figure 8. Maternal inheritance of antibodies. In each plot, points are measured log-MFI values for adults and juveniles in mother-pup pairs for each of the three antibody tests. Lines represent the predicted mean relationship between the two and their associated 95% confidence intervals.

3. Discussion

We found serological evidence for regular circulation of multiple viruses in *P. medius* populations in Bangladesh. These included Nipah virus, which spills over from bat populations to humans in the region, as well as a filovirus and a rubulavirus. It was common for bats to have antibodies against multiple viruses, and more common in adults than juveniles. Co-immunity patterns among pre-weaned juveniles mirrored those in adults, reflecting maternal inheritance patterns. Patterns of co-immunity were not random, with antibodies against all three virus types being positively correlated within bats.

The dynamic patterns in population seroprevalence were distinct for the three viral groups. The rubulavirus exhibited the most regular and consistent patterns. Some juveniles in these populations inherit maternal antibodies against the rubulavirus, which wane over the first six months of their life, after which a regular annual epidemic occurs in the juvenile population, causing a steep rise in juvenile seroprevalence by the end of their first year. High adult seroprevalence is likely the consequence of most bats being exposed to the disease as juveniles. When adult seroprevalence drops, as it did in 2009 to 2011, it rapidly rebounds during the same season as juveniles, indicating the same regular epidemics affect both adults and juveniles. We expect circulation and shedding of the rubulavirus to be strongest in the late winter and early spring months in these populations. Mortlock at al.[39] found irregular patterns of rubulavirus shedding in *Rousettus* bats over one year (via PCR detection in pooled urine samples), including a peak during a period of presumed antibody waning, though only within a one-year study.

An outstanding question is how the rubulavirus is maintained in the population despite high seroprevalence in inter-epidemic periods. Recrudescence is one possibility. Evidence for latent infections and recrudescence has been found for Nipah virus in *Pteropus vampyrus*[40], Hendra virus in *P. alecto and P. poliocephalus*[41], and Lagos bat lyssavirus and African henipavirus in *Eidolon helvum*[42]. Another possibility is re-importation. In concurrent work with this study, we found that bat home

ranges overlapped with nearby colonies so as to form a meta-population[34], allowing occasional infection from outside bats, as has been shown to maintain Hendra virus in *Pteropus* populations[43,44].

The identity of the rubulavirus in this population is unclear. Our test was designed for Menangle virus, which has been found in multiple *Pteropus* species in Australia[45,46]. At least 11 distinct Paramyxoviruses have been found in *P. medius* in Bangladesh alone: Nipah virus and ten uncharacterized species, including six Rubulaviruses closely related to Menangle virus and the Tioman virus[9,47]. It is possible that the serological patterns observed represent antibodies against a complex of multiple Rubulaviruses, though the regular interannual patterns in seroprevalence would indicate that they are operating similarly.

Nipah virus serodynamics exhibited a different pattern. We found serodynamics consistent with little to no circulation amongst juveniles. Instead, each cohort of juveniles appeared to inherit some maternal antibodies - maternal inheritance was most consistent for Nipah virus in our mother-pup comparisons - and population seroprevalence declined over their first year for all cohorts. Adults experienced several outbreaks, but Nipah virus serodynamics did not exhibit any seasonal patterns, rather, circulation events occurred in irregular intervals one to two years apart. In Thailand, Nipah virus shedding in *Pteropus lylei* was found to vary in time over two years and this pattern varied by strain; Bangladesh-strain Nipah virus shedding clustered in spring months, but only over two years[28]. Human Nipah virus outbreaks in Bangladesh, on the other hand, exhibit seasonality associated with the palm-sap consumption, the most frequently implicated spillover pathway[48].

We had repeated positive detections of filovirus antibodies, using a test designed for Ebola-Zaire virus. This is the first detection of filovirus antibodies in *P. medius* in Bangladesh, though they have been found in *R. leschenaultii* in the country[49]. This could be one of several known filoviruses or an unknown species. There have been several recent findings of new Ebola-like filoviruses in bats extending across Africa and Asia. These include Bombali virus in Sierra Leone[50], and Měnglà virus in *Rousettus* bats in China[51]. Ebola-Reston virus was found in multiple bat species (*M. australis*, *C. brachyotis* and *Ch. plicata*) in the Philippines[52]. Marburg virus and Ebola-Zaire virus may have been detected in Sierra Leone and Liberia (unpublished,[53,54]). Serological evidence of filoviruses in bats has been found in multiple bat species in Central[12,55] and Western[56] African countries, Singapore [57], China[58], as well as Trinidad[59]. Our finding here adds to the evidence of broad host and geographic host ranges for filoviruses.

Several components of the serological patterns of filovirus antibodies are of interest. Young juveniles were almost all seronegative, and seroprevalence rose over the course of the first six months all years, then declined in the latter half of the year. Adult seroprevalence remained steady despite these regular juvenile outbreaks near 50% over the course of the five-year study. Adult seroprevalence against the filovirus also did not exhibit regular annual cycles, though the one period of detectable increase, in mid-2010, coincided with one of the regular seasonal periods of increase among juveniles. One possible hypothesis explaining this pattern is that the filovirus may exhibit low transmissibility in this system, only circulating when seroprevalence is near zero in young juveniles. It is also possible that bats may exhibit weak or waning filovirus antibody response but remain immune. In *R. aegyptiacus*, infected with the Marburg virus, antibody waning post-infection was found to be rapid, but bats retained protective immunity even with very low antibody titers[60,61]. If a similar mechanism occurs in this population, this could explain low seroprevalence in adults despite regular apparent periods of circulation in juveniles. The regular drop of seroprevalence in juveniles following the peak is consistent with this explanation.

However, if adults remain immune to the filovirus despite low antibody titers, this immunity does not appear to transfer to juveniles, as seroprevalence is near-zero among newborns and circulation in juveniles begins soon after birth. The relationship of pup/dam MFI found for the filovirus antibodies suggests that there may be differential inheritance of antibodies and possibly differential maternally derived immunity. If pups derive weaker immunity from dams than for other viruses, this may provide another mechanism for maintenance of the virus. It possible that patterns in filovirus antibody detection are an artifact of our test, specific to Ebola-Zaire virus, and that temporal trends are in part reflective of differential cross-reaction with viruses in this bat population.

Number: 1 Author: Hume Field Date: 8/24/2022 9:59:00 AM
And recently in Australia.
Barr et al. J Gen Virol. 2022 Aug;103(8).
doi: 10.1099/jgv.0.001785.

However, while this may modify estimates of overall seroprevalence, the differential pup/dam MFI relationship, and consistent rise in seroprevalence among young juveniles, would require the test to exhibit differential sensitivity by age.

Serodynamics in our two one-year studies were broadly similar to those found in the five-year study. We found weak evidence for differences in serodynamics between sites for the filovirus in adults, and the rubulavirus for adults and juveniles. However, limited sample numbers and the shorter study period limit our ability to generalize about seasonal patterns. In a concurrent study[34], we found that flying foxes home ranges in Bangladesh extended an average of 50 km from their roosts. The roosts at Chakoria and Ramnagar, the sites of the one-year studies, are approximately 225 km apart. Thus, we would not expect the dynamics of these sites to be strongly coupled. It is plausible that inter- and even intra-annual dynamics of these viruses could be similar if weak coupling and/or environmental factors drive some of the dynamics. More detailed and longer-term studies at multiple sites in parallel are needed to better understand spatiotemporal variation in viral circulation.

Regular co-circulation of these viruses indicates the potential for viral interactions to affect host-viral dynamics. Viral interactions in hosts – whether direct during co-infection or indirect from infection in different periods – can enhance or suppress pathogen mortality, fecundity, or transmission, via competition for host nutritional or cellular resources, or via immune-mediation involving cross-immunity or antibody-dependent enhancement, and these interactions structure viral communities[26]. These interactions can have complex, even opposite effects when scaled to the population[27]. Here, we found positive correlations between serostatus against different viruses. External factors may also affect these relationships. For instance, all three viruses appear to have circulated in adults in mid-2010. It is possible that common factors like population density and/or nutrition availability[62] affected transmission of multiple viruses.

While rich observational serological data reveal these patterns, greater study is required to characterize these viruses and their effects on the host population and potential for spillover, as well as the degree and mechanisms of interactions. Our inferred periods of viral circulation point to optimal sub-populations and times to sample this population to detect viral shedding and isolate these viruses, and potentially capture co-infected hosts.

Extended longitudinal sampling also sheds light on viral dynamics not found in short-term studies. One- to two-year studies can identify temporal patterns in serology or viral prevalence and shedding, but establishing patterns or variation in seasonality requires extended, multi-year sampling. In our case, while Nipah virus outbreaks occurred in the same season in two years, it was apparent from the longer time series that there was no distinct seasonal pattern in dynamics. The consistency of the filovirus and rubulavirus patterns required repeated years of sampling to establish. Similarly, we were able to understand these patterns far better by separating juvenile and adult patterns, which we would be unable to distinguish in pooled samples.

While such extended individual-capture longitudinal studies are resource-intense, understanding the joint circulation of multiple viruses can be accomplished via multiplex immunoassays such as those used here. The continuous measures from these assays also have the potential to identify key patterns such as the differential inheritance of filovirus antibodies we identified here. Interpretation of these values is challenging and the relationship between immune status, antibody titer, and measured fluorescence is complex[32], but they have much potential to shed light on mechanistic drivers of disease circulation.

Methods

Field collection

We conducted a five-year longitudinal study sampling *P. medius* bats quarterly and two one-year studies in different locations, sampling monthly. All capture and sampling methods were approved by Tufts University IACUC protocol #G929-07 and icddr,b animal ethical review (protocol 2006-012). For the five-year study, we sampled from a roost complex near Faridpur, Bangladesh, as previously described in Epstein, et al. [34] The area of the roost complex consists of patchy forest

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Number: 1 Author: Steve Luby Date: 9/2/2022 10:41:00 AM

As noted above, this important detail needs to be mentioned sooner.

Number: 2 Author: Steve Luby Date: 9/2/2022 10:44:00 AM

In addition to uncertainty over which specific viruses these assays are detecting antibodies against, which is addressed earlier in the discussion, other limitations to scientific inference that seem to me important to discuss include:
there were only two sites to assess spatial heterogeneity. A more robust assessment would require more sites.
Although five years is longer than usually studied, it is too short of a time for measuring temporal dynamics that might play out over longer time periods.

Number: 3 Author: Steve Luby Date: 9/2/2022 10:43:00 AM

Seems a weak concluding statement. I recommend a stronger statement of implications and way forward. To my mind, identification of the specific viruses that are driving these antibody responses would be the highest priority.

Number: 4 Author: Steve Luby Date: 9/2/2022 9:47:00 AM

I recommend listing the local IRB first.

interspersed with agrarian human settlements and large rice paddies. Sampling occurred from July 2007 to November 2012 approximately every three months. The bat colony regularly shifted amongst roosts within the 80km^2 roost complex over the period of the study. Sampling occurred at the largest occupied roost found each quarterly sampling trip, sampling at different roosts within the complex across consecutive sampling nights if required to capture a sufficient number of individuals.

Approximately 100 bats were captured at each sampling event, over 7-10 days. We captured bats with a 10x15m mist net between 11pm and 5am each night as bats returned from foraging until the count of 100 was reached.

In the one-year longitudinal studies, sampling was undertaken in two roost complexes in Ramnagar and Chakhoria, Bangladesh between April 2010 and May 2011. Monthly sampling of approximately 40 bats in each location was performed to obtain data at a finer temporal scale. Details of collection are otherwise as described for the five-year study.

For each study, we recorded each bat's age class, reproductive status, weight, size, and body condition. We collected up to 3.0 mL of blood from each bat's brachial vein into serum tubes with serum clot activator (Vaccutainer, USA). Blood tubes were stored vertically on ice packs in a cold box and serum was allowed to separate overnight. Serum was drawn from the tube after 24 hours and placed in a screw-top cryovial (Corning, USA) and stored in a liquid nitrogen dewar (Princeton Cryogenics, NJ, USA).

As part of a larger study to detect viral RNA, we also collected urine, rectal, and oropharyngeal swabs and pooled urine samples from under colonies. Further details can be found in Epstein, et al. [34]

Serological Assays

Sera from the longitudinal studies were sent to the Australian Animal Health Laboratory and gamma irradiated upon receipt. We used a bead-based microsphere assay that specifically detects antibodies to the soluble attachment glycoproteins[57,63,64] for a panel of viruses. Beads coated with each protein were mixed with sera at a dilution of 1:100. Biotinylated Protein A/G and Streptavidin-PE were then used to detect bound antibody. Beads were interrogated by lasers in a BioRad BioPlex machine and the results recorded as the Median Fluorescent Intensity (MFI) of 100 beads. We report here results for Nipah, Ebola-Zaire, and Menangle, the only three for which we established regular positive results.

While the Nipah virus has been detected in this population[65] and the specificity of the Nipah test is well-established [66], the Ebola-Zaire and Menangle virus tests are cross-reactive with other Ebola and rubulavirus species[67,68]. Thus we refer to these as tests for filovirus and Rubulavirus.

Data Analysis

We determined individual bat serostatus using Bayesian mixture models[69] fit on pooled data across all three longitudinal studies, calculating a cutoff of log-MFI as the point of equal probability between the smallest and second-smallest cluster of equal distributions for each assay.

To determine correlations of serostatus in bats across the three viruses, we fit a Bayesian multivariate probit model[70,71] which allows estimation of the joint outcomes (serostatus against each virus) and the correlation between the outcomes. We included age and sex variables to account for these effects on serostatus.

To examine time-varying changes in population seroprevalence, we fit binomial generalized additive mixed models (GAMMs)[72,73] to the time-series of serostatus measurements. For the five-year study, we fit separate models for adults and juveniles and for each immunoassay. For adults, we fit a model for seroprevalence dynamics over the course of the whole study. This treats the adult population as a single unit, though individuals within the population may turn over via migration, death and recruitment. We included both long-term and annual cyclic components for the multi-year time series of measurements. For juveniles, we treated each year's cohort of new recruits as separate populations with commonalities in overall first-year dynamics. The dynamics of each year's juvenile cohort were modeled as random-effect splines, deviating from an overall mean splines for juveniles

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Number: 1 Author: Steve Luby Date: 9/2/2022 9:49:00 AM

Error 5.4

Number: 2 Author: Wang Linfa Date: 8/21/2022 10:50:00 AM

I am not 100% sure about this. All serological tests (the NiV test included) will detect cross-RX antibodies from related viruses. For NiV, you can argue that longitudinal surveillance has NOT detected any related viruses in the study populations, but we can't say that the Ab test is specific to NiV.

Number: 3 Author: Steve Luby Date: 9/2/2022 9:53:00 AM

Why should we assume that the second smallest cluster is associated with the genuine presence or absence of antibody?

over their first year. To estimate periods of peak viral circulation within the population, we calculated the derivatives of model-derived seroprevalence over time. We sampled these from GAMM posterior distributions and classified periods with >95% of samples with positive derivatives - that is, increasing population seroprevalence - as periods of viral circulation. We also calculated strength and timing seasonality for each virus as the maximum rate of seroprevalence increase and the date at which this maximum occurred, again sampling these values from the model posterior, and calculating mean and high-density posterior interval (HDPI) values.

For the one-year studies, we fit models primarily to detect differences in time-varying changes between sites. We fit binomial generalized additive models (GAMs) to the monthly serostatus measurements with separate models for each immunoassay. We included a separate, fixed-term spline in each model for age (adult or juvenile) and location (Chakhoria or Ramnagar). The trends in serodynamics for each virus in each age group were compared between locations to test for spatial differences[74]. Juveniles identified as being from the previous year's cohort were excluded from the analysis.

To examine patterns of maternal inheritance, we used GAMs to fit a relationship of log-MFI between adult lactating females and their attached pups for each viral assay. We limited these to data from the five-year longitudinal study.

Data Availability: Raw data from this study and reproducible code used to perform analyses is available at the Zenodo Scientific Archive [Zenodo DOI to be generated] and also on GitHub at https://github.com/ecohealthalliance/bangladesh-bat-serodynamics

Author Contributions: Conceptualization: N.R. and J.H.E..; methodology, N.R., L-FW and J.H.E.; software, N.R. and S.H.; formal analysis, N.R. and S.H.; investigation, J.H.E., A.I. and L-FW….; resources, X.X.; data curation, N.R and J.H.E; writing—original draft preparation, N.R. J.H.E., and S.H.; writing—review and editing, HEF, X.X.; visualization, N.R. supervision, N.R. and J.H.E.; project administration, J.H.E.; funding acquisition, P.D., L-FW and J.H.E

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Number: 1 Author: Steve Luby Date: 9/2/2022 9:57:00 AM
How did you assess model fit?

Number: 2 Author: Noam Ross Date: 8/10/2022 12:19:00 PM
All co-authors please insert yourselves as appropriate here. A summary of contribution types can be found here: https://img.mdpi.org/data/contributor-role-instruction.pdf

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Co-circulation dynamics of henipaviruses, filoviruses and rubulaviruses in a bat population

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Abstract: Bats are hosts for a variety of viruses, some with potential to cause human disease. *Pteropus medius* are fruit bats that live in close association with humans in Bangladesh and are a known source of Nipah virus and other viruses of unknown pathogenic risk. Here we report on dynamics of seroprevalence against multiple viruses in a population of *P. medius* in Bangladesh over five years. We found evidence of regular circulation of Nipah virus, a rubulavirus, and a filovirus. Nipah virus circulated primarily among adult bats, with no seasonal patterns. The rubulavirus exhibited annual cycles in juveniles and near universal seroprevalence in adults. The filovirus exhibited annual cycles and antibody waning in juveniles and little circulation but lower seroprevalence amongst adults. We found no evidence for spatial differences in Nipah virus dynamics, but weak evidence for differences with other viruses. Host-viral interactions result in very different viral circulation patterns in the same individual bats and environments.

Keywords: bats; Nipah virus; filovirus; Rubulavirus; *Pteropus medius*; Bangladesh; serology; disease dynamics; generalized additive models

Introduction

Bats have been associated with a large diversity of viruses, several of which have been linked to human epidemics [1-3]. The discovery of Severe Acute Respiratory Syndrome (SARS)-related coronaviruses (CoVs) in Chinese horseshoe bats (*Rhinolophus spp.*) in 2004 and subsequent discoveries of related viruses[4,5] including close relatives of SARS-CoV-2[6], along with studies of other high consequence pathogens such as Ebola virus, Marburg virus, Nipah virus, Hendra virus have led to questions about whether and why bats were disproportionately represented among mammals as reservoirs for zoonotic viruses[1,2,7].

Bat species can carry diverse viruses which circulate simultaneously within single populations [8-11]. *Rousettus aegyptiacus* is a natural reservoir of Marburg virus, and IgG antibodies against Ebola Zaire virus have also been detected in this bat in Central Africa, suggesting that it may play a role in the circulation of multiple filoviruses[12,13]. In addition to these filoviruses, a novel zoonotic and pathogenic paramyxovirus, Sosuga virus, was also found in Ugandan *R. aegyptiacus* populations[14].

Summary of Comments on Email 6 - Attachment 2 - Ross-et-al_bangladesh-bats-cocirculation-serology_2022-08-11_LW_HF.pdf

Page: 1

Number: 1 Author: Hume Field Date: 8/24/2022 9:20:00 AM

Pls add my UQ affiliation Noam.
'School of Veterinary Science, The University of Queensland, Gatton 4343 Australia'

Number: 2 Author: Wang Linfa Date: 8/21/2022 10:57:00 AM

It's better to include my CSIRO affiliation here as the work started when I was still associated with AAHL

Number: 3 Author: Noam Ross Date: 8/10/2022 12:38:00 PM

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Eidolon helvum, another pteropodid bat with a broad geographic range in Africa, has been found to carry a lyssavirus, a henipavirus, and several other viruses of unknown pathogenicity[15]. Various surveillance efforts have found diverse viruses from with the same viral family in various bat species[5,16,17], and other studies have used metagenomic approaches to look broadly at viral diversity within individual bat species[9,11,18-21].

Studies of viral diversity in a single taxonomic host group can inform evolutionary history of viruses and their relationships to specific hosts[6,22] and inform public health strategies[23]. However, detection of a zoonotic virus in an animal host is insufficient to determine whether the associated host species is a natural reservoir for the virus, or to characterize the demographic, physiological, or abiotic factors that may influence the timing of viral shedding and therefore risk of spillover to other species, including domestic animals and humans[24]. For these, more detailed demographic and longitudinal studies are required, especially in cases of multiple viruses, as direct or indirect interactions between multiple pathogens may affect disease presence, prevalence, and dynamics[25-27]. Some longitudinal studies of zoonotic viruses in bats have provided insight into the timing of viral infection and shedding, generally of single viruses. Nipah virus in *Pteropus lylei* in Thailand has been observed to have annual shedding patterns [28], whereas Hendra virus, which is closely related to Nipah virus and is carried by multiple pteropid bat species in Australia, has asynchronous and non-periodic cycles which appear to be influenced by localized factors such as specific bat species abundance and climatic factors [29]. Bi-annual pulses of Marburg virus shedding were observed in Uganda, coinciding with synchronous birth pulses in *R. aegyptiacus*[30]. Serological data is often valuable in understanding disease dynamics and transmission[31-33], especially in cases where direct detection and incidence rates of viruses are low[34].

Pteropus sp. are reservoirs of henipaviruses, a group of mostly zoonotic, lethal neurotropic viruses that include Nipah, Hendra, Cedar, Ghana and Mojiang virus[35-37]. In Bangladesh and India, *P. medius* is a reservoir of Nipah virus, which has spilled over to human populations repeatedly[38]. Viruses from eight other viral families have been detected in *P. medius* in Bangladesh[9]. The epidemiology of these other viruses is far less characterized, and little is known about their interactions or zoonotic potential.

Here, we report on the dynamics of seroprevalence against three types of viruses - Nipah virus, a filovirus, and a rubulavirus - circulating simultaneously in *P. medius* populations in three sites in Bangladesh. We conducted a longitudinal study over a period of five years, and two shorter, one-year studies for comparative seasonal data. These studies enable us to develop a detailed picture of the seasonal and demographic serological patterns, and infer the extent and periodicity of viral circulation. We find that Nipah virus circulates primarily among adults without distinct seasonality, while the rubulavirus exhibits strong annual dynamics characteristic of juvenile epidemics driven by waning maternal antibodies. The filovirus, the first detected in *P. medius* in Bangladesh, also circulated in juveniles but exhibited distinct patterns antibody waning and limited maternal inheritance.

2. Results

Bat Dynamics and Demographics

We conducted a five-year longitudinal study sampling *P. medius* bats quarterly from a roost complex in the vicinity of Faridpur, Bangladesh, and also two parallel one-year studies sampling bats monthly from roosts in Chakhoria and Ramnagar, Bangladesh. In the five-year study, we sampled and tested serology of 1,886 bats: 1,224 adults, 623 free-flying juveniles, and 39 weaning juveniles (captured attached to adult females) over 19 sampling events (Figure 1). During the-one-year studies, 919 bats were sampled: 435 in Chakhoria (251 adults, 144 free-flying juveniles and 40 weaning juveniles) and 484 in Ramnagar (277 adults, 164 free-flying juveniles and 43 weaning juveniles (Figure S1). Nearly all juveniles (as determined by examination of maturation of sex organs) were 14 months old or less and could be assigned to a birth cohort based on size. Pregnant and juvenile bats were captured more frequently during the late spring and summer months. Mother-pup pairs were all

Page: 2

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To be consistent with other section headings in Results

captured during April and May in the five-year study whilst in the one-year-studies a small number were also captured in June and July. Pregnant females were captured between November and April/May. Lactating females were found between April and July.

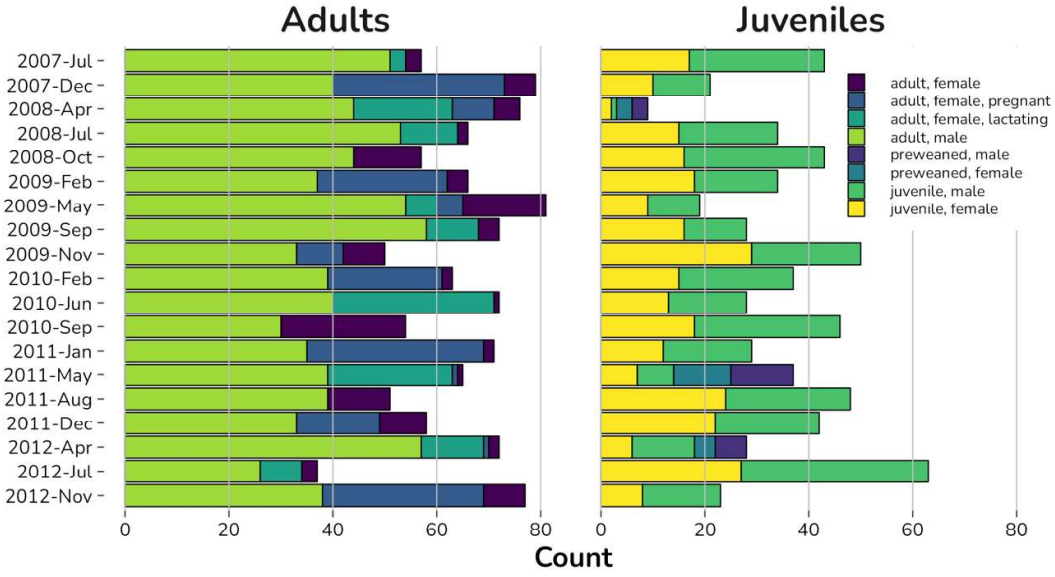

Figure 1. Demographics of bats captured in longitudinal sampling. Pregnant females were captured in months from November to June, lactating females were captured in months from April to July. Females with pre-weaning juveniles attached were found from April to May.

Patterns of Immunity and Co-immunity

We found bats were seropositive for antibodies against the henipavirus (HenipVsero+), a filovirus (EbVsero+), and a rubulavirus (MenVsero+).. Among adult bats, antibodies against the rubulavirus were common; 1,314 of 1752 adult bats were MenVsero+. Antibodies against Nipah virus or filovirus were less common – 1,031 were NiVsero+ and 921 were EbVsero+. Among the 921 juveniles, 192 were MenVsero+, 311 were NiVsero+ and 348 were EbVsero+. Of the 122 pre-weaned juveniles, 43 were MenVsero+, 52 were NiVsero+, and 32 were EbVsero+.

Co-exposure to multiple viruses was common (Figure 2). Over half (1,173) of the 1752 adults had antibodies against more than one of the three viruses, and 493 had antibodies against all three. Among the 921 juveniles, 210 had antibodies against more than one, and 39 had antibodies all three. 38 of 122 pre-weaned juveniles had more than one of the three antibodies, with seven having all three.

We found correlations between serostatus between all three pairs of viruses in a model accounting for bat age, sex, and study location, with all viruses tending to co-occur with each other more than would be expected than if they were distributed independently among bats. Nipah virus and filovirus antibodies had a standardized covariance of 0.13 (95% posterior interval 0.08-0.20). Nipah virus and rubulavirus antibodies were also more likely to occur together, with a covariance of 0.15 (0.09-0.21). Filovirus and rubulavirus antibodies had a covariance of 0.23 (0.17-0.29).

Page: 3

Number: 1 Author: Wang Linfa Date: 8/21/2022 10:48:00 AM

I think it is better to treat the three group of viruses equally as you have done for the title. It makes no difference whether we use NiV or Henipavirus in the context of the current study. Luminex-based assays targeting binding antibodies will in theory detect cross-RX antibodies in all three cases. See also comments in Methods

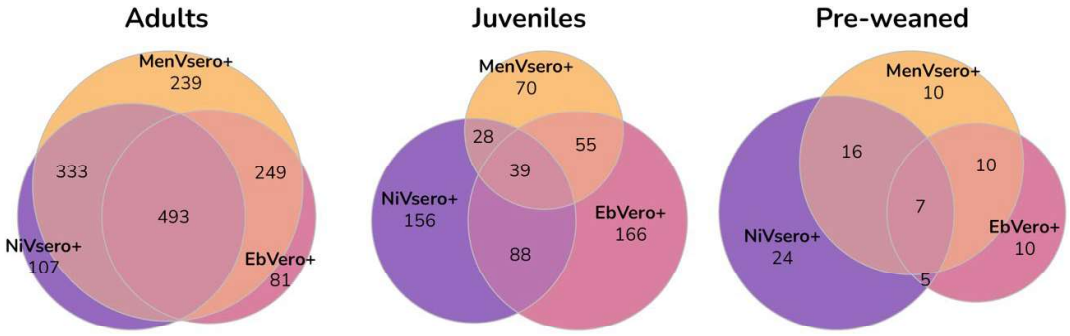

Figure 2. Detection of IgG antibodies against multiple viruses in individual *Pteropus medius*: Venn diagrams for adult, juvenile, and pre-weaned bats testing positive for antibodies against Nipah virus, filovirus, and Rubulavirus. Numbers under labels are counts of bats with only those viruses, numbers in overlapping areas represent number of bats detected with multiple viruses.

Serodynamics

Dynamics of population seroprevalence were different across the viral types. In adults, population seroprevalence against Nipah virus decreased over the study period from 78% (66%-88%) to 50% (40-62%) (Figure 3). Three periods of distinct increase in seroprevalence stood out in the inter-annual signal: early 2008, early 2009, and the second half of 2010. Nipah seroprevalence showed very weak seasonal patterns (Figure 4). (Nipah dynamics were previously reported in Epstein, et al. [34]).

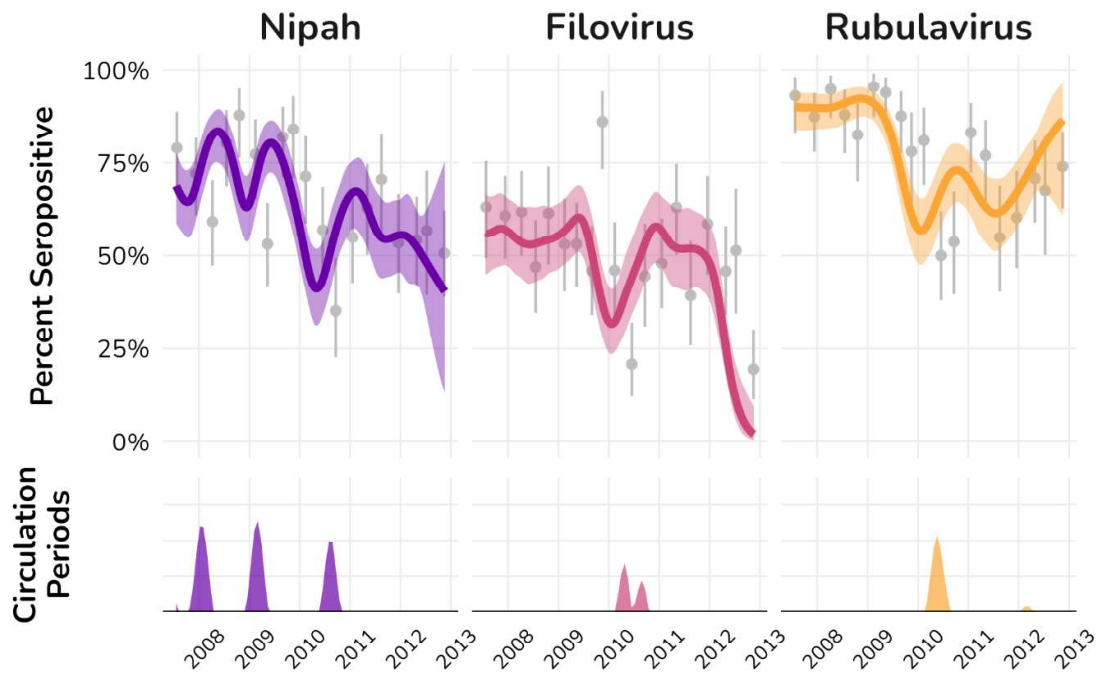

Figure 3. Inter-annual serodynamics in adult bats. Top: Measured and modeled population seroprevalence in adults over the term of the study. X-axis ticks mark the first day of the year. Grey points and bars represent measured population seroprevalence from individual sampling events on and 95% exact binomial confidence intervals. Thick lines and shaded areas represent the inter-annual (non-seasonal) spline component of a generalized additive mixed model (GAMM) of serodynamics and 95% confidence intervals for the mean pattern over time. Bottom: Circulation periods, when modeled slopes of the GAMM splines are positive for >95% of 1000 draws from the model posterior.

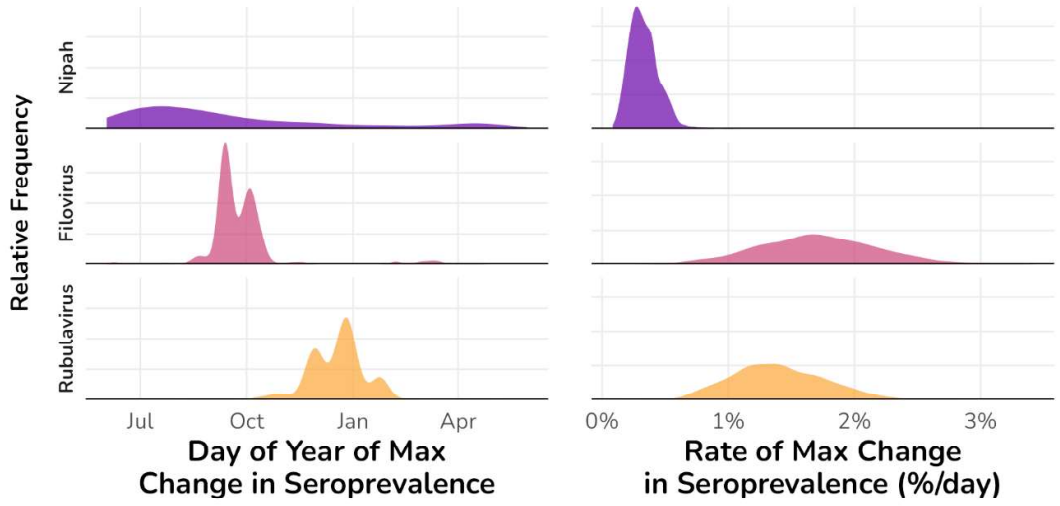

Figure 4. Timing and strength of seasonality of viral circulation in adults. Plots show posterior densities of the date for which increase in seroprevalence is greatest (left), and the rate of increase (right), for adult bats, drawn from GAMM models of seasonality. Densities are of these quantities derived from 1000 samples of model posteriors.

Among juveniles, we examined the dynamics of each cohort over their first year. For Nipah virus, the average cohort had 44% (32%-58%) seroprevalence in June, which consistently decreased over the course of the year to 20% (10%-36%) (Fig. 5). Year-to-year dynamics varied little, with overall declining seroprevalence in all years. No part of the year exhibited regular increases in seroprevalence for juveniles; the maximum rate of increase was indistinguishable from zero (Fig. 6).

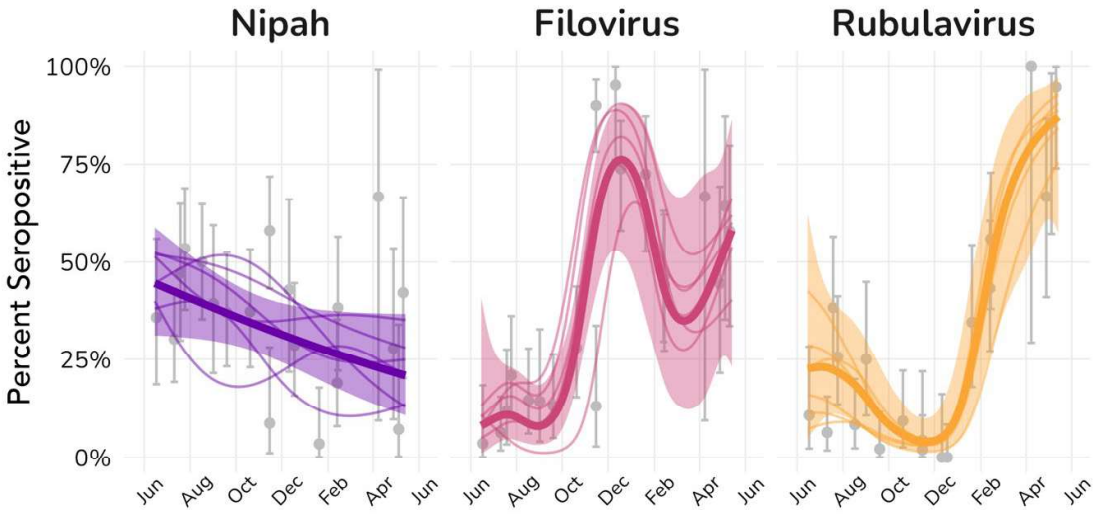

Figure 5. Serodynamics in juvenile bats in five-year study. Measured and modeled population seroprevalence in juvenile bats caught over their first year of life. Sampling events from multiple years are transposed onto a single year starting June (the earliest month free-flying young-of-the-year juveniles were captured). X-axis ticks mark the first day of each month. Grey points and bars represent measured population seroprevalence from individual sampling events on and 95% exact binomial confidence intervals. Thick lines and shaded areas represent GAMM-modeled average serodynamics across all years and 95% confidence intervals for the mean effect. Thin lines represent individual-year deviations from the average year pattern.

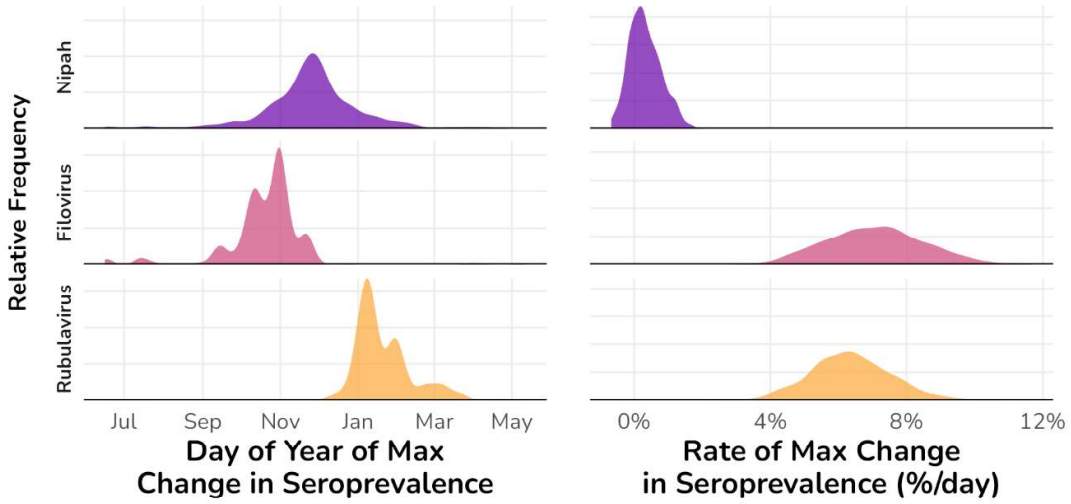

Figure 6. Timing and strength of seasonality of viral circulation in juveniles over the five-year study. Plots show posterior densities of the date for which increase in seroprevalence is greatest (left), and the rate of increase (right), for juvenile bats. Densities are of these quantities derived from 1000 samples of model posteriors.

Filovirus seroprevalence exhibited different patterns. Among adults, seroprevalence against filovirus was lower than for Nipah virus or Rubulavirus. As with Nipah virus, filovirus seroprevalence decreased over the study period, from 64% (50%-76%) to 20% (12%-30%), and had periods of distinct increases in spring/summer 2010 (concurrently with a circulation period for Nipah virus) (Fig. 3). filovirus seroprevalence did not exhibit cyclic annual patterns in adults (Fig. 4).

Among juveniles within the average year, filovirus seroprevalence started very low in June at 8% (2%-40%), but increased to a peak of 44% (32%-58%) in juveniles approximately six months of age before declining. (Fig 5). This pattern was consistent across years; though the peak varied in size, it consistently occurred in December or January. The average date with the greatest rate of seroprevalence increase was Oct 31 (Jul 14-Nov 25), and the maximum rate of increase was 7.1%/day (4.5%/day-9.9%/day).

Rubulavirus seroprevalence had a distinct dynamic pattern. Among adults, rubulavirus seroprevalence was high at 92% (82%-98%), and remained high through the end of the period at 74% (62%-84%), though there were some temporary periods of decline. There were distinct periods of increasing circulation in early 2010 and early 2012. Only for the rubulavirus did adult seroprevalence exhibit patterns of seasonality. Periods of increasing seroprevalence in adults exhibited strong seasonality of 1.6%/day (1.0%/day-2.4%/day) increase on peak days, with the mean peak date occurring on Dec 15 (Oct 15-Feb 1)

In juveniles, seroprevalence against the rubulavirus in the average year was 22% (4%-62%) in the youngest bats in June, though in individual years this could range from 7%-43%. In all years juvenile seroprevalence declined to a low of 1.7% (0.6%, 8.3%) that occurred between Oct 9 and Dec 11, then increased starting in winter, reaching high values in yearling juveniles of 88% (56%-99%) The peak rate of increase for juvenile rubulavirus seroprevalence was 6.4%/day (4.2%-8.8%) and occurred Jan 9 (Dec 21-Mar 16).

Spatial Comparisons

Seroprevalence trends in both adult and juvenile bats within the one-year studies in Chakhoria and Ramnagar broadly mirrored those in the five-year study in Faridpur (Supplementary Figure 2). In adults, Nipah virus seroprevalence increased in both Ramnagar and Chakhoria over the course of April 2010-May 2011, consistent with the period of increased seroprevalence observed in late 2010 in

the Faridpur. In Ramnagar, the seroprevalence in adults was 41% (24%-61%) in April 2010, rising to 60% (36%-81%) in May 2011 whilst in Chakhoria it rose from 26% (10%-48%) to 60% (36%-81%) over the same period. The trend in juveniles was as described for the five-year study.

For the filovirus, the seroprevalence in the one-year studies was higher than the reported average in the five-year study, with seroprevalence ranging between 41% (21%-64%) and 75% (51%-91%) in Ramnagar and between 48% (26%-70%) and 100% (83%-100%) in Chakhoria. A period of distinct increase in spring/summer 2010 was described in the five-year study which overlaps the timing of the one-year studies and may explain this difference in seroprevalence. In Ramnagar the serodynamics in both adults and juveniles were as described for the five-year study. In Chakhoria, there were two periods of increasing seroprevalence in adults over the course of the year. The first of these occurred in late 2010 when the adult seroprevalence increased from 48% (28%-69%) in August 2010 to 100% (83%-100%) in October 2010. The second period of increase occurred at the end of the study period in Spring 2011 with seroprevalence rising from 48% (26%-70%) to 75% (51%-91%) between early March 2011 to late April 2011.

Rubulavirus seroprevalence was high in adults throughout the one-year studies, ranging from 50% (27%-73%) to 95% (76%-100%) in Ramnagar and 29% (11%-52%) to 100% (83%-100%) in Chakhoria, with the lowest values observed in September 2010 in Ramnagar and December 2010/January 2011 in Chakhoria. These decreases were followed by rapid increases back to high seroprevalence, coincident with increasing levels in juveniles. The pattern in juveniles was as described in the five-year study, with seroprevalence estimates in yearlings in April 2011 of 80% (28%-99%) in both locations.

Comparison of the seroprevalence trends within each age group and virus between the two one-year study locations did not support spatial differences in the timing of changes in seroprevalence (Figure 7). Only for the filovirus in adults was there some evidence of a difference in trend between locations coinciding with the period of increased seroprevalence observed in adults in Chakhoria at the end of 2010.

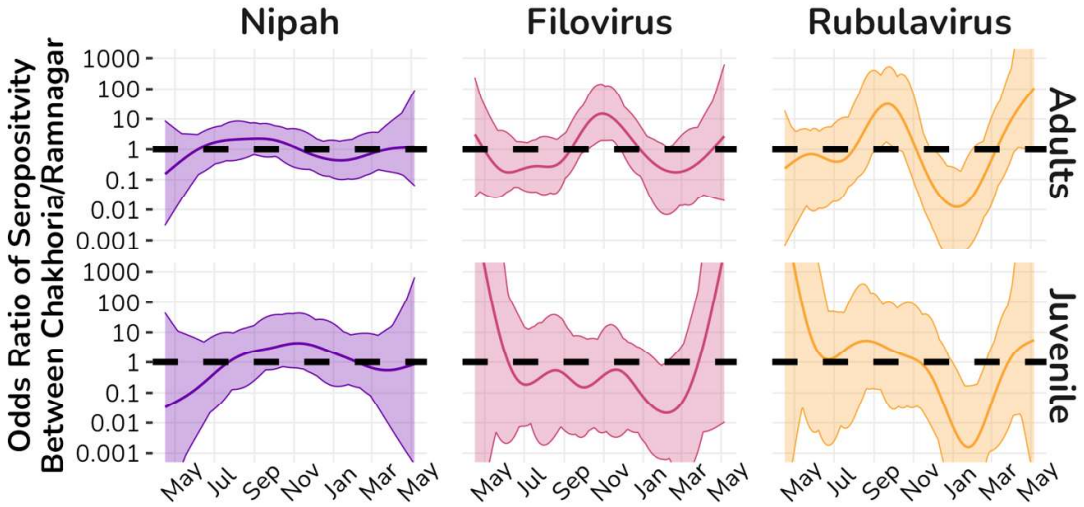

Figure 7. Comparison of splines for seroprevalence trends between locations in adults and juveniles over one-year study. Thick lines and shaded errors represent the estimated difference in trends and their associated 95% confidence intervals. The horizontal dashed line is set at zero. Where the confidence interval excludes zero, there is evidence for a significant difference in the serodynamics between the Ramnagar and Chakhoria populations over that period.

Maternal Inheritance of antibodies

We examined the relationship between mother and pup antibody titers, as measured by our Luminex assays (see Methods). This relationship varied between viruses (Figure 7). For Nipah virus,

the relationship was nonlinear due to clustering at high and low values, but largely followed a 1:1 relationship between mother and pup antibody titers. For the rubulavirus the relationship was near-linear and near 1:1. For the filovirus, though, the relationship between MFI values measured in mothers and their pups fell well below the 1:1 line, indicating pups having low inheritance of antibodies against the filovirus relative to the other two viruses.

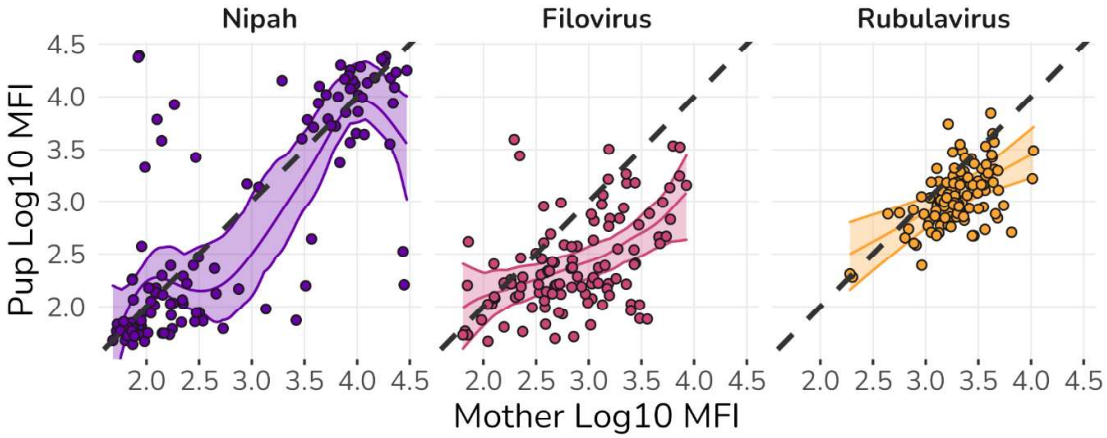

Figure 8. Maternal inheritance of antibodies. In each plot, points are measured log-MFI values for adults and juveniles in mother-pup pairs for each of the three antibody tests. Lines represent the predicted mean relationship between the two and their associated 95% confidence intervals.

3. Discussion

We found serological evidence for regular circulation of multiple viruses in *P. medius* populations in Bangladesh. These included Nipah virus, which spills over from bat populations to humans in the region, as well as a filovirus and a rubulavirus. It was common for bats to have antibodies against multiple viruses, and more common in adults than juveniles. Co-immunity patterns among pre-weaned juveniles mirrored those in adults, reflecting maternal inheritance patterns. Patterns of co-immunity were not random, with antibodies against all three virus types being positively correlated within bats.

The dynamic patterns in population seroprevalence were distinct for the three viral groups. The rubulavirus exhibited the most regular and consistent patterns. Some juveniles in these populations inherit maternal antibodies against the rubulavirus, which wane over the first six months of their life, after which a regular annual epidemic occurs in the juvenile population, causing a steep rise in juvenile seroprevalence by the end of their first year. High adult seroprevalence is likely the consequence of most bats being exposed to the disease as juveniles. When adult seroprevalence drops, as it did in 2009 to 2011, it rapidly rebounds during the same season as juveniles, indicating the same regular epidemics affect both adults and juveniles. We expect circulation and shedding of the rubulavirus to be strongest in the late winter and early spring months in these populations. Mortlock at al.[39] found irregular patterns of rubulavirus shedding in *Rousettus* bats over one year (via PCR detection in pooled urine samples), including a peak during a period of presumed antibody waning, though only within a one-year study.

An outstanding question is how the rubulavirus is maintained in the population despite high seroprevalence in inter-epidemic periods. Recrudescence is one possibility. Evidence for latent infections and recrudescence has been found for Nipah virus in *Pteropus vampyrus*[40], Hendra virus in *P. alecto and P. poliocephalus*[41], and Lagos bat lyssavirus and African henipavirus in *Eidolon helvum*[42]. Another possibility is re-importation. In concurrent work with this study, we found that bat home

ranges overlapped with nearby colonies so as to form a meta-population[34], allowing occasional infection from outside bats, as has been shown to maintain Hendra virus in *Pteropus* populations[43,44].

The identity of the rubulavirus in this population is unclear. Our test was designed for Menangle virus, which has been found in multiple *Pteropus* species in Australia[45,46]. At least 11 distinct Paramyxoviruses have been found in *P. medius* in Bangladesh alone: Nipah virus and ten uncharacterized species, including six Rubulaviruses closely related to Menangle virus and the Tioman virus[9,47]. It is possible that the serological patterns observed represent antibodies against a complex of multiple Rubulaviruses, though the regular interannual patterns in seroprevalence would indicate that they are operating similarly.

Nipah virus serodynamics exhibited a different pattern. We found serodynamics consistent with little to no circulation amongst juveniles. Instead, each cohort of juveniles appeared to inherit some maternal antibodies - maternal inheritance was most consistent for Nipah virus in our mother-pup comparisons - and population seroprevalence declined over their first year for all cohorts. Adults experienced several outbreaks, but Nipah virus serodynamics did not exhibit any seasonal patterns, rather, circulation events occurred in irregular intervals one to two years apart. In Thailand, Nipah virus shedding in *Pteropus lylei* was found to vary in time over two years and this pattern varied by strain; Bangladesh-strain Nipah virus shedding clustered in spring months, but only over two years[28]. Human Nipah virus outbreaks in Bangladesh, on the other hand, exhibit seasonality associated with the palm-sap consumption, the most likely spillover mechanism[48].

We had repeated positive detections of filovirus antibodies, using a test designed for Ebola-Zaire virus. This is the first detection of filovirus antibodies in *P. medius* in Bangladesh, though they have been found in *R. leschenaultii* in the country[49]. This could be one of several known filoviruses or an unknown species. There have been several recent findings of new Ebola-like filoviruses in bats extending across Africa and Asia. These include Bombali virus in Sierra Leone[50], and Měnglà virus in *Rousettus* bats in China[51]. Ebola-Reston virus was found in multiple bat species (*M. australis*, *C. brachyotis* and *Ch. plicata*) in the Philippines[52]. Marburg virus and Ebola-Zaire virus may have been detected in Sierra Leone and Liberia (unpublished,[53,54]). Serological evidence of filoviruses in bats has been found in multiple bat species in Central[12,55] and Western[56] African countries, Singapore [57], China[58], as well as Trinidad[59]. Our finding here adds to the evidence of broad host and geographic host ranges for filoviruses.

Several components of the serological patterns of filovirus antibodies are of interest. Young juveniles were almost all seronegative, and seroprevalence rose over the course of the first six months all years, then declined in the latter half of the year. Adult seroprevalence remained steady despite these regular juvenile outbreaks near 50% over the course of the five-year study. Adult seroprevalence against the filovirus also did not exhibit regular annual cycles, though the one period of detectable increase, in mid-2010, coincided with one of the regular seasonal periods of increase among juveniles. One possible hypothesis explaining this pattern is that the filovirus may exhibit low transmissibility in this system, only circulating when seroprevalence is near zero in young juveniles. It is also possible that bats may exhibit weak or waning filovirus antibody response but remain immune. In *R. aegyptiacus*, infected with the Marburg virus, antibody waning post-infection was found to be rapid, but bats retained protective immunity even with very low antibody titers[60,61]. If a similar mechanism occurs in this population, this could explain low seroprevalence in adults despite regular apparent periods of circulation in juveniles. The regular drop of seroprevalence in juveniles following the peak is consistent with this explanation.

However, if adults remain immune to the filovirus despite low antibody titers, this immunity does not appear to transfer to juveniles, as seroprevalence is near-zero among newborns and circulation in juveniles begins soon after birth. The relationship of pup/dam MFI found for the filovirus antibodies suggests that there may be differential inheritance of antibodies and possibly differential maternally derived immunity. If pups derive weaker immunity from dams than for other viruses, this may provide another mechanism for maintenance of the virus. It possible that patterns in filovirus antibody detection are an artifact of our test, specific to Ebola-Zaire virus, and that temporal trends are in part reflective of differential cross-reaction with viruses in this bat population.

Number: 1 Author: Hume Field Date: 8/24/2022 9:59:00 AM
And recently in Australia.
Barr et al. J Gen Virol. 2022 Aug;103(8).
doi: 10.1099/jgv.0.001785.

However, while this may modify estimates of overall seroprevalence, the differential pup/dam MFI relationship, and consistent rise in seroprevalence among young juveniles, would require the test to exhibit differential sensitivity by age.

Serodynamics in our two one-year studies were broadly similar to those found in the five-year study. We found weak evidence for differences in serodynamics between sites for the filovirus in adults, and the rubulavirus for adults and juveniles. However, limited sample numbers and the shorter study period limit our ability to generalize about seasonal patterns. In a concurrent study[34], we found that flying foxes home ranges in Bangladesh extended an average of 50 km from their roosts. The roosts at Chakoria and Ramnagar, the sites of the one-year studies, are approximately 225 km apart. Thus, we would not expect the dynamics of these sites to be strongly coupled. It is plausible that inter- and even intra-annual dynamics of these viruses could be similar if weak coupling and/or environmental factors drive some of the dynamics. More detailed and longer-term studies at multiple sites in parallel are needed to better understand spatiotemporal variation in viral circulation.

Regular co-circulation of these viruses indicates the potential for viral interactions to affect host-viral dynamics. Viral interactions in hosts – whether direct during co-infection or indirect from infection in different periods – can enhance or suppress pathogen mortality, fecundity, or transmission, via competition for host nutritional or cellular resources, or via immune-mediation involving cross-immunity or antibody-dependent enhancement, and these interactions structure viral communities[26]. These interactions can have complex, even opposite effects when scaled to the population[27]. Here, we found positive correlations between serostatus against different viruses. External factors may also affect these relationships. For instance, all three viruses appear to have circulated in adults in mid-2010. It is possible that common factors like population density and/or nutrition availability[62] affected transmission of multiple viruses.

While rich observational serological data reveal these patterns, greater study is required to characterize these viruses and their effects on the host population and potential for spillover, as well as the degree and mechanisms of interactions. Our inferred periods of viral circulation point to optimal sub-populations and times to sample this population to detect viral shedding and isolate these viruses, and potentially capture co-infected hosts.

Extended longitudinal sampling also sheds light on viral dynamics not found in short-term studies. One- to two-year studies can identify temporal patterns in serology or viral prevalence and shedding, but establishing patterns or variation in seasonality requires extended, multi-year sampling. In our case, while Nipah virus outbreaks occurred in the same season in two years, it was apparent from the longer time series that there was no distinct seasonal pattern in dynamics. The consistency of the filovirus and rubulavirus patterns required repeated years of sampling to establish. Similarly, we were able to understand these patterns far better by separating juvenile and adult patterns, which we would be unable to distinguish in pooled samples.

While such extended individual-capture longitudinal studies are resource-intense, understanding the joint circulation of multiple viruses can be accomplished via multiplex immunoassays such as those used here. The continuous measures from these assays also have the potential to identify key patterns such as the differential inheritance of filovirus antibodies we identified here. Interpretation of these values is challenging and the relationship between immune status, antibody titer, and measured fluorescence is complex[32], but they have much potential to shed light on mechanistic drivers of disease circulation.

Methods

Field collection

We conducted a five-year longitudinal study sampling *P. medius* bats quarterly and two one-year studies in different locations, sampling monthly. All capture and sampling methods were approved by Tufts University IACUC protocol #G929-07 and icddr,b animal ethical review (protocol 2006-012). For the five-year study, we sampled from a roost complex near Faridpur, Bangladesh, as previously described in Epstein, et al. [34] The area of the roost complex consists of patchy forest

interspersed with agrarian human settlements and large rice paddies. Sampling occurred from July 2007 to November 2012 approximately every three months. The bat colony regularly shifted amongst roosts within the 80km^2 roost complex over the period of the study. Sampling occurred at the largest occupied roost found each quarterly sampling trip, sampling at different roosts within the complex across consecutive sampling nights if required to capture a sufficient number of individuals.

Approximately 100 bats were captured at each sampling event, which lasted 7-10 days. We captured bats with a 10x15m mist net between 11pm and 5am each night as bats returned from foraging until the count of 100 was reached.

In the one-year longitudinal studies, sampling was undertaken in two roost complexes in Ramnagar and Chakhoria, Bangladesh between April 2010 and May 2011. Monthly sampling of approximately 40 bats in each location was performed to obtain data at a finer temporal scale. Details of collection are otherwise as described for the five-year study.

For each study, we recorded each bat's age class, reproductive status, weight, size, and body condition. We collected up to 3.0 mL of blood from each bat's brachial vein into serum tubes with serum clot activator (Vaccutainer, USA). Blood tubes were stored vertically on ice packs in a cold box and serum was allowed to separate overnight. Serum was drawn from the tube after 24 hours and placed in a screw-top cryovial (Corning, USA) and stored in a liquid nitrogen dewar (Princeton Cryogenics, NJ, USA).

As part of a larger study to detect viral RNA, we also collected urine, rectal, and oropharyngeal swabs and pooled urine samples from under colonies. Further details can be found in Epstein, et al. [34]

Serological Assays

Sera from the longitudinal studies were sent to the Australian Animal Health Laboratory and gamma irradiated upon receipt. We used a bead-based microsphere assay that specifically detects antibodies to the soluble attachment glycoproteins[57,63,64] for a panel of viruses. Beads coated with each protein were mixed with sera at a dilution of 1:100. Biotinylated Protein A/G and Streptavidin-PE were then used to detect bound antibody. Beads were interrogated by lasers in a BioRad BioPlex machine and the results recorded as the Median Fluorescent Intensity (MFI) of 100 beads. We report here results for Nipah, Ebola-Zaire, and Menangle, the only three for which we established regular positive results.

While the Nipah virus has been detected in this population[65] and the specificity of the Nipah test is well-established [66], the Ebola-Zaire and Menangle virus tests are cross-reactive with other Ebola and rubulavirus species[67,68]. Thus we refer to these as tests for filovirus and Rubulavirus.

Data Analysis

We determined individual bat serostatus using Bayesian mixture models[69] fit on pooled data across all three longitudinal studies, calculating a cutoff of log-MFI as the point of equal probability between the smallest and second-smallest cluster of equal distributions for each assay.

To determine correlations of serostatus in bats across the three viruses, we fit a Bayesian multivariate probit model[70,71] which allows estimation of the joint outcomes (serostatus against each virus) and the correlation between the outcomes. We included age and sex variables to account for these effects on serostatus.

To examine time-varying changes in population seroprevalence, we fit binomial generalized additive mixed models (GAMMs)[72,73] to the time-series of serostatus measurements. For the five-year study, we fit separate models for adults and juveniles and for each immunoassay. For adults, we fit a model for seroprevalence dynamics over the course of the whole study. This treats the adult population as a single unit, though individuals within the population may turn over via migration, death and recruitment. We included both long-term and annual cyclic components for the multi-year time series of measurements. For juveniles, we treated each year's cohort of new recruits as separate populations with commonalities in overall first-year dynamics. The dynamics of each year's juvenile cohort were modeled as random-effect splines, deviating from an overall mean splines for juveniles

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Number: 1 Author: Wang Linfa Date: 8/21/2022 10:50:00 AM

I am not 100% sure about this. All serological tests (the NiV test included) will detect cross-RX antibodies from related viruses. For NiV, you can argue that longitudinal surveillance has NOT detected any related viruses in the study populations, but we can't say that the Ab test is specific to NiV.

over their first year. To estimate periods of peak viral circulation within the population, we calculated the derivatives of model-derived seroprevalence over time. We sampled these from GAMM posterior distributions and classified periods with >95% of samples with positive derivatives - that is, increasing population seroprevalence - as periods of viral circulation. We also calculated strength and timing seasonality for each virus as the maximum rate of seroprevalence increase and the date at which this maximum occurred, again sampling these values from the model posterior, and calculating mean and high-density posterior interval (HDPI) values.

For the one-year studies, we fit models primarily to detect differences in time-varying changes between sites. We fit binomial generalized additive models (GAMs) to the monthly serostatus measurements with separate models for each immunoassay. We included a separate, fixed-term spline in each model for age (adult or juvenile) and location (Chakhoria or Ramnagar). The trends in serodynamics for each virus in each age group were compared between locations to test for spatial differences[74]. Juveniles identified as being from the previous year's cohort were excluded from the analysis.

To examine patterns of maternal inheritance, we used GAMs to fit a relationship of log-MFI between adult lactating females and their attached pups for each viral assay. We limited these to data from the five-year longitudinal study.

Data Availability: Raw data from this study and reproducible code used to perform analyses is available at the Zenodo Scientific Archive [Zenodo DOI to be generated] and also on GitHub at https://github.com/ecohealthalliance/bangladesh-bat-serodynamics

Author Contributions: Conceptualization: N.R. and J.H.E..; methodology, N.R., L-FW and J.H.E.; software, N.R. and S.H.; formal analysis, N.R. and S.H.; investigation, J.H.E., A.I. and L-FW….; resources, X.X.; data curation, N.R and J.H.E; writing—original draft preparation, N.R. J.H.E., and S.H.; writing—review and editing, HEF, X.X.; visualization, N.R. supervision, N.R. and J.H.E.; project administration, J.H.E.; funding acquisition, P.D., L-FW and J.H.E

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All co-authors please insert yourselves as appropriate here. A summary of contribution types can be found here: https://img.mdpi.org/data/contributor-role-instruction.pdf

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From:	Broder, Christopher on behalf of Broder, Christopher <christopher.broder@usuhs.edu>
To:	epstein@ecohealthalliance.org
Subject:	Fwd: Draft manuscript: Co-circulation dynamics of viruses in a bat population
Date:	Saturday, September 3, 2022 2:11:17 PM
Attachments:	Ross-et-al_bangladesh-bats-cocirculation-serology_2022-08-11_LW_HF_sl.docx

hi Jon,

Happy Labor day weekend.

What is the story on the screening here? The
paper reads only NiV EBOV and MenV attachment proteins used
so I am assuming all our proteins. NiV-sG / EBO sGp / MeV-sHN ?
What the entire panel of proteins used? Linfa's comment about saying
heniv sero+ and not just NiV only matters if the other sG beads were used
and what the data looks like.

technically, sGp EBO trimer, is attachment+fusion Glycoprotein,
but the sHN if used this was a new project of both Eric and I working with Linda to construct and we
shipped to Ina Smith, Jenn and Gary in early 18'

I think we need to see all the raw data

Chris

---------- Forwarded message ---------
From: **Steve Luby** <sluby@stanford.edu>
Date: Fri, Sep 2, 2022 at 3:08 PM
Subject: Re: Draft manuscript: Co-circulation dynamics of viruses in a bat population
To: Jon Epstein <epstein@ecohealthalliance.org>
Cc: Ariful Islam <arif@ecohealthalliance.org>, Hayes, Sarah <sarah.hayes16@imperial.ac.uk>, A. Marm Kilpatrick <akilpatr@ucsc.edu>, Kevin Olival, PhD <olival@ecohealthalliance.org>, Emily Gurley <egurley1@jhu.edu>, Dr. Jahangir Hossain <jhossain@mrc.gm>, Gary Crameri <garycrameri1@gmail.com>, Linfa Wang <linfa.wang@duke-nus.edu.sg>, Christopher Broder <christopher.broder@usuhs.edu>, Peter Daszak <daszak@ecohealthalliance.org>, Noam Ross <ross@ecohealthalliance.org>, Madeline Salino <salino@ecohealthalliance.org>, <hume.field@ecohealthalliance.org>

Hi Jon,

Thanks for sharing this interesting work. Kudos to Noam and the team for pulling all of this together.

Attached are my comments.

To reduce the amount of my time and improve the thoroughness of my review, I use error codes when I identify common errors within a scientific document. A full description of the error and strategies for addressing it can be found in a scientific writing guide written by Dorothy Southern and I (Pathway to Publishing-A Guide to Quantitative Writing in the Health

Sciences) published as an Open Access ebook through Springer. It can be downloaded from here: https://link.springer.com/book/10.1007/978-3-030-98175-4

Steve

hume.field@ecohealthalliance.org wrote on 8/23/2022 6:40 PM:

> Great to see Jon, Noam. Reads well, nice graphics. Thanks for co-authorship.. my edits added to Linfa's version.
>
> Regards all
>
> Hume
>
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>
> Adjunct Professor | **University of Queensland** | Australia
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From: Jon Epstein <epstein@ecohealthalliance.org>
Sent: Saturday, 20 August 2022 1:22 AM
To: Ariful Islam <arif@ecohealthalliance.org>; Hayes, Sarah <sarah.hayes16@imperial.ac.uk>; A. Marm Kilpatrick <akilpatr@ucsc.edu>; Kevin Olival, PhD <olival@ecohealthalliance.org>; Emily Gurley <egurley1@jhu.edu>; Dr. Jahangir Hossain <jhossain@mrc.gm>; Hume Field <hume.field@ecohealthalliance.org>; Gary Crameri <garycrameri1@gmail.com>; Linfa Wang <linfa.wang@duke-nus.edu.sg>; Stephen Luby <sluby@stanford.edu>; Christopher Broder <christopher.broder@usuhs.edu>; Peter Daszak <daszak@ecohealthalliance.org>
Cc: Noam Ross <ross@ecohealthalliance.org>; Madeline Salino <salino@ecohealthalliance.org>
Subject: Draft manuscript: Co-circulation dynamics of viruses in a bat population

Colleagues,

I hope you're all doing well. I'm excited to share a draft manuscript, led by Noam, that details the serodynamics of henipaviruses, filoviruses and rubulaviruses in a population of *Pteropus medius*. We plan to submit this to *Nature Communications* in September. Please send Noam and I your comments by **September 10th**.

Cheers,

Jon

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Jonathan H. Epstein DVM, MPH, PhD

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EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation

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(b) (6) [redacted]

Co-circulation dynamics of henipaviruses, filoviruses and rubulaviruses in a bat population

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Abstract: Bats are hosts for a variety of viruses, some with potential to cause human disease. *Pteropus medius* are fruit bats that live in close association with humans in Bangladesh and are a known source of Nipah virus and other viruses of unknown pathogenic risk. Here we report on dynamics of seroprevalence against multiple viruses in a population of *P. medius* in Bangladesh over five years. We found evidence of regular circulation of Nipah virus, a rubulavirus, and a filovirus. Nipah virus circulated primarily among adult bats, with no seasonal patterns. The rubulavirus exhibited annual cycles in juveniles and near universal seroprevalence in adults. The filovirus exhibited annual cycles and antibody waning in juveniles and little circulation but lower seroprevalence amongst adults. We found no evidence for spatial differences in Nipah virus dynamics, but weak evidence for differences with other viruses. Host-viral interactions result in very different viral circulation patterns in the same individual bats and environments.

Keywords: bats; Nipah virus; filovirus; Rubulavirus; *Pteropus medius*; Bangladesh; serology; disease dynamics; generalized additive models

Introduction

Bats have been associated with a large diversity of viruses, several of which have been linked to human epidemics [1-3]. The discovery of Severe Acute Respiratory Syndrome (SARS)-related coronaviruses (CoVs) in Chinese horseshoe bats (*Rhinolophus spp.*) in 2004 and subsequent discoveries of related viruses[4,5] including close relatives of SARS-CoV-2[6], along with studies of other high consequence pathogens such as Ebola virus, Marburg virus, Nipah virus, Hendra virus have led to questions about whether and why bats were disproportionately represented among mammals as reservoirs for zoonotic viruses[1,2,7].

Bat species can carry diverse viruses which circulate simultaneously within single populations [8-11]. *Rousettus aegyptiacus* is a natural reservoir of Marburg virus, and IgG antibodies against Ebola Zaire virus have also been detected in this bat in Central Africa, suggesting that it may play a role in the circulation of multiple filoviruses[12,13]. In addition to these filoviruses, a novel zoonotic and pathogenic paramyxovirus, Sosuga virus, was also found in Ugandan *R. aegyptiacus* populations[14].

Summary of Comments on Email 7 - Attachment 1 - Ross-et-al_bangladesh-bats-cocirculation-serology_2022-08-11_LW (002).pdf

Page: 1

Number: 1 Author: Wang Linfa Date: 8/21/2022 10:57:00 AM

It's better to include my CSIRO affiliation here as the work started when I was still associated with AAHL

Number: 2 Author: Noam Ross Date: 8/10/2022 12:38:00 PM

Please check that your affiliation is as it should be!

Eidolon helvum, another pteropodid bat with a broad geographic range in Africa, has been found to carry a lyssavirus, a henipavirus, and several other viruses of unknown pathogenicity[15]. Various surveillance efforts have found diverse viruses from with the same viral family in various bat species[5,16,17], and other studies have used metagenomic approaches to look broadly at viral diversity within individual bat species[9,11,18-21].

Studies of viral diversity in a single taxonomic host group can inform evolutionary history of viruses and their relationships to specific hosts[6,22] and inform public health strategies[23]. However, detection of a zoonotic virus in an animal host is insufficient to determine whether the associated host species is a natural reservoir for the virus, or to characterize the demographic, physiological, or abiotic factors that may influence the timing of viral shedding and therefore risk of spillover to other species, including domestic animals and humans[24]. For these, more detailed demographic and longitudinal studies are required, especially in cases of multiple viruses, as direct or indirect interactions between multiple pathogens may affect disease presence, prevalence, and dynamics[25-27]. Some longitudinal studies of zoonotic viruses in bats have provided insight into the timing of viral infection and shedding, generally of single viruses. Nipah virus in *Pteropus lylei* in Thailand has been observed to have annual shedding patterns [28], whereas Hendra virus, which is closely related to Nipah virus and is carried by multiple pteropid bat species in Australia, has asynchronous and non-periodic cycles which appear to be influenced by localized factors such as specific bat species abundance and climatic factors [29]. Bi-annual pulses of Marburg virus shedding were observed in Uganda, coinciding with synchronous birth pulses in *R. aegyptiacus*[30]. Serological data is often valuable in understanding disease dynamics and transmission[31-33], especially in cases where direct detection and incidence rates of viruses are low[34].

Pteropus sp. are reservoirs of henipaviruses, a group of mostly zoonotic, lethal neurotropic viruses that include Nipah, Hendra, Cedar, Ghana and Mojiang virus[35-37]. In Bangladesh and India, *P. medius* is a reservoir of Nipah virus, which has spilled over to human populations repeatedly[38]. Viruses from eight other viral families have been detected in *P. medius* in Bangladesh[9]. The epidemiology of these other viruses is far less characterized, and little is known about their interactions or zoonotic potential.

Here, we report on the dynamics of seroprevalence against three types of viruses - Nipah virus, a filovirus, and a rubulavirus - circulating simultaneously in *P. medius* populations in three sites in Bangladesh. We conducted a longitudinal study over a period of five years, and two shorter, one-year studies for comparative seasonal data. These studies enable us to develop a detailed picture of the seasonal and demographic serological patterns, and infer the extent and periodicity of viral circulation. We find that Nipah virus circulates primarily among adults without distinct seasonality, while the rubulavirus exhibits strong annual dynamics characteristic of juvenile epidemics driven by waning maternal antibodies. The filovirus, the first detected in *P. medius* in Bangladesh, also circulated in juveniles but exhibited distinct patterns antibody waning and limited maternal inheritance.

2. Results

Bat Dynamics and Demographics[1]

We conducted a five-year longitudinal study sampling *P. medius* bats quarterly from a roost complex in the vicinity of Faridpur, Bangladesh, and also two parallel one-year studies sampling bats monthly from roosts in Chakhoria and Ramnagar, Bangladesh. In the five-year study, we sampled and tested serology of 1,886 bats: 1,224 adults, 623 free-flying juveniles, and 39 weaning juveniles (captured attached to adult females) over 19 sampling events (Figure 1). During the-one-year studies, 919 bats were sampled: 435 in Chakhoria (251 adults, 144 free-flying juveniles and 40 weaning juveniles) and 484 in Ramnagar (277 adults, 164 free-flying juveniles and 43 weaning juveniles (Figure S1). Nearly all juveniles (as determined by examination of maturation of sex organs) were 14 months old or less and could be assigned to a birth cohort based on size. Pregnant and juvenile bats were captured more frequently during the late spring and summer months. Mother-pup pairs were all

Number: 1 Author: Wang Linfa Date: 8/21/2022 10:37:00 AM
To be consistent with other section headings in Results

captured during April and May in the five-year study whilst in the one-year-studies a small number were also captured in June and July. Pregnant females were captured between November and April/May. Lactating females were found between April and July.

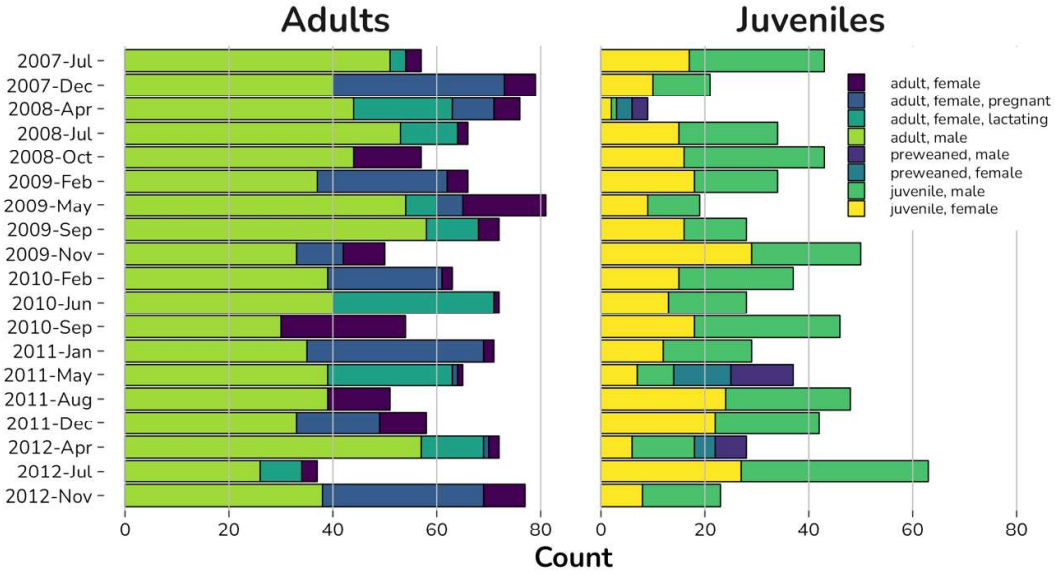

Figure 1. Demographics of bats captured in longitudinal sampling. Pregnant females were captured in months from November to June, lactating females were captured in months from April to July. Females with pre-weaning juveniles attached were found from April to May.

Patterns of Immunity and Co-immunity

We found bats were seropositive for antibodies against the henipavirus (HenipVsero+), a filovirus (EbVsero+), and a rubulavirus (MenVsero+).. Among adult bats, antibodies against the rubulavirus were common; 1,314 of 1752 adult bats were MenVsero+. Antibodies against Nipah virus or filovirus were less common – 1,031 were NiVsero+ and 921 were EbVsero+. Among the 921 juveniles, 192 were MenVsero+, 311 were NiVsero+ and 348 were EbVsero+. Of the 122 pre-weaned juveniles, 43 were MenVsero+, 52 were NiVsero+, and 32 were EbVsero+.

Co-exposure to multiple viruses was common (Figure 2). Over half (1,173) of the 1752 adults had antibodies against more than one of the three viruses, and 493 had antibodies against all three. Among the 921 juveniles, 210 had antibodies against more than one, and 39 had antibodies all three. 38 of 122 pre-weaned juveniles had more than one of the three antibodies, with seven having all three.

We found correlations between serostatus between all three pairs of viruses in a model accounting for bat age, sex, and study location, with all viruses tending to co-occur with each other more than would be expected than if they were distributed independently among bats. Nipah virus and filovirus antibodies had a standardized covariance of 0.13 (95% posterior interval 0.08-0.20). Nipah virus and rubulavirus antibodies were also more likely to occur together, with a covariance of 0.15 (0.09-0.21). Filovirus and rubulavirus antibodies had a covariance of 0.23 (0.17-0.29).

Page: 3

Number: 1 Author: Wang Linfa Date: 8/21/2022 10:48:00 AM

I think it is better to treat the three group of viruses equally as you have done for the title. It makes no difference whether we use NiV or Henipavirus in the context of the current study. Luminex-based assays targeting binding antibodies will in theory detect cross-RX antibodies in all three cases. See also comments in Methods

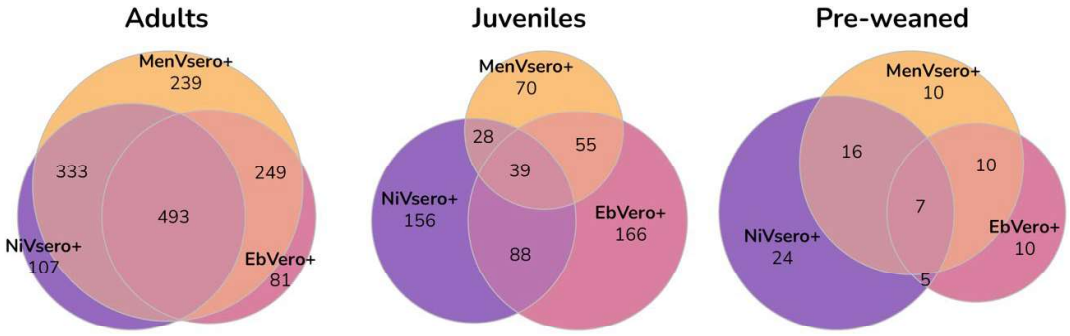

Figure 2. Detection of IgG antibodies against multiple viruses in individual *Pteropus medius*: Venn diagrams for adult, juvenile, and pre-weaned bats testing positive for antibodies against Nipah virus, filovirus, and Rubulavirus. Numbers under labels are counts of bats with only those viruses, numbers in overlapping areas represent number of bats detected with multiple viruses.

Serodynamics

Dynamics of population seroprevalence were different across the viral types. In adults, population seroprevalence against Nipah virus decreased over the study period from 78% (66%-88%) to 50% (40-62%) (Figure 3). Three periods of distinct increase in seroprevalence stood out in the inter-annual signal: early 2008, early 2009, and the second half of 2010. Nipah seroprevalence showed very weak seasonal patterns (Figure 4). (Nipah dynamics were previously reported in in Epstein, et al. [34]).

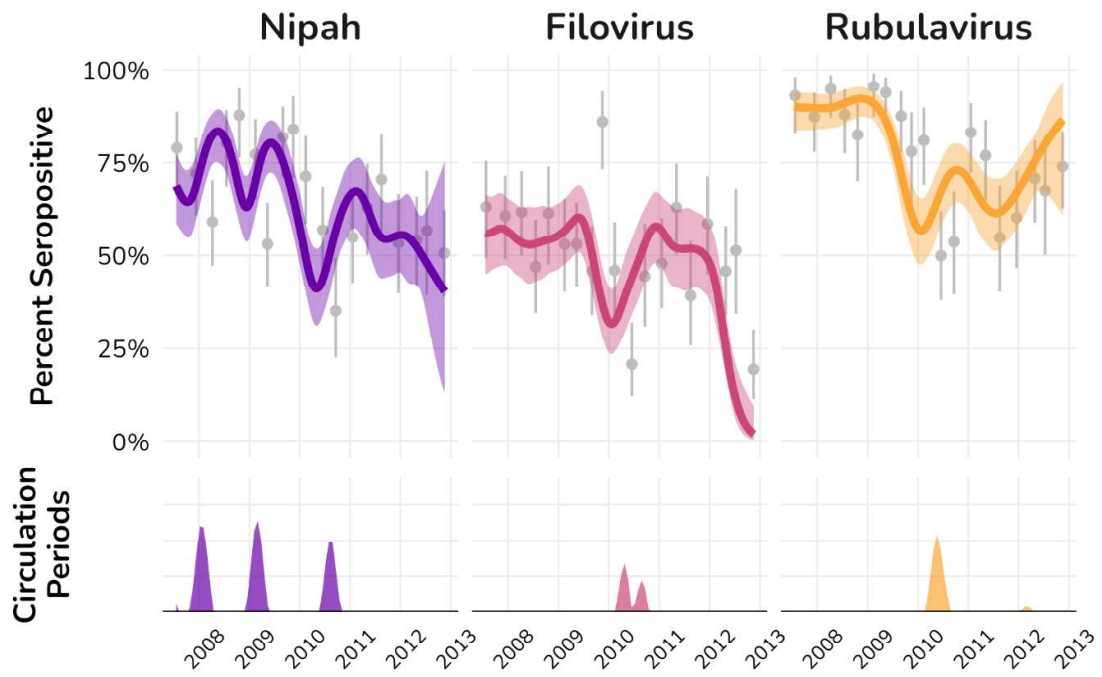

Figure 3. Inter-annual serodynamics in adult bats. Top: Measured and modeled population seroprevalence in adults over the term of the study. X-axis ticks mark the first day of the year. Grey points and bars represent measured population seroprevalence from individual sampling events on and 95% exact binomial confidence intervals. Thick lines and shaded areas represent the inter-annual (non-seasonal) spline component of a generalized additive mixed model (GAMM) of serodynamics and 95% confidence intervals for the mean pattern over time. Bottom: Circulation periods, when modeled slopes of the GAMM splines are positive for >95% of 1000 draws from the model posterior.

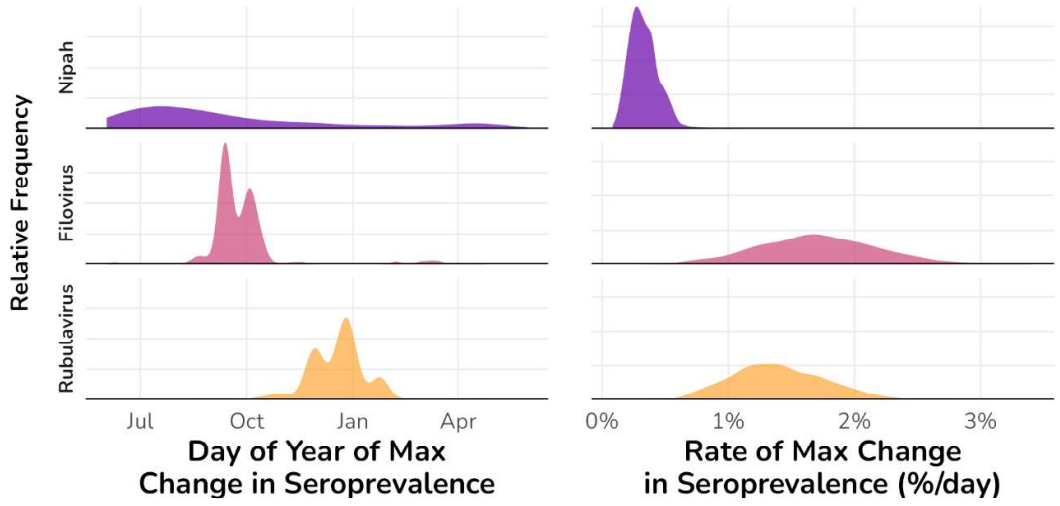

Figure 4. Timing and strength of seasonality of viral circulation in adults. Plots show posterior densities of the date for which increase in seroprevalence is greatest (left), and the rate of increase (right), for adult bats, drawn from GAMM models of seasonality. Densities are of these quantities derived from 1000 samples of model posteriors.

Among juveniles, we examined the dynamics of each cohort over their first year. For Nipah virus, the average cohort had 44% (32%-58%) seroprevalence in June, which consistently decreased over the course of the year to 20% (10%-36%) (Fig. 5). Year-to-year dynamics varied little, with overall declining seroprevalence in all years. No part of the year exhibited regular increases in seroprevalence for juveniles; the maximum rate of increase was indistinguishable from zero (Fig. 6).

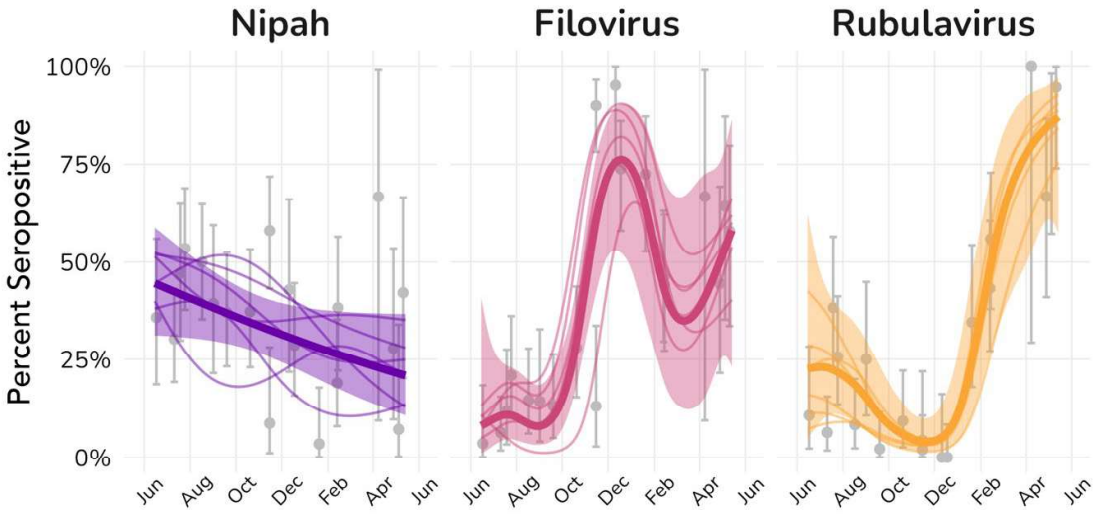

Figure 5. Serodynamics in juvenile bats in five-year study. Measured and modeled population seroprevalence in juvenile bats caught over their first year of life. Sampling events from multiple years are transposed onto a single year starting June (the earliest month free-flying young-of-the-year juveniles were captured). X-axis ticks mark the first day of each month. Grey points and bars represent measured population seroprevalence from individual sampling events on and 95% exact binomial confidence intervals. Thick lines and shaded areas represent GAMM-modeled average serodynamics across all years and 95% confidence intervals for the mean effect. Thin lines represent individual-year deviations from the average year pattern.

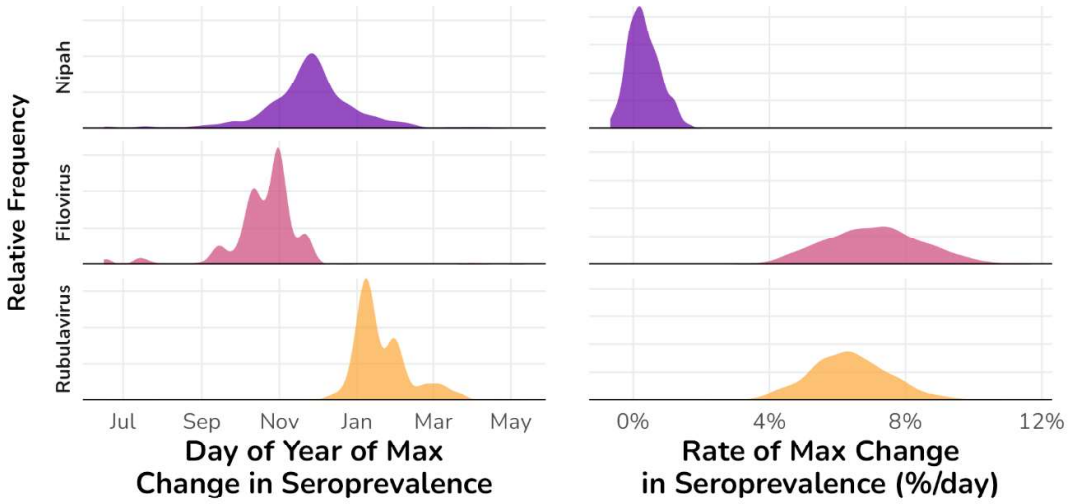

Figure 6. Timing and strength of seasonality of viral circulation in juveniles over the five-year study. Plots show posterior densities of the date for which increase in seroprevalence is greatest (left), and the rate of increase (right), for juvenile bats. Densities are of these quantities derived from 1000 samples of model posteriors.

Filovirus seroprevalence exhibited different patterns. Among adults, seroprevalence against filovirus was lower than for Nipah virus or Rubulavirus. As with Nipah virus, filovirus seroprevalence decreased over the study period, from 64% (50%-76%) to 20% (12%-30%), and had periods of distinct increases in spring/summer 2010 (concurrently with a circulation period for Nipah virus) (Fig. 3). filovirus seroprevalence did not exhibit cyclic annual patterns in adults (Fig. 4).

Among juveniles within the average year, filovirus seroprevalence started very low in June at 8% (2%-40%), but increased to a peak of 44% (32%-58%) in juveniles approximately six months of age before declining. (Fig 5). This pattern was consistent across years; though the peak varied in size, it consistently occurred in December or January. The average date with the greatest rate of seroprevalence increase was Oct 31 (Jul 14-Nov 25), and the maximum rate of increase was 7.1%/day (4.5%/day-9.9%/day).

Rubulavirus seroprevalence had a distinct dynamic pattern. Among adults, rubulavirus seroprevalence was high at 92% (82%-98%), and remained high through the end of the period at 74% (62%-84%), though there were some temporary periods of decline. There were distinct periods of increasing circulation in early 2010 and early 2012. Only for the rubulavirus did adult seroprevalence exhibit patterns of seasonality. Periods of increasing seroprevalence in adults exhibited strong seasonality of 1.6%/day (1.0%/day-2.4%/day) increase on peak days, with the mean peak date occurring on Dec 15 (Oct 15-Feb 1)

In juveniles, seroprevalence against the rubulavirus in the average year was 22% (4%-62%) in the youngest bats in June, though in individual years this could range from 7%-43%. In all years juvenile seroprevalence declined to a low of 1.7% (0.6%, 8.3%) that occurred between Oct 9 and Dec 11, then increased starting in winter, reaching high values in yearling juveniles of 88% (56%-99%) The peak rate of increase for juvenile rubulavirus seroprevalence was 6.4%/day (4.2%-8.8%) and occurred Jan 9 (Dec 21-Mar 16).

Spatial Comparisons

Seroprevalence trends in both adult and juvenile bats within the one-year studies in Chakhoria and Ramnagar broadly mirrored those in the five-year study in Faridpur (Supplementary Figure 2). In adults, Nipah virus seroprevalence increased in both Ramnagar and Chakhoria over the course of April 2010-May 2011, consistent with the period of increased seroprevalence observed in late 2010 in

the Faridpur. In Ramnagar, the seroprevalence in adults was 41% (24%-61%) in April 2010, rising to 60% (36%-81%) in May 2011 whilst in Chakhoria it rose from 26% (10%-48%) to 60% (36%-81%) over the same period. The trend in juveniles was as described for the five-year study.

For the filovirus, the seroprevalence in the one-year studies was higher than the reported average in the five-year study, with seroprevalence ranging between 41% (21%-64%) and 75% (51%-91%) in Ramnagar and between 48% (26%-70%) and 100% (83%-100%) in Chakhoria. A period of distinct increase in spring/summer 2010 was described in the five-year study which overlaps the timing of the one-year studies and may explain this difference in seroprevalence. In Ramnagar the serodynamics in both adults and juveniles were as described for the five-year study. In Chakhoria, there were two periods of increasing seroprevalence in adults over the course of the year. The first of these occurred in late 2010 when the adult seroprevalence increased from 48% (28%-69%) in August 2010 to 100% (83%-100%) in October 2010. The second period of increase occurred at the end of the study period in Spring 2011 with seroprevalence rising from 48% (26%-70%) to 75% (51%-91%) between early March 2011 to late April 2011.

Rubulavirus seroprevalence was high in adults throughout the one-year studies, ranging from 50% (27%-73%) to 95% (76%-100%) in Ramnagar and 29% (11%-52%) to 100% (83%-100%) in Chakhoria, with the lowest values observed in September 2010 in Ramnagar and December 2010/January 2011 in Chakhoria. These decreases were followed by rapid increases back to high seroprevalence, coincident with increasing levels in juveniles. The pattern in juveniles was as described in the five-year study, with seroprevalence estimates in yearlings in April 2011 of 80% (28%-99%) in both locations.

Comparison of the seroprevalence trends within each age group and virus between the two one-year study locations did not support spatial differences in the timing of changes in seroprevalence (Figure 7). Only for the filovirus in adults was there some evidence of a difference in trend between locations coinciding with the period of increased seroprevalence observed in adults in Chakhoria at the end of 2010.

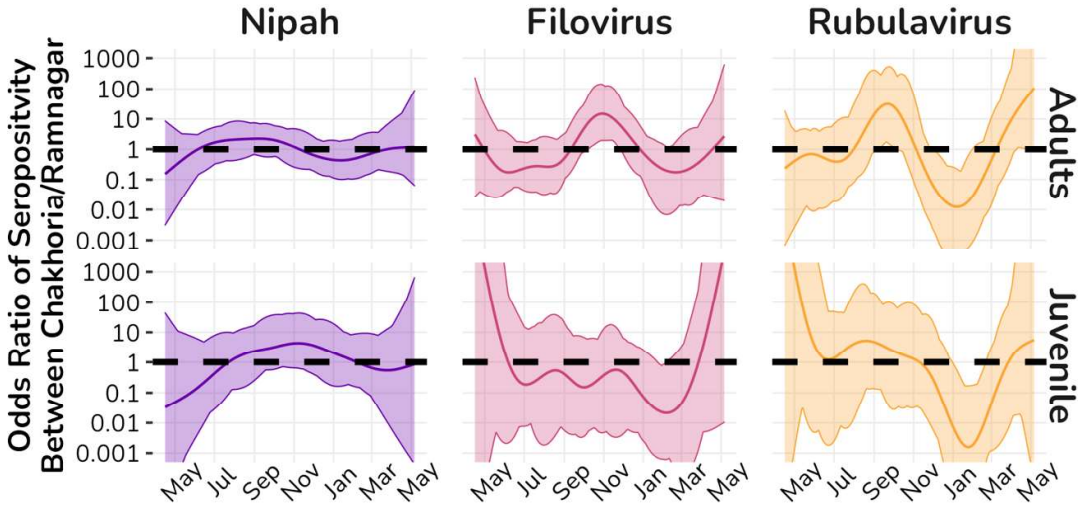

Figure 7. Comparison of splines for seroprevalence trends between locations in adults and juveniles over one-year study. Thick lines and shaded errors represent the estimated difference in trends and their associated 95% confidence intervals. The horizontal dashed line is set at zero. Where the confidence interval excludes zero, there is evidence for a significant difference in the serodynamics between the Ramnagar and Chakhoria populations over that period.

Maternal Inheritance of antibodies

We examined the relationship between mother and pup antibody titers, as measured by our Luminex assays (see Methods). This relationship varied between viruses (Figure 7). For Nipah virus,

the relationship was nonlinear due to clustering at high and low values, but largely followed a 1:1 relationship between mother and pup antibody titers. For the rubulavirus the relationship was near-linear and near 1:1. For the filovirus, though, the relationship between MFI values measured in mothers and their pups fell well below the 1:1 line, indicating pups having low inheritance of antibodies against the filovirus relative to the other two viruses.

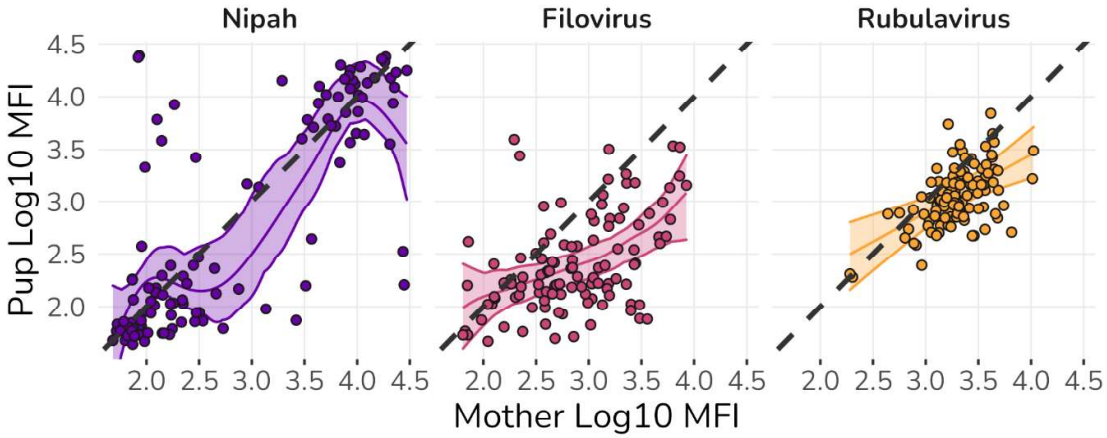

Figure 8. Maternal inheritance of antibodies. In each plot, points are measured log-MFI values for adults and juveniles in mother-pup pairs for each of the three antibody tests. Lines represent the predicted mean relationship between the two and their associated 95% confidence intervals.

3. Discussion

We found serological evidence for regular circulation of multiple viruses in *P. medius* populations in Bangladesh. These included Nipah virus, which spills over from bat populations to humans in the region, as well as a filovirus and a Rubulavirus. It was common for bats to have antibodies against multiple viruses, and more common in adults than juveniles. Co-immunity patterns among pre-weaned juveniles mirrored those in adults, reflecting maternal inheritance patterns. Patterns of co-immunity were not random, with antibodies against all three virus types being positively correlated within bats.

The dynamic patterns in population seroprevalence were distinct for the three viral groups. The rubulavirus exhibited the most regular and consistent patterns. Some juveniles in these populations inherit maternal antibodies against the Rubulavirus, which wane over the first six months of their life, after which a regular annual epidemic occurs in the juvenile population, causing a steep rise in juvenile seroprevalence by the end of their first year. High adult seroprevalence is likely the consequence of most bats being exposed to the disease as juveniles. When adult seroprevalence drops, as it did in 2009 to 2011, it rapidly rebounds during the same season as juveniles, indicating the same regular epidemics affect both adults and juveniles. We expect circulation and shedding of the rubulavirus to be strongest in the late winter and early spring months in these populations. Mortlock at al.[39] found irregular patterns of rubulavirus shedding in *Rousettus* bats over one year (via PCR detection in pooled urine samples), including a peak during a period of presumed antibody waning, though only within a one-year study.

An outstanding question is how the rubulavirus is maintained in the population despite high seroprevalence in inter-epidemic periods. Recrudescence is one possibility. Evidence for latent infections and recrudescence has been found for Nipah virus in *Pteropus vampyrus*[40], Hendra virus in *P. Medius*[41], and Lagos bat lyssavirus and African henipavirus in *Eidolon helvum*[42]. Another possibility is re-importation. In concurrent work with this study, we found that bat home ranges overlapped

with nearby colonies so as to form a meta-population[34], allowing occasional infection from outside bats, as has been shown to maintain Hendra virus in *Pteropus* populations[43,44].

The identity of the rubulavirus in this population is unclear. Our test was designed for Menangle virus, which has been found in *P. medius* in Australia[45,46]. At least 11 distinct Paramyxoviruses have been found in *P. medius* in Bangladesh alone: Nipah virus and ten uncharacterized species, including six Rubulaviruses closely related to Menangle virus and the Tioman virus[9,47]. It is possible that the serological patterns observed represent antibodies against a complex of multiple Rubulaviruses, though the regular interannual patterns in seroprevalence would indicate that they are operating similarly.

Nipah virus serodynamics exhibited a different pattern. We found serodynamics consistent with little to no circulation amongst juveniles. Instead, each cohort of juveniles appeared to inherit some maternal antibodies - maternal inheritance was most consistent for Nipah virus in our mother-pup comparisons - and population seroprevalence declined over their first year for all cohorts. Adults experienced several outbreaks, but Nipah virus serodynamics did not exhibit any seasonal patterns, rather, circulation events occurred in irregular intervals one to two years apart. In Thailand, Nipah virus shedding in *Pteropus lylei* was found to vary in time over two years and this pattern varied by strain; Bangladesh-strain Nipah virus shedding clustered in spring months, but only over two years[28]. Human Nipah virus outbreaks in Bangladesh, on the other hand, exhibit seasonality associated with the palm-sap consumption, the most likely spillover mechanism[48].

We had repeated positive detections of filovirus antibodies, using a test designed for Ebola-Zaire virus. This is the first detection of filovirus antibodies in *P. medius* in Bangladesh, though they have been found in *R. leschenaultii* in the country[49]. This could be one of several known filoviruses or an unknown species. There have been several recent findings of new Ebola-like filoviruses in bats extending across Africa and Asia. These include Bombali virus in Sierra Leone[50], and Měnglà virus in *Rousettus* bats in China[51]. Ebola-Reston virus was found in multiple bat species (*M. australis*, *C. brachyotis* and *Ch. plicata*) in the Philippines[52]. Marburg virus and Ebola-Zaire virus may have been detected in Sierra Leone and Liberia (unpublished,[53,54]). Serological evidence of filoviruses in bats has been found in multiple bat species in Central[12,55] and Western[56] African countries, Singapore [57], China[58], as well as Trinidad[59]. Our finding here adds to the evidence of broad host and geographic host ranges for filoviruses.

Several components of the serological patterns of filovirus antibodies are of interest. Young juveniles were almost all seronegative, and seroprevalence rose over the course of the first six months all years, then declined in the latter half of the year. Adult seroprevalence remained steady despite these regular juvenile outbreaks near 50% over the course of the five-year study. Adult seroprevalence against the filovirus also did not exhibit regular annual cycles, though the one period of detectable increase, in mid-2010, coincided with one of the regular seasonal periods of increase among juveniles. One possible hypothesis explaining this pattern is that the filovirus may exhibit low transmissibility in this system, only circulating when seroprevalence is near zero in young juveniles. It is also possible that bats may exhibit weak or waning filovirus antibody response but remain immune. In *R. aegyptiacus*, infected with the Marburg virus, antibody waning post-infection was found to be rapid, but bats retained protective immunity even with very low antibody titers[60,61]. If a similar mechanism occurs in this population, this could explain low seroprevalence in adults despite regular apparent periods of circulation in juveniles. The regular drop of seroprevalence in juveniles following the peak is consistent with this explanation.

However, if adults remain immune to the filovirus despite low antibody titers, this immunity does not appear to transfer to juveniles, as seroprevalence is near-zero among newborns and circulation in juveniles begins soon after birth. The relationship of pup/dam MFI found for the filovirus antibodies suggests that there may be differential inheritance of antibodies and possibly differential maternally derived immunity. If pups derive weaker immunity from dams than for other viruses, this may provide another mechanism for maintenance of the virus. It possible that patterns in filovirus antibody detection are an artifact of our test, specific to Ebola-Zaire virus, and that temporal trends are in part reflective of differential cross-reaction with viruses in this bat population.

However, while this may modify estimates of overall seroprevalence, the differential pup/dam MFI relationship, and consistent rise in seroprevalence among young juveniles, would require the test to exhibit differential sensitivity by age.

Serodynamics in our two one-year studies were broadly similar to those found in the five-year study. We found weak evidence for differences in serodynamics between sites for the filovirus in adults, and the rubulavirus for adults and juveniles. However, limited sample numbers and the shorter study period limit our ability to generalize about seasonal patterns. In a concurrent study[34], we found that flying foxes home ranges in Bangladesh extended an average of 50 km from their roosts. The roosts at Chakoria and Ramnagar, the sites of the one-year studies, are approximately 225 km apart. Thus, we would not expect the dynamics of these sites to be strongly coupled. It is plausible that inter- and even intra-annual dynamics of these viruses could be similar if weak coupling and/or environmental factors drive some of the dynamics. More detailed and longer-term studies at multiple sites in parallel are needed to better understand spatiotemporal variation in viral circulation.

Regular co-circulation of these viruses indicates the potential for viral interactions to affect host-viral dynamics. Viral interactions in hosts – whether direct during co-infection or indirect from infection in different periods – can enhance or suppress pathogen mortality, fecundity, or transmission, via competition for host nutritional or cellular resources, or via immune-mediation involving cross-immunity or antibody-dependent enhancement, and these interactions structure viral communities[26]. These interactions can have complex, even opposite effects when scaled to the population[27]. Here, we found positive correlations between serostatus against different viruses. External factors may also affect these relationships. For instance, all three viruses appear to have circulated in adults in mid-2010. It is possible that common factors like population density and/or nutrition availability[62] affected transmission of multiple viruses.

While rich observational serological data reveal these patterns, greater study is required to characterize these viruses and their effects on the host population and potential for spillover, as well as the degree and mechanisms of interactions. Our inferred periods of viral circulation point to optimal sub-populations and times to sample this population to detect viral shedding and isolate these viruses, and potentially capture co-infected hosts.

Extended longitudinal sampling also sheds light on viral dynamics not found in short-term studies. One- to two-year studies can identify temporal patterns in serology or viral prevalence and shedding, but establishing patterns or variation in seasonality requires extended, multi-year sampling. In our case, while Nipah virus outbreaks occurred in the same season in two years, it was apparent from the longer time series that there was no distinct seasonal pattern in dynamics. The consistency of the filovirus and rubulavirus patterns required repeated years of sampling to establish. Similarly, we were able to understand these patterns far better by separating juvenile and adult patterns, which we would be unable to distinguish in pooled samples.

While such extended individual-capture longitudinal studies are resource-intense, understanding the joint circulation of multiple viruses can be accomplished via multiplex immunoassays such as those used here. The continuous measures from these assays also have the potential to identify key patterns such as the differential inheritance of filovirus antibodies we identified here. Interpretation of these values is challenging and the relationship between immune status, antibody titer, and measured fluorescence is complex[32], but they have much potential to shed light on mechanistic drivers of disease circulation.

Methods

Field collection

We conducted a five-year longitudinal study sampling *P. medius* bats quarterly and two one-year studies in different locations, sampling monthly. All capture and sampling methods were approved by Tufts University IACUC protocol #G929-07 and icddr,b animal ethical review (protocol 2006-012). For the five-year study, we sampled from a roost complex near Faridpur, Bangladesh, as previously described in Epstein, et al. [34] The area of the roost complex consists of patchy forest

interspersed with agrarian human settlements and large rice paddies. Sampling occurred from July 2007 to November 2012 approximately every three months. The bat colony regularly shifted amongst roosts within the 80km^2 roost complex over the period of the study. Sampling occurred at the largest occupied roost found each quarterly sampling trip, sampling at different roosts within the complex across consecutive sampling nights if required to capture a sufficient number of individuals.

Approximately 100 bats were captured at each sampling event, which lasted 7-10 days. We captured bats with a 10x15m mist net between 11pm and 5am each night as bats returned from foraging until the count of 100 was reached.

In the one-year longitudinal studies, sampling was undertaken in two roost complexes in Ramnagar and Chakhoria, Bangladesh between April 2010 and May 2011. Monthly sampling of approximately 40 bats in each location was performed to obtain data at a finer temporal scale. Details of collection are otherwise as described for the five-year study.

For each study, we recorded each bat's age class, reproductive status, weight, size, and body condition. We collected up to 3.0 mL of blood from each bat's brachial vein into serum tubes with serum clot activator (Vaccutainer, USA). Blood tubes were stored vertically on ice packs in a cold box and serum was allowed to separate overnight. Serum was drawn from the tube after 24 hours and placed in a screw-top cryovial (Corning, USA) and stored in a liquid nitrogen dewar (Princeton Cryogenics, NJ, USA).

As part of a larger study to detect viral RNA, we also collected urine, rectal, and oropharyngeal swabs and pooled urine samples from under colonies. Further details can be found in Epstein, et al. [34]

Serological Assays

Sera from the longitudinal studies were sent to the Australian Animal Health Laboratory and gamma irradiated upon receipt. We used a bead-based microsphere assay that specifically detects antibodies to the soluble attachment glycoproteins[57,63,64] for a panel of viruses. Beads coated with each protein were mixed with sera at a dilution of 1:100. Biotinylated Protein A/G and Streptavidin-PE were then used to detect bound antibody. Beads were interrogated by lasers in a BioRad BioPlex machine and the results recorded as the Median Fluorescent Intensity (MFI) of 100 beads. We report here results for Nipah, Ebola-Zaire, and Menangle, the only three for which we established regular positive results.

While the Nipah virus has been detected in this population[65] and the specificity of the Nipah test is well-established [66], the Ebola-Zaire and Menangle virus tests are cross-reactive with other Ebola and rubulavirus species[67,68]. Thus we refer to these as tests for filovirus and Rubulavirus.

Data Analysis

We determined individual bat serostatus using Bayesian mixture models[69] fit on pooled data across all three longitudinal studies, calculating a cutoff of log-MFI as the point of equal probability between the smallest and second-smallest cluster of equal distributions for each assay.

To determine correlations of serostatus in bats across the three viruses, we fit a Bayesian multivariate probit model[70,71] which allows estimation of the joint outcomes (serostatus against each virus) and the correlation between the outcomes. We included age and sex variables to account for these effects on serostatus.

To examine time-varying changes in population seroprevalence, we fit binomial generalized additive mixed models (GAMMs)[72,73] to the time-series of serostatus measurements. For the five-year study, we fit separate models for adults and juveniles and for each immunoassay. For adults, we fit a model for seroprevalence dynamics over the course of the whole study. This treats the adult population as a single unit, though individuals within the population may turn over via migration, death and recruitment. We included both long-term and annual cyclic components for the multi-year time series of measurements. For juveniles, we treated each year's cohort of new recruits as separate populations with commonalities in overall first-year dynamics. The dynamics of each year's juvenile cohort were modeled as random-effect splines, deviating from an overall mean splines for juveniles

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I am not 100% sure about this. All serological tests (the NiV test included) will detect cross-RX antibodies from related viruses. For NiV, you can argue that longitudinal surveillance has NOT detected any related viruses in the study populations, but we can't say that the Ab test is specific to NiV.

over their first year. To estimate periods of peak viral circulation within the population, we calculated the derivatives of model-derived seroprevalence over time. We sampled these from GAMM posterior distributions and classified periods with >95% of samples with positive derivatives - that is, increasing population seroprevalence - as periods of viral circulation. We also calculated strength and timing seasonality for each virus as the maximum rate of seroprevalence increase and the date at which this maximum occurred, again sampling these values from the model posterior, and calculating mean and high-density posterior interval (HDPI) values.

For the one-year studies, we fit models primarily to detect differences in time-varying changes between sites. We fit binomial generalized additive models (GAMs) to the monthly serostatus measurements with separate models for each immunoassay. We included a separate, fixed-term spline in each model for age (adult or juvenile) and location (Chakhoria or Ramnagar). The trends in serodynamics for each virus in each age group were compared between locations to test for spatial differences[74]. Juveniles identified as being from the previous year's cohort were excluded from the analysis.

To examine patterns of maternal inheritance, we used GAMs to fit a relationship of log-MFI between adult lactating females and their attached pups for each viral assay. We limited these to data from the five-year longitudinal study.

Data Availability: Raw data from this study and reproducible code used to perform analyses is available at the Zenodo Scientific Archive [Zenodo DOI to be generated] and also on GitHub at https://github.com/ecohealthalliance/bangladesh-bat-serodynamics

Author Contributions: Conceptualization: N.R. and J.H.E..; methodology, N.R., L-FW and J.H.E.; software, N.R. and S.H.; formal analysis, N.R. and S.H.; investigation, J.H.E., A.I. and L-FW….; resources, X.X.; data curation, N.R and J.H.E; writing—original draft preparation, N.R. J.H.E., and S.H.; writing—review and editing, X.X.; visualization, N.R. supervision, N.R. and J.H.E.; project administration, J.H.E.; funding acquisition, P.D., L-FW and J.H.E

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73 Pedersen, E. J., Miller, D. L., Simpson, G. L. & Ross, N. Hierarchical generalized additive models: an introduction with mgcv. Report No. e27320v1, (PeerJ Preprints, 2018).

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From:	Wang Linfa on behalf of Wang Linfa <linfa.wang@duke-nus.edu.sg>
To:	Jon Epstein; Ariful Islam; Hayes, Sarah; A. Marm Kilpatrick; Kevin Olival, PhD; Emily Gurley; Dr. Jahangir Hossain; Hume Field; Gary Crameri; Stephen Luby; Christopher Broder; Peter Daszak
Cc:	Noam Ross; Madeline Salino
Subject:	RE: Draft manuscript: Co-circulation dynamics of viruses in a bat population
Date:	Sunday, August 21, 2022 1:47:19 AM
Attachments:	Ross-et-al_bangladesh-bats-cocirculation-serology_2022-08-11_LW.docx

Dear Jon and all,

It is great to see this in a ready to go form after so much effort put into this longitudinal study.

See my edits/comments in the attached. My main suggestion is to treat all three groups the same, i.e., at the genus level.

@Steve @Emily: I finally made my trip to Bangladesh (and icddr,b) working on a BMGF-funded pathogen genomics project. I "challenged" the new ED to host the Nipah@25 in 2024 there and he said he will do his best to make sure it happens. So hopefully we an all meet again in Bangladesh in 2024.

Cheers,

LF

Linfa (Lin-Fa) WANG, PhD FTSE FAAM
Professor
Programme in Emerging Infectious Disease
Duke-NUS Medical School,
8 College Road, Singapore 169857
Tel: +65 6516 8397

From: Jon Epstein <epstein@ecohealthalliance.org>
Sent: Friday, 19 August 2022 11:22 PM
To: Ariful Islam <arif@ecohealthalliance.org>; Hayes, Sarah <sarah.hayes16@imperial.ac.uk>; A. Marm Kilpatrick <akilpatr@ucsc.edu>; Kevin Olival, PhD <olival@ecohealthalliance.org>; Emily Gurley <egurley1@jhu.edu>; Dr. Jahangir Hossain <jhossain@mrc.gm>; Hume Field <hume.field@ecohealthalliance.org>; Gary Crameri <garycrameri1@gmail.com>; Wang Linfa <linfa.wang@duke-nus.edu.sg>; Stephen Luby <sluby@stanford.edu>; Christopher Broder <christopher.broder@usuhs.edu>; Peter Daszak <daszak@ecohealthalliance.org>
Cc: Noam Ross <ross@ecohealthalliance.org>; Madeline Salino <salino@ecohealthalliance.org>
Subject: Draft manuscript: Co-circulation dynamics of viruses in a bat population

Colleagues,

I hope you're all doing well. I'm excited to share a draft manuscript, led by Noam, that details the serodynamics of henipaviruses, filoviruses and rubulaviruses in a population of *Pteropus medius.* We plan to submit this to *Nature Communications* in September. Please send Noam and I your comments by **September 10th**.

Cheers,
Jon

--
Jonathan H. Epstein DVM, MPH, PhD
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Twitter: @epsteinjon
EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation

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EID-SEARCH
Emerging Infectious Diseases
South East Asia Research Collaboration Hub

CREID
Centers for Research in Emerging Infectious Diseases

Centers for Research in Emerging Infectious Diseases (CREID) Network

Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH)

2022 Report to the External Advisory Committee

August 19, October

Table of Contents

Guidance: 7-page limit for EAC Report
(excluding cover page and TOC)

Section 1. Executive Summary

The Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH) is a multidisciplinary center focused on analyzing zoonotic disease risk in a key EID hotspot region. Our aims are to 1) characterize diverse coronaviruses, paramyxoviruses, filoviruses, and other viral pathogens in wildlife; 2) assess the frequency and causes of their spillover; & 3) identify viral etiologies of undiagnosed 'cryptic' outbreaks in Thailand, Malaysia, and Singapore. Highlights of research and significant findings during the reporting period (October 2021 – August 2022) include:

To be updated with bullet points by Peter…

Section 2: Research Center Overview

A. Specific Aims

The overarching goal of the Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH) is to characterize the diversity of key viral pathogens in wildlife, assess the frequency and causes of their spillover, and identify viral etiologies of undiagnosed 'cryptic' outbreaks in people. The research goals of the EID-SEARCH follow three specific aims designed to advance our understanding of spillover and outbreak risk for novel viruses in a globally important EID hotspot, strengthen in-country research capacity, and enhance international collaboration:

- Specific Aim 1: Identify, characterize, and rank spillover risk of high zoonotic potential viruses from wildlife.
- Specific Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays.
- Specific Aim 3: Identify and characterize the viral etiology of 'cryptic' outbreaks in clinical cohorts.

B. Partners and Staffing

EID-SEARCH includes leaders in the field of emerging viral pathogens at key US institutions (EcoHealth Alliance PI Peter Daszak; University of North Carolina Co-I Ralph Baric; Uniformed Service University Co-Is Christopher Broder and Eric Laing), and in Thailand (Chulalongkorn University Co-Is Supaporn Wacharapluesadee and Opass Putcharoen), Singapore (Duke-NUS Medical School Co-I Linfa Wang), and the three major Malaysian administrative regions (Conservation Medicine Co-I Tom Hughes). These organizations represent the 'hub' of the center, with an additional, more informal collaborative network spanning >50 clinics, laboratories, and research institutes across almost all Southeast Asian countries. By enhancing and focusing research within the three hub countries, and coordinating communication among the network, we envision EID-SEARCH acting as an early warning system for outbreaks; a way to exchange information, reagents, samples, and technology; and a collaborative powerhouse for translational research. EID-SEARCH is supported by a multidisciplinary team of researchers at our partner institutions, with skills in epidemiology, clinical management, molecular biology, virology, wildlife biology, and advanced data and statistical analyses. The long collaborative history between the key personnel and partner institutions is a significant asset that can be deployed to support outbreak research response in the region.

C. Research Sites

The three core (hub) countries for the EID-SEARCH are Thailand, Malaysia, and Singapore, *expanded to Viet Nam and Cambodia through the CREID Pilot Research Program*. These stretch through one of the

most significant foci of EID risk globally. Additionally, we have assembled a greater network of EID-SEARCH partners that includes >50 clinics, laboratories, and research institutes across almost all Southeast Asian countries – stretching from Nepal to the Philippines. Our targeted field research includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sarawak, Sabah, Viet Nam, and Cambodia. The central location of our hub countries in mainland Southeast Asia allows us to sample representative wildlife species that occur in other countries across the greater biogeographic region, including India, Bangladesh, Myanmar, Southwest China, Laos, Cambodia, Philippines, and Indonesia.

Section 3: Research Center Progress and Accomplishments

Progress and Accomplishments

- Organized by Aim
- Focus on key accomplishments
- Address outbreak research response if applicable
- Address how the 2021 EAC recommendations have been incorporated (*see Appendix A for 2021 EAC report*)

A. Projects and Studies

- Have you reverted to your original (pre-pandemic) CREID research agenda in the last year? If not, how has your research agenda changed in the last year due to the ongoing impact of SARS-CoV-2?
- What additional changes to your CREID research agenda have taken place in the last year?

Aim 1: Identify, characterize, and rank spillover risk of high zoonotic potential viruses from wildlife

- **Field surveillance among wild bats, rodents, and non-human primates.** We have collected 14,302 specimens from 2,055 animals of 31 bat species, one non-human primate (NHP) species, and five rodent species from six sites at high-risk human-wildlife interfaces in Malaysia and Thailand. (Table 1)

	Thailand	**Malaysia**
No. of collected specimens	3,862	14,171
No. of individuals	454 bats, 106 rodents	2,000 bats
No. of species	6 (bats), 5 (rodents)	24 (bats)
No. of sites	5	4
No. of tested individuals	407	1,332
No. PCR tests for coronaviruses (CoVs), paramyxoviruses (PMVs), filoviruses (FLVs), influenza viruses (IVs)	4,070	9,228
Identified viruses	3 new CoVs from 11 bats 3 known PMVs from 4 rodents 4 new PMVs from 11 bats	5 new CoVs from 112 bats 4 new PMVs from 10 bats 2 known PMVs from 2 bats
No. of specimen for multiplex immunoassay for FLVs, CoV and Henipavirus	100 macaques 92 rodents	720 bats

Table 1. EID-SEARCH viral surveillance in wildlife in Malaysia and Thailand.

- **Discovery of novel SARS-like beta-coronavirus in bats in Thailand**. The whole genomes revealed a 91.72% nucleotide similarity to SARS-CoV-2. Phylogenetic analysis suggested that this virus is closer to pangolin CoV-GD-1 than to SARS-CoV-2; however, the RBD is more similar to that of bat-CoV RaTG13, suggesting that this new virus may also bind to mammalian ACE2 receptors. Whole-genome sequencing of other new CoVs from Thailand and Malaysia is ongoing.

Summary of Comments on Email 8 - Attachment 1 - 2022 EAC Report_EID-SEARCH_draft v01-EDL-KR-SN_15Aug22 (002).pdf

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Number: 1 Author: Microsoft Office User Date: 8/2/2022 8:27:00 AM

Footnote about what “specimens” includes, how were 14,000 specimens obtained from 2,000 bats. 1 bat -> blood sample, oral swab, rectal swab, body swab, ectoparasite removed, wing puncture, etc…

Number: 2 Author: Microsoft Office User Date: 8/2/2022 8:23:00 AM

I’m not a mol. evol. geneticist but the way this is laid out in the table gives equal weight to a full genome, Thailand 1 new CoV, and distinct RdRp NT sequences that are used to create phylogenetic trees. I don’t think I’ll ever understand how the virology field uses small NT reads to discover new viruses. I suggest adding another row for “full virus genome sequenced” and a footnote about the methods used to determine how a new virus has been genetically identified.

Number: 3 Author: Sasiprapa Ninwattana Date: 8/15/2022 5:48:00 PM

Based on complete whole genome sequences (3 samples) and partial whole genome sequences (8 samples)

Number: 4 Author: Microsoft Office User Date: 8/2/2022 9:56:00 AM

Same species? Details.

Number: 5 Author: Sasiprapa Ninwattana Date: 8/13/2022 11:22:00 AM

Samples collected in year 1 (31 March 2021)

Number: 6 Author: Microsoft Office User Date: 8/2/2022 8:30:00 AM

Check ICTV guideline, I thought it was established to use, e.g., bat SARS-related coronavirus (bat SARSr-CoV). The use of beta-coronavirus widens the focus away from sarbecoviruses.

- **Genome-wide CRISPR knockout screen identifies essential factor for HKU2/SADS-CoV infection.** Using a genome-wide CRISPR knockout screen, we identified placenta-associated 8 protein (PLAC8) as an essential host factor for SADS-CoV infection of human cells. Infection is prevented in PLAC8 knockouts and can be restored by transiently transfecting PLAC8 from multiple species, including human, pig, mouse, and bat. Trypsin treatment of SADS-CoV bypasses the PLAC8-dependent pathway and partially rescues SADS-CoV infection. PLAC8 is involved in both early entry and later dissemination of SADS-CoV infection and PLAC8 KO cells have: 1) delayed and reduced viral subgenomic RNA expression and 2) diminished cell-to-cell viral spreading of infected cells. *These results suggest that PLAC8 is a promising target for antiviral development for the potential pandemic SADS-CoV virus*.
- **Host range, transmissibility, and antigenicity of a pangolin coronavirus.** Using a synthetically derived infectious cDNA clone, we recovered the wildtype pangolin (Pg) CoV-GD and derivatives encoding indicator genes. Like SARS-CoV-2, PgCoV-GD virus efficiently uses human and many other orthologous mammalian ACE2 receptors for entry, shares a similarly broad host range *in vitro*, and displays altered sensitivity to host proteases. PgCoV-GD replicates to slightly higher to lower titers in primary human cells derived from the proximal to distal lung, respectively, and with reduced fitness compared to SARS-CoV-2. PgCoV-GD also replicates efficiently in hamsters and could transmit via the aerosol route, but at a lower frequency than SARS-CoV2. Efficient PgCoV-GD replication in primary nasal airway epithelial cells and transmission in the hamster 5highlights the high emergence potential of this virus. However, PgCoV-GD is efficiently neutralized by COVID-19 patient sera and many commercially available SARS-CoV-2 therapeutic antibodies, but not by human antibodies targeting the spike N-terminal domain. Furthermore, a pan-sarbecovirus antibody, ADG-2, and a SARS-CoV-2 prefusion stabilized spike protein (S2P) recombinant protein vaccine protected mice in a PgCoV-GD replication model. Lastly, we showed that cross-neutralizing antibodies in pre-immune SARS-CoV-2 humans and the benefit of current COVID-19 countermeasures should impede its ability to emerge and spread globally in humans. *This research helps build FDA-approved therapeutic antibodies against a range of CoVs with emergence potential and shows that FDA-approved SARS-CoV-2 vaccines also protect animals from PgCoV-GD when challenged in vivo*.

Aims 2 & 3: Identify evidence and analyze risk factors for viral spillover in high-risk communities and clinical cohorts, and characterize viral etiology of 'cryptic' outbreaks

- **Incorporation our bat-CoV spike proteins into the multi-family multiplex-based immunoassay (MMIA)**. Specific bat-CoV spike proteins including bat SARSr-CoVs RaT13, bat SARSr-CoV ZXC21, bat MERSr-CoV PDF2180, and HKU9 were designed as pre-fusion stabilized spike glycoprotein ectodomain trimers (S-2P). These were spike protein antigens were incorporated into our 17-plex henipavirus/filovirus-focused serology assay expanding the representation of virus targets to include priority coronaviruses such as SARS-CoV-2 and bat SARSr-CoVs/bat MERSr-CoV. SOPs were revised and updated to reflect best practices that were optimized during Year 1.

- **Community serology surveillance.** We initiated the human surveillance work in Thailand, with 56 participants enrolled at one at-risk community site, and 2 individuals from a clinic site, with a total of 58 participants enrolled. Serology testing of samples from 56 participants from the community site showed positive for henipaviruses (HNVs) (family *Paramyxoviridae*), filoviruses (FLVs), and CoVs (Fig. 1). Human sera were tested in the multi-family MMIA for serologic evidence of previous infection by Asiatic FLVs, HNVs, and CoV. We observed minimal reactivity and IgG binding with the NiV receptor-binding protein (RBP), implying that subclinical cryptic NiV infections had not occurred in these

Page: 5

Number: 1 Author: Microsoft Office User Date: 8/2/2022 8:29:00 AM
The fusion data Chee Wah presented indicated that it does bind to and mediate cellular entry/fusion via the human ACE-2 orthologue.

Number: 2 Author: Microsoft Office User Date: 8/2/2022 8:35:00 AM
? not sure what this means. Were there lower viral loads in the respiratory tract? Is aerosol route correct, or should it be respiratory secretions? There is a lot of data in this section, is there a reference to a pre-print or article?

Number: 3 Author: Microsoft Office User Date: 8/2/2022 8:33:00 AM
List these?

Number: 4 Author: Microsoft Office User Date: 8/2/2022 8:37:00 AM
This is pretty nuanced. Do the commercially available mAbs that neutralized PgCoV-GD target the RBD or Q neutralizing epitopes in the S glycoprotein and not the NTD?

Number: 5 Author: Microsoft Office User Date: 8/2/2022 8:33:00 AM
Is this an FDA EUA or authorized treatment?

Number: 6 Author: Microsoft Office User Date: 8/2/2022 8:32:00 AM
This is something not FDA EUA? Will stakeholders understand what S-2P etc means? This is what NovaVax is making.

Number: 7 Author: Microsoft Office User Date: 8/2/2022 8:39:00 AM
Replication, not infection or disease model? What did these mAb and vaccine protect from, infection?

Number: 8 Author: Microsoft Office User Date: 8/2/2022 8:41:00 AM
What does this mean, sera from humans with memory to seasonal beta-human coronaviruses can cross-neutralize PgCoV-GD, by binding recognition to the S2 fusion domains?

Number: 9 Author: Microsoft Office User Date: 8/2/2022 8:43:00 AM
I didn't see any mention of FDA-approved antibody therapies or vaccine countermeasures being tested in the sentences before.

Tighten this up with explicit details.

participants (Figure 1B). One human serum sample possessed IgG that was reactive with envelope glycoproteins (GP) from Ebola virus, Bundibugyo virus, Bombali virus, Sudan virus, and Lloviu virus, consistent with a serologic pattern of cross-reactivity among phylogenetically-related ebolaviruses (Figure 1A). A diversity of genetically and antigenically uncharacterized Asiatic filoviruses is likely to exist, and this serology results is consistent with other serology-focused projects in the region. The antigenic relationships and contribution of glycoepitopes to heterotypic cross-reactions among ebolaviruses and broadly within FLVs remains poorly understood, follow-up testing will explore whether broad cross-reactions with FLVs antigens is driven by glycosylated epitopes that may be more conserved across the ebolaviruses than protein sequence similarity/identify would imply. Lastly, human sera displayed maximum preferential reactivity with SARS-CoV-2 spike protein, consistent with COVID-19 vaccination status of participants. At the low testing titer, cross-reactions with phylogenetically-related bat sarbecoviruses was expectedly observed (Figure 1C). Interestingly, two participants had sera IgG that bound to the bat MERSr-CoV PDF2180, which does not utilize the orthologous human DPP4/CD20 receptor for cellular entry, and it quite distinct from the sarbecoviruses.

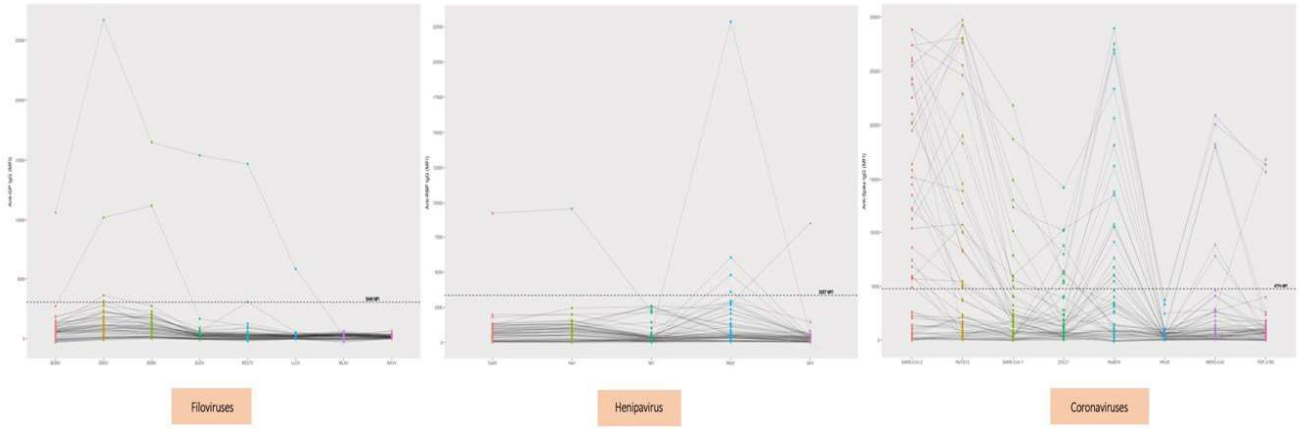

Fig. 1 Serology testing results for FLVs, HNVs, and CoVs among community participants in Thailand (n=56).

- **<u>Design and application of pan-sarbecovirus multiplex surrogate virus neutralization test (sVNT)</u>** that covers SARS-CoV-2 and variants of concern (Alpha, Beta, Gamma, Delta, Mu, Lambda, Omicron BA.1 and BA.2), clade-2 sarbecoviruses (BANAL-52, BANAL-263, GD-1, RaTG13, GX-P5L), clade-1 sarbecoviruses (Rs2018B, LYRa11, WIV-1, RsSHC014, Rs4231), SARS-CoV-1, and *the newly discovered bat SARSr-CoV we recently sequenced from Thailand.* This assay allows for the discrimination of past SARS-CoV-2 infection from that of other sarbecoviruses in human and animal samples, which will be critical for identifying cryptic spillover of sarbecoviruses in people. It also has the potential to aid in identifying the progenitor of SARS-CoV-2 in animals.

- **<u>Optimization and establishment of pan-henipavirus multiplex sVNT at Chulalongkorn University.</u>** We also optimized an sVNT for Nipah virus and provided enough material to characterize Nipah virus IgG positive serum samples for neutralizing antibodies via a virus-free/cell-culture free bead-based serology assay.

Page: 6

Number: 1 Author: Microsoft Office User Date: 8/2/2022 8:45:00 AM
What does this mean?

Number: 2 Author: Sasiprapa Ninwattana Date: 8/15/2022 5:50:00 PM
I re-tested the two serum samples that were weakly positive for NiV and it turned out that both of them showed seronegative.

Number: 3 Author: Microsoft Office User Date: 8/2/2022 8:45:00 AM
These panels are showing the same thing, pick one format and make these bigger. I prefer the “connected” lines because it shows the that it’s the same individual.

Number: 4 Author: Sasiprapa Ninwattana Date: 8/15/2022 5:53:00 PM
I plotted new graphs because I re-tested some of samples that were positive to confirm the results.

Number: 5 Author: Microsoft Office User Date: 8/2/2022 2:54:00 PM
Needs a proper legend.

Outbreak investigation and preparedness in Thailand

- The Ministry of Public Health in Thailand requested support from EID-SEARCH for a viral diarrhea outbreak investigation in Chanthaburi province. We received samples from both Prapokklao Hospital (regional hospital) and Khlong Khut Health-Promoting hospital collected from 59 healthcare workers and students who had diarrhea during Dec. 2021-Feb. 2022. PCR tests were conducted for six gastrointestinal viruses causing gastrointestinal tract infections including adenovirus, astrovirus, norovirus GI, norovirus GII, rotavirus, and sapovirus. 32 samples **(54.24%)** were positive for norovirus GII. Further genomic characterization of the detected norovirus GII will be performed, allowing for comparison with noroviruses found in other provinces in Thailand.
- In preparedness for Monkeypox outbreaks, EID-SEARCH's lab partner in Thailand coordinated with the Department of Medical Science, MOPH, National lab to establish the Monkeypox testing platform in April 2022 and detected the first Monkeypox case in Thailand in July 2022.

B. Dissemination of Results

- Summarize dissemination plan
- Include number of peer-reviewed publications; highlight those in high-impact journals
- Number and content of presentations on CREID research, including audience

EID-SEARCH members were invited to present the project work at government and inter-government briefings (> 10), conference and university lectures (>13), panels and webinars (>18), and public interviews (>6) in Thailand, Malaysia, the US, regionally and internationally on the trends in global disease emergence, zoonotic surveillance, pandemic control and prevention, and research results from this project research activities. Nine (9) peer-reviewed paper has been published or with preprint available from EID-SEARCH work, two (2) manuscripts are currently under revision, and three (3) manuscripts are in preparation for submission*, including papers on.. please highlight the high-impact ones*. We organized a workshop with 55 scientists and government officials from Thailand, Malaysia, Singapore, Bangladesh, Cambodia, Indonesia, Lao PDR, Myanmar, Nepal, Vietnam, USA, and Australia to present EID-SEARCH work, share knowledge, strengthen research capacity, and foster scientific collaborations in Southeast Asia.

C. Training and Capacity Building Activities

- Summarize capacity building plan
- Include number of training activities and number of participants for each training
- Address how capacity building activities were adapted due to COVID-19 and long-term changes as a result of the pandemic

During this reporting period, XX training sessions with a total of XX participants were conducted by EID-SEARCH partners in Thailand, Malaysia, and Vietnam for project members, local government partners, universities, research institutes, and NGOs. These training focused on biosafety (x training x participants by x), applying new technologies (x training x participants by x), and field and laboratory SOPs (x training x participants by x), to strengthen in-country research capacity and improve information sharing. Both in-person and online training was conducted to accommodate COVID conditions. As travel and COVID-19 restrictions have begun to be lifted, students, postdocs, and all EID-SEARCH research staff have been provided with professional development opportunities to travel to attend conferences, workshops, and other in-person training activities. *To be updated with numbers….*

Page: 7

Number: 1 Author: Sasiprapa Ninwattana Date: 8/15/2022 5:28:00 PM

As mentioned earlier, I re-tested the two serum samples that were weakly positive for NiV and it turned out that both of them showed seronegative. So I'm not sure that should this part still be included.

Number: 2 Author: Microsoft Office User Date: 8/2/2022 8:51:00 AM

If not including the PMID, I'd at least add the Surrname, First Initial, et al. J. Infect Dis. 2022.

Number: 3 Author: Sasiprapa Ninwattana Date: 8/13/2022 11:02:00 AM

1. Our paper "Rapid and Simultaneous Detection of Omicron and Other Concerned SARS-CoV-2 Variants (Alpha, Beta, and Delta) in Clinical Specimens Using Multiplex PCR MassARRAY Technology" is under revision

2. "Homologous or Heterologous COVID-19 Booster Regimens Significantly Impact Sero-Neutralization of SARS-CoV-2 Virus and its Variants" has been accepted for publication in vaccines journal

D. Challenges and Proposed Solutions

- Bottlenecks to CREID-related research that might be like those that you could face when pivoting research activities in direct response to an EID outbreak. What solutions could be or have you put in place in advance to solve those challenges? How is being part of the CREID Network mitigating those or not?

The significant challenges we encountered during the reporting period were travel restrictions to our field partner countries and within countries, as well as a large number of hospitalized patients in local hospitals in Thailand and Malaysia due to COVID-19 that delayed human participant enrollment at targeted community and hospital sites and the subsequent laboratory analysis we anticipated in Year 2. To address these issues, we worked closely with in-country partners and stakeholders to continue assessing the situations (i.e., safety and research priorities of study sites) to ensure a rapid start of enrollment and focused on the refinement of assays to be ready for human surveillance. Meanwhile, to prevent a possible supply shortage of PPE and swabs as happened in early Year 2, we have identified supply sources to ensure we can procure the necessary consumables without significant delays. With travel restrictions in Southeast Asian countries now beginning to be lifted, international travel of US-based staff to our field sites has begun (April 2022).

E. Examples of Network Collaboration

- How has the work/research of your RC been affected by or involved collaboration with other members of the CREID Network?
- Examples: highlight joint publications, joint activities, sharing materials and data, benefits of Working Groups, etc.

Through the participation of different CREID Working Groups, EID-SEARCH members participated in the network's outbreak responses, capacity building, and cross-center collaboration by sharing existing local partnerships, techniques, and relevant resources.

- For outbreak responses, EID-SEARCH has been actively participating in the network's response to Monkeypox by providing local partnerships and monitoring platforms in DRC and Thailand.
- For lab assay sharing, EID-SEARCH partner Uniformed Services University is working with UWARN PIs, Dr. Robert Cross and Dr. Scott Weaver, to establish a MTA with UTMB and regional partners in Liberia and Nigeria for the multi-family FLV/HNV MMIA for anti-EBOV serology testing. Enquiries has been made with Dr. Mark Page and the National Institute for Biological Standards and Control (NIBSC) regarding additional MARV and NiV serology standards.
- We are contacted by multiple Research Centers regarding the Field Biosafety Manual presented at the CREID Scientific Meeting by EID-SEARCH senior field veterinarian, the Manual and corresponding training resources will be made available to all CREID members once it's completed with peer review. Marc, please feel to edit…
- EID-SEARCH are also committed to providing our expertise on CCHF, Henipavirus, RVF related research by connecting CREID members with our research partners.

Section 4: Future Directions

- Plan for the next reporting period.
- How is your Research Center adapting to the April 2022 NIAID Program Priorities and how does this change (or not) your planned activities in the next year? *See Appendix B for NIAID Program Priorities*.

- Moving forward, what is the greatest opportunity your RC has to advance the knowledge base for emerging infectious diseases? What are the significant knowledge gaps that the RC can advance research on?

In the next reporting period, we will follow the research plans laid out in our proposal with the specific actions to **continue expanding our knowledge of emerging and re-emerging infectious diseases and better prepare to respond to outbreak/pandemic threats that align with the CREID network research priorities:**

Specific Aim 1: Identify, characterize, and rank spillover risk of high zoonotic potential viruses from wildlife.

- Wildlife (bats, rodents, and non-human primates) sampling at selected sites, with increased efforts for rodents and non-human primates and potential longitudinal sampling at a few selected sites to analyze patterns of viral shedding and spillover risk.
- Continue to develop predictive host-range models and spatial risk models to prioritize sampling sites and species in Years 3-5 of the project.
- Conduct viral family-level molecular screening of collected wildlife samples for coronaviruses, filoviruses, and paramyxoviruses.
- Perform serology testing on the wildlife samples or coronaviruses, filoviruses, and henipavirus.
- Continue developing a more comprehensive multiplex CoV RBD-based sVNT covering newly identified sarboviruses and additional merbecoviruses to allow the evaluation of neutralizing activity in serum samples against a much broader and more comprehensive range of RBDs.
- Conduct whole-genome and spike glycoprotein sequencing in countries with newly identified viruses.
- Continue research to further characterize a subset of novel viruses including virus isolation, human cell infection experiments, and animal modeling for viral pathogenesis studies, including pangolin GD strain, BANAL-52, and other significant viruses identified in Y1-2.
- Develop zoonotic risk analyses for relevant viruses, including the analysis of Spike and whole-genome sequences to predict host range using a variety of in silico approaches, model the host range, geographic distribution, extent of overlap with high-density human populations, etc. using existing apps/models to assess potential zoonotic disease risk

Specific Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays

- Increase the number of sites and enrolled participants for community surveillance among at-risk populations for biological and behavioral data collection in Thailand, Peninsular Malaysia, and Sabah Malaysia
- Continue to identify community sites concurrent with animal surveillance sites (Specific Aim 1).
- Perform serology testing of collected human samples with optimized multiplex assays for coronaviruses, filoviruses, and paramyxoviruses
- Begin epidemiological analyses of biological and behavioral data.

Specific Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts

- Start enrolling participants at selected hospital/clinic sites for syndromic surveillance at two sites in Thailand, and identified sites in Sabah, Malaysia and Peninsular Malaysia, Malaysia
- Continue to identify potential clinical cohorts and sites in the catchment of the community (Specific Aim 2) and animal surveillance (Specific Aim 1) sites.

- Start serological and molecular testing on collected human samples.
- Start viral characterization work if any novel viruses are identified.

Through the work under Specific Aims 2&3, we are engaging multiple disciplines of social science, anthropology, health policy, and behavioral science towards EID risk mitigation strategies on key human-animal interfaces, we will share relevant research methods (e.g., behavioral questionnaires) and best practices with the CREID Network members.

Training and capacity-building activities among in-country team members, especially young EIDs researchers, to strengthen domestic, regional, and international capacity and readiness to efficiently undertake the research required in response to emerging or re-emerging threats. Available training materials or resources will be made available to the CREID Network.

- Conduct annual field biosafety and animal surveillance training (bats, rodents, non-human primates) with lectures and discussion as well as hands-on guidance.
- Conduct training in enriched RNAseq protocols for coronavirus whole-genome sequencing.
- Conduct training in traditional viral whole-genome sequencing using primer walking for paramyxoviruses.
- Conduct virtual and in-person viral phylogenetic and metagenomics analysis training.
- Trial the use of the portable Oxford Nanopore MinION platform as an in-house sequencing solution in Malaysia.
- Continue training on multiplex microsphere immunoassay (MMIA) data analysis and interpretation.
- Conduct refresh training on human research regarding survey design, interview skills, and data analysis.
- Finalize the standard operating procedures (SOP) manual for emerging infectious disease research field biosafety for peer-review, publication, and broad distribution.
- Continue sharing research resources (protocols, SOPs, technologies) and results within the scientific community and local government in a timely manner.
- Organize meetings and workshops with scientists from Southeast Asia to identify common challenges and opportunities for emerging infectious disease research in the region (November 2022).
- Initiate exchange training for young researchers among EID-SEARCH partners for NGS and other laboratory technology transfers.
- Support in-country outbreak research and response.

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Number: 1 Author: Sasiprapa Ninwattana Date: 8/15/2022 5:31:00 PM
Would it be possible to propose training on RBD and/or spike glycoprotein design and coupling?

Appendix A: 2021 EAC Report

Centers for Research in Emerging Infectious Diseases (CREID)
CREID External Advisory Committee (EAC) Summary
In follow up to the CREID Network Annual Meeting
November 3-5, 2021 (virtual meeting)

Prepared by Mark Feinberg, MD, PhD on behalf of the CREID EAC

Overview of EAC Perspectives and Recommendations

The EAC members were unanimous in their assessment that the CREID Network team made very impressive and positive progress in their first year of this very important and timely new NIAID/DMID initiative. While the amount of progress realized in starting up a new global collaborative research network would be highly laudable overall in any circumstance, the EAC members were even further impressed by the fact that the initiation of the CREID Network coincided with the emergence of the COVID-19 pandemic (with all of its attendant challenges). This coincidence provided a clear initial focus of many of the CREID efforts on SARS-CoV-2-related topics, but it also provided very strong validation for the original rationale for establishment of the CREID Network by the NIAID/DMID leadership as a means of fostering global networks for research collaboration on emerging infectious disease threats.

While the EAC members identified a number of areas where additional attention and effort will be beneficial to maximize the impact of the CREID Network, and key to fostering the sustainability of its efforts, our expectations for what the Network has the potential to accomplish were raised by the strong foundation that the CREID PIs and collaborators have already put in place in their first year. We were also very impressed with the clear commitment, thoughtfulness and inclusive approach of the CC leadership, and with the expertise, passion, and experience of the broader CREID Network community of scientists. At the heart of the CREID is a commitment to new models of collaborative science that links investigators from around the world to understand, prepare for, and respond to emerging infectious disease threats. As such, the extent to which CREID utilizes current best practices for collaborative research and pioneers new ones; embraces the essential role played by investigators working in countries at risk for disease emergence (including resource-limited settings); partners in new and more effective and mutually trusting ways with governments, normative agencies and other emerging infectious disease-focused research organizations; exemplifies and communicates the value of scientific research in providing solutions to emerging infectious disease threats to governments and the public; and delivers on its commitment to genuine research capacity strengthening and sustainability will be critical measures for evaluating the future success of the CREID. The EAC recognizes that this is a very ambitious agenda filled with often challenging objectives, and that the inherent nature of time-limited NIH funding mechanisms presents additional challenges to their potential realization.

In considering these issues, the EAC felt that significant progress has been made in the first year of the CREID Network, but that additional opportunities exist for the PIs and CREID investigators to be even more intentional and strategic about their collaborative research efforts. The CREID Network has the opportunity to establish a number of positive precedents in models of effective global collaborative science where the “whole is truly greater than the sum of its parts”; by demonstrating new approaches for the “decolonization” of international research; and by delivering on a strong commitment to diversity and inclusion in all of its endeavors. In many ways, achieving these goals will require innovation in collaborative research models within a large global network, and not just in delivering innovative, high quality scientific research results. The EAC members recognize the challenge of finding the right balance between investigator- and RC-initiated science with broader strategies and shared goals of a large collaborative network, but also note that doing so will require an overarching strategy, effective

communication tools and ongoing performance monitoring and adjustment. The EAC members also recognize that the important Network goals of research capacity strengthening and sustainability are not trivial undertakings and that they are very promising, but also rather novel objectives, within the context of NIH-supported research programs. These are additional important areas where the CREID CC and RC leadership can further articulate a respectful, inclusive, realistic, achievable and measurable strategy.

In all of these areas, the EAC recommends that the CREID investigators further develop and implement (a) a clear, well-defined and shared set of Network priorities (that links the work across RCs) and key performance indicators (KPIs) to measure performance toward realizing them, (b) a shared strategic vision and plan that connects the research efforts being prioritized and conducted across the Network centers, (c) a mechanism to share best practices across the Coordinating Center (CC) and Research Centers and (d) a proactive approach to identify collaborative research opportunities, fill gaps, avoid duplication and pursue synergies both within the CREID Network and, importantly, with the large and growing number of other organization working on preparing for and responding to emerging infectious disease outbreaks. Clarity and specificity in these efforts will be beneficial, as well as adopting an appropriate sense of realism about what can be accomplished by the CREID Network itself and where the new partnerships it implements with other organizations working in the emerging infectious disease arena will be essential. In this regard, given the complexity of the emerging infectious disease landscape, the CREID Network's ability to have the most significant impact will likely depend on the connections it establishes with other stakeholders and the innovation it drives in conceptualizing and delivering on effective new collaborative endeavors it develops with other capable partners. Towards this end, the Network would benefit from developing a thorough understanding of the ecosystem of potential partners and a defined approach to engage with the most relevant and promising ones. In addition, while the CREID Network includes collaborators from many centers and countries, its true global scope is limited by gaps in its geographic coverage (eg, countries in Asia, the Middle East, Northern Africa, Australia, etc...including those where risk of pathogen emergence are significant). Understanding if the CREID Network will be limited by its current geographic reach or plans to work to build additional connections (perhaps via strategic partnerships) in other regions will be beneficial from both practical and strategic levels.

Given that the readiness of the CREID Network for future outbreak response (and to be able to perform research in the midst of an outbreak when many challenges prevail) and for pandemic preparedness is a priority for NIAID/DMID, it will be important for the Network to clearly define how they best proactively plan to be able to promptly implement critical research programs when a new pathogen emerges or a previously known one reemerges. It will also be beneficial for the CREID CC and RCs to develop a clear plan (including protocols for coordination, implementation, data sharing and communication) to enable the Network to be able to respond expeditiously to an emerging infectious disease threat. Towards this end, tabletop exercises (or similar) to "pressure test" Network approaches and identify gaps/opportunities for improvement are worth considering.
The EAC is excited about what the CREID CC and RCs, and all of the collaborating CREID investigators, have accomplished in their first year of work and look forward to seeing the innovations and insights the Network will deliver in the future. The EAC recognizes that the CREID Network cannot realistically be "all things for all people", and should not strive to be. WE therefore also look forward to seeing how your Network priorities are further articulated, implemented and measured moving forward.

Additional EAC Impressions

The EAC felt that the Executive Summary of the CREID Network Annual Meeting provided an accurate overview of the meeting proceedings and of the EAC feedback provided at the end of the meeting proceedings. Some specific additional impressions/requests shared by EAC members include:

- Gaining a better understanding of how CREID investigators are interfacing with local governments and ministries, public health authorities (eg, WHO, US CDC, Africa CDC, etc), normative agencies will be helpful, and how learnings of effective approaches should be shared across the Network. In addition, optimization of engagements with other research organizations, enabling disciplines (eg, social science, health economics, health policy, ethics), as well as the medical countermeasures community, will be helpful
- Gaining a better understanding of the experience to date in data sharing efforts and how these can be optimized
- Gaining a better understanding of how bilateral sharing of research materials and specimens can be optimized (given the role of host institutions and government policies) and how these might operate during an outbreak/pandemic
- How the CREID investigators view and are approaching their educational efforts with governments, political leaders and the public, and sharing of best practices for engagement and trust-building
- How the CREID Network is engaging with industry partners that both supply enabling tools and technologies and who may also assist in the development and commercialization of CREID-advanced innovations (eg, diagnostics)
- Opportunities exist to demonstrate the CREID commitment to decolonization of global health (with increased attention to having the voices from LMIC-based investigators better balanced with those of US-based investigators)
- Opportunities exist to better model the Network's and the NIH's commitment to DEI objectives (with increased attention to full representation in all aspects of the work…including presentations, leadership groups, pilot research awards)
- Opportunities for enhancing epidemiology, bioinformatics, digital tools and modeling capacity
- Gaining a better understanding of the role of social and cultural aspects of the outbreak response and communication, and exploring innovative approaches to address them
- Understanding how engagement with governments and other local stakeholders is being pursued to advance capacity strengthening and sustainability goals
- Further articulation of rapid response collaboration frameworks
- Gaining a better understanding of how local priorities influence the prioritization decisions of the CC and RCs
- Gaining a better understanding/"needs assessment" of capacity gaps (eg, BSL-3 training and labs) and how these are being addressed via strategic connections or focused investment
- How "parachute or safari research" is avoided, and how local researchers are engaged as full partners/leaders
- Definition of opportunities to maximize speed and efficiency in research implementation efforts (eg, US permits and in-country regulations, IRB delays, key relationship gaps) needed for prompt outbreak responses
- Value of developing a clear vision and plan for sustainability that maximizes the connection with and contributions of other partners and local governments
- South-South collaboration should be applauded and fostered
- Shared EAC interest in seeing the CREID Network's progress in ongoing database and biorepository workstreams

Appendix B: NIAID CREID Network Program Priorities (April 2022)

(4 main priorities all carry equal weight and are intended to cover research and activities to prepare for and respond to emerging and re-emerging threats.)

- **Conduct innovative research to expand our knowledge of emerging and re-emerging infectious diseases and better prepare to respond to outbreak/pandemic threats**
    - Research Priorities:
        - Pathogen discovery & characterization
        - Pathogen/host surveillance
        - Pathogen transmission
        - Pathogenesis & immunologic responses in the host
        - Natural history & contemporary clinical disease
        - Develop reagents & diagnostic/detection assays
    - Engage in coordinated, Network wide outbreak/pandemic research readiness, differentiated from standard public health responses
- **Establish a collaborative, strategic, and preemptive research Network to ensure coordination of efforts across the Network**
    - Centralize communication
    - Conduct Network wide capacity inventory
    - Establish effective data harmonization and platforms for sharing
    - Harmonize sample collection, biorepositories, and network sharing/agreements
    - Share laboratory assay best practices and protocols
    - Share reagents, diagnostic/detection assays, and other resources
- **Develop and expand flexible domestic and international capacity and readiness to efficiently undertake research required in response to emerging or re-emerging threats**
    - Prioritize and engage with key stakeholders that will enhance network capabilities and integrate into the global health landscape
    - Strengthen and enhance research collaboration and partnerships globally
    - Pivot programmatic priorities and resources when needed for a timely response to emerging or re-emerging pathogen threats
        - Develop protocols for coordination, implementation, data sharing, and communication.
        - Execute tabletop exercises to test, refine, and maintain Network readiness
    - Formulate novel strategies to detect, control, and prevent outbreaks
        - Translate findings to downstream partners to facilitate development of medical countermeasures or preventive interventions
        - Develop adaptable risk assessment tools for evaluating a pathogen threat's potential for significant emergence and impact
- **Contribute to the development of the next generation of emerging/re-emerging infectious disease scientists and leaders**
    - Develop and maintain a pilot research program
    - Foster a diverse and inclusive research environment and program

EID-SEARCH
Emerging Infectious Diseases
South East Asia Research Collaboration Hub

CREID
Centers for Research in Emerging Infectious Diseases

Centers for Research in Emerging Infectious Diseases (CREID) Network

Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH)

2022 Report to the External Advisory Committee

August 19, October

Table of Contents

Guidance: 7-page limit for EAC Report
(excluding cover page and TOC)

Section 1. Executive Summary

The Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH) is a multidisciplinary center focused on analyzing zoonotic disease risk in a key EID hotspot region. Our aims are to 1) characterize diverse coronaviruses, paramyxoviruses, filoviruses, and other viral pathogens in wildlife; 2) assess the frequency and causes of their spillover; & 3) identify viral etiologies of undiagnosed 'cryptic' outbreaks in Thailand, Malaysia, and Singapore. Highlights of research and significant findings during the reporting period (October 2021 – August 2022) include:

To be updated with bullet points by Peter…

Section 2: Research Center Overview

A. Specific Aims

The overarching goal of the Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH) is to characterize the diversity of key viral pathogens in wildlife, assess the frequency and causes of their spillover, and identify viral etiologies of undiagnosed 'cryptic' outbreaks in people. The research goals of the EID-SEARCH follow three specific aims designed to advance our understanding of spillover and outbreak risk for novel viruses in a globally important EID hotspot, strengthen in-country research capacity, and enhance international collaboration:

- Specific Aim 1: Identify, characterize, and rank spillover risk of high zoonotic potential viruses from wildlife.
- Specific Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays.
- Specific Aim 3: Identify and characterize the viral etiology of 'cryptic' outbreaks in clinical cohorts.

B. Partners and Staffing

EID-SEARCH includes leaders in the field of emerging viral pathogens at key US institutions (EcoHealth Alliance PI Peter Daszak; University of North Carolina Co-I Ralph Baric; Uniformed Service University Co-Is Christopher Broder and Eric Laing), and in Thailand (Chulalongkorn University Co-Is Supaporn Wacharapluesadee and Opass Putcharoen), Singapore (Duke-NUS Medical School Co-I Linfa Wang), and the three major Malaysian administrative regions (Conservation Medicine Co-I Tom Hughes). These organizations represent the 'hub' of the center, with an additional, more informal collaborative network spanning >50 clinics, laboratories, and research institutes across almost all Southeast Asian countries. By enhancing and focusing research within the three hub countries, and coordinating communication among the network, we envision EID-SEARCH acting as an early warning system for outbreaks; a way to exchange information, reagents, samples, and technology; and a collaborative powerhouse for translational research. EID-SEARCH is supported by a multidisciplinary team of researchers at our partner institutions, with skills in epidemiology, clinical management, molecular biology, virology, wildlife biology, and advanced data and statistical analyses. The long collaborative history between the key personnel and partner institutions is a significant asset that can be deployed to support outbreak research response in the region.

C. Research Sites

The three core (hub) countries for the EID-SEARCH are Thailand, Malaysia, and Singapore, *expanded to Viet Nam and Cambodia through the CREID Pilot Research Program*. These stretch through one of the

most significant foci of EID risk globally. Additionally, we have assembled a greater network of EID-SEARCH partners that includes >50 clinics, laboratories, and research institutes across almost all Southeast Asian countries – stretching from Nepal to the Philippines. Our targeted field research includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sarawak, Sabah, Viet Nam, and Cambodia. The central location of our hub countries in mainland Southeast Asia allows us to sample representative wildlife species that occur in other countries across the greater biogeographic region, including India, Bangladesh, Myanmar, Southwest China, Laos, Cambodia, Philippines, and Indonesia.

Section 3: Research Center Progress and Accomplishments

Progress and Accomplishments

- Organized by Aim
- Focus on key accomplishments
- Address outbreak research response if applicable
- Address how the 2021 EAC recommendations have been incorporated (*see Appendix A for 2021 EAC report*)

A. Projects and Studies

- Have you reverted to your original (pre-pandemic) CREID research agenda in the last year? If not, how has your research agenda changed in the last year due to the ongoing impact of SARS-CoV-2?
- What additional changes to your CREID research agenda have taken place in the last year?

Aim 1: Identify, characterize, and rank spillover risk of high zoonotic potential viruses from wildlife

- **Field surveillance among wild bats, rodents, and non-human primates.** We have collected 14,302 specimens from 2,055 animals of 31 bat species, one non-human primate (NHP) species, and five rodent species from six sites at high-risk human-wildlife interfaces in Malaysia and Thailand. (Table 1)

	Thailand	**Malaysia**
No. of collected specimens	3,862	14,171
No. of individuals	454 bats, 106 rodents	2,000 bats
No. of species	6 (bats), 5 (rodents)	24 (bats)
No. of sites	5	4
No. of tested individuals	407	1,332
No. PCR tests for coronaviruses (CoVs), paramyxoviruses (PMVs), filoviruses (FLVs), influenza viruses (IVs)	4,070	9,228
Identified viruses	3 new CoVs from 11 bats 3 known PMVs from 4 rodents 4 new PMVs from 11 bats	5 new CoVs from 112 bats 4 new PMVs from 10 bats 2 known PMVs from 2 bats
No. of specimen for multiplex immunoassay for FLVs, CoV and Henipavirus	100 macaques 92 rodents	720 bats

Table 1. EID-SEARCH viral surveillance in wildlife in Malaysia and Thailand.

- **Discovery of novel SARS-like beta-coronavirus in bats in Thailand**. The whole genomes revealed a 91.72% nucleotide similarity to SARS-CoV-2. Phylogenetic analysis suggested that this virus is closer to pangolin CoV-GD-1 than to SARS-CoV-2; however, the RBD is more similar to that of bat-CoV RaTG13, suggesting that this new virus may also bind to mammalian ACE2 receptors. Whole-genome sequencing of other new CoVs from Thailand and Malaysia is ongoing.

Summary of Comments on Email 8 - Attachment 2 - 2022 EAC Report_EID-SEARCH_draft v01-EDL-KR-SN_15Aug22.pdf

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Number: 1 Author: Microsoft Office User Date: 8/2/2022 8:27:00 AM

Footnote about what “specimens” includes, how were 14,000 specimens obtained from 2,000 bats. 1 bat -> blood sample, oral swab, rectal swab, body swab, ectoparasite removed, wing puncture, etc…

Number: 2 Author: Microsoft Office User Date: 8/2/2022 8:23:00 AM

I’m not a mol. evol. geneticist but the way this is laid out in the table gives equal weight to a full genome, Thailand 1 new CoV, and distinct RdRp NT sequences that are used to create phylogenetic trees. I don’t think I’ll ever understand how the virology field uses small NT reads to discover new viruses. I suggest adding another row for “full virus genome sequenced” and a footnote about the methods used to determine how a new virus has been genetically identified.

Number: 3 Author: Sasiprapa Ninwattana Date: 8/15/2022 5:48:00 PM

Based on complete whole genome sequences (3 samples) and partial whole genome sequences (8 samples)

Number: 4 Author: Microsoft Office User Date: 8/2/2022 9:56:00 AM

Same species? Details.

Number: 5 Author: Sasiprapa Ninwattana Date: 8/13/2022 11:22:00 AM

Samples collected in year 1 (31 March 2021)

Number: 6 Author: Microsoft Office User Date: 8/2/2022 8:30:00 AM

Check ICTV guideline, I thought it was established to use, e.g., bat SARS-related coronavirus (bat SARSr-CoV). The use of beta-coronavirus widens the focus away from sarbecoviruses.

- **Genome-wide CRISPR knockout screen identifies essential factor for HKU2/SADS-CoV infection.** Using a genome-wide CRISPR knockout screen, we identified placenta-associated 8 protein (PLAC8) as an essential host factor for SADS-CoV infection of human cells. Infection is prevented in PLAC8 knockouts and can be restored by transiently transfecting PLAC8 from multiple species, including human, pig, mouse, and bat. Trypsin treatment of SADS-CoV bypasses the PLAC8-dependent pathway and partially rescues SADS-CoV infection. PLAC8 is involved in both early entry and later dissemination of SADS-CoV infection and PLAC8 KO cells have: 1) delayed and reduced viral subgenomic RNA expression and 2) diminished cell-to-cell viral spreading of infected cells. *These results suggest that PLAC8 is a promising target for antiviral development for the potential pandemic SADS-CoV virus*.
- **Host range, transmissibility, and antigenicity of a pangolin coronavirus.** Using a synthetically derived infectious cDNA clone, we recovered the wildtype pangolin (Pg) CoV-GD and derivatives encoding indicator genes. Like SARS-CoV-2, PgCoV-GD virus efficiently uses human and many other orthologous mammalian ACE2 receptors for entry, shares a similarly broad host range *in vitro*, and displays altered sensitivity to host proteases. PgCoV-GD replicates to slightly higher to lower titers in primary human cells derived from the proximal to distal lung, respectively, and with reduced fitness compared to SARS-CoV-2. PgCoV-GD also replicates efficiently in hamsters and could transmit via the aerosol route, but at a lower frequency than SARS-CoV2. Efficient PgCoV-GD replication in primary nasal airway epithelial cells and transmission in the hamster 5highlights the high emergence potential of this virus. However, PgCoV-GD is efficiently neutralized by COVID-19 patient sera and many commercially available SARS-CoV-2 therapeutic antibodies, but not by human antibodies targeting the spike N-terminal domain. Furthermore, a pan-sarbecovirus antibody, ADG-2, and a SARS-CoV-2 prefusion stabilized spike protein (S2P) recombinant protein vaccine protected mice in a PgCoV-GD replication model. Lastly, we showed that cross-neutralizing antibodies in pre-immune SARS-CoV-2 humans and the benefit of current COVID-19 countermeasures should impede its ability to emerge and spread globally in humans. *This research helps build FDA-approved therapeutic antibodies against a range of CoVs with emergence potential and shows that FDA-approved SARS-CoV-2 vaccines also protect animals from PgCoV-GD when challenged in vivo*.

Aims 2 & 3: Identify evidence and analyze risk factors for viral spillover in high-risk communities and clinical cohorts, and characterize viral etiology of 'cryptic' outbreaks

- **Incorporation our bat-CoV spike proteins into the multi-family multiplex-based immunoassay (MMIA)**. Specific bat-CoV spike proteins including bat SARSr-CoVs RaT13, bat SARSr-CoV ZXC21, bat MERSr-CoV PDF2180, and HKU9 were designed as pre-fusion stabilized spike glycoprotein ectodomain trimers (S-2P). These were spike protein antigens were incorporated into our 17-plex henipavirus/filovirus-focused serology assay expanding the representation of virus targets to include priority coronaviruses such as SARS-CoV-2 and bat SARSr-CoVs/bat MERSr-CoV. SOPs were revised and updated to reflect best practices that were optimized during Year 1.

- **Community serology surveillance.** We initiated the human surveillance work in Thailand, with 56 participants enrolled at one at-risk community site, and 2 individuals from a clinic site, with a total of 58 participants enrolled. Serology testing of samples from 56 participants from the community site showed positive for henipaviruses (HNVs) (family *Paramyxoviridae*), filoviruses (FLVs), and CoVs (Fig. 1). Human sera were tested in the multi-family MMIA for serologic evidence of previous infection by Asiatic FLVs, HNVs, and CoV. We observed minimal reactivity and IgG binding with the NiV receptor-binding protein (RBP), implying that subclinical cryptic NiV infections had not occurred in these

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Number: 1 Author: Microsoft Office User Date: 8/2/2022 8:29:00 AM
The fusion data Chee Wah presented indicated that it does bind to and mediate cellular entry/fusion via the human ACE-2 orthologue.

Number: 2 Author: Microsoft Office User Date: 8/2/2022 8:35:00 AM
? not sure what this means. Were there lower viral loads in the respiratory tract? Is aerosol route correct, or should it be respiratory secretions? There is a lot of data in this section, is there a reference to a pre-print or article?

Number: 3 Author: Microsoft Office User Date: 8/2/2022 8:33:00 AM
List these?

Number: 4 Author: Microsoft Office User Date: 8/2/2022 8:37:00 AM
This is pretty nuanced. Do the commercially available mAbs that neutralized PgCoV-GD target the RBD or Q neutralizing epitopes in the S glycoprotein and not the NTD?

Number: 5 Author: Microsoft Office User Date: 8/2/2022 8:33:00 AM
Is this an FDA EUA or authorized treatment?

Number: 6 Author: Microsoft Office User Date: 8/2/2022 8:32:00 AM
This is something not FDA EUA? Will stakeholders understand what S-2P etc means? This is what NovaVax is making.

Number: 7 Author: Microsoft Office User Date: 8/2/2022 8:39:00 AM
Replication, not infection or disease model? What did these mAb and vaccine protect from, infection?

Number: 8 Author: Microsoft Office User Date: 8/2/2022 8:41:00 AM
What does this mean, sera from humans with memory to seasonal beta-human coronaviruses can cross-neutralize PgCoV-GD, by binding recognition to the S2 fusion domains?

Number: 9 Author: Microsoft Office User Date: 8/2/2022 8:43:00 AM
I didn't see any mention of FDA-approved antibody therapies or vaccine countermeasures being tested in the sentences before.

Tighten this up with explicit details.

participants (Figure 1B). One human serum sample possessed IgG that was reactive with envelope glycoproteins (GP) from Ebola virus, Bundibugyo virus, Bombali virus, Sudan virus, and Lloviu virus, consistent with a serologic pattern of cross-reactivity among phylogenetically-related ebolaviruses (Figure 1A). A diversity of genetically and antigenically uncharacterized Asiatic filoviruses is likely to exist, and this serology results is consistent with other serology-focused projects in the region. The antigenic relationships and contribution of glycoepitopes to heterotypic cross-reactions among ebolaviruses and broadly within FLVs remains poorly understood, follow-up testing will explore whether broad cross-reactions with FLVs antigens is driven by glycosylated epitopes that may be more conserved across the ebolaviruses than protein sequence similarity/identify would imply. Lastly, human sera displayed maximum preferential reactivity with SARS-CoV-2 spike protein, consistent with COVID-19 vaccination status of participants. At the low testing titer, cross-reactions with phylogenetically-related bat sarbecoviruses was expectedly observed (Figure 1C). Interestingly, two participants had sera IgG that bound to the bat MERSr-CoV PDF2180, which does not utilize the orthologous human DPP4/CD20 receptor for cellular entry, and it quite distinct from the sarbecoviruses.

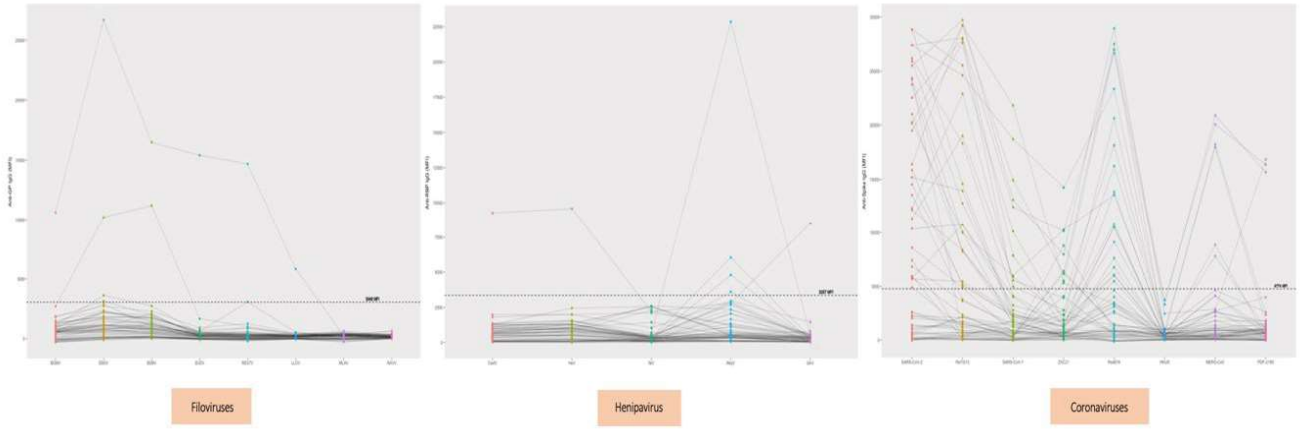

Fig. 1 Serology testing results for FLVs, HNVs, and CoVs among community participants in Thailand (n=56).

- **<u>Design and application of pan-sarbecovirus multiplex surrogate virus neutralization test (sVNT)</u>** that covers SARS-CoV-2 and variants of concern (Alpha, Beta, Gamma, Delta, Mu, Lambda, Omicron BA.1 and BA.2), clade-2 sarbecoviruses (BANAL-52, BANAL-263, GD-1, RaTG13, GX-P5L), clade-1 sarbecoviruses (Rs2018B, LYRa11, WIV-1, RsSHC014, Rs4231), SARS-CoV-1, and *the newly discovered bat SARSr-CoV we recently sequenced from Thailand.* This assay allows for the discrimination of past SARS-CoV-2 infection from that of other sarbecoviruses in human and animal samples, which will be critical for identifying cryptic spillover of sarbecoviruses in people. It also has the potential to aid in identifying the progenitor of SARS-CoV-2 in animals.

- **<u>Optimization and establishment of pan-henipavirus multiplex sVNT at Chulalongkorn University.</u>** We also optimized an sVNT for Nipah virus and provided enough material to characterize Nipah virus IgG positive serum samples for neutralizing antibodies via a virus-free/cell-culture free bead-based serology assay.

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Number: 1 Author: Microsoft Office User Date: 8/2/2022 8:45:00 AM
What does this mean?

Number: 2 Author: Sasiprapa Ninwattana Date: 8/15/2022 5:50:00 PM
I re-tested the two serum samples that were weakly positive for NiV and it turned out that both of them showed seronegative.

Number: 3 Author: Microsoft Office User Date: 8/2/2022 8:45:00 AM
These panels are showing the same thing, pick one format and make these bigger. I prefer the “connected” lines because it shows the that it’s the same individual.

Number: 4 Author: Sasiprapa Ninwattana Date: 8/15/2022 5:53:00 PM
I plotted new graphs because I re-tested some of samples that were positive to confirm the results.

Number: 5 Author: Microsoft Office User Date: 8/2/2022 2:54:00 PM
Needs a proper legend.

Outbreak investigation and preparedness in Thailand

- The Ministry of Public Health in Thailand requested support from EID-SEARCH for a viral diarrhea outbreak investigation in Chanthaburi province. We received samples from both Prapokklao Hospital (regional hospital) and Khlong Khut Health-Promoting hospital collected from 59 healthcare workers and students who had diarrhea during Dec. 2021-Feb. 2022. PCR tests were conducted for six gastrointestinal viruses causing gastrointestinal tract infections including adenovirus, astrovirus, norovirus GI, norovirus GII, rotavirus, and sapovirus. 32 samples **(54.24%)** were positive for norovirus GII. Further genomic characterization of the detected norovirus GII will be performed, allowing for comparison with noroviruses found in other provinces in Thailand.
- In preparedness for Monkeypox outbreaks, EID-SEARCH's lab partner in Thailand coordinated with the Department of Medical Science, MOPH, National lab to establish the Monkeypox testing platform in April 2022 and detected the first Monkeypox case in Thailand in July 2022.

B. Dissemination of Results

- Summarize dissemination plan
- Include number of peer-reviewed publications; highlight those in high-impact journals
- Number and content of presentations on CREID research, including audience

EID-SEARCH members were invited to present the project work at government and inter-government briefings (> 10), conference and university lectures (>13), panels and webinars (>18), and public interviews (>6) in Thailand, Malaysia, the US, regionally and internationally on the trends in global disease emergence, zoonotic surveillance, pandemic control and prevention, and research results from this project research activities. Nine (9) peer-reviewed paper has been published or with preprint available from EID-SEARCH work, two (2) manuscripts are currently under revision, and three (3) manuscripts are in preparation for submission*, including papers on.. please highlight the high-impact ones*. We organized a workshop with 55 scientists and government officials from Thailand, Malaysia, Singapore, Bangladesh, Cambodia, Indonesia, Lao PDR, Myanmar, Nepal, Vietnam, USA, and Australia to present EID-SEARCH work, share knowledge, strengthen research capacity, and foster scientific collaborations in Southeast Asia.

C. Training and Capacity Building Activities

- Summarize capacity building plan
- Include number of training activities and number of participants for each training
- Address how capacity building activities were adapted due to COVID-19 and long-term changes as a result of the pandemic

During this reporting period, XX training sessions with a total of XX participants were conducted by EID-SEARCH partners in Thailand, Malaysia, and Vietnam for project members, local government partners, universities, research institutes, and NGOs. These training focused on biosafety (x training x participants by x), applying new technologies (x training x participants by x), and field and laboratory SOPs (x training x participants by x), to strengthen in-country research capacity and improve information sharing. Both in-person and online training was conducted to accommodate COVID conditions. As travel and COVID-19 restrictions have begun to be lifted, students, postdocs, and all EID-SEARCH research staff have been provided with professional development opportunities to travel to attend conferences, workshops, and other in-person training activities. *To be updated with numbers….*

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Number: 1 Author: Sasiprapa Ninwattana Date: 8/15/2022 5:28:00 PM

As mentioned earlier, I re-tested the two serum samples that were weakly positive for NiV and it turned out that both of them showed seronegative. So I'm not sure that should this part still be included.

Number: 2 Author: Microsoft Office User Date: 8/2/2022 8:51:00 AM

If not including the PMID, I'd at least add the Surrname, First Initial, et al. J. Infect Dis. 2022.

Number: 3 Author: Sasiprapa Ninwattana Date: 8/13/2022 11:02:00 AM

1. Our paper "Rapid and Simultaneous Detection of Omicron and Other Concerned SARS-CoV-2 Variants (Alpha, Beta, and Delta) in Clinical Specimens Using Multiplex PCR MassARRAY Technology" is under revision

2. "Homologous or Heterologous COVID-19 Booster Regimens Significantly Impact Sero-Neutralization of SARS-CoV-2 Virus and its Variants" has been accepted for publication in vaccines journal

D. Challenges and Proposed Solutions

- Bottlenecks to CREID-related research that might be like those that you could face when pivoting research activities in direct response to an EID outbreak. What solutions could be or have you put in place in advance to solve those challenges? How is being part of the CREID Network mitigating those or not?

The significant challenges we encountered during the reporting period were travel restrictions to our field partner countries and within countries, as well as a large number of hospitalized patients in local hospitals in Thailand and Malaysia due to COVID-19 that delayed human participant enrollment at targeted community and hospital sites and the subsequent laboratory analysis we anticipated in Year 2. To address these issues, we worked closely with in-country partners and stakeholders to continue assessing the situations (i.e., safety and research priorities of study sites) to ensure a rapid start of enrollment and focused on the refinement of assays to be ready for human surveillance. Meanwhile, to prevent a possible supply shortage of PPE and swabs as happened in early Year 2, we have identified supply sources to ensure we can procure the necessary consumables without significant delays. With travel restrictions in Southeast Asian countries now beginning to be lifted, international travel of US-based staff to our field sites has begun (April 2022).

E. Examples of Network Collaboration

- How has the work/research of your RC been affected by or involved collaboration with other members of the CREID Network?
- Examples: highlight joint publications, joint activities, sharing materials and data, benefits of Working Groups, etc.

Through the participation of different CREID Working Groups, EID-SEARCH members participated in the network's outbreak responses, capacity building, and cross-center collaboration by sharing existing local partnerships, techniques, and relevant resources.

- For outbreak responses, EID-SEARCH has been actively participating in the network's response to Monkeypox by providing local partnerships and monitoring platforms in DRC and Thailand.
- For lab assay sharing, EID-SEARCH partner Uniformed Services University is working with UWARN PIs, Dr. Robert Cross and Dr. Scott Weaver, to establish a MTA with UTMB and regional partners in Liberia and Nigeria for the multi-family FLV/HNV MMIA for anti-EBOV serology testing. Enquiries has been made with Dr. Mark Page and the National Institute for Biological Standards and Control (NIBSC) regarding additional MARV and NiV serology standards.
- We are contacted by multiple Research Centers regarding the Field Biosafety Manual presented at the CREID Scientific Meeting by EID-SEARCH senior field veterinarian, the Manual and corresponding training resources will be made available to all CREID members once it's completed with peer review. Marc, please feel to edit…
- EID-SEARCH are also committed to providing our expertise on CCHF, Henipavirus, RVF related research by connecting CREID members with our research partners.

Section 4: Future Directions

- Plan for the next reporting period.
- How is your Research Center adapting to the April 2022 NIAID Program Priorities and how does this change (or not) your planned activities in the next year? *See Appendix B for NIAID Program Priorities*.

- Moving forward, what is the greatest opportunity your RC has to advance the knowledge base for emerging infectious diseases? What are the significant knowledge gaps that the RC can advance research on?

In the next reporting period, we will follow the research plans laid out in our proposal with the specific actions to **continue expanding our knowledge of emerging and re-emerging infectious diseases and better prepare to respond to outbreak/pandemic threats that align with the CREID network research priorities:**

Specific Aim 1: Identify, characterize, and rank spillover risk of high zoonotic potential viruses from wildlife.

- Wildlife (bats, rodents, and non-human primates) sampling at selected sites, with increased efforts for rodents and non-human primates and potential longitudinal sampling at a few selected sites to analyze patterns of viral shedding and spillover risk.
- Continue to develop predictive host-range models and spatial risk models to prioritize sampling sites and species in Years 3-5 of the project.
- Conduct viral family-level molecular screening of collected wildlife samples for coronaviruses, filoviruses, and paramyxoviruses.
- Perform serology testing on the wildlife samples or coronaviruses, filoviruses, and henipavirus.
- Continue developing a more comprehensive multiplex CoV RBD-based sVNT covering newly identified sarboviruses and additional merbecoviruses to allow the evaluation of neutralizing activity in serum samples against a much broader and more comprehensive range of RBDs.
- Conduct whole-genome and spike glycoprotein sequencing in countries with newly identified viruses.
- Continue research to further characterize a subset of novel viruses including virus isolation, human cell infection experiments, and animal modeling for viral pathogenesis studies, including pangolin GD strain, BANAL-52, and other significant viruses identified in Y1-2.
- Develop zoonotic risk analyses for relevant viruses, including the analysis of Spike and whole-genome sequences to predict host range using a variety of in silico approaches, model the host range, geographic distribution, extent of overlap with high-density human populations, etc. using existing apps/models to assess potential zoonotic disease risk

Specific Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays

- Increase the number of sites and enrolled participants for community surveillance among at-risk populations for biological and behavioral data collection in Thailand, Peninsular Malaysia, and Sabah Malaysia
- Continue to identify community sites concurrent with animal surveillance sites (Specific Aim 1).
- Perform serology testing of collected human samples with optimized multiplex assays for coronaviruses, filoviruses, and paramyxoviruses
- Begin epidemiological analyses of biological and behavioral data.

Specific Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts

- Start enrolling participants at selected hospital/clinic sites for syndromic surveillance at two sites in Thailand, and identified sites in Sabah, Malaysia and Peninsular Malaysia, Malaysia
- Continue to identify potential clinical cohorts and sites in the catchment of the community (Specific Aim 2) and animal surveillance (Specific Aim 1) sites.

- Start serological and molecular testing on collected human samples.
- Start viral characterization work if any novel viruses are identified.

Through the work under Specific Aims 2&3, we are engaging multiple disciplines of social science, anthropology, health policy, and behavioral science towards EID risk mitigation strategies on key human-animal interfaces, we will share relevant research methods (e.g., behavioral questionnaires) and best practices with the CREID Network members.

Training and capacity-building activities among in-country team members, especially young EIDs researchers, to strengthen domestic, regional, and international capacity and readiness to efficiently undertake the research required in response to emerging or re-emerging threats. Available training materials or resources will be made available to the CREID Network.

- Conduct annual field biosafety and animal surveillance training (bats, rodents, non-human primates) with lectures and discussion as well as hands-on guidance.
- Conduct training in enriched RNAseq protocols for coronavirus whole-genome sequencing.
- Conduct training in traditional viral whole-genome sequencing using primer walking for paramyxoviruses.
- Conduct virtual and in-person viral phylogenetic and metagenomics analysis training.
- Trial the use of the portable Oxford Nanopore MinION platform as an in-house sequencing solution in Malaysia.
- Continue training on multiplex microsphere immunoassay (MMIA) data analysis and interpretation.
- Conduct refresh training on human research regarding survey design, interview skills, and data analysis.
- Finalize the standard operating procedures (SOP) manual for emerging infectious disease research field biosafety for peer-review, publication, and broad distribution.
- Continue sharing research resources (protocols, SOPs, technologies) and results within the scientific community and local government in a timely manner.
- Organize meetings and workshops with scientists from Southeast Asia to identify common challenges and opportunities for emerging infectious disease research in the region (November 2022).
- Initiate exchange training for young researchers among EID-SEARCH partners for NGS and other laboratory technology transfers.
- Support in-country outbreak research and response.

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Number: 1 Author: Sasiprapa Ninwattana Date: 8/15/2022 5:31:00 PM
Would it be possible to propose training on RBD and/or spike glycoprotein design and coupling?

Appendix A: 2021 EAC Report

Centers for Research in Emerging Infectious Diseases (CREID)
CREID External Advisory Committee (EAC) Summary
In follow up to the CREID Network Annual Meeting
November 3-5, 2021 (virtual meeting)

Prepared by Mark Feinberg, MD, PhD on behalf of the CREID EAC

Overview of EAC Perspectives and Recommendations

The EAC members were unanimous in their assessment that the CREID Network team made very impressive and positive progress in their first year of this very important and timely new NIAID/DMID initiative. While the amount of progress realized in starting up a new global collaborative research network would be highly laudable overall in any circumstance, the EAC members were even further impressed by the fact that the initiation of the CREID Network coincided with the emergence of the COVID-19 pandemic (with all of its attendant challenges). This coincidence provided a clear initial focus of many of the CREID efforts on SARS-CoV-2-related topics, but it also provided very strong validation for the original rationale for establishment of the CREID Network by the NIAID/DMID leadership as a means of fostering global networks for research collaboration on emerging infectious disease threats.

While the EAC members identified a number of areas where additional attention and effort will be beneficial to maximize the impact of the CREID Network, and key to fostering the sustainability of its efforts, our expectations for what the Network has the potential to accomplish were raised by the strong foundation that the CREID PIs and collaborators have already put in place in their first year. We were also very impressed with the clear commitment, thoughtfulness and inclusive approach of the CC leadership, and with the expertise, passion, and experience of the broader CREID Network community of scientists. At the heart of the CREID is a commitment to new models of collaborative science that links investigators from around the world to understand, prepare for, and respond to emerging infectious disease threats. As such, the extent to which CREID utilizes current best practices for collaborative research and pioneers new ones; embraces the essential role played by investigators working in countries at risk for disease emergence (including resource-limited settings); partners in new and more effective and mutually trusting ways with governments, normative agencies and other emerging infectious disease-focused research organizations; exemplifies and communicates the value of scientific research in providing solutions to emerging infectious disease threats to governments and the public; and delivers on its commitment to genuine research capacity strengthening and sustainability will be critical measures for evaluating the future success of the CREID. The EAC recognizes that this is a very ambitious agenda filled with often challenging objectives, and that the inherent nature of time-limited NIH funding mechanisms presents additional challenges to their potential realization.

In considering these issues, the EAC felt that significant progress has been made in the first year of the CREID Network, but that additional opportunities exist for the PIs and CREID investigators to be even more intentional and strategic about their collaborative research efforts. The CREID Network has the opportunity to establish a number of positive precedents in models of effective global collaborative science where the “whole is truly greater than the sum of its parts”; by demonstrating new approaches for the “decolonization” of international research; and by delivering on a strong commitment to diversity and inclusion in all of its endeavors. In many ways, achieving these goals will require innovation in collaborative research models within a large global network, and not just in delivering innovative, high quality scientific research results. The EAC members recognize the challenge of finding the right balance between investigator- and RC-initiated science with broader strategies and shared goals of a large collaborative network, but also note that doing so will require an overarching strategy, effective

communication tools and ongoing performance monitoring and adjustment. The EAC members also recognize that the important Network goals of research capacity strengthening and sustainability are not trivial undertakings and that they are very promising, but also rather novel objectives, within the context of NIH-supported research programs. These are additional important areas where the CREID CC and RC leadership can further articulate a respectful, inclusive, realistic, achievable and measurable strategy.

In all of these areas, the EAC recommends that the CREID investigators further develop and implement (a) a clear, well-defined and shared set of Network priorities (that links the work across RCs) and key performance indicators (KPIs) to measure performance toward realizing them, (b) a shared strategic vision and plan that connects the research efforts being prioritized and conducted across the Network centers, (c) a mechanism to share best practices across the Coordinating Center (CC) and Research Centers and (d) a proactive approach to identify collaborative research opportunities, fill gaps, avoid duplication and pursue synergies both within the CREID Network and, importantly, with the large and growing number of other organization working on preparing for and responding to emerging infectious disease outbreaks. Clarity and specificity in these efforts will be beneficial, as well as adopting an appropriate sense of realism about what can be accomplished by the CREID Network itself and where the new partnerships it implements with other organizations working in the emerging infectious disease arena will be essential. In this regard, given the complexity of the emerging infectious disease landscape, the CREID Network's ability to have the most significant impact will likely depend on the connections it establishes with other stakeholders and the innovation it drives in conceptualizing and delivering on effective new collaborative endeavors it develops with other capable partners. Towards this end, the Network would benefit from developing a thorough understanding of the ecosystem of potential partners and a defined approach to engage with the most relevant and promising ones. In addition, while the CREID Network includes collaborators from many centers and countries, its true global scope is limited by gaps in its geographic coverage (eg, countries in Asia, the Middle East, Northern Africa, Australia, etc...including those where risk of pathogen emergence are significant). Understanding if the CREID Network will be limited by its current geographic reach or plans to work to build additional connections (perhaps via strategic partnerships) in other regions will be beneficial from both practical and strategic levels.

Given that the readiness of the CREID Network for future outbreak response (and to be able to perform research in the midst of an outbreak when many challenges prevail) and for pandemic preparedness is a priority for NIAID/DMID, it will be important for the Network to clearly define how they best proactively plan to be able to promptly implement critical research programs when a new pathogen emerges or a previously known one reemerges. It will also be beneficial for the CREID CC and RCs to develop a clear plan (including protocols for coordination, implementation, data sharing and communication) to enable the Network to be able to respond expeditiously to an emerging infectious disease threat. Towards this end, tabletop exercises (or similar) to "pressure test" Network approaches and identify gaps/opportunities for improvement are worth considering.
The EAC is excited about what the CREID CC and RCs, and all of the collaborating CREID investigators, have accomplished in their first year of work and look forward to seeing the innovations and insights the Network will deliver in the future. The EAC recognizes that the CREID Network cannot realistically be "all things for all people", and should not strive to be. WE therefore also look forward to seeing how your Network priorities are further articulated, implemented and measured moving forward.

Additional EAC Impressions

The EAC felt that the Executive Summary of the CREID Network Annual Meeting provided an accurate overview of the meeting proceedings and of the EAC feedback provided at the end of the meeting proceedings. Some specific additional impressions/requests shared by EAC members include:

- Gaining a better understanding of how CREID investigators are interfacing with local governments and ministries, public health authorities (eg, WHO, US CDC, Africa CDC, etc), normative agencies will be helpful, and how learnings of effective approaches should be shared across the Network. In addition, optimization of engagements with other research organizations, enabling disciplines (eg, social science, health economics, health policy, ethics), as well as the medical countermeasures community, will be helpful
- Gaining a better understanding of the experience to date in data sharing efforts and how these can be optimized
- Gaining a better understanding of how bilateral sharing of research materials and specimens can be optimized (given the role of host institutions and government policies) and how these might operate during an outbreak/pandemic
- How the CREID investigators view and are approaching their educational efforts with governments, political leaders and the public, and sharing of best practices for engagement and trust-building
- How the CREID Network is engaging with industry partners that both supply enabling tools and technologies and who may also assist in the development and commercialization of CREID-advanced innovations (eg, diagnostics)
- Opportunities exist to demonstrate the CREID commitment to decolonization of global health (with increased attention to having the voices from LMIC-based investigators better balanced with those of US-based investigators)
- Opportunities exist to better model the Network's and the NIH's commitment to DEI objectives (with increased attention to full representation in all aspects of the work…including presentations, leadership groups, pilot research awards)
- Opportunities for enhancing epidemiology, bioinformatics, digital tools and modeling capacity
- Gaining a better understanding of the role of social and cultural aspects of the outbreak response and communication, and exploring innovative approaches to address them
- Understanding how engagement with governments and other local stakeholders is being pursued to advance capacity strengthening and sustainability goals
- Further articulation of rapid response collaboration frameworks
- Gaining a better understanding of how local priorities influence the prioritization decisions of the CC and RCs
- Gaining a better understanding/"needs assessment" of capacity gaps (eg, BSL-3 training and labs) and how these are being addressed via strategic connections or focused investment
- How "parachute or safari research" is avoided, and how local researchers are engaged as full partners/leaders
- Definition of opportunities to maximize speed and efficiency in research implementation efforts (eg, US permits and in-country regulations, IRB delays, key relationship gaps) needed for prompt outbreak responses
- Value of developing a clear vision and plan for sustainability that maximizes the connection with and contributions of other partners and local governments
- South-South collaboration should be applauded and fostered
- Shared EAC interest in seeing the CREID Network's progress in ongoing database and biorepository workstreams

Appendix B: NIAID CREID Network Program Priorities (April 2022)

(4 main priorities all carry equal weight and are intended to cover research and activities to prepare for and respond to emerging and re-emerging threats.)

- **Conduct innovative research to expand our knowledge of emerging and re-emerging infectious diseases and better prepare to respond to outbreak/pandemic threats**
    - Research Priorities:
        - Pathogen discovery & characterization
        - Pathogen/host surveillance
        - Pathogen transmission
        - Pathogenesis & immunologic responses in the host
        - Natural history & contemporary clinical disease
        - Develop reagents & diagnostic/detection assays
    - Engage in coordinated, Network wide outbreak/pandemic research readiness, differentiated from standard public health responses
- **Establish a collaborative, strategic, and preemptive research Network to ensure coordination of efforts across the Network**
    - Centralize communication
    - Conduct Network wide capacity inventory
    - Establish effective data harmonization and platforms for sharing
    - Harmonize sample collection, biorepositories, and network sharing/agreements
    - Share laboratory assay best practices and protocols
    - Share reagents, diagnostic/detection assays, and other resources
- **Develop and expand flexible domestic and international capacity and readiness to efficiently undertake research required in response to emerging or re-emerging threats**
    - Prioritize and engage with key stakeholders that will enhance network capabilities and integrate into the global health landscape
    - Strengthen and enhance research collaboration and partnerships globally
    - Pivot programmatic priorities and resources when needed for a timely response to emerging or re-emerging pathogen threats
        - Develop protocols for coordination, implementation, data sharing, and communication.
        - Execute tabletop exercises to test, refine, and maintain Network readiness
    - Formulate novel strategies to detect, control, and prevent outbreaks
        - Translate findings to downstream partners to facilitate development of medical countermeasures or preventive interventions
        - Develop adaptable risk assessment tools for evaluating a pathogen threat's potential for significant emergence and impact
- **Contribute to the development of the next generation of emerging/re-emerging infectious disease scientists and leaders**
    - Develop and maintain a pilot research program
    - Foster a diverse and inclusive research environment and program

DRAFT AGENDA

Hybrid Meeting
Washington DC region and Zoom

DAY 1 | Wednesday, September 21	
Time (ET)	Session
7:30-9:00	**Research Center meetings** *Participants: Research Center members*
09:00-09:15	**TableTop Exercise: Introduction** *In-person Participants: External Advisory Committee (EAC), DMID, Network Leadership* *Virtual Participants: Network members*
09:15-12:00	**Business Meeting** *Participants: EAC, DMID, Network Leadership* ▪ Potential topics: Operationalizing vision to achieve Network priorities, key performance indicators, pivoting resources, mentorship/development
12:00-13:00	**Lunch**
13:00-14:00	**Opening, Priorities, and Vision** *Participants: EAC, DMID, Network members, USG, and select external stakeholders* ▪ Welcome (Chris & Nikos) ▪ Opening, CREID Role and Network Priorities (DMID) ▪ CREID Network Shared Vision (SC reps x 2)
14:00-14:15	**Break**
14:15-16:45	**Collaboration and Engagement** *Participants: EAC, DMID, Network members, USG, and select external stakeholders* ▪ Collaboration for Outbreak Research Response ▪ Cross-RC collaboration: Additional opportunities, benefits, and needs ▪ NIH Networks, USG, and External Collaborations ▪ Benefit sharing, preparing for downstream translation
16:45-17:00	**Day 1 Wrap up** (1 RC and/or 1 DMID rep)

DAY 2 | Thursday, September 23	
Time (ET)	Session
07:30-09:00	**Research Center meetings** *Participants: Research Center members*
08:30-10:40	**TableTop Exercise: Session II** *Participants: EAC, DMID, Network members*
10:40-11:00	**Break**
11:00-12:00	**CREID Year 2 Review Panels** 5 RCs x 10 min ea., Q&A/discussion x 10 min *Participants: EAC, DMID, Network members*
12:00-13:00	**Lunch**
13:00-14:00	**CREID Year 2 Review Panels (cont.)** 5 RCs x 10 min ea., Q&A/discussion x 10 min *Participants: EAC, DMID, Network members*
14:00-15:10	**Abstract-Driven Session** *Participants: EAC, DMID, Network members*
15:10-15:30	**Break**
15:30-16:40	**Abstract-Driven Session** *Participants: EAC, DMID, Network members*
16:40-17:00	**Day 2 Takeaways** (1 RC and/or 1 DMID rep) *Participants: EAC, DMID, Network members*

DAY 3 | Friday, September 23	
Time (ET)	Session
07:30-09:00	Research Center meetings *Participants: Research Center members*
08:30-10:40	TableTop Exercise: Session III *Participants: EAC, DMID, Network members*
10:40-11:00	Break
11:00-12:00	Pilot Awardees Presentations (in-person and virtual) *Participants: EAC, DMID, Network members*
12:00-13:00	Lunch
13:00-15:10	Abstract-Driven Session *Participants: EAC, DMID, Network members*
15:10-15:30	Break
15:30-16:00	Abstract-Driven Session *Participants: EAC, DMID, Network members*
16:00-17:00	DMID and EAC Wrap-Up *Participants: EAC, DMID, Network members*

EID-SEARCH
Emerging Infectious Diseases
South East Asia Research Collaboration Hub

CREID
Centers for Research in Emerging Infectious Diseases

Centers for Research in Emerging Infectious Diseases (CREID) Network

Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH)

2022 Report to the External Advisory Committee

August 19, October

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Guidance: 7-page limit for EAC Report
(excluding cover page and TOC)

Section 1. Executive Summary

The Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH) is a multidisciplinary center focused on analyzing zoonotic disease risk in a key EID hotspot region. Our aims are to 1) characterize diverse coronaviruses, paramyxoviruses, filoviruses, and other viral pathogens in wildlife; 2) assess the frequency and causes of their spillover; & 3) identify viral etiologies of undiagnosed 'cryptic' outbreaks in Thailand, Malaysia, and Singapore. Highlights of research and significant findings during the reporting period (October 2021 – August 2022) include:

To be updated with bullet points by Peter…

Section 2: Research Center Overview

A. Specific Aims

The overarching goal of the Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH) is to characterize the diversity of key viral pathogens in wildlife, assess the frequency and causes of their spillover, and identify viral etiologies of undiagnosed 'cryptic' outbreaks in people. The research goals of the EID-SEARCH follow three specific aims designed to advance our understanding of spillover and outbreak risk for novel viruses in a globally important EID hotspot, strengthen in-country research capacity, and enhance international collaboration:

- Specific Aim 1: Identify, characterize, and rank spillover risk of high zoonotic potential viruses from wildlife.
- Specific Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays.
- Specific Aim 3: Identify and characterize the viral etiology of 'cryptic' outbreaks in clinical cohorts.

B. Partners and Staffing

EID-SEARCH includes leaders in the field of emerging viral pathogens at key US institutions (EcoHealth Alliance PI Peter Daszak; University of North Carolina Co-I Ralph Baric; Uniformed Service University Co-Is Christopher Broder and Eric Laing), and in Thailand (Chulalongkorn University Co-Is Supaporn Wacharapluesadee and Opass Putcharoen), Singapore (Duke-NUS Medical School Co-I Linfa Wang), and the three major Malaysian administrative regions (Conservation Medicine Co-I Tom Hughes). These organizations represent the 'hub' of the center, with an additional, more informal collaborative network spanning >50 clinics, laboratories, and research institutes across almost all Southeast Asian countries. By enhancing and focusing research within the three hub countries, and coordinating communication among the network, we envision EID-SEARCH acting as an early warning system for outbreaks; a way to exchange information, reagents, samples, and technology; and a collaborative powerhouse for translational research. EID-SEARCH is supported by a multidisciplinary team of researchers at our partner institutions, with skills in epidemiology, clinical management, molecular biology, virology, wildlife biology, and advanced data and statistical analyses. The long collaborative history between the key personnel and partner institutions is a significant asset that can be deployed to support outbreak research response in the region.

C. Research Sites

The three core (hub) countries for the EID-SEARCH are Thailand, Malaysia, and Singapore, *expanded to Viet Nam and Cambodia through the CREID Pilot Research Program*. These stretch through one of the

most significant foci of EID risk globally. Additionally, we have assembled a greater network of EID-SEARCH partners that includes >50 clinics, laboratories, and research institutes across almost all Southeast Asian countries – stretching from Nepal to the Philippines. Our targeted field research includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sarawak, Sabah, Viet Nam, and Cambodia. The central location of our hub countries in mainland Southeast Asia allows us to sample representative wildlife species that occur in other countries across the greater biogeographic region, including India, Bangladesh, Myanmar, Southwest China, Laos, Cambodia, Philippines, and Indonesia.

Section 3: Research Center Progress and Accomplishments

Progress and Accomplishments

- Organized by Aim
- Focus on key accomplishments
- Address outbreak research response if applicable
- Address how the 2021 EAC recommendations have been incorporated (*see Appendix A for 2021 EAC report*)

A. Projects and Studies

- Have you reverted to your original (pre-pandemic) CREID research agenda in the last year? If not, how has your research agenda changed in the last year due to the ongoing impact of SARS-CoV-2?
- What additional changes to your CREID research agenda have taken place in the last year?

Aim 1: Identify, characterize, and rank spillover risk of high zoonotic potential viruses from wildlife

- **Field surveillance among wild bats, rodents, and non-human primates.** We have collected 14,302 specimens from 2,055 animals of 31 bat species, one non-human primate (NHP) species, and five rodent species from six sites at high-risk human-wildlife interfaces in Malaysia and Thailand. (Table 1)

	Thailand	Malaysia
No. of collected specimens	3,862	14,171
No. of individuals	454 bats, 106 rodents	2,000 bats
No. of species	6 (bats), 5 (rodents)	24 (bats)
No. of sites	5	4
No. of tested individuals	407	1,332
No. PCR tests for coronaviruses (CoVs), paramyxoviruses (PMVs), filoviruses (FLVs), influenza viruses (IVs)	4,070	9,228
Identified viruses	1 new CoV from 3 bats 3 known PMVs from 4 rodents 4 new PMVs from 11 bats	5 new CoVs from 112 bats 4 new PMVs from 10 bats 2 known PMVs from 2 bats
No. of specimen for multiplex immunoassay for FLVs and Henipavirus	-	720 bats

Table 1. EID-SEARCH viral surveillance in wildlife in Malaysia and Thailand.

- **Discovery of novel SARS-like beta-coronavirus in bats in Thailand**. The whole genomes revealed a 91.72% nucleotide similarity to SARS-CoV-2. Phylogenetic analysis suggested that this virus is closer to pangolin CoV-GD-1 than to SARS-CoV-2; however, the RBD is more similar to that of bat-CoV RaTG13, suggesting that this new virus may also bind to mammalian ACE2 receptors. Whole-genome sequencing of other new CoVs from Thailand and Malaysia is ongoing.
- **Genome-wide CRISPR knockout screen identifies essential factor for HKU2/SADS-CoV infection.** Using a genome-wide CRISPR knockout screen, we identified placenta-associated 8 protein (PLAC8) as

an essential host factor for SADS-CoV infection of human cells. Infection is prevented in PLAC8 knockouts and can be restored by transiently transfecting PLAC8 from multiple species, including human, pig, mouse, and bat. Trypsin treatment of SADS-CoV bypasses the PLAC8-dependent pathway and partially rescues SADS-CoV infection. PLAC8 is involved in both early entry and later dissemination of SADS-CoV infection and PLAC8 KO cells have: 1) delayed and reduced viral subgenomic RNA expression and 2) diminished cell-to-cell viral spreading of infected cells. *These results suggest that PLAC8 is a promising target for antiviral development for the potential pandemic SADS-CoV virus*.

- **Host range, transmissibility, and antigenicity of a pangolin coronavirus.** Using a synthetically derived infectious cDNA clone, we recovered the wildtype pangolin (Pg) CoV-GD and derivatives encoding indicator genes. Like SARS-CoV-2, PgCoV-GD virus efficiently uses human and many other mammalian ACE2 receptors for entry, shares a similarly broad host range *in vitro*, and displays altered sensitivity to host proteases. PgCoV replicates to slightly higher to lower titers in primary human cells derived from the proximal to distal lung, respectively, and with reduced fitness compared to SARS-CoV-2. PgCoV also replicates efficiently in hamsters and could transmit via the aerosol route, but at a lower frequency than SARS-CoV2. PgCoV is efficiently neutralized by COVID-19 patient sera and many commercially available SARS-CoV-2 therapeutic antibodies, but not by human antibodies targeting the spike N-terminal domain. A pan-Sarbecovirus antibody ADG-2 and SARS-CoV-2 S2P recombinant protein vaccine protected mice in a PgCoV replication model; however, efficient PgCoV replication in primary nasal airway epithelial cells and transmission in the hamster highlights the high emergence potential of this virus. We showed that cross-neutralizing antibodies in pre-immune SARS-CoV-2 humans and the benefit of current COVID-19 countermeasures should impede its ability to emerge and spread globally in humans. *This research helps build FDA-approved therapeutic antibodies against a range of CoVs with emergence potential and shows that FDA-approved SARS-CoV-2 vaccines also protect animals from PgCoV when challenged in vivo*.

Aims 2 & 3: Identify evidence and analyze risk factors for viral spillover in high-risk communities and clinical cohorts, and characterize viral etiology of 'cryptic' outbreaks

- **Community serology surveillance.** We initiated the human surveillance work in Thailand, with 56 participants enrolled at one at-risk community site, and 2 individuals from a clinic site, with a total of 58 participants enrolled. Serology testing of samples from 56 participants from the community site showed positive for PMVs, Henipavirus, and CoVs (Fig. 1)

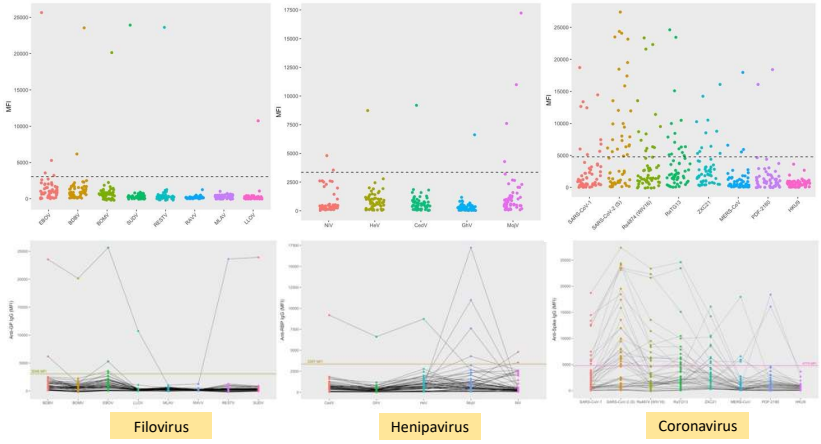

Fig. 1 Serology testing results for FLVs, Henipavirus, and CoVs among community participants in Thailand (n=56)

- **Design and application of multiplex surrogate virus neutralization test (sVNT) platform** that covers SARS-CoV-2 and variants of concern (Alpha, Beta, Gamma, Delta, Mu, Lambda, Omicron BA.1 and

BA.2), clade-2 sarbecoviruses (BANAL-52, BANAL-263, GD-1, RaTG13, GX-P5L), clade-1 sarbecoviruses (Rs2018B, LYRa11, WIV-1, RsSHC014, Rs4231), SARS-CoV-1, and *the newly discovered bat CoV we recently sequenced from Thailand.* This assay allows for the discrimination of past SARS-CoV-2 infection from that of other sarbecoviruses in human and animal samples, which will be critical for identifying cryptic spillover of sarbecoviruses in people. It also has the potential to aid in identifying the progenitor of SARS-CoV-2 in animals.

- **Designed and secured the expression of bat-CoV spike proteins for incorporation into our multiplex (multi-family) serological assay**. Specific bat-borne CoV spike proteins include bat SARSr-CoVs RaT13, ZXC21, bat MERSr-CoV PDF2180, and HKU9. These were incorporated into our 17-plex henipavirus/filovirus-focused serology assay to include SARS-CoV-2 and bat-borne CoV antigens. We also optimized an sVNT for Nipah virus and provided enough material to characterize Nipah virus IgG positive serum samples neutralizing antibodies. SOPs were revised and updated to reflect best practices that were optimized during Year 1.

Outbreak investigation and preparedness in Thailand

- The Ministry of Public Health in Thailand requested support from EID-SEARCH for a viral diarrhea outbreak investigation in Chanthaburi province. We received samples from both Prapokklao Hospital (regional hospital) and Khlong Khut Health-Promoting hospital collected from 59 healthcare workers and students who had diarrhea during Dec. 2021-Feb. 2022. PCR tests were conducted for six gastrointestinal viruses causing gastrointestinal tract infections including adenovirus, astrovirus, norovirus GI, norovirus GII, rotavirus, and sapovirus. 32 samples **(54.24%)** were positive for norovirus GII. Further genomic characterization of the detected norovirus GII will be performed, allowing for comparison with noroviruses found in other provinces in Thailand.
- In preparedness for Monkeypox outbreaks, EID-SEARCH's lab partner in Thailand coordinated with the Department of Medical Science, MOPH, National lab to establish the Monkeypox testing platform in April 2022 and detected the first Monkeypox case in Thailand in July 2022.

B. Dissemination of Results

- Summarize dissemination plan
- Include number of peer-reviewed publications; highlight those in high-impact journals
- Number and content of presentations on CREID research, including audience

EID-SEARCH members were invited to present the project work at government and inter-government briefings (> 10), conference and university lectures (>13), panels and webinars (>18), and public interviews (>6) in Thailand, Malaysia, the US, regionally and internationally on the trends in global disease emergence, zoonotic surveillance, pandemic control and prevention, and research results from this project research activities. Nine (9) peer-reviewed paper has been published or with preprint available from EID-SEARCH work, two (2) manuscripts are currently under revision, and three (3) manuscripts are in preparation for submission*, including papers on.. please highlight the high-impact ones.* We organized a workshop with 55 scientists and government officials from Thailand, Malaysia, Singapore, Bangladesh, Cambodia, Indonesia, Lao PDR, Myanmar, Nepal, Vietnam, USA, and Australia to present EID-SEARCH work, share knowledge, strengthen research capacity, and foster scientific collaborations in Southeast Asia.

C. Training and Capacity Building Activities

- Summarize capacity building plan
- Include number of training activities and number of participants for each training
- Address how capacity building activities were adapted due to COVID-19 and long-term changes as a result of the pandemic

During this reporting period, XX training sessions with a total of XX participants were conducted by EID-SEARCH partners in Thailand, Malaysia, and Vietnam for project members, local government partners, universities, research institutes, and NGOs. These training focused on biosafety (x training x participants by x), applying new technologies (x training x participants by x), and field and laboratory SOPs (x training x participants by x), to strengthen in-country research capacity and improve information sharing. Both in-person and online training was conducted to accommodate COVID conditions. As travel and COVID-19 restrictions have begun to be lifted, students, postdocs, and all EID-SEARCH research staff have been provided with professional development opportunities to travel to attend conferences, workshops, and other in-person training activities. *To be updated with numbers….*

D. Challenges and Proposed Solutions

- Bottlenecks to CREID-related research that might be like those that you could face when pivoting research activities in direct response to an EID outbreak. What solutions could be or have you put in place in advance to solve those challenges? How is being part of the CREID Network mitigating those or not?

The significant challenges we encountered during the reporting period were travel restrictions to our field partner countries and within countries, as well as a large number of hospitalized patients in local hospitals in Thailand and Malaysia due to COVID-19 that delayed human participant enrollment at targeted community and hospital sites and the subsequent laboratory analysis we anticipated in Year 2. To address these issues, we worked closely with in-country partners and stakeholders to continue assessing the situations (i.e., safety and research priorities of study sites) to ensure a rapid start of enrollment and focused on the refinement of assays to be ready for human surveillance. Meanwhile, to prevent a possible supply shortage of PPE and swabs as happened in early Year 2, we have identified supply sources to ensure we can procure the necessary consumables without significant delays. With travel restrictions in Southeast Asian countries now beginning to be lifted, international travel of US-based staff to our field sites has begun (April 2022).

E. Examples of Network Collaboration

- How has the work/research of your RC been affected by or involved collaboration with other members of the CREID Network?
- Examples: highlight joint publications, joint activities, sharing materials and data, benefits of Working Groups, etc.

Through the participation of different CREID Working Groups, EID-SEARCH members participated in the network's outbreak responses, capacity building, and cross-center collaboration by sharing existing local partnerships, techniques, and relevant resources.

- For outbreak responses, EID-SEARCH has been actively participating in the network's response to Monkeypox by providing local partnerships and monitoring platforms in DRC and Thailand.
- For lab assay sharing, EID-SEARCH partner Uniformed Services University is working with UWAEN to share the multiplex microsphere immunoassay (MMIA) with the regional partners for EBOV serology testing, and plan to work with the National Institute for Biological Standards and Control (NIBSC) to… Eric, please feel free to edit to add more info

- We are contacted by multiple Research Centers regarding the Field Biosafety Manual presented at the CREID Scientific Meeting by EID-SEARCH senior field veterinarian, the Manual and corresponding training resources will be made available to all CREID members once it's completed with peer review. Marc, please feel to edit…
- EID-SEARCH are also committed to providing our expertise on CCHF, Henipavirus, RVF related research by connecting CREID members with our research partners.

Section 4: Future Directions

- Plan for the next reporting period.
- How is your Research Center adapting to the April 2022 NIAID Program Priorities and how does this change (or not) your planned activities in the next year? *See Appendix B for NIAID Program Priorities*.
- Moving forward, what is the greatest opportunity your RC has to advance the knowledge base for emerging infectious diseases? What are the significant knowledge gaps that the RC can advance research on?

In the next reporting period, we will follow the research plans laid out in our proposal with the specific actions to **continue expanding our knowledge of emerging and re-emerging infectious diseases and better prepare to respond to outbreak/pandemic threats that align with the CREID network research priorities:**

Specific Aim 1: Identify, characterize, and rank spillover risk of high zoonotic potential viruses from wildlife.

- Wildlife (bats, rodents, and non-human primates) sampling at selected sites, with increased efforts for rodents and non-human primates and potential longitudinal sampling at a few selected sites to analyze patterns of viral shedding and spillover risk.
- Continue to develop predictive host-range models and spatial risk models to prioritize sampling sites and species in Years 3-5 of the project.
- Conduct viral family-level molecular screening of collected wildlife samples for coronaviruses, filoviruses, and paramyxoviruses.
- Perform serology testing on the wildlife samples or coronaviruses, filoviruses, and henipavirus.
- Continue developing a more comprehensive multiplex CoV RBD-based sVNT covering newly identified sarboviruses and additional merbecoviruses to allow the evaluation of neutralizing activity in serum samples against a much broader and more comprehensive range of RBDs.
- Conduct whole-genome and spike glycoprotein sequencing in countries with newly identified viruses.
- Continue research to further characterize a subset of novel viruses including virus isolation, human cell infection experiments, and animal modeling for viral pathogenesis studies, including pangolin GD strain, BANAL-52, and other significant viruses identified in Y1-2.
- Develop zoonotic risk analyses for relevant viruses, including the analysis of Spike and whole-genome sequences to predict host range using a variety of in silico approaches, model the host range, geographic distribution, extent of overlap with high-density human populations, etc. using existing apps/models to assess potential zoonotic disease risk

Specific Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays

- Increase the number of sites and enrolled participants for community surveillance among at-risk populations for biological and behavioral data collection in Thailand, Peninsular Malaysia, and Sabah Malaysia
- Continue to identify community sites concurrent with animal surveillance sites (Specific Aim 1).
- Perform serology testing of collected human samples with optimized multiplex assays for coronaviruses, filoviruses, and paramyxoviruses
- Begin epidemiological analyses of biological and behavioral data.

Specific Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts

- Start enrolling participants at selected hospital/clinic sites for syndromic surveillance at two sites in Thailand, and identified sites in Sabah, Malaysia and Peninsular Malaysia, Malaysia
- Continue to identify potential clinical cohorts and sites in the catchment of the community (Specific Aim 2) and animal surveillance (Specific Aim 1) sites.
- Start serological and molecular testing on collected human samples.
- Start viral characterization work if any novel viruses are identified.

Through the work under Specific Aims 2&3, we are engaging multiple disciplines of social science, anthropology, health policy, and behavioral science towards EID risk mitigation strategies on key human-animal interfaces, we will share relevant research methods (e.g., behavioral questionnaires) and best practices with the CREID Network members.

Training and capacity-building activities among in-country team members, especially young EIDs researchers, to strengthen domestic, regional, and international capacity and readiness to efficiently undertake the research required in response to emerging or re-emerging threats. Available training materials or resources will be made available to the CREID Network.

- Conduct annual field biosafety and animal surveillance training (bats, rodents, non-human primates) with lectures and discussion as well as hands-on guidance.
- Conduct training in enriched RNAseq protocols for coronavirus whole-genome sequencing.
- Conduct training in traditional viral whole-genome sequencing using primer walking for paramyxoviruses.
- Conduct virtual and in-person viral phylogenetic and metagenomics analysis training.
- Trial the use of the portable Oxford Nanopore MinION platform as an in-house sequencing solution in Malaysia.
- Continue training on multiplex microsphere immunoassay (MMIA) data analysis and interpretation.
- Conduct refresh training on human research regarding survey design, interview skills, and data analysis.
- Finalize the standard operating procedures (SOP) manual for emerging infectious disease research field biosafety for peer-review, publication, and broad distribution.
- Continue sharing research resources (protocols, SOPs, technologies) and results within the scientific community and local government in a timely manner.
- Organize meetings and workshops with scientists from Southeast Asia to identify common challenges and opportunities for emerging infectious disease research in the region (November 2022).
- Initiate exchange training for young researchers among EID-SEARCH partners for NGS and other laboratory technology transfers.
- Support in-country outbreak research and response.

Appendix A: 2021 EAC Report

Centers for Research in Emerging Infectious Diseases (CREID)
CREID External Advisory Committee (EAC) Summary
In follow up to the CREID Network Annual Meeting
November 3-5, 2021 (virtual meeting)

Prepared by Mark Feinberg, MD, PhD on behalf of the CREID EAC

Overview of EAC Perspectives and Recommendations

The EAC members were unanimous in their assessment that the CREID Network team made very impressive and positive progress in their first year of this very important and timely new NIAID/DMID initiative. While the amount of progress realized in starting up a new global collaborative research network would be highly laudable overall in any circumstance, the EAC members were even further impressed by the fact that the initiation of the CREID Network coincided with the emergence of the COVID-19 pandemic (with all of its attendant challenges). This coincidence provided a clear initial focus of many of the CREID efforts on SARS-CoV-2-related topics, but it also provided very strong validation for the original rationale for establishment of the CREID Network by the NIAID/DMID leadership as a means of fostering global networks for research collaboration on emerging infectious disease threats.

While the EAC members identified a number of areas where additional attention and effort will be beneficial to maximize the impact of the CREID Network, and key to fostering the sustainability of its efforts, our expectations for what the Network has the potential to accomplish were raised by the strong foundation that the CREID PIs and collaborators have already put in place in their first year. We were also very impressed with the clear commitment, thoughtfulness and inclusive approach of the CC leadership, and with the expertise, passion, and experience of the broader CREID Network community of scientists. At the heart of the CREID is a commitment to new models of collaborative science that links investigators from around the world to understand, prepare for, and respond to emerging infectious disease threats. As such, the extent to which CREID utilizes current best practices for collaborative research and pioneers new ones; embraces the essential role played by investigators working in countries at risk for disease emergence (including resource-limited settings); partners in new and more effective and mutually trusting ways with governments, normative agencies and other emerging infectious disease-focused research organizations; exemplifies and communicates the value of scientific research in providing solutions to emerging infectious disease threats to governments and the public; and delivers on its commitment to genuine research capacity strengthening and sustainability will be critical measures for evaluating the future success of the CREID. The EAC recognizes that this is a very ambitious agenda filled with often challenging objectives, and that the inherent nature of time-limited NIH funding mechanisms presents additional challenges to their potential realization.

In considering these issues, the EAC felt that significant progress has been made in the first year of the CREID Network, but that additional opportunities exist for the PIs and CREID investigators to be even more intentional and strategic about their collaborative research efforts. The CREID Network has the opportunity to establish a number of positive precedents in models of effective global collaborative science where the “whole is truly greater than the sum of its parts”; by demonstrating new approaches for the “decolonization” of international research; and by delivering on a strong commitment to diversity and inclusion in all of its endeavors. In many ways, achieving these goals will require innovation in collaborative research models within a large global network, and not just in delivering innovative, high quality scientific research results. The EAC members recognize the challenge of finding the right balance between investigator- and RC-initiated science with broader strategies and shared goals of a large collaborative network, but also note that doing so will require an overarching strategy, effective

communication tools and ongoing performance monitoring and adjustment. The EAC members also recognize that the important Network goals of research capacity strengthening and sustainability are not trivial undertakings and that they are very promising, but also rather novel objectives, within the context of NIH-supported research programs. These are additional important areas where the CREID CC and RC leadership can further articulate a respectful, inclusive, realistic, achievable and measurable strategy.

In all of these areas, the EAC recommends that the CREID investigators further develop and implement (a) a clear, well-defined and shared set of Network priorities (that links the work across RCs) and key performance indicators (KPIs) to measure performance toward realizing them, (b) a shared strategic vision and plan that connects the research efforts being prioritized and conducted across the Network centers, (c) a mechanism to share best practices across the Coordinating Center (CC) and Research Centers and (d) a proactive approach to identify collaborative research opportunities, fill gaps, avoid duplication and pursue synergies both within the CREID Network and, importantly, with the large and growing number of other organization working on preparing for and responding to emerging infectious disease outbreaks. Clarity and specificity in these efforts will be beneficial, as well as adopting an appropriate sense of realism about what can be accomplished by the CREID Network itself and where the new partnerships it implements with other organizations working in the emerging infectious disease arena will be essential. In this regard, given the complexity of the emerging infectious disease landscape, the CREID Network's ability to have the most significant impact will likely depend on the connections it establishes with other stakeholders and the innovation it drives in conceptualizing and delivering on effective new collaborative endeavors it develops with other capable partners. Towards this end, the Network would benefit from developing a thorough understanding of the ecosystem of potential partners and a defined approach to engage with the most relevant and promising ones. In addition, while the CREID Network includes collaborators from many centers and countries, its true global scope is limited by gaps in its geographic coverage (eg, countries in Asia, the Middle East, Northern Africa, Australia, etc...including those where risk of pathogen emergence are significant). Understanding if the CREID Network will be limited by its current geographic reach or plans to work to build additional connections (perhaps via strategic partnerships) in other regions will be beneficial from both practical and strategic levels.

Given that the readiness of the CREID Network for future outbreak response (and to be able to perform research in the midst of an outbreak when many challenges prevail) and for pandemic preparedness is a priority for NIAID/DMID, it will be important for the Network to clearly define how they best proactively plan to be able to promptly implement critical research programs when a new pathogen emerges or a previously known one reemerges. It will also be beneficial for the CREID CC and RCs to develop a clear plan (including protocols for coordination, implementation, data sharing and communication) to enable the Network to be able to respond expeditiously to an emerging infectious disease threat. Towards this end, tabletop exercises (or similar) to "pressure test" Network approaches and identify gaps/opportunities for improvement are worth considering.
The EAC is excited about what the CREID CC and RCs, and all of the collaborating CREID investigators, have accomplished in their first year of work and look forward to seeing the innovations and insights the Network will deliver in the future. The EAC recognizes that the CREID Network cannot realistically be "all things for all people", and should not strive to be. WE therefore also look forward to seeing how your Network priorities are further articulated, implemented and measured moving forward.

Additional EAC Impressions

The EAC felt that the Executive Summary of the CREID Network Annual Meeting provided an accurate overview of the meeting proceedings and of the EAC feedback provided at the end of the meeting proceedings. Some specific additional impressions/requests shared by EAC members include:

- Gaining a better understanding of how CREID investigators are interfacing with local governments and ministries, public health authorities (eg, WHO, US CDC, Africa CDC, etc), normative agencies will be helpful, and how learnings of effective approaches should be shared across the Network. In addition, optimization of engagements with other research organizations, enabling disciplines (eg, social science, health economics, health policy, ethics), as well as the medical countermeasures community, will be helpful
- Gaining a better understanding of the experience to date in data sharing efforts and how these can be optimized
- Gaining a better understanding of how bilateral sharing of research materials and specimens can be optimized (given the role of host institutions and government policies) and how these might operate during an outbreak/pandemic
- How the CREID investigators view and are approaching their educational efforts with governments, political leaders and the public, and sharing of best practices for engagement and trust-building
- How the CREID Network is engaging with industry partners that both supply enabling tools and technologies and who may also assist in the development and commercialization of CREID-advanced innovations (eg, diagnostics)
- Opportunities exist to demonstrate the CREID commitment to decolonization of global health (with increased attention to having the voices from LMIC-based investigators better balanced with those of US-based investigators)
- Opportunities exist to better model the Network's and the NIH's commitment to DEI objectives (with increased attention to full representation in all aspects of the work…including presentations, leadership groups, pilot research awards)
- Opportunities for enhancing epidemiology, bioinformatics, digital tools and modeling capacity
- Gaining a better understanding of the role of social and cultural aspects of the outbreak response and communication, and exploring innovative approaches to address them
- Understanding how engagement with governments and other local stakeholders is being pursued to advance capacity strengthening and sustainability goals
- Further articulation of rapid response collaboration frameworks
- Gaining a better understanding of how local priorities influence the prioritization decisions of the CC and RCs
- Gaining a better understanding/"needs assessment" of capacity gaps (eg, BSL-3 training and labs) and how these are being addressed via strategic connections or focused investment
- How "parachute or safari research" is avoided, and how local researchers are engaged as full partners/leaders
- Definition of opportunities to maximize speed and efficiency in research implementation efforts (eg, US permits and in-country regulations, IRB delays, key relationship gaps) needed for prompt outbreak responses
- Value of developing a clear vision and plan for sustainability that maximizes the connection with and contributions of other partners and local governments
- South-South collaboration should be applauded and fostered
- Shared EAC interest in seeing the CREID Network's progress in ongoing database and biorepository workstreams

Appendix B: NIAID CREID Network Program Priorities (April 2022)

(4 main priorities all carry equal weight and are intended to cover research and activities to prepare for and respond to emerging and re-emerging threats.)

- **Conduct innovative research to expand our knowledge of emerging and re-emerging infectious diseases and better prepare to respond to outbreak/pandemic threats**
    - Research Priorities:
        - Pathogen discovery & characterization
        - Pathogen/host surveillance
        - Pathogen transmission
        - Pathogenesis & immunologic responses in the host
        - Natural history & contemporary clinical disease
        - Develop reagents & diagnostic/detection assays
    - Engage in coordinated, Network wide outbreak/pandemic research readiness, differentiated from standard public health responses
- **Establish a collaborative, strategic, and preemptive research Network to ensure coordination of efforts across the Network**
    - Centralize communication
    - Conduct Network wide capacity inventory
    - Establish effective data harmonization and platforms for sharing
    - Harmonize sample collection, biorepositories, and network sharing/agreements
    - Share laboratory assay best practices and protocols
    - Share reagents, diagnostic/detection assays, and other resources
- **Develop and expand flexible domestic and international capacity and readiness to efficiently undertake research required in response to emerging or re-emerging threats**
    - Prioritize and engage with key stakeholders that will enhance network capabilities and integrate into the global health landscape
    - Strengthen and enhance research collaboration and partnerships globally
    - Pivot programmatic priorities and resources when needed for a timely response to emerging or re-emerging pathogen threats
        - Develop protocols for coordination, implementation, data sharing, and communication.
        - Execute tabletop exercises to test, refine, and maintain Network readiness
    - Formulate novel strategies to detect, control, and prevent outbreaks
        - Translate findings to downstream partners to facilitate development of medical countermeasures or preventive interventions
        - Develop adaptable risk assessment tools for evaluating a pathogen threat's potential for significant emergence and impact
- **Contribute to the development of the next generation of emerging/re-emerging infectious disease scientists and leaders**
    - Develop and maintain a pilot research program
    - Foster a diverse and inclusive research environment and program

From:	Sasiprapa Ninwattana on behalf of Sasiprapa Ninwattana <sasiprapa.n@outlook.com>
To:	Hongying Li
Cc:	Laing, Eric; Supaporn Wacharapluesadee; Kevin Olival; Cadhla Firth; Broder, Christopher; Opass ID; Peter Daszak; Aleksei Chmura
Subject:	Re: For you to edit and review by August 15 Monday_CREID EAC report
Date:	Monday, August 15, 2022 7:47:58 AM
Attachments:	2022 EAC Report_EID-SEARCH_draft v01-EDL-KR-SN_15Aug22.docx

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Dear Hongying,

Apologies for the delayed response.
Attached please find the edited report from our side. Here are some changes that we have made, further detail can be found in the report.

- Number of identified viruses
- Number of specimens for MMIA
- Serology testing graphs (I repeated some of the samples that were positive to confirm the results, most of the samples are still positive **except** the two samples that were weakly positive for NiV)
- Number of manuscripts

Please let me know if you require any further information/clarification.

Best regards,
Bow

Sasiprapa Ninwattana
Research coordinator,
Thai Red Cross Emerging Infectious Diseases Clinical Centre,
King Chulalongkorn Memorial Hospital
Faculty of Medicine, Chulalongkorn University
Rama4 road, Patumwan, Bangkok, Thailand 10330

> On 9 Aug BE 2565, at 01:20, Hongying Li <li@ecohealthalliance.org> wrote:
>
> Thank you so much, Eric, for your quick response!
>
> Sending a reminder that please review and edit the document **by August 15 next Monday.** Or if you don't have anything to add, please simply reply to approve the content for submission. Thank you very much!!
>
> Best regards,
> Hongying
>
> **Hongying Li, MPH**
> *Senior Program Manager & Research Scientist*
>
> EcoHealth Alliance
> 520 Eighth Avenue, Ste. 1200

New York, NY 10018

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EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.

On Tue, Aug 2, 2022 at 3:36 PM Laing, Eric <eric.laing@usuhs.edu> wrote:

> Hi Hongying,
>
> Some edits and suggestions are attached.
>
> - Eric
>
> Eric D. Laing, Ph.D.
> Assistant Professor
> Department of Microbiology and Immunology
> Uniformed Services University
> 4301 Jones Bridge Road
> Bethesda, MD 20814
> cell: (301) 980-8192
> office: (301) 295-3419
> lab: (301) 295-9618
>
> eric.laing@usuhs.edu
>
> On Tue, Aug 2, 2022 at 1:23 AM Hongying Li <li@ecohealthalliance.org> wrote:
>
> > Dear All,
> >
> > As mentioned at the EID-SEARCH meeting in July, we'll submit an External Advisory Committee (EAC) report for our Research Center in August, so the EAC can evaluate our work as an individual Research Center and the overall CREID Network to give feedback at the CREID annual meeting with DMID, USG, and other stakeholders in September.
> >
> > Attached please find the very first draft report for your edit and review. We'll greatly appreciate everyone's input on incorporating the EAC recommendations and aligning with NIAID CREID Network Program Priorities as described in the Appendix.
> >
> > For unpublished data, I tried to keep the information general, will also make sure the CREID keeps all information for internal use only (to the EAC), but please let me know if you have any concerns.
> >
> > We hope to submit the report on August 19, and your feedback **by August 15 Monday** will be appreciated! Thank you all very much in advance!

All the best,
Hongying

Hongying Li, MPH
Senior Program Manager & Research Scientist

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EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.

From: Hongying Li on behalf of Hongying Li <li@ecohealthalliance.org>
To: Spencer Sterling
Cc: Laing, Eric; Broder, Christopher; Peter Daszak; Kevin Olival; Cadhla Firth
Subject: Re: CREID 2022 Annual Meeting: Call for Abstracts
Date: Wednesday, May 4, 2022 9:51:09 AM

Thank you, Eric and Spencer, for the information!

Wonderful to hear all these results, we should encourage Bow to submit an abstract and present. I'll take note of this and remind you all to discuss it at the lab meeting next Wednesday.

- Hongying

On Tue, May 3, 2022 at 8:11 PM Spencer Sterling <spencer.sterling.ctr@usuhs.edu> wrote:

> Chu's guano farmer set has baseline (pre-vaccination, not sure if it's pre-pandemic) and two yearly follow up points. I think it's about 54 farmers, all have been vaccinated. I want to say they are mostly Sinovac but some are Astra, and I don't know if there were any cross-vaccinations.
>
> I recall Chu talking about a separate healthcare worker cohort at one point but I don't know if that is still on the table.
>
> -S
>
> > On May 3, 2022, at 7:55 PM, Laing, Eric <eric.laing@usuhs.edu> wrote:
> >
> > Hi Hongying,
> >
> > I can focus on the bat-cov multiplex development, but we only have SARS-CoV-2 validated sera and research studies outside the scope of EID-SEARCH. We'd need some data from Bow (who should be the lead), and it can be data related to guano farmers, pre-COVID-19, or vaccine recipients.
> >
> > There are 3 virus families being screened in the multiplex - there should be at least 2 stories (filo/henipa and sars-2/covid) coming from Bow/Chu's team a Chula. I've been meaning to email Chu about their covid-19 cohort, I have this memory that they have some vaccine recipients (Spencer, am I remembering this correctly?). My research team has been involved in Pfizer vaccine response research, and head-head comparison with Moderna. Thailand is mostly vaccinated with AZ and Sinovax, a product comparison would be pretty easy to write-up since the assay is a bridge.
> >
> > - Eric

Eric D. Laing, Ph.D.
Assistant Professor
Department of Microbiology and Immunology
Uniformed Services University
4301 Jones Bridge Road
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eric.laing@usuhs.edu

On Mon, May 2, 2022 at 9:52 PM Hongying Li <li@ecohealthalliance.org> wrote:

> Thank you, Eric.
>
> Assay development related to EID-SEARCH may fit into the topic of "New techniques, or novel approaches, to sampling, detection, and characterization of pathogen risk" if you would like to present the methodology, but understand it's better to present with sample testing results from the project.
>
> As you suggested, we have shared this with the teams at CM and Chulalongkorn, hope to hear from them soon and work together with you on the abstracts if they're interested to present some preliminary findings from the serological testing. We may check in with Tom about this next Wednesday at the lab meeting (if you and Cadhla agree).
>
> Thanks,
> Hongying
>
> On Mon, May 2, 2022 at 9:09 PM Laing, Eric <eric.laing@usuhs.edu> wrote:
>
> > Hi Hongying,
> >
> > Serology testing is underway in Chulalongkorn, but I think that Chu's lead, Bow, would be the appropriate SEARCH researcher to draft an abstract. Spencer mentioned that at the previous month's 'lab meeting', Tom had concluded testing some serologies.
> >
> > Spencer and I are happy to help with results analysis and drafting abstracts from either Chulalongkorn or CM, LLC/EHA, but we don't have access to any data from our side.
> >
> > - Eric
> >
> > Eric D. Laing, Ph.D.
> > Assistant Professor
> > Department of Microbiology and Immunology
> > Uniformed Services University
> > 4301 Jones Bridge Road

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On Mon, May 2, 2022 at 9:54 AM Hongying Li <li@ecohealthalliance.org> wrote:

> Good Morning, Chris, Eric, and Spencer,
>
> The CREID Network is holding the annual meeting on September 21-23 in Washington DC, there will be sessions to present scientific findings supported by the CREID, and we need to submit abstracts for the presentations.
>
> Please see the Call for Abstraction information below, it would be great if anyone from your team can submit an abstract for presentation, if you are busy, it will be a terrific opportunity for graduate students or postdocs, too. Please consider this and feel free to contact me if any questions.
>
> The meeting will be hybrid, and we'll let you know once we received the final agenda, so we can discuss travel and meeting in person if you will be available during that time.
>
> Best,
> Hongying
> ---------- Forwarded message ---------
> From: **Macoubray, Aaron** <amacoubray@rti.org>
> Date: Wed, Apr 27, 2022 at 3:12 PM
> Subject: CREID 2022 Annual Meeting: Call for Abstracts
> To: amy.aegypti <amy.aegypti@gmail.com>, Anavaj SAKUNTABHAI <anavaj.sakuntabhai@pasteur.fr>, Christine Kreuder Johnson <ckjohnson@ucdavis.edu>, Christopher M Barker <cmbarker@ucdavis.edu>, Eva HARRIS <eharris@berkeley.edu>, Michael J Gale <mgale@uw.edu>, colomaj <colomaj@berkeley.edu>, Kathryn Hanley <khanley@nmsu.edu>, Kristian G. Andersen <kga1978@gmail.com>, Lark L A Coffey <lcoffey@ucdavis.edu>, Njenga, M. Kariuki <mkariuki.njenga@wsu.edu>, Pardis Sabeti <pardis@broadinstitute.org>, Peter Daszak <daszak@ecohealthalliance.org>, Peter Rabinowitz <peterr7@uw.edu>, Garry, Robert F <rfgarry@tulane.edu>, Tierra Smiley Evans <tsmevans@ucdavis.edu>, WVanVoorhis <WVanVoorhis@medicine.washington.edu>, Vasilakis, Nikolaos <nivasila@utmb.edu>, Wang, David <davewang@wustl.edu>, Judith N Wasserheit <jwasserh@uw.edu>, Weaver, Scott <sweaver@utmb.edu>
> Cc: Aleksei Chmura <chmura@ecohealthalliance.org>, Lynn K. Barrett <lynnbob@uw.edu>, Claudia Sanchez San Martin <csanchezsanmartin@berkeley.edu>, Dawa, Jeanette

<jeanette.dawa@wsu.edu>, Hongying Li <li@ecohealthalliance.org>, Michelle McGraw <mmcgraw@scripps.edu>, Nicole R Gardner <nrgardner@ucdavis.edu>, Paredes, Anne <anneparedes@wustl.edu>, Kelly PRIFTI <kelly.prifti@pasteur.fr>, Weldon, Caroline <caweldon@utmb.edu>, Van Vliet, Gretchen <gvanvliet@rti.org>, Vandergrift, Nathan <nvandergrift@rti.org>, Gregory Sempowski <greg.sempowski@duke.edu>, Wagner, Danielle <wagner@rti.org>, Hilary Bouton-Verville <hilary.bouton-verville@duke.edu>, Heather Lynch, Ph.D. <heather.lynch@duke.edu>, Reithinger, Richard <reithinger@rti.org>, Hemingway-Foday, Jennifer <hemingway@rti.org>, Beaubien, Candice (NIH/NIAID) [E] <candice.beaubien@nih.gov>, Dyall, Julie (NIH/NIAID) [E] <dyallj@niaid.nih.gov>, Florese, Ruth (NIH/NIAID) [E] <ruth.florese@nih.gov>, Patterson, Jean (NIH/NIAID) [E] <jean.patterson@nih.gov>, Woodson, Sara (NIH/NIAID) [E] <sara.woodson@nih.gov>, Batsuli, Nefer <nbatsuli@rti.org>, Megan Averill <megan.averill@duke.edu>

Dear CREID Network Leadership,

Thanks to your feedback, we will not be placing any limits on the number of allowed abstract submissions. Any submissions not selected for a rapid, virtual poster presentations or longer oral presentations will have the opportunity to create e-posters/slides to be shared with the CREID Network.

Below, please find the updated call for abstracts for the CREID Network's 2022 Annual Meeting. The meeting will include multiple abstract-driven scientific sessions. Each CREID Research Center is requested to submit at least one abstract by May 17, per the guidelines below.

Thank you,

CREID Network 2022 Annual Meeting Organizing Committee

2022 Annual Meeting: Call for Abstracts

The CREID Network 2022 Annual Meeting (September 21-23, in person and online) will include multiple abstract-driven scientific sessions. Each CREID Research Center is requested to submit at least one abstract, per the guidelines below. Abstracts will be assigned for rapid/lightning talks, longer oral presentations, or e-posters/slides that will be shared with the CREID Network. A committee will convene to review the content of the abstracts and determine the assigned format. More information on the review committee process is forthcoming. Submission template is attached.

Due	May 17, 2022
Length	Abstract body (excluding title and authors): 250 words max
Focus	Abstracts should focus on work supported by CREID, including but not limited to: ▪ Innovative research on pathogen discovery and characterization, pathogen/host surveillance, transmission, pathogenesis, host immunological response, natural history, or related assays or reagents; ▪ New techniques, or novel approaches, to sampling, detection, and characterization of pathogen risk; ▪ Preparation for, or results of, research conducted in response to an outbreak; ▪ Novel strategies to translate related findings downstream Selected abstracts will be grouped into sessions based on content/focus.
Format	As the 2022 Meeting will be hybrid, both in-person and virtual presentations are welcome. Abstracts may be selected for a rapid, virtual poster presentation, a full (>10 min) oral presentation, or for e-posters/slides to be shared with the CREID Network. Assigned format and presentation times will be determined based on the content and number of submissions.
Authors/ Presenters	Authors may include investigators and staff from CREID Research Centers, sites, and partners. Women and those from low- and middle-income countries (LMICs) are strongly encouraged to author and present.

	Submissions with authors from multiple Research Centers are welcome.
Submissions	Each Research Center is asked to submit 1-4 abstracts. Each abstracts should include a title, presenters/authors (names, institutional affiliations, emails, and CREID Research Center), and body (250 words max). See basic template, attached. Send to info@creid-network.org by May 17, 2022.

Aaron Macoubray

RTI International

Public Health Analyst

Global Public Health Impact Center

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A. COVER PAGE

Project Title: Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia	
Grant Number: 5U01AI151797-03	**Project/Grant Period:** 06/17/2020 - 05/31/2025
Reporting Period: 06/01/2021 - 05/31/2022	**Requested Budget Period:** 06/01/2022 - 05/31/2023
Report Term Frequency: Annual	**Date Submitted:** 04/01/2022
Program Director/Principal Investigator Information: PETER DASZAK , PHD BS **Phone Number:** 212 380 4460 **Email:** daszak@ecohealthalliance.org	**Recipient Organization:** ECOHEALTH ALLIANCE, INC. ECOHEALTH ALLIANCE, INC. 520 EIGHTH AVENUE NEW YORK, NY 100181620 **DUNS:** 077090066 **EIN:** 1311726494A1 **RECIPIENT ID:**
Change of Contact PD/PI: NA	
Administrative Official: ALEKSEI CHMURA 460 W 34th St., 17th Floor New York, NY 10001 **Phone number:** 1.212.380.4473 **Email:** chmura@ecohealthalliance.org	**Signing Official:** ALEKSEI CHMURA 460 W 34th St., 17th Floor New York, NY 10001 **Phone number:** 1.212.380.4473 **Email:** chmura@ecohealthalliance.org
Human Subjects: Yes **HS Exempt:** NA **Exemption Number:** **Phase III Clinical Trial:** NA	**Vertebrate Animals:** Yes
hESC: No	**Inventions/Patents:** No

B. ACCOMPLISHMENTS

B.1 WHAT ARE THE MAJOR GOALS OF THE PROJECT?

Southeast Asia is one of the world's highest-risk EID hotspots, and the origin of the SARS pandemic, repeated outbreaks of novel influenza strains, and the spillover of dangerous viral pathogens such as the Nipah virus. It is a wildlife 'megadiversity' region, where a rapidly expanding human population is increasing contact with wildlife, and increasing the risk of zoonotic disease outbreaks. The overarching goal of this proposal is to launch the Emerging Infectious Diseases - South East Asia Research Collaboration Hub (EID-SEARCH) to analyze the diversity of key viral pathogens in wildlife, the frequency and causes of their spillover, and to identify viral etiologies of undiagnosed 'cryptic' outbreaks in people. EID-SEARCH includes leaders in the field of emerging viral pathogens at key US institutions, and in Thailand, Singapore, and the 3 major Malaysian administrative regions, whose collaborative networks span >50 clinics, laboratories, and research institutes across almost all SE Asian countries. This hub, and the network, will act as an early warning system for outbreaks - a way to exchange information, reagents, samples, and technology, and a collaborative power-house for translational research. The long-term collaboration among the key personnel and multidisciplinary skillsets from epidemiology, clinical management, lab analysis, through wildlife biology and data analysis will act as significant assets when deployed to help counter outbreaks in the region. The research goals of this EIDRC follow three specific aims:

Specific Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife. We will: 1) analyze some of the tens of thousands of archived wildlife samples at our disposal, conduct geographically- and taxonomically-targeted field surveillance in wild mammals (bats, rodents, primates), and use serological & PCR assays to identify known high-profile zoonotic pathogens, or close relatives with potential to infect people; 2) biologically characterize novel viruses that our analyses suggest have high spillover and pandemic potential; and 3) conduct in vitro receptor binding assays and cell culture experiments, and in vivo animal model infections using humanized mice and the collaborative cross mouse to assess their potential to infect people and cause disease.

Specific Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays. Assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities and approaches that can deal with the low statistical probability of identifying rare events. To achieve this, we will 1) conduct targeted cross-sectional serological surveys of human communities with extremely high geographic and cultural, occupational, and behavioral exposure to wildlife-origin viruses; 2) design and deploy novel serological assays to identify baseline spillover of known or novel CoVs, PMVs, and FVs in these populations; and 3) analyze and test hypotheses on the occupational, cultural and other risk factors for spillover (e.g. hunting wildlife).

Specific Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts. Our prior work provides substantial evidence of spillover leading to undiagnosed illness in people in the region. To test if these represent 'cryptic' outbreaks of novel viruses, we will conduct syndromic surveillance at regional clinics for the communities sampled in SA2. We will: 1) enroll and collect biological samples, and detailed survey data on risk factors, from patients presenting with influenza-like illness, severe respiratory illness, encephalitis, and other specific symptoms; 2) conduct molecular and follow-up serological diagnostic assays to test causal links between their syndromes and known and novel viral agents identified in SA1. Where viruses are identified, we will attempt to isolate and characterize them, then use the survey data, ecological and phylogenetic analyses to identify likely reservoir hosts/spillover pathways and inform intervention programs.

This research will advance our understanding of the risk of novel viral emergence in a uniquely important region. It will strengthen in-country research capacity by linking local infectious disease scientists with an international collaborative network that has proven capacity to conduct this work and produce significant findings. These include testing of tens of thousands of samples from wildlife, humans, and livestock in the region; discovery of hundreds of novel viruses from zoonotic viral families in wildlife; outbreak investigations in rural communities across SE Asia; discovery of the bat-origin of SARSr-CoVs; discovery of a novel bat-origin SADS-CoV killing >25,000 pigs in S. China; and development of novel serological and molecular assays for high-impact viruses, and state-of-the-art in vitro and in vivo assays to characterize viral pathogenic potential. This body of collaborative research provides proof-of-concept that EID-SEARCH has the background, collaborative network, experience, and skillset to act as a unique early warning system for novel EIDs of any etiology threatening to emerge in this hottest of the EID

hotspots.

B.1.a Have the major goals changed since the initial competing award or previous report?

No

B.2 WHAT WAS ACCOMPLISHED UNDER THESE GOALS?

File Uploaded : B.2 Accomplishments_Final.pdf

B.3 COMPETITIVE REVISIONS/ADMINISTRATIVE SUPPLEMENTS

For this reporting period, is there one or more Revision/Supplement associated with this award for which reporting is required?

No

B.4 WHAT OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT HAS THE PROJECT PROVIDED?

File Uploaded : B.4 Training and Professional Development_Final.pdf

B.5 HOW HAVE THE RESULTS BEEN DISSEMINATED TO COMMUNITIES OF INTEREST?

PI Daszak, and Co-investigators Wacharapluesadee, Hughes, Olival, Wang, Baric, Broder, Laing, and other key personnel from the EID-SEARCH were invited to give lectures, keynote speeches, government and inter-government briefings, and public interviews in Thailand, Malaysia, US, Southeast Asia, and internationally on the trends in global disease emergence, zoonotic surveillance, pandemic control and prevention, and research results from this project research activities.

Government and inter-government agency briefings
• Advising US GAO on risks and benefits of viral characterization
• Advising US GAO on drivers of emerging infectious diseases
• Meeting with the Bureau of Epidemiology, Department of Disease Control of Thailand on COVID detection and outbreak investigation.
• Meeting with the Sabah Wildlife Department (SWD) in Malaysia for project briefing and ongoing collaboration.
• Meeting with the Deputy Director General at Ministry of Health (MOH) in Malaysia for project briefing and human research planning
• Meeting with the new US Ambassador in Malaysia to discuss project and relevant research in Malaysia.
• Report to the World Health Organization (WHO) on how to utilize this moment to improve genomic sequencing for CoVs worldwide.
• Report to Newham City Council in the UK on the emergence of new zoonotic diseases to advise on new policies set forth in the wake of COVID-19.
• Meeting with the Wildlife Health Genetic and Forensic Laboratory and Economic Officer of U.S. Embassy in Malaysia for project briefing and planning.
• Meeting with the Wildlife Health Genetic and Forensic Laboratory and Director of Sabah State Health Department and Director of Kota Kinabalu Public Health Lab for ongoing collaboration and syndromic surveillance in Malaysia.

Panels, workshop, webinars discussion
• Organized a regional workshop with scientists from Thailand, Malaysia, Singapore, Bangladesh, Cambodia, Indonesia, Lao PDR, Myanmar, Nepal, Vietnam, USA, Australia on the challenges and opportunities for emerging infectious diseases research in Southeast Asia.

• Wildlife Crime Panel at the Wilson Center, "Emerging zoonoses and the wildlife trade"
• The Association of Medical Technologist of Thailand, "COVID-19 variants"
• Australasian Society for Infectious Diseases Zoonoses, "Lessons for the next zoonotic pandemic"
• PREZODE International scientific workshop, "Introduction of the CREID Network and EID-SEARCH"
• The Science and Practice of Networks at the National Academy of Sciences
• Global Research and Innovation Forum, WHO, "Increasing risks at the human-animal interface: Research needs for risk reduction and prevention"
• World Zoonoses Day webinar by UPM, MyoHUN and USAID, Malaysia

Conference and university lectures
• Thailand One Health University Network International - Short Course on Ecosystem Health, "One Health Social Innovations for EIDs research"
• Global Bat Network of Networks (GBatNet), "Scientific priority setting for bat disease surveillance and research"
• Chulalongkorn University, "All about COVID-19 detection, multidisciplinary team"
• American Society of Tropical Medicine and Hygiene's annual meeting, "Surveillance for zoonotic coronavirus discovery"
• University of Rochester to the Goergen Institute, "How can we analyze pandemics to predict and prevent the next one"
• Duke-NUS Emerging Infectious Diseases (EID) Programme Seminar, "SARS-related CoVs provide a unique test-case of a global strategy to predict & prevent pandemics"
• Mahidol University, "One Health for emerging infectious diseases"
• CU-TU-BUU-BSRU, Burapha University (Thailand), "Roles of medical technologist in Disease X"
• Global Health Program, University of Geneva, "Understand zoonotic risk at human-wildlife interaction"
• Ecology and Evolution of Infectious Diseases Conference, "A strategy to assess spillover risk of bat SARS-related coronaviruses in Southeast Asia"
• American Public Health Association annual meeting, "Behavioral-biological surveillance of emerging infectious diseases among a dynamic cohort in Thailand"
• Columbia University, "Understanding zoonotic spillover and risk"

Public outreach and interviews
• Interview with Dr. Sanjay Gupta for an upcoming documentary on COVID-19 origins on the risk of viruses emerging from wildlife and how we can prepare for the next pandemic through modeling and surveillance to address hotspots for disease
• Podcast with Viertausendhertz on the strategy to assess spillover risk of bat SARS-related coronaviruses in Southeast Asia https://viertausendhertz.de/pan31/
• Closest known relatives of virus behind COVID-19 found in Laos https://www.nature.com/articles/d41586-021-02596-2
• Bats carrying coronaviruses closely related to the one that causes Covid-19 found outside China https://www.straitstimes.com/singapore/health/bats-carrying-coronaviruses-closely-related-to-virus-which-causes-covid-19-found
• Study finds coronavirus-related bat viruses in Thailand https://www.modernhealthcare.com/safety-quality/study-finds-coronavirus-related-bat-viruses-thailand
• Bat coronaviruses may infect up to 400,000 people in China and Southeast Asia every year https://www.telegraph.co.uk/global-health/science-and-disease/bat-coronaviruses-may-infect-400000-people-china-southeast-asia/

B.6 WHAT DO YOU PLAN TO DO DURING THE NEXT REPORTING PERIOD TO ACCOMPLISH THE GOALS?

In the next reporting period, we will follow the research plans laid out in our proposal with the specific actions as follows (no modifications of the scope of work):

Specific Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife
1.1 Wildlife (bats, rodents, non-human primates) sampling at selected sites.
1.1.1 First 6 months, testing and analysis of all samples; refine sampling strategies to target under-sampled species; 2nd 6 months, targeted sampling.
1.1.2 Increase sampling efforts for rodents and non-human primates.

1.1.3 Conduct longitudinal sampling at few selected sites to analyze patterns of viral shedding and spillover risk.
1.2 Continue to develop predictive host-range models and spatial risk models to prioritize sampling sites and species in Years 3-5 of the project.
1.3 Conduct viral family-level molecular screening of collected wildlife samples for coronaviruses, filoviruses, and paramyxoviruses.
1.4 Perform serology testing on the wildlife samples collected in Y1-3.
1.4.1 Perform serological characterization of Sarbecovirus-positive samples using surrogate virus neutralization test (sVNT) RBD-based assay.
1.4.2 Perform additional serological characterization of Henipavirus-positive samples using sVNT glycoprotein-based assay.
1.4.3 Continue developing a more comprehensive multiplex CoV RBD-based sVNT covering newly identified sarboviruses and additional merbecoviruses to allow the evaluation of neutralizing activity in
serum samples against a much broader and more comprehensive range of RBDs.
1.4.4 Validate, and initiate in-country use of Filovirus sVNT glycoprotein RBD assay.
1.5 Conduct whole-genome and spike glycoprotein sequencing in countries with newly identified viruses.
1.6 Continue research to further characterize a subset of novel viruses including virus isolation, human cell infection experiments, and animal modeling for viral pathogenesis studies.
1.6.1 Rescue additional pangolin viruses that are genetically more distant from SARS-CoV-2 and the pangolin GD strain, as well as synthetically reconstruct and characterize the BANAL-52 isolate using in
vitro and in vivo approaches.
1.6.2 Review novel bat sarbecovirus full genomes that have been identified in Y1-2 for synthetic recovery.
1.7 Develop zoonotic risk analyses for relevant viruses
1.7.1 Analyze Spike and whole-genome sequences to predict host range using a variety of in silico approaches
1.7.2 Model the host range, geographic distribution, extent of overlap with high-density human populations, etc. using existing apps/models to assess potential zoonotic disease risk
1.7.3 Improve data workflow to integrate viral characterization results into zoonotic virus prediction models.

Specific Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays
2.1 Increase the number of sites and enrolled participants for community surveillance among at-risk populations collect biological and behavioral data collection.
2.1.1 Enroll human participants from a site in Thailand where Henipavirus serology positives were identified among bats
2.1.2 Continue enrolling human participants from bat guano collection site(s) in Thailand.
2.1.3 Enroll human participants from high wildlife exposure communities, Peninsular Malaysia.
2.1.4 Enroll human participants from high wildlife exposure communities, Kinabatangan, Sabah, Malaysia
2.2 Continue to identify community sites concurrent with animal surveillance sites (Specific Aim 1).
2.3 Perform serology testing of collected human samples with optimized multiplex assays for coronaviruses, filoviruses, and paramyxoviruses (1.3.1 - 1.3.4).
2.3.1 Perform additional serological characterization using virus family-specific svNTs for positive samples
2.4 Begin epidemiological analyses of biological and behavioral data.

Specific Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts
3.1 Start enrolling participants at selected hospital/clinic sites for syndromic surveillance.
3.1.1 Continue enrolling human participants at King Chulalongkorn Memorial Hospital, Thailand
3.1.2 Enroll human participants in Sabah, Malaysia
3.1.3 Enroll human participants in Peninsular Malaysia, Malaysia
3.2 Continue to identify potential clinical cohorts and sites in the catchment of the community (Specific Aim 2) and animal surveillance (Specific Aim 1) sites.
3.3 Start serological and molecular testing on collected human samples.
3.4 Start viral characterization work if any novel viruses are identified.

Training and capacity building activities to achieve project goals
• Conduct annual field biosafety and animal surveillance training (bats, rodents, non-human primates) with lectures and

discussion as well as hands-on guidance. In situ training for in-country teams is planned in 2022 to accommodate the ease of COVID-19 travel restrictions. (1.1)
• Conduct training in enriched RNAseq protocols for coronavirus whole-genome sequencing (1.5)
• Conduct training in traditional viral whole-genome sequencing using primer walking for paramyxoviruses (1.5)
• Conduct virtual and in-person viral phylogenetic and metagenomics analysis training (1.5)
• Trial the use of the portable Oxford Nanopore MinION platform as an in-house sequencing solution in Malaysia (1.5)
• Continue training on multiplex microsphere immunoassay (MMIA) data analysis and interpretation (1.3)
• Conduct refresh training on human research regarding survey design, interview skills, and data analysis (2.1, 2.3, 3.1)

Additional activities
• Finalize the standard operating procedures (SOP) manual for emerging infectious disease research field biosafety for peer-review, publication, and broad distribution.
• Continue sharing research resources (protocols, SOPs, technologies) and results within the scientific community and local government in a timely manner.
• Organize meetings and workshops with scientists from Southeast Asia to identify common challenges and opportunities for emerging infectious disease research in the region.
• Continue supporting in-country outbreak research and response as requested.

The Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH) is a multidisciplinary research center with aims to: 1) characterize diverse coronaviruses, paramyxoviruses, filoviruses, and other viral pathogens in wildlife; 2) assess the frequency and causes of their spillover; & 3) identify viral etiologies of undiagnosed 'cryptic' outbreaks. Despite COVID-19 lockdowns and restrictions, we have fulfilled our project goals for the period of 06/01/2021 – 05/31/2022, with the following highlighted accomplishments:

Targeted Surveillance

	Thailand	**Malaysia**
Animal		
No. of collected specimens	3,862	14,171
No. of individuals	454 bats, 106 rodents	2,000 bats
No. of species	6 (bats), 5 (rodents)	24 (bats)
No. of sites	5	4
No. of tested individuals	407	1,332
No. PCR tests for coronaviruses (CoVs), paramyxoviruses (PMVs), filoviruses (FLVs), influenza viruses (IVs)	4,070	9,228
Identified viruses	1 new CoV from 3 bats; 3 known PMVs from 4 rodents; 4 new PMVs from 11 bats	5 new CoVs from 112 bats; 4 new PMVs from 10 bats; 2 known PMVs from 2 bats
No. of specimen for multiplex immunoassay for FLVs and henipavirus	-	720 (bats)
Human		
No. of enrolled human participants	56 community; 2 clinic; 59 outbreak	-
No. of site	1(community), 1(clinic)	-

- Human surveillance began during this reporting period in Thailand, but overall was delayed due to COVID-19 travel restrictions and safety concerns at community sites, and limited human resources at hospital sites. Testing of previously collected human specimens is ongoing.

Viral Characterization

- Analysis of one complete and two near-complete whole genome sequences of a novel SARS-like betacoronavirus identified in three bat specimens collected in Thailand revealed a 91.72% nucleotide similarity to SARS-CoV-2. Phylogenetic analysis suggested that this virus is closer to pangolin CoV-GD-1 than to SARS-CoV-2; however, the RBD is more similar to that of bat-CoV RaTG13, suggesting that this new virus may also bind to mammalian ACE2 receptors. Whole-genome sequencing of other new CoVs is ongoing. (Fig. 1)

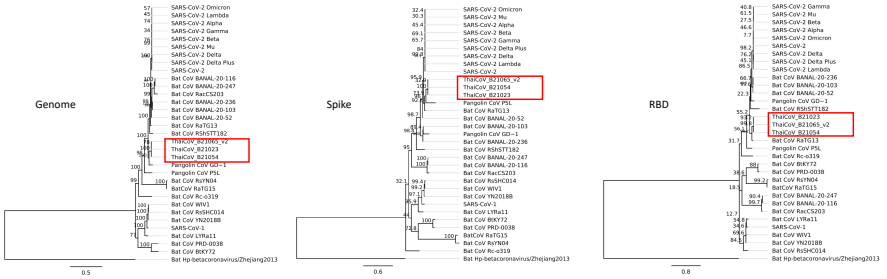

Figure 1. Close similarity of a newly discovered bat SARSr-CoV from Thailand to SARS-CoV-2 and relatives, including RaTG13.

- **Genome-wide CRISPR knockout screen identifies essential factor for HKU2/SADS-CoV infection.** Using a genome-wide CRISPR knockout screen, we identified placenta-associated 8 protein (PLAC8) as an essential host factor for SADS-CoV infection of human cells. Infection is prevented in PLAC8 knockouts and can be restored by transiently transfecting PLAC8 from multiple species, including human, pig, mouse, and bat. Trypsin treatment of SADS-CoV bypasses the PLAC8 dependent pathway and partially rescues SADS-CoV infection. PLAC8 is involved in both early entry and later dissemination of SADS-CoV infection and PLAC8 KO cells have: 1) delayed and reduced viral subgenomic RNA expression and 2) diminished cell-to-cell viral spreading of infected cells. These results suggest that PLAC8 **is a promising target for antiviral development for the potential pandemic SADS-CoV virus.**
- **Host Range, Transmissibility and Antigenicity of a Pangolin Coronavirus.** Using a synthetically derived infectious cDNA clone, we recovered the wildtype pangolin (Pg) CoV-GD and derivatives encoding

indicator genes. Like SARS-CoV-2, PgCoV-GD virus efficiently uses human and many other mammalian ACE2 receptors for entry, shares a similarly broad host range *in vitro*, and displays altered sensitivity to host proteases. PgCoV replicates to slightly higher to lower titers in primary human cells derived from the proximal to distal lung, respectively, and with reduced fitness compared to SARS-CoV-2. PgCoV also replicates efficiently in hamsters and could transmit via the aerosol route, but at a lower frequency than SARS-CoV2. PgCoV is efficiently neutralized by COVID-19 patient sera and many commercially available SARS-CoV-2 therapeutic antibodies, but not by human antibodies targeting the spike N-terminal domain. A pan-Sarbecovirus antibody ADG-2 and SARS-CoV-2 S2P recombinant protein vaccine protected mice in a PgCoV replication model; however, efficient PgCoV replication in primary nasal airway epithelial cells and transmission in the hamster highlights the high emergence potential of this virus. We showed that cross-neutralizing antibodies in pre-immune SARS-CoV-2 humans and the benefit of current COVID-19 countermeasures should impede its ability to emerge and spread globally in humans. **This research helps build FDA-approved therapeutic antibodies against a range of CoVs with emergence potential, and shows that FDA-approved SARS-CoV-2 vaccines also protect animals from PgCoV when challenged *in vivo*.**

Assay Development

- We established a **multiplex surrogate virus neutralization test (sVNT) platform** that covers SARS-CoV-2 and variants of concern (Alpha, Beta, Gamma, Delta, Mu, Lambda, Omicron BA.1 and BA.2), clade-2 sarbecoviruses (BANAL-52, BANAL-263, GD-1, RaTG13, GX-P5L), clade-1 sarbecoviruses (Rs2018B, LYRa11, WIV-1, RsSHC014, Rs4231), SARS-CoV-1, and *the newly discovered bat CoV we recently sequenced from Thailand.* This assay allows for the discrimination of past SARS-CoV-2 infection from that of other sarbecoviruses in human and animal samples, which will be critical for identifying cryptic spillover of sarbecoviruses in people. It also has potential to aid in identifying the progenitor of SARS-CoV-2 in animals.
- We designed and secured the expression of **bat-CoV spike proteins for incorporation into our multiplex (multi-family) serological assay**. Specific bat-borne CoV spike proteins include bat SARSr-CoVs RaT13, ZXC21, bat MERSr-CoV PDF2180, and HKU9. These were incorporated into our 17-plex henipavirus/filovirus-focused serology assay to include SARS-CoV-2 and bat-borne CoV antigens. We also optimized an sVNT for Nipah virus and provided enough material to characterize Nipah virus IgG positive serum samples neutralizing antibodies. SOPs were revised and updated to reflect best practices that were optimized during Year 1.

Outbreak Investigation in Thailand

- The Ministry of Public Health in Thailand requested our support for a viral diarrhea outbreak investigation in Chanthaburi province. We received samples from both Prapokklao Hospital (regional hospital) and Khlong Khut Health-Promoting hospital collected from 59 healthcare workers and students who had diarrhea during Dec. 2021-Feb. 2022. PCR test were conducted for six gastrointestinal viruses causing gastrointestinal tract infections including adenovirus, astrovirus, norovirus GI, norovirus GII, rotavirus and sapovirus. 32 samples **(54.24%)** were positive for norovirus GII. Further genomic characterization of the detected norovirus GII will be performed, allowing for comparison with noroviruses found in other provinces in Thailand.

Research Network and Capacity Building

- To fulfill the goal of building a regional EID research and response network and capacity, we have worked with scientists and government officials from Thailand, Malaysia, Singapore, Bangladesh, Cambodia, Indonesia, Lao PDR, Myanmar, Nepal, Vietnam, USA, and Australia via meetings, workshops, training, pilot research project development, data analysis, and knowledge exchange, to strengthen research capacity and foster scientific collaborations in Southeast Asia. *(Section B.4 and B.5)*
- The collaborations under this project have generated 14 scientific articles for peer-review journals (six published, five under review/revision, three in preparation) during the reporting period, field and lab research protocols, training materials, and technologies that are shared and transferred beyond this project in the region. *(Section C.1 and C.3)*

During this reporting period, we conducted a series of trainings with EID-SEARCH project partners and in-country stakeholders in Thailand, Malaysia, and Vietnam from local government, universities, research institutes, and NGOs. These focused on biosafety, applying new technologies, and field and laboratory SOPs to strengthen in-country research capacity and improve information sharing. As travel and COVID-19 restrictions have begun to be lifted, students, postdocs, and all EID-SEARCH research staff have been provided with professional development opportunities to travel to attend conferences, workshops, and other in-person training activities. Highlighted trainings are as follows:

June - July 2021 Biosafety training at the Molecular Zoonosis Lab in the National Wildlife Forensic Lab (NWFL) and Wildlife Health, Genetic and Forensic Laboratory (WHGFL) focusing on biosafety guidelines and all biosafety documents (lab and field SOPs, risk assessments, and safety manual) for EID-SEARCH project biosafety lab evaluation. Risk assessment training was conducted based on the WHO lab biosafety manual 4th edition was conducted for 11 Conservation Medicine and three other local NGO staff. The training was provided by Prof Stuart Blacksell of MORU, Thailand.

The Wildlife Health, Genetic and Forensic Laboratory (WHGFL) in Sabah was certified for the eighth year in a row as a BSL- 2 laboratory (July 2021), the molecular Zoonosis Laboratory at the Department of Wildlife and National Parks of Peninsular Malaysia (PERHILITAN)'s new National Wildlife Forensic Laboratory (NWFL) was certified for the fifth year in a row as a BSL-2 laboratory (September 2021), in accordance with the Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition (June 2020) which is the US standard for laboratory specifications.

June 2021 Online training among Conservation Medicine staff regarding advancement in wireless temperature monitoring in Malaysia for clinical syndromic surveillance.

September 2021 Online biosafety training session among field team members from Conservation Medicine (Malaysia), Chulalongkorn University (Thailand) focusing on:

- Identification of health risks (disease, routes of exposure, field equipment, medical supplies, climate, trauma, etc.)
- Understanding how to mitigate risks (personal health, immuniztions, site risk analysis, personal protective equipment [selection, donning, doffing], disinfection, & disposal)
- Responding to health threats (pathogen exposure, trauma, envenomation, physical exhaustion)

September 2021 Training of EID-SEARCH Thailand team on using Airtable to facilitate real-time project data sharing.

September 2021 User training with standard operating procedures and safe use of biosafety cabinets among 18 members from Conservation Medicine, Universiti Malaysia Sabah, PERHILITAN, and a local NGO in Malaysia.

October 2021 Serology data analysis training was provided to Conservation Medicine staff, Wildlife Health, Genetics and Forensic Laboratory (WHGFL), Chulalongkorn University laboratory team members on using R Studio to analyze laboratory data from multiplex microsphere immunoassay (MMIA) testing.

November 2021 On-site lectures and hands-on training on biosafety, human safety, safe animal handling and sampling, safe sample transportation training for 3 PERHILITAN Science Officers at National Wildlife Forensic Laboratory. The objective of this training was to build capacity for those involved with the bio-surveillance of wildlife species and for them to better understand the distribution of these pathogens and train researchers on how to collect samples safely and securely in the field with a focus on biosafety and biosecurity.

November 2021 Virtual training among Conservation Medicine staff on nanopore sequencing. This training explores the functions of nanopore sequencing, a technology that enables direct, real-time analysis of long DNA or RNA fragments.

January 2022 Room differential pressure training for three Conservation Medicine staff in Malaysia. The objective of this training was how to use manometers in a laboratory to help us monitor room pressures at PERHILITAN and Sabah Wildlife Department (SWD) laboratories.

March 2022 Training among two PERHILITAN Science Officers on Bio-Plex/Luminex that consists of theories on lab safety, introduction to Bio-Plex and multiplex immunoassay, Bio-Plex best practices and maintenance and raw data handling, and practical training on operating the Bio-Plex machine and running the filovirus and Henipavirus multiplex immunoassay on samples collected in Malaysia.

April 2022 On-site lectures and hands-on training to members from Oxford University Clinical Research Unit (OUCRU) and Institute of Ecology and Biological Resources (IEBR) (Vietnam) on:

- Field biosafety
- Site assessment
- Bat handling and sampling

Regular meetings and training on serologic testing were provided to Thailand and Malaysia partners on using surrogate virus neutralization test (sVNT) and multiplex microsphere immunoassay (MMIA) developed by EID-SEARCH partners.

C. PRODUCTS

C.1 PUBLICATIONS

Are there publications or manuscripts accepted for publication in a journal or other publication (e.g., book, one-time publication, monograph) during the reporting period resulting directly from this award?

Yes

Publications Reported for this Reporting Period

Public Access Compliance	Citation
Complete	Edwards CE, Yount BL, Graham RL, Leist SR, Hou YJ, Dinnon KH 3rd, Sims AC, Swanstrom J, Gully K, Scobey TD, Cooley MR, Currie CG, Randell SH, Baric RS. Swine acute diarrhea syndrome coronavirus replication in primary human cells reveals potential susceptibility to infection. Proceedings of the National Academy of Sciences of the United States of America. 2020 October 27;117(43):26915-26925. PubMed PMID: 33046644; PubMed Central PMCID: PMC7604506; DOI: 10.1073/pnas.2001046117.
Complete	Putcharoen O, Wacharapluesadee S, Chia WN, Paitoonpong L, Tan CW, Suwanpimolkul G, Jantarabenjakul W, Ruchisrisarod C, Wanthong P, Sophonphan J, Chariyavilaskul P, Wang LF, Hemachudha T. Early detection of neutralizing antibodies against SARS-CoV-2 in COVID-19 patients in Thailand. PloS one. 2021;16(2):e0246864. PubMed PMID: 33577615; PubMed Central PMCID: PMC7880427; DOI: 10.1371/journal.pone.0246864.
N/A: Not Journal	Wells HL, Loh E, Nava A, Lee M, Lee J, Sukor JR, Navarrete-Macias I, Liang E, Firth C, Epstein J, Rostal M, Zambrana-Torrelio C, Murray K, Daszak P, Goldstein T, Mazet JA, Lee B, Hughes T, Durigon E, Anthony SJ. Taxonomic classification methods reveal a new subgenus in the paramyxovirus subfamily Orthoparamyxovirinae. bioRxiv [Preprint]. 2021 October 13. Available from: https://www.biorxiv.org/content/10.1101/2021.10.12.464153v1.abstract.
Complete	Wacharapluesadee S, Tan CW, Maneeorn P, Duengkae P, Zhu F, Joyjinda Y, Kaewpom T, Chia WN, Ampoot W, Lim BL, Worachotsueptrakun K, Chen VC, Sirichan N, Ruchisrisarod C, Rodpan A, Noradechanon K, Phaichana T, Jantarat N, Thongnumchaima B, Tu C, Crameri G, Stokes MM, Hemachudha T, Wang LF. Evidence for SARS-CoV-2 related coronaviruses circulating in bats and pangolins in Southeast Asia. Nature communications. 2021 February 9;12(1):972. PubMed PMID: 33563978; PubMed Central PMCID: PMC7873279; DOI: 10.1038/s41467-021-21240-1.
Complete	Esther CR Jr, Kimura KS, Mikami Y, Edwards CE, Das SR, Freeman MH, Strickland BA, Brown HM, Wessinger BC, Gupta VC, Von Wahlde K, Sheng Q, Huang LC, Bacon DR, Kimple AJ, Ceppe AS, Kato T, Pickles RJ, Randell SH, Baric RS, Turner JH, Boucher RC. Pharmacokinetic-based failure of a detergent virucidal for SARS-COV-2 nasal infections. Research square. 2021 May 14. PubMed PMID: 34013253; PubMed Central PMCID: PMC8132247; DOI: 10.21203/rs.3.rs-500168/v1.
Complete	Wacharapluesadee S, Ghai S, Duengkae P, Manee-Orn P, Thanapongtharm W, Saraya AW, Yingsakmongkon S, Joyjinda Y, Suradhat S, Ampoot W, Nuansrichay B, Kaewpom T, Tantilertcharoen R, Rodpan A, Wongsathapornchai K, Ponpinit T, Buathong R, Bunprakob S, Damrongwatanapokin S, Ruchiseesarod C, Petcharat S, Kalpravidh W, Olival KJ, Stokes MM, Hemachudha T. Two decades of one health surveillance of Nipah virus in Thailand. One health outlook. 2021 July 5;3(1):12. PubMed PMID: 34218820; PubMed Central PMCID: PMC8255096; DOI: 10.1186/s42522-021-00044-9.
Complete	Sánchez CA, Li H, Phelps KL, Zambrana-Torrelio C, Wang LF, Olival KJ, Daszak P. A strategy

	to assess spillover risk of bat SARS-related coronaviruses in Southeast Asia. medRxiv : the preprint server for health sciences. 2021 September 14. PubMed PMID: 34545371; PubMed Central PMCID: PMC8452109; DOI: 10.1101/2021.09.09.21263359.
Complete	Carlson CJ, Farrell MJ, Grange Z, Han BA, Mollentze N, Phelan AL, Rasmussen AL, Albery GF, Bett B, Brett-Major DM, Cohen LE, Dallas T, Eskew EA, Fagre AC, Forbes KM, Gibb R, Halabi S, Hammer CC, Katz R, Kindrachuk J, Muylaert RL, Nutter FB, Ogola J, Olival KJ, Rourke M, Ryan SJ, Ross N, Seifert SN, Sironen T, Standley CJ, Taylor K, Venter M, Webala PW. The future of zoonotic risk prediction. Philosophical transactions of the Royal Society of London. Series B, Biological sciences. 2021 November 8;376(1837):20200358. PubMed PMID: 34538140; PubMed Central PMCID: PMC8450624; DOI: 10.1098/rstb.2020.0358.
Complete	Phumee A, Wacharapluesadee S, Petcharat S, Siriyasatien P. A new cluster of rhabdovirus detected in field-caught sand flies (Diptera: Psychodidae: Phlebotominae) collected from southern Thailand. Parasites & vectors. 2021 November 8;14(1):569. PubMed PMID: 34749797; PubMed Central PMCID: PMC8576998; DOI: 10.1186/s13071-021-05047-z.
Complete	Hunsawong T, Fernandez S, Buathong R, Khadthasrima N, Rungrojchareonkit K, Lohachanakul J, Suthangkornkul R, Tayong K, Huang AT, Klungthong C, Chinnawirotpisan P, Poolpanichupatam Y, Jones AR, Lombardini ED, Wacharapluesadee S, Putcharoen O. Limited and Short-Lasting Virus Neutralizing Titers Induced by Inactivated SARS-CoV-2 Vaccine. Emerging infectious diseases. 2021 December;27(12):3178-3180. PubMed PMID: 34559045; PubMed Central PMCID: PMC8632161; DOI: 10.3201/eid2712.211772.
Complete	Becker J, Stanifer ML, Leist SR, Stolp B, Maiakovska O, West A, Wiedtke E, Börner K, Ghanem A, Ambiel I, Tse LV, Fackler OT, Baric RS, Boulant S, Grimm D. Ex vivo and in vivo suppression of SARS-CoV-2 with combinatorial AAV/RNAi expression vectors. Molecular therapy : the journal of the American Society of Gene Therapy. 2022 January 14. PubMed PMID: 35038579; PubMed Central PMCID: PMC8758558; DOI: 10.1016/j.ymthe.2022.01.024.
In Process at NIHMS	Esther CR Jr, Kimura KS, Mikami Y, Edwards CE, Das SR, Freeman MH, Strickland BA, Brown HM, Wessinger BC, Gupta VC, Von Wahlde K, Sheng Q, Huang LC, Bacon DR, Kimple AJ, Ceppe AS, Kato T, Pickles RJ, Randell SH, Baric RS, Turner JH, Boucher RC. Pharmacokinetic-based failure of a detergent virucidal for severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) nasal infections: A preclinical study and randomized controlled trial. International forum of allergy & rhinology. 2022 January 18. PubMed PMID: 35040594; DOI: 10.1002/alr.22975.
Non-Compliant	Tse LV, Meganck RM, Yount BL, Hou YJ, Munt JE, Adams LE, Dong S, Baric RS. Genome-wide CRISPR Knockout Screen Identified PLAC8 as an Essential Factor for SADS-CoVs Infection. The Proceedings of the National Academy of Sciences. Forthcoming.

C.2 WEBSITE(S) OR OTHER INTERNET SITE(S)

NOTHING TO REPORT

C.3 TECHNOLOGIES OR TECHNIQUES

Category	Explanation
Protocols	Protocols regarding surrogate virus neutralization test Tan CW, Chia WN, Qin X, Liu P, Chen MI, Tiu C, Hu Z, Chen VC, Young BE, Sia WR, Tan YJ, Foo R, Yi Y, Lye DC, Anderson DE, Wang LF. A SARS-CoV-2 surrogate virus

	neutralization test based on antibody-mediated blockage of ACE2-spike protein-protein interaction. Nature Biotechnology 2020, 38: 1073-1078. Tan CW, Chia WN, Young BE, Zhu F, Lim BL, Sia WR, Thein TL, Chen MIC, Leo YS, Lye D, Wang LF. Pan-sarbecovirus neutralizing antibodies in BNT162b2-immunized SARS-CoV-2 survivors. New England Journal of Medicine 2021385:1401-1406. Wang LF, Tan CW, Chia WN, Zhu F, Young BE, Chantasrisawad N, Hwa SH, Yeoh AYY, Lim BL, Yap WC, Pada SK, Tan SY, Jantarabenjakul W, Chen S, Zhang J, Mah YY, Chen V, Chen MIC, Wacharapluesadee S, Commit-Kzn Team, Putcharoen O, Lye D. Differential escape of neutralizing antibodies by SARS-CoV-2 Omicron and pre-emergent sarbecoviruses. Researchsquare 2022, doi:10.21203/rs.3-1362541/v1 (preprint).
Protocols	Field biosafety guidance EID-SEARCH is developing a comprehensive field biosafety manual that provides explicit direction on protection measures in the field accounting for differences across field projects in local contexts. The areas of focus include: Identification of health risks (disease, routes of exposure, field equipment, medical supplies, climate, trauma, etc.) Understanding how to mitigate risks (personal health, immunizations, site risk analysis, personal protective equipment [selection, donning, doffing], disinfection, & disposal) Responding to health threats (pathogen exposure, trauma, envenomation, physical exhaustion) Field biosafety information is being collated for sharing in several forms of documents and media including 1) A comprehensive standard operating procedure (SOP) manual that is under internal peer-review, that will be shared broadly with the CREID network in Y3 of our project; 2) Lecture presentations with recorded voiceovers; 3) Quick reference guides for field use; 4) Video demonstrations, and 5) Web-based photo guides with descriptors.
Protocols	Standard protocols for the preservation of voucher bat specimens, including guidance on: Circumstances in which an individual should be euthanized and preserved as a voucher specimen to confirm host species identification, document new locality information for a species, or if a new species is suspected based on the opinion of wildlife experts involved with fieldwork Euthanasia methods for different taxonomic groups that are approved by the American Veterinary Medical Association Preparation techniques, including “wet” (alcohol) preparation which is most appropriate under field conditions Proper specimen labeling conventions and management of associated metadata

	Deposition of voucher specimens at in-country natural history museums An expanded version is under development to include additional host taxa that is anticipated to be shared with the CREID Network.

C.4 INVENTIONS, PATENT APPLICATIONS, AND/OR LICENSES

Have inventions, patent applications and/or licenses resulted from the award during the reporting period? No

If yes, has this information been previously provided to the PHS or to the official responsible for patent matters at the grantee organization? No

C.5 OTHER PRODUCTS AND RESOURCE SHARING

NOTHING TO REPORT

From:	Hongying Li on behalf of Hongying Li <li@ecohealthalliance.org>
To:	Tom Hughes; Supaporn Wacharapluesadee; Sasiprapa Ninwattana; eric.laing_usuhs; Broder, Christopher; Baric, Ralph S; Baric, Toni C; wang linfa; Tan Chee Wah
Cc:	Boxley, Kimberly; Omotunde (Mo) Osilesi - Henry Jackson; Jennifer Jackson; Ong Xin Mei; Loh Jian Yun; Peter Daszak; Kevin Olival; Aleksei Chmura; Cadhla Firth; Parrish, Kalyn; McGlaughon, Ben; Su Yadana
Subject:	NIH EID-SEARCH Year 2 Progress Report_submitted
Date:	Sunday, April 3, 2022 11:16:43 PM
Attachments:	RPPR10427219 Y2 Annual Report As Submitted_short.pdf

Dear All,

Attached please find a copy of the submitted Year 2 Progress Report (technical part), thank you very much for your wonderful work, and for sending updates to complete the report!

As we are approaching the end of Year 2 on May 31, we will follow up closely on the Year 2 funding expenses and invoices in the next couple of weeks.

Sincerely,
Hongying

Hongying Li, MPH
Senior Program Manager & Research Scientist

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EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.

On Mon, Feb 7, 2022 at 2:02 PM Hongying Li <li@ecohealthalliance.org> wrote:

> Dear Partners,
>
> The NIH Research Performance Progress Report (reporting period 06/01/2021-05/31/2022) for EID-SEARCH (No. 5U01AI151797) is due on April 1, 2022. I'm sending this email as an initial notification in case your institution needs some time to proceed and prepare a report, but the information required for this report is majorly focused on the research findings and plans, very minimal on administration unless you have any major changes on key/senior personnel.
>
> Attached please find a template with the different sections we would like to get updates from you as part of the report to submit. Any relevant work for each section is welcomed (don't worry about the languages or word limits), but I labeled some sections as *required where are the most important to concentrate.
>
> We would greatly appreciate it if you can send us updates by **March 21, 2022, Monday** so that we can have a few days to edit and combine all things together to submit.
>
> Please feel free to let me know if you have any questions. You will also receive reminders

from me on March 01 and March 14 if it's still too early and you're waiting for more results to include in the report. Thank you very much!!

Sincerely,
Hongying

Hongying Li, MPH
Senior Program Coordinator & Research Scientist

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EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.

Abstract
Keystone Symposium: Lessons from the Pandemic: Responding to Emerging Zoonotic Viral Diseases.

Integrating Nipah virus surveillance, outbreak investigation and research in Bangladesh

Jonathan H. Epstein[1], Ariful Islam[1], Tahmina Shirin[2], Md Ziaur Rahman[3], Maria Kaczmarek[1], Noam Ross[1], Eric Laing[4], Sarah Munro[1], Kevin J. Olival[1], Christopher C. Broder[4], Vincent J. Munster[5], Peter Daszak[1], Meerjady S. Flora[6]

1. EcoHealth Alliance, New York, USA
2. Institute for Epidemiology, Disease Control and Research, Dhaka, Bangladesh
3. Icddr,b, Dhaka, Bangladesh
4. Uniformed Services University, Maryland, USA
5. National Institutes of Health, National Institute of Allergy and Infectious Disease, Rocky Mountain Laboratories. Montana, USA
6. Government of Bangladesh Directorate General of Health Services, Dhaka, Bangladesh

Nipah virus is an emerging zoonotic paramyxovirus that has repeatedly caused outbreaks of encephalitis associated with high case fatality rates in South Asia. Surveillance for Nipah and related henipaviruses across their geographic distribution is generally lacking, and this group of viruses has the potential to cause significant outbreaks in people and domestic animals. In Bangladesh, Nipah virus outbreaks have been reported nearly every year since 2001. *Pteropus medius*, a common and abundant frugivorous bat, is the natural reservoir for Nipah virus in Bangladesh and spillover occurs primarily through the consumption of date palm sap contaminated with bat excreta. Human-to-human transmission has been a typical feature of Nipah virus outbreaks, although observed transmissibility has, so far, been relatively low. Yet little is known about the genetic diversity of henipaviruses in bats, the relationship between viral genotype and clinical or epidemiological characteristics, or the frequency of spillover. To address these knowledge gaps and ultimately develop more effective interventions to prevent Nipah virus outbreaks, the government of Bangladesh and its partners have implemented a multidisciplinary, One Health approach to Nipah virus surveillance, outbreak response and research, combining longitudinal studies of Nipah virus in bats, contemporaneous human and animal testing during outbreak investigations, behavioral risk assessments, virological studies and serological surveys. Here we describe a current model for integrated surveillance and research designed to better understand, respond to, and ultimately prevent spillover of a high consequence, emerging zoonotic virus.

KEYSTONE SYMPOSIA
on Molecular and Cellular Biology

Lessons from the Pandemic: Responding to Emerging Zoonotic Viral Diseases (D3)

April 10-13, 2022 • Snowbird Resort • Snowbird, UT, USA

Scientific Organizers: Linfa Wang, Sarah Catherine Gilbert and William E. Dowling

Supported by the Directors' Fund

Global Health Travel Award Deadline: March 21, 2022 / Scholarship Deadline: January 17, 2022 / Abstract Deadline: January 17, 2022 / Discounted Registration Deadline: February 10, 2022

SUNDAY, APRIL 10

Arrival and Registration

MONDAY, APRIL 11

Welcome and Keynote Address

Anthony S. Fauci, NIAID, National Institutes of Health, USA
Remote Presentation: The Good, Bad and Ugly Aspects of COVID-19 Responses

Challenges in Balancing Outbreak Investigation and Basic Research

Maria D. Van Kerkhove, World Health Organization, Switzerland
Challenges in Responding to COVID-19

John N. Nkengasong, Africa Centres for Disease Control and Prevention, Ethiopia
Remote Presentation: Talk Title to be Announced

Speaker to be Announced

Short Talks Chosen from Abstracts

Early Warning and Reporting for Emerging Zoonotic Diseases

Lawrence C. Madoff, University of Massachusetts Medical School and ProMED, USA
WHO and ProMED: Have We got the Early Warning Systems Right?

Linfa Wang, Duke-NUS Medical School, Singapore
Are we ready for SARS-CoV-3?

Short Talks Chosen from Abstracts

Poster Session 1

TUESDAY, APRIL 12

Rapid Development and Deployment of Diagnostics in an Outbreak Setting

Daniel G. Bausch, FIND, Switzerland
The 100 Day Mission for Pandemic Diagnostics: The Marathon Before the Sprint

Christian T. Happi, Redeemer's University, Nigeria
Experience in Assay Development: From Lassa to COVID-19

Speaker to be Announced

Short Talks Chosen from Abstracts

Career Roundtable

Pathogen Biology and Model Systems

César Muñoz-Fontela, Bernhard-Nocht-Institute for Tropical Medicine, Germany
Filoviruses and Arenaviruses

Florian Krammer, Icahn School of Medicine at Mount Sinai, USA
Influenza

Thomas W. Geisbert, University of Texas Medical Branch, USA
NHP Models for Hemorrhagic Fever Viruses

Emmie de Wit, NIAID, National Institutes of Health, USA
Models for Coronaviruses and Henipaviruses

Short Talks Chosen from Abstracts

Poster Session 2

WEDNESDAY, APRIL 13

Countermeasures: Vaccines, Therapeutics and Rapid Manufacturing I

William E. Dowling, Coalition for Epidemic Preparedness Innovations, USA
COVAX and CEPI Disease "X" Platforms

Sarah Catherine Gilbert, University of Oxford, UK
ChadOx1 Vaccines against Different Viral Targets

Kizzmekia S. Corbett, Harvard T.H. Chan School of Public Health, USA
Coronavirus Vaccines - NIAID Pandemic Preparedness Program

Sue Ann Costa Clemens, Oxford University, UK
Testing a Pandemic Vaccine during a Pandemic

Short Talks Chosen from Abstracts

Countermeasures: Vaccines, Therapeutics and Rapid Manufacturing II

Catherine Green, University of Oxford, UK
Developing a Highly Transferrable Vaccine Manufacturing Process for Worldwide Use

Kathrin U. Jansen, Pfizer, USA
Vaccine Manufacturing for Public Health Emergencies

Erica Ollmann Saphire, La Jolla Institute for Immunology, USA
Hemorrhagic Fever Consortium and Coronavirus Immunotherapy Consortium

Dan Hartman, Bill and Melinda Gates Foundation, USA
Therapeutics Accelerator

Short Talks Chosen from Abstracts

Meeting Wrap-Up: Outcomes and Future Directions (Organizers)

THURSDAY, APRIL 14

Departure

* Session Chair † Invited but not yet accepted Program current as of ***January 14, 2022.*** Meal formats are based on meeting venue.
For the most up-to-date details, visit ***https://www.keystonesymposia.org.***

From:	Kevin Olival on behalf of Kevin Olival <olival@ecohealthalliance.org>
To:	Jon Epstein
Cc:	Ariful Islam; Dr. Tahmina Shirin IEDCR; Dr.Mohammad Ziaur Rahman; Maryska Kaczmarek; Noam Ross; Eric Laing; Sarah Munro; Christopher Broder; Munster, Vincent (NIH/NIAID) [E]; Peter Daszak; Meerjady Sabrina Flora, PhD; Madeline Salino
Subject:	Re: Urgent: Time sensitive: abstract for Keystone Symposia. please respond by Monday 3pm
Date:	Saturday, January 15, 2022 1:49:53 PM
Attachments:	Keystone Nipah Abstract d1.docx ATT00002.bin Keystone symposia program.pdf ATT00004.bin

---

Thanks Jon, I'm good with this as well, thanks for spearheading.

Kevin J. Olival, PhD
Vice President for Research

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EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.

> On Jan 15, 2022, at 2:43 AM, Jon Epstein <epstein@ecohealthalliance.org> wrote:
>
> Dear colleagues,
> I've just become aware of an upcoming Keystone Symposium on pandemic prevention being co-organized by CEPI.
> www.keystonesymposia.org/conferences/conference-listing/meeting?eventid=6862
>
> I think it would be a good opportunity to submit an abstract for a short talk to showcase our Nipah virus work. The deadline for submission is Monday night (Eastern Time)
>
> With apologies for the short notice, I would be grateful if you would review the attached abstract and send me your consent to be a co-author, along with any comments, by Monday 3pm.
>
> Cheers,
> Jon
>
> --
>
> Jonathan H. Epstein DVM, MPH, PhD

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PROJECT DESCRIPTION

Introduction and Background

The spillover and spread of novel, zoonotic (animal origin) pathogens can have tragic societal and economic impacts, as is evidenced by the ongoing COVID-19 pandemic. The emergence of SARS-CoV-2 (the virus that causes COVID-19) is the latest in a series of viral emergence events that have accelerated in recent decades. In just the last year, as the world continues to respond and adapt to COVID-19, we have seen other deadly zoonotic viruses re-emerge in the human population, including Ebola and Marburg in Guinea[1,2], Nipah virus in India[3], Rift Valley Fever virus in Kenya[4], and novel strains of influenza in China[5]. Most emerging infectious diseases (EIDs) originate in wildlife hosts and are transmitted across species boundaries, or ("spill over"), when livestock or human populations increase contact with wildlife through factors including land use change, agricultural intensification, and the wildlife trade[6-8]. Zoonotic spillover is a dynamic process determined by many steps including 'pre-emergence' viral evolution, diversification, and transmission among wildlife hosts; 'human exposure' via direct or indirect contact between humans, livestock, and wildlife; 'post-exposure' limits to cell entry, host range, human-to-human transmission; and population level virus amplification and spread. Once efficiently transmitted, EIDs spread rapidly through globalized travel and trade networks, threatening countries with high population growth, urbanization and international trade (**Fig 1**).

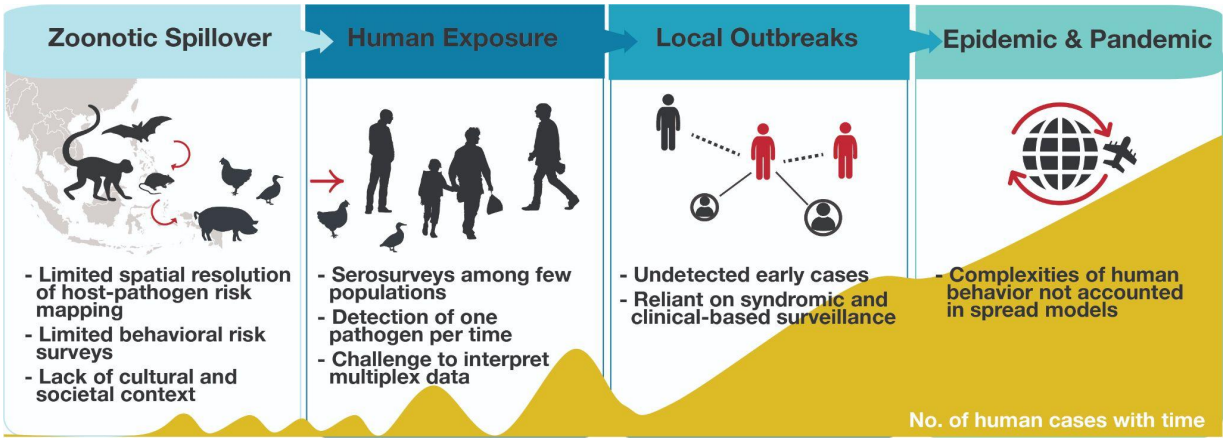

Fig 1. Four stages of zoonotic disease emergence and spread, with gaps in knowledge at each stage. Our proposed project will develop and pilot novel, multidisciplinary and technological advances to address critical gaps in the first three stages of emergence, when pandemic prevention is possible.

Novel human EIDs are typically only discovered when they cause severe or unusual symptoms, when astute clinicians are linked with laboratories capable of pathogen discovery research, and after they have been transmitted from person to person (after which control becomes more difficult)[8,9]. Emerging human viruses characterized to date are a small sample of hundreds of thousands of predicted zoonotic viruses in nature but undiscovered due to limited surveillance capacity globally[10]. New research has upended the paradigm that zoonotic disease spillover is a rare event. A combination of more advanced molecular and serological assays, improved laboratory capacity around the world, and targeted surveillance of "high risk" human populations found evidence of frequent zoonotic virus transmission and infection, even in the absence of notable disease outbreaks. Often these spillover events lead to "dead end" infections in people or limited "stuttering" chains of transmission, a phenomenon referred to as "viral chatter"[11]. Community-based serological surveys from our group and others have illuminated relatively frequent and previously-unknown instances of zoonotic virus spillover including Henipaviruses in bat hunters from Cameroon[12], SARS-related CoVs in rural communities living near caves in China (pre-2019), and Ebola virus exposure in DRC[13] and even Southeast Asia[14]

Innovative and multidisciplinary approaches are needed to disrupt the emergence of diseases at the "viral chatter" stage and further upstream at the human-animal interface to prevent epidemics and pandemics before they are out of control. While we have made significant scientific advances over the last ~15 years in understanding where and through what mechanisms novel zoonotic viruses spill over into the human population[6,15-18], there **are still fundamental knowledge and implementation gaps that stymie our ability to preempt and prevent future disease emergence events**.

Our Grand Challenge Problem

Prior to 2019, EcoHealth Alliance (EHA) and our partners demonstrated that SARS-related coronaviruses (SARSr-CoVs) in China pose a clear and present danger for a future human outbreak. We conducted extensive viral discovery in wildlife from China and Southeast Asia, phylogenetic analysis, and host-range experiments to show that certain bat species harbor a diverse assemblage of SARSr-CoVs[19,20], as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics[21-24]. We conducted behavioral risk factor surveys and serological sampling from human populations living close to caves and found serological evidence of SARSr-CoV spillover in people exposed to wildlife[25]. Building on this body of knowledge, we estimate bat-origin SARSr-CoVs infect 50,000 people in Southeast Asia each year[26]. While confidence intervals around this median estimate are large, our sensitivity analyses identify key area for future research to improve these spillover risk models: quantification and better contextual understanding of human-bat contact, the role of wildlife farming and livestock hosts, scaling up human serological surveys, and better data on sero-dynamics following exposure[26].

Despite this strong body of evidence for SARSr-CoV spillover risk prior to the emergence of SARS-CoV-2, surveillance was not conducted across a wide enough geography, nor at a temporal or spatial scale to inform actionable disease mitigation. Despite large-scale efforts to strengthen zoonotic virus early warning systems, like the 10-year, 30 country USAID PREDICT program[27], led by a global consortium including EHA, current methods and systems cannot capture and disseminate enough data quickly enough to enable prevention measures at national or global scales. This is especially so for multiple and novel viral threats, and in large countries with heterogeneous human populations, diverse wildlife fauna, and known ecological and societal risk factors for emergence. Approaches to massively scale up zoonotic spillover surveillance are needed.

Our grand challenge: "How can we capture actionable data about the risk of zoonotic spillover and spread at the scale and speed required to prevent outbreaks?" How can we leverage tools developed by computer scientists, data engineers, and technology companies to collect data from large, diverse populations and geographies to advance zoonotic spillover intelligence? How do we incorporate new insights from medical anthropology, sociology, wildlife biology, disease ecology, molecular virology, biomedical assay design, and data science to pioneer accelerate data collection and interpretation for zoonotic disease prediction and prevention? We propose to build a collaborative research and training network to confront this challenge, that will scale up to a future *Center for Spillover Intelligence (CSI)*. Our network will deeply integrate computer scientists, engineers, and data scientists using machine learning and other novel technological approaches together with experts in emerging disease and social-behavioral research. New insights from social, behavioral, and anthropological research make it clear that the emergence and future trajectory of human epidemics require that we recognize potential biological risks alongside risks posed by cultural and societal factors[28-30]. Our cross-disciplinary, multi-institution project will combine innovative research activities with training and education programs to facilitate to scale-up the collection and interpretation of data to inform where, how, and what zoonotic viruses are poised to emerge – a field we collectively refer to as

spillover intelligence. Our inclusion of social-behavioral researchers, data ethicists, and inter-governmental EID policymakers will ensure that our aims and approaches are culturally and locally relevant, and align with our ultimate goal of improving global health.

Our Vision: We envision a world where diffuse data streams can be combined and interpreted in near real-time to disrupt the spillover and spread of future zoonotic viruses. We envision diverse leaders and scientific disciplines coming together to advance spillover intelligence and transform the ways we think about, monitor, and respond to global viral threats.

Our Team

We have assembled a multidisciplinary team of experts to lead our development project and to create and grow the Center for Spillover Intelligence (CSI). Our **Leadership Team (PI, coPIs, Senior Personnel)** includes established scientists and early career leaders working across the fields of emerging infectious diseases, computer and data science, engineering, mathematical modeling, virology, technology and biotechnology, data ethics, social science, and medical anthropology. Our project will be led by EHA (PI Olival) building off the foundational pandemic prediction work our institution has developed over the last decade, and include new partnerships and collaborations. These include with computer scientists and engineers from University of Rochester (UR) and behavioral anthropologists from Georgetown University (GU). We will develop and pilot new technologies for field biosurveillance and data interpretation with Uniformed Services University (USU). Our leadership team is ethnically, culturally, and geographically diverse, including US-based researchers who originate from and/or currently conduct their primary research in global EID hotspot regions[6,15], e.g. South Asia (Hoque); Latin America (Mateos); Africa (Mendenhall); and Southeast Asia and the Pacific (Olival). Our geographic and disciplinary diversity, including long-standing international collaborators from each region, make our team well-equipped to address spillover at a global scale, while incorporating the nuance of local context.

PI Olival (EHA) is a disease ecologist and evolutionary biologist with a primary interest in developing novel analyses to target zoonoses surveillance and viral discovery. He currently leads two regional biosurveillance projects in Southeast and Western Asia, and recently served as Modeling & Analytics Coordinator for the USAID PREDICT project[27] overseeing a multi-institution team developing pandemic prediction strategies using public and project-specific data from 30 countries[6,16,31-36]. **coPI Mendenhall** (GU) is a medical anthropologist and Professor at the Edmund A. Walsh School of Foreign Service. Mendenhall works at the intersection of social, psychological, and biological lives, attempting to understand what makes people sick, why, and where. Mendenhall is currently a PI of NIH Fogarty International Center grant “Soweto Syndemics”, the first ever study to evaluate a syndemic from the ground up. **coPI Hoque** (UR) is an Associate Professor of Computer Science working at the intersection of Human-centric Computing (HCC) and Artificial Intelligence with a focus on multimodal machine learning with applications in health and wellness. Hoque is currently a PI of NSF NRT grant, and received NSF CAREER and CRII grant. Hoque has been identified as an ‘emerging leader of health and medicine’ at the National Academy of Medicine (NAM). **coPI Mateos** (UR) is Assoc. Professor of Electrical and Computer Engineering and a Data Science Fellow, his research focuses on algorithmic foundations, analysis, application of (graph) signal processing tools to the study of large-scale and dynamic networks. He has pioneered foundational work in network topology inference, online learning from graph streams, and interpretable representation learning for unstructured data. **coPI Ross** (EHA) is Principal Scientist for Computational Research. His research includes model development to interpret complex spatio-temporal disease surveillance data in heterogeneous populations, real-time community and hospital testing, and simulating and forecasting epidemic dynamics over populations, space, and networks. He is PI on awards from DHS Science & Technology Directorate and the Defense Threat Reduction Agency

(DTRA) for designing disease forecasting systems. **SP Mendelsohn** (EHA) is a data scientist specializing in the development and application of machine-learning models to draw insights into the occurrence and spread of emerging infectious diseases. With a focus on reproducible research methods and data management, she has been the lead analyst on data driven disease forecasting and behavioral survey-based projects characterizing animal-human contact patterns. **SP Daszak** (EHA) is an EID expert who has studied the process of disease emergence for decades and pioneered groundbreaking work in "hotspot" risk mapping to target surveillance. **SP Broder and Laing** (USU) are experts in the design of multiplex serological assays, molecular virology, and implementation of field-based human and wildlife biosurveillance strategies. **OP Busa** is a biotechnology consultant with a specialty in developing lateral flow assays and optical readers. **OP Pompe** is the Research Manager for Facebook Data for Good, who specializes in applying human mobility datasets from social media feeds and data privacy and ethical issues.

Intellectual Merit

The primary intellectual merit of our proposed project is in **developing and piloting innovative approaches to rapid and large-scale spillover intelligence across previously siloed scientific domains** including biology, computer science, electrical engineering, and social-behavioral research. We apply new theoretical advances and convergent research to address our grand challenge of capturing actionable data about the risk of zoonotic spillover and early spread at a scale and speed required to prevent outbreaks. We will identify gaps and solutions for step-level changes in zoonotic spillover prediction via three multidisciplinary working groups that will engage in pilot projects, organize workshops, and engage students in immersive training. Our pilot research projects will serve as a test bed for applying new theoretical frameworks to advance spillover intelligence test scalability in future work. For example, in Aim 1 we will transform decades-old anthropological approaches and pilot cost-effective methods to collect large samples of quantitative and qualitative behavioral data via unique public-private partnership with Facebook; and use image recognition and artificial intelligence to quantify and map human-wildlife contact globally. In Aim 2 we will develop rapid bioinformatics pipelines to process and interpret rich data from multiplex serological surveillance; and develop the first field-forward lateral-flow assay for Nipah virus antibody detection. In Aim 3 will use advances in network science, anomaly detection, and machine learning to detect changes in human mobility patterns that may portent spillover (e.g. increase congregation around wildlife markets) and that signal early epidemic spread after the point of spillover.

Broader impacts

This proposal could potentially transform our understanding of zoonotic spillover risks using survey, multimedia, and mobility data on a global scale, including aggregated and anonymized data from ~3B active Facebook users. Insights and intellectual outputs from our working group activities will have direct benefits to public health by informing disease mitigation strategies for pandemic prevention. All outputs from our three Working Group activities, including workshop materials and code and analyses from pilot projects, will be made publicly available (**see Data Management Plan**), and published as both peer-reviewed manuscripts and popular press articles for wider dissemination. EHA's *Alliance,* includes a network of over 70 partner institutions in 30+ countries, presents a unique opportunity to work with local stakeholders in emerging virus 'hotspots' and validate models using data sets obtained through our long-standing collaboration with government partners including human health, agriculture, and environmental sectors. Our education mission consists of multiple workshops, hackathons, monthly seminar series, and access to state-of-the-art visualization facilities.

We will train a new generation of graduate and undergraduate students in understanding medical anthropology, disease surveillance, and data and network science so as to become

future leaders in spillover intelligence. We will do so via a unique partnership between the academic (UR, GU, USU), non-profit (EHA), and private (FB) sectors, allowing students to apply lessons learned from classroom settings to the real-world. Students will participate in project workshops, hackathons, monthly seminar series, and have access to state-of-the-art visualization and computing facilities. Our recruitment will focus on including >50% women and underrepresented minorities in science and engineering. This planning grant will be a path towards developing an interdisciplinary curriculum, including cross-training courses across institutions, and hands-on experience via internships and research exchanges. Through the planning grant, a new MS data science track on zoonotic surveillance will be established at UR, the first step in our plans to expand our Education and Training plan should we move towards a Center-level award in the future.

Education and Training Plan

Our Leadership Team will serve as mentors for our **CSI Student Training Program** that will offer a fellowship stipend to cross-train 20 Master's level graduate students via semester-long projects associated with proposed pilot research activities (3-4 students per project) and 3-week research exchanges between our institutions (UR, GU, EHA, USU, 4-8 students from each). The research exchange builds off EHA's successful NSF-funded EcoHealthNet training program (2011-21) and will take place in either the Summer of 2022 or 2023. We will link with currently-funded international programs at our respective institutions (e.g. Gui^{2}de in Nairobi, Kenya run by GU; and the NIH funded CREID research pilot program with EHA's EID-SEARCH project in Southeast Asia) to support students who desire to gain international, hands-on research experience as part of their research exchange. The focus of each research exchange will be on cross-disciplinary training, hands-on experience working with experts outside student's area of study, and big-picture strategic thinking in pandemic prevention. Students will be drawn from respective departments across our institutions: students with a computational background and with interests in interdisciplinary work will be recruited from UR's cross-department Goergen Institute for Data Science, Anthropology and International Affairs students from GU, and Emerging Disease and Virology students from USU. EHA will recruit Ecology, Evolutionary Biology, and Environmental sciences students from universities where we have active partnerships, including Columbia University's E3B department where PI Olival and SP Daszak serve as adjunct faculty. We will explore specific mentorship opportunities with data scientists from Facebook. If awarded, we will additionally apply for funds through NSF's Research Experiences for Undergraduates (REU) program, with the goal of supporting summer stipend for 1 undergraduate per institution or the maximum allowable. We will host a joint monthly virtual seminar series (2 semesters long) open to graduate and undergraduate students from our respective institutions, with dedicated student "lab meetings" before each seminar. Two CSI students from diverse disciplines will lead each lab meeting discussion based on a deep read of relevant publications that each speaker will distribute in advance. The first semester of the seminar series will be led by our Leadership Team and Working Group experts, and the second semester will be for CSI Graduate students to present results or current challenges related to their pilot research projects. We hope to encourage these students to pursue Ph.D. studies. If we are successful at establishing a center through our planning grant, we should have access to a trained cohort of students with appropriate multidisciplinary skillset.

Project Overview

Our 18-month research agenda aims to understand how new methods and technologies can improve the efficiency, intensity, speed, and scope of zoonotic disease surveillance. **We have structured our development grant around three primary research aims** that will guide our interdisciplinary research and training activities. We will establish a working group composed of multi-disciplinary experts for each primary aim that will be co-led by members of our Leadership

Team. We will develop pilot research projects that capture different parts of the disease spillover process focused on specific geographies, and lead 3-day intensive workshops, monthly student seminars, and student training activities that cut across our inter-linked research aims.

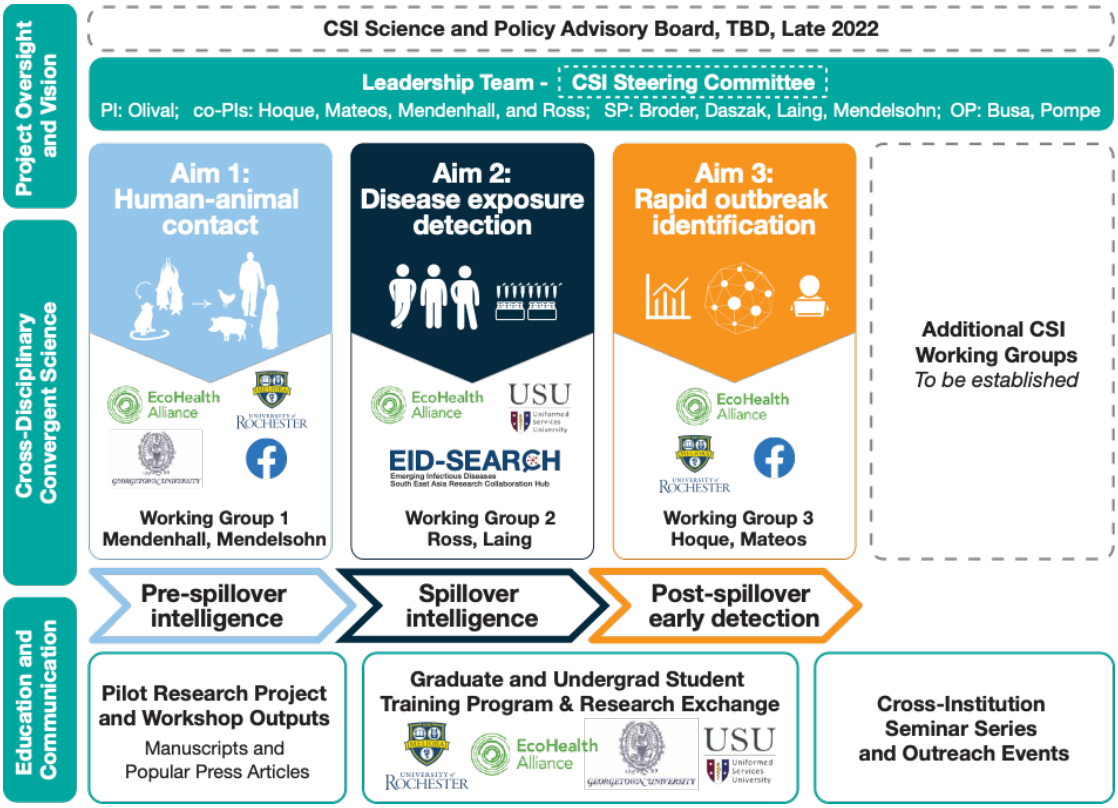

Fig 2. Project overview schematic showing our project management; three primary aims with graphical representation of pilot research activities to scale up and advance spillover intelligence; institutional partners leading activities for each aim including designated working group leads, and student education and communication activities, including pilot research projects, graduate student research exchanges, seminars, and other outreach activities. Our development level project is built to scale to a Center level award (the Center for Spillover Intelligence, CSI) as noted with the dashed outline boxes, including the future addition of other CSI Working Groups, a Science and Policy Advisory Board, and transitioning our current Leadership Team (PI, coPIs and SP) into a more formal Steering Committee for oversight.

Aim 1: Measuring human-animal contact at scale

Interactions among humans, wildlife, and livestock create interfaces for pathogen spillover and are proximate risk factors for the emergence of all directly-transmitted zoonotic diseases.[15,37] Human-animal contact is commonplace around the world, with many factors mediating the type and frequency of contact, including cultural practices, environmental conditions, food security, and occupational demands[37]. In regions of the world considered prone to spillover--forested tropical landscapes with high biodiversity and recent land-use changes--understanding human-animal contact is crucial to pandemic prevention[6]. To measure these interactions and characterize associated behaviors, beliefs, and knowledge, researchers often implement resource-intensive surveys in key communities[38,39]. While crucial to understanding human-animal contact dynamics, on-the-ground surveys cannot yield scalable data that is comparable across populations. Often, in place of surveys, researchers conducting analyses at broad scales rely on coarse estimates of contact such as the joint density of human and animal populations.

Our working group will uniquely combine social scientists, disease ecologists, and computational scientists to increase the scale at which we can directly measure human-animal contact. We will develop and implement two pilot research projects. The first will use novel, social-media-based survey approaches to reach larger populations at higher frequencies while maintaining the cultural sensitivity and relevance of on-the-ground surveys. Working collaboratively with cross-disciplinary researchers, we will design surveys to understand social behaviors, cultural perceptions, and occupational hazards that may pose zoonotic spillover risk to individuals and communities. We will target geographies based on our existing spatial risk modeling work that maps infectious disease spillover potential and the ecological distributions of key reservoir host species[6,16,26]. Second, we will conduct pilot research on the use of social-media-based image recognition algorithms to identify locations of human contact with key species known to host high pathogen diversity. This pilot will include a collaboration effort to test the efficacy of such technology for understanding taxon-specific contact patterns globally, and to bring multidisciplinary context to meaningfully interpret the outputs (including species validation with taxonomic experts). If successful, will use these data on frequency of animal contact from image data to identify locations for additional survey data collection.

Aim 1 Pilot Research Projects: We will develop two pilot projects to assess the potential of 1a) social-media dispensed surveys and 1b) passive surveillance using artificial intelligence for image recognition to capture information about human-animal contact. We will target populations at wildland interfaces where contact with wildlife is likely high but internet penetration may be mixed.

1a. Social-media dispensed surveys to quantify and contextualize human-animal contact
We will pilot social-media dispensed, large scale population surveys about perceptions of and interactions with animals. We will optimize the design of these surveys for mobile platforms to facilitate higher response rates, and will also explore the possibility of longitudinal sampling (of either individuals or populations) to understand changes in conditions and responses over time. Survey questions will be based on a combination of EHA's past experience designing on-the-ground human-animal contact surveys[38,40,41] and ideas generated from our proposed working group activities involving anthropologists, data scientists, and disease ecologists who have worked extensively in the geographic and societal contexts in which we focus our work. We will design surveys that account for populations and individuals holding different beliefs, carrying different histories, and living in different ecologies. We will survey demographically and geographically diverse populations to capture direct and indirect animal-contact patterns and behaviors, and to capture the role that international, state, and local policy plays in individuals' lives. Further, we will aim to identify particular hesitancies among individuals to interact with technologies or other public health interventions.

We will explore multiple online platforms at the start of our project for survey administration. Given our unique public-private partnership with Facebook for many components of this project, using the Facebook platform to disseminate our pilot survey is likely to be a strong option, especially given Facebook's global reach (nearly half of the world's population, ~3 billion users), which introduces opportunities for digital interaction across class, gender, ethnicity, and cultural divides. We will leverage Facebook's extensive experience designing and administering surveys, especially for mobile devices, both directly on its platform (e.g. Yale Climate Change opinion survey) or off-platform (e.g. Carnegie Mellon University COVID-19 surveys). Facebook also has extensive privacy infrastructure and data-review policies to ensure anonymity and data security.

We will also explore creating games that users will be encouraged to play online to enable us to learn more about how they think about host species, viral threats, environment, and

health/disease. For example, users may be given a list of 30 images and will be asked to put them into categories and describe what each category means. Adapting these "pile sorting" anthropological methods[42] to an online interface and for spillover risk will be a novel application with potential for a massive scale-up of data around cultural and conceptual dimensions of zoonotic disease risk. Data generated from this exercise will generate an ethnographic understanding of people's experiences and thought processes, typically locally mediated.

We understand that there are geographic and demographic gaps in internet and social media use, and will incorporate bias corrections into our study design. We will initially focus on Southeast Asia, a region with a high likelihood of future zoonotic disease emergence and where EHA has good coverage of on-the-ground surveillance programs to ground truth online data collection[6], and expand to new geographies as we develop Center-level activities.

1b. Image recognition of zoonotic disease host species

We will pilot image-based surveillance of Facebook and Instagram feeds to attempt to identify human contact with key zoonotic disease host species and to explore the limits of taxonomic and behavioral recognition in images. We will work with Facebook Data for Good (see **Letter of Collaboration**) to adapt and refine their existing machine vision algorithms to target key taxa and to assess the efficacy of different approaches, working within strict guidelines and ethical standards for data de-identification. While data will be de-identified, we will use location meta-data, allowing us to not only quantify the type of species and contact, but where it occurred. There are multiple image libraries that may serve as AI training datasets, including iNaturalist, the American Society of Mammologists, and Integrated Digitized Biocollections (iDigBio). We will iteratively implement and test algorithms starting with broad taxonomic groups (e.g., bats) and will explore the extent to which our approaches can identify finer taxonomic resolutions down to the genus or species level. In addition to taxa identification, we will explore image recognition of features that may suggest specific types of human-animal contact, such as cages or market settings. We will use text and image metadata and explore using hashtags associated with images to boost the ability of our algorithms to identify key taxa.

This work will involve collaboration between machine learning scientists, biologists/ecologists, and social scientists. We will follow all data anonymity and privacy guidelines from Facebook and will also discuss data handling ethics in our working group. We will assess the ability of passive surveillance with image recognition to target surveys to populations that have higher frequency of contact with wildlife, and ultimately to target biological surveillance of human and animal populations. We will consider both the accuracy of the image recognition algorithms as well as implications for survey distributions, results interpretation, and ethical data practices. We will also compare the spatial distribution of human-animal contact patterns derived from image recognition with previously generated spillover risk maps based on joint occurrences of animal and human distributions (e.g., our SARS-related CoV spillover risk[26]).

Working Group 1 Activities: Our working group will convene 12-15 experts from the social sciences, disease ecology, and computational sciences for an intensive, 3-day workshop. We will structure the workshop to focus first on data ethics in human behavioral surveying and image recognition surveillance and secondly on survey design and analysis planning, with specific attention to accounting for gaps or biases in survey distributions. We will center the workshop on data collection that addresses long term social, ecological, and economic realities to understand how people perceive, experience, and interact with the risk of and repercussions from spillover events, rather than technological "quick fixes". We will also address experiences and challenges in engaging longtime ethnographers and local leaders to understand what's at stake for communities engaging with online and field surveillance programs. Our working group will help mentor students part of the CSI Student Training Program, to facilitate information sharing and idea generation among undergraduate and master-level students. Working group 1

will also contribute to the CSI Seminar Series with cross-disciplinary topics such AI image recognition (lead by a data scientist and bat biologist) and human-behavioral survey design (lead by an epidemiologist and medical anthropologist).

Partners, Initial Working Group 1 Members, and Roles: EHA (SP Mendelsohn): survey design and implementation, image recognition algorithm development and testing, novel data analyses. Facebook Data for Good (OP Pompe): survey implementation, image recognition algorithm development and testing, data ethics. University of Rochester (coPI Hoque): Image recognition algorithm development and testing. Georgetown University (coPI Mendenhall): Survey design with focus on ethnography and anthropology; online free listing/pile sorting game design; help lead expansion of WG to include emerging international leaders in social-behavioral science (e.g. Dr. Edna Bosire from Kenya). We will invite additional leaders from our extensive funded collaborations to be part of the Working Group for Aim 1.

Aim 2: Detecting zoonotic disease exposure at scale

Animal-human contact is the necessary condition for spillover of potential pandemic, directly-transmitted viruses. While a small fraction of such encounters result in exposure or infection, such exposures are frequent given the total sum of encounters. Some viruses such as Lassa spill over thousands of times per year[43]. Most spillover infections are never reported and rarely spread widely, and a very rare subset become widespread in the human population[44]. In areas of frequent spillover, the eco-evolutionary dynamics of "stuttering chains" of infection are what give rise to epidemics and pandemics[45]. Identifying populations where frequent exposure occurs is critical for deploying preventative measures and following viruses upstream to their wildlife hosts. Yet even large-scale surveillance projects cover too small populations and produce far too few detections to determine the most exposed geographic, demographic, or occupational groups at an actionable scale. This is for two reasons: field and laboratory cost of surveilling populations interacting with wildlife interface, and low detection rates of widely-used molecular PCR or metagenomic approaches.

We aim to drastically increase the scale at which populations can be monitored for exposure to animal-origin viruses by developing and piloting novel tools and analyses needed to implement large-scale serological surveys. While metagenomic methods have been favored for their ability to capture genetic sequences of previously unknown viruses, serological methods can be used to detect exposure to known and unknown viruses using multiplex assays tailored with a mixture of sensitivities and specificities[46]. Serological tools can detect antibodies for months or years after viral infection, rather than the limited window of days or weeks using molecular methods, and thus can provide a much richer profile of populations where pandemics may emerge[47].

Aim 2 Pilot Research Projects: In this planning period, we will begin pilots to test the feasibility of two complementary approaches to enabling serological screening at large scales: 2a) the creation of rapid bioinformatics pipelines to interpret high-dimensional serological data from large streams of surveillance data, and 2b) the development of rapid field serological tests for wildlife-origin diseases.

2a. Rapid bioinformatics for high-throughput, multiplex serology Members of our team (SP Laing, Broder) have developed multiplex serology tests that can be used to screen for exposure for many species and variants of filo-, henipa-, and coronaviruses simultaneously, and screening pipelines that can process samples at the scale of tens of thousands of samples. However, these tests pose challenges for interpretation, as high-dimensional results from multiplex bead or chip assays frequently contain signals of cross-reaction and autocorrelation (**Fig 3**). Through cross-disciplinary research between molecular virologists, serologists, mathematical modelers and data scientists on our team, we will create models to interpret this high-dimensional data and integrate them into a rapid bioinformatic pipeline that identifies

individuals and populations to known viruses as well as flagging serological signals indicative of novel viruses. We will make use of data collected under our ongoing NIH and DTRA funded biosurveillance activities (Laing, Broder, Ross, Olival, Daszak) to develop detailed reaction/cross reaction profiles of a broad panel of zoonotic viruses to serological tests. We will build mechanistic, hierarchical Bayesian models that allow us to determine whether serological "profiles" are more likely to represent antibodies of known viruses, mixtures of multiple known viruses, or previously uncharacterized viruses. We will extend techniques of antigenic mapping[48], using serological profiles to estimate phenotypic similarity of new viruses to known viral families, determine the correlation with phylogenetic relatedness, and use these measures to screen new viruses for pandemic potential. Using simulation experiments, we will test reduced-form, simple models against fully structured models so as to develop statistically robust procedures to interpret these data without high-performance computation.

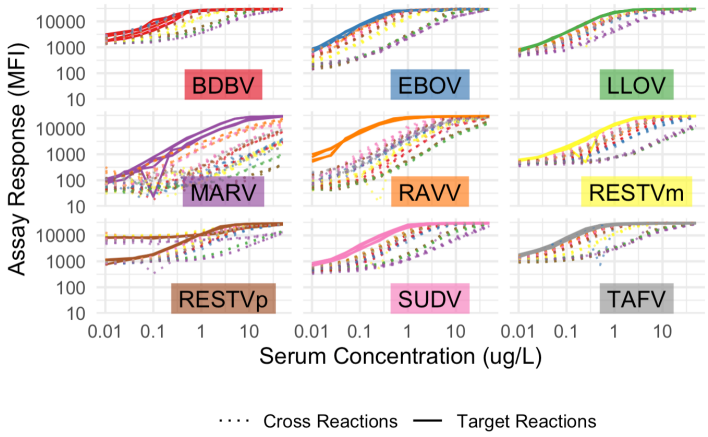

Fig 3*.* Preliminary data: Reaction-cross reaction plots of multiplex Luminex assay response to antibodies for closely-related filoviruses.

Scaling center operations: This pilot project will produce methods and a proof-of-concept pipeline for high-throughput analyses. We will leverage EHA's Emerging Infectious Disease – Southeast Asia Research Hub (EID-SEARCH) project, a NIH-funded Center for Research in Emerging Infectious Diseases (PI Daszak) who's partners include USU, Duke NUS, Chulalongkorn University in Thailand, and various clinics and institutions in Malaysia – currently screening large numbers of human samples (>10K/yr) for various coronavirus, filovirus, and paramyxovirus antibodies. In the future the CSI will implement surveillance studies screening these sample streams using multiplex serology, and utilize the analytical and bioinformatic tools we develop in the next 18 months. As part of our planning activities, we will establish protocols and approvals for this work.

2b. Development of a field-ready semi-quantitative lateral flow assays for an emerging zoonotic virus (Nipah virus)

Lateral flow assays (LFAs) are low-cost tests where reagents printed on cellulose strips react with a sample that flows via diffusion. LFAs are capable of providing quantitative results (in proportion to antibody titer) when produced via high-quality manufacturing and with carefully designed and calibrated optical scans[49]. LFAs can be used to provide diagnostics at low cost at the point of care and by consumers. Lack of commercial-scale applications has prevented LFAs from being used for serological testing for novel or emerging disease. To our knowledge, no studies have used LFAs for zoonotic virus serosurveillance in the field.

We will develop a proof-of-concept LFA for Nipah Virus (NiV). NiV has a high mortality rate and is considered to have pandemic potential that is ranked by CEPI as one of 7 high pandemic risk pathogens[50]. The range of NiV's primary wildlife hosts (*Pteropus spp,* fruit bats) cover South and Southeast Asia but human cases have only been detected in Malaysia, Bangladesh, and the Philippines. Spillovers occur regularly in Bangladesh, but are rarely reported in large portions of the country[51] and reporting is strongly associated with health care accessibility[52]. Broad-scale serological monitoring has the potential to identify populations from which

pandemic strains may emerge. Through previous work, members of our working group (Laing, Broder) have developed multiplex serological assays for NiV[53] and the soluble proteins and reagents required for an LFA. We have also added experience in engineering, quality control and commercialization of LFAs (Busa, industry consultant) to our working group. For the pilot, we will engage with commercial manufacturers to create and test a prototype Nipah LFA and a protocol for smartphone-based optical reading of quantitative responses.

Scaling center operations: The pilot will create a prototype LFAs. As part of full center operations under CSI, we will aim to scale up production and test LFAs efficacy using control sera and field deployment as part of EID-SEARCH. We will additionally assess feasibility and cost of deployment, and compare performance against current laboratory-based assays.

Working Group 2 Activities: In addition to the two pilots above, working group members will contribute to our CSI monthly seminar series, presenting on topics including high-dimensional data models, field serology, serological assay development, laboratory product design, and public-private partnerships and commercialization. We will conduct two online workshops on serological data analysis, jointly with undergraduate, masters, and doctoral students at USU and UR, leveraging partnerships with field researchers currently working in serological surveillance. Our working group will lead a 3-day intensive workshop, where we will recruit additional participants with expertise in serological assay design and surveillance, immunology, emerging zoonoses, and focus discussion on key barriers to scaling serological surveillance for known and unknown zoonotic viruses.

Partners, Initial Working Group 2 Members, and Roles: EHA (coPI Ross): Model and pipeline design, software optimization. Uniformed Services University (SP Laing, SP Broder): data and model interpretation, reagent manufacture, assay design. SP Busa: device manufacturing, quality control, industry outreach and partnership development. We will recruit additional participants from our partner network engaged in serological field studies as well as industry representatives to be part of the Working Group for Aim 2.

Aim 3: Rapidly Identifying Anomalous Health Events

In early stages of epidemic spread, contact and mobility-induced behavioral data as well as communication patterns may provide a signal of health-seeking behavior, indicative of an outbreak cluster. We contend that these signals may be detectable in large-scale data streams of population mobility and contact, or in disease reporting available through telecommunications and health care providers. Timely detection requires approaches that can manage data at scale, analyze high-dimensional, spatio-temporally correlated streaming data in (pseudo) real time, and distinguish between known signals (e.g., typical influenza-related activity) and novel spillover-related signatures that we wish to unveil, characterize, and explain in order to trigger the necessary public policy responses.

This desideratum calls for fundamental methodological and computational advances, along with epidemiologically-relevant data sources, to describe how humans are moving and interacting across physical space with unprecedented temporal resolution and geographical coverage. Traditional flow-based mobility datasets used to parameterize epidemiological models have been aggregated and formatted in the form of origin-destination (OD) matrices. While valuable towards understanding population flows between regions of interest, OD matrices lack information on how much time a traveler spends in any given location. For spillover detection, duration of time spent in potentially risky geographical areas is key towards deciphering actionable contact and exposure patterns[54]. Moreover, recent studies have shown that flow counts between pairs of geographical areas can be loosely correlated with, and not representative of, epidemiology-relevant mixing patterns[55]. Instead, these couplings among subpopulations are measured by effective contact rates, meaning how often people from each

region come into contact in a way that is sufficient to transmit a disease. To address these challenges, our consortium will partner with the Facebook Data for Good team and leverage two recently assembled global-scale datasets - Activity Space Maps and Colocation Maps[54,55].

Our ambitious goal in this aim is to markedly advance the surveillance infrastructure available to predict anomalous health-related events before a major outbreak occurs. This differs from existing approaches that have focused on measuring the causal impact of mobility-oriented interventions. While undoubtedly a multi-faceted endeavor, in this planning grant we will take initial, but significant steps with an emphasis on network-centric, computational analyses of dynamic mobility and interaction data at a global scale. By developing the appropriate statistical tools, we will support the ultimate goal of characterizing early pandemic "fingerprints" or signatures that can be extracted from these global datasets.

Aim 3 Pilot Research Projects: Working closely with Facebook Data for Good (see Letter of Collaboration from Alex Pompe), this working group will pilot data-driven research activities to explore the feasibility of extracting early (post spillover) signals of epidemic onset from Activity Space Maps and Colocation Maps. Our multi-disciplinary working group will bring together leading experts in systems engineering, AI for healthcare, data science, mathematical epidemiology, graph signal and information processing, privacy-preserving mobility data generation and management, human behavioral modeling, and environmental science. Our current team, will instigate the cross-pollination of ideas leading to center-level activities while making significant inroads towards our grand challenge. We propose two specific pilot projects:

3a. Spatio-temporal monitoring of spillover-induced activity anomalies in Southeast Asia
Activity Space Maps (ASMs) are a novel global-scale movement and mobility data set which describes how people distribute their time through geographic space.[54] They are designed to complement existing digitally-collected mobility datasets by quantifying the amount of time that people spend in different locations. Accordingly, this information is valuable for estimating the duration of contact with the environment and the potential risk of exposure to disease -- something critical especially early after spillover. It also offers the unique possibility of developing and testing sequential change-point detection algorithms to monitor multiple spatial grid cells of interest (informed, for instance, by outcomes of the image-recognition effort under Aim 1). We will target areas known to include health care facilities in pursuit of abnormal trends of (increased) activity. Moreover, using ASMs we will examine flow and temporal activity anomalies around our expert-annotated wildlife and wet market locations, e.g.[56]. Said events are expected if there are human infections, or significant associated changes in behavior around these sites early after spillover. Our initial efforts during this planning grant will be focused on Southeast Asia, which on top of its epidemiological relevance is one of the regions where Facebook ASMs have the most coverage. We will target Indonesia, where EHA has extensive on-the-ground efforts tracing networks of wildlife trade as well as market sites.[56] We will also tap into the resources stemming from EHA's Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH) to identify clinics and market sites of interest in Thailand and Malaysia. Some key challenges we will address include accounting for the implicit biases in data collection associated with the construction of ASMs,[54] and their subsequent effects on the statistical algorithms that we will develop. Our Working Group will address additional challenges of data interpretation of anomaly detection analyses with input from emerging disease experts and epidemiologists. To calibrate our models, we will bring to bear recent activity data before and after the SARS-CoV-2 outbreak in those locations, in addition to historical influenza seasonal trends extending further back in time. With the prospect of new ASM data being officially released in the last quarter of 2021, *we are in a privileged position to be the first multi-disciplinary team to leverage this invaluable global-scale resource to advance our understanding of zoonotic spillover risk and its immediate effect on human mobility.*

3b. Online network change-point detection in dynamic, global-scale Colocation Maps
Activity data are complemented by *Colocation Maps (CMs),* which estimate how often people from different regions worldwide are colocated. In particular, for a pair of geographic regions *i* and *j* (e.g., counties in the US), CMs estimate the probability that a randomly chosen person from *i* and a randomly chosen person from *j* are simultaneously located in the same place during a randomly chosen minute in a given week[55]. In other words, CMs are global-scale, dynamic network datasets with colocation probabilities encoded as edge weights. Thus, they are well suited to parametrize metapopulation models of disease spread[57]. or to measure temporal changes in interactions between people from different regions[58]. To monitor said changes as part of our second pilot activity in this aim, we will build on our recent advances in online (i.e., sequential) algorithms for anomaly and change-point detection from network streams[59,60]; see also[61-63]. Our approach starkly contrasts with most existing graph change-point detection approaches, which either rely on extensive computation[64], or require storing and processing the entire observed CM time series[65]. We will prototype and test a lightweight online change-point detection algorithm with quantifiable performance guarantees, that is also explainable since it relies on interpretable node embeddings[59,66]. The latter feature will be leveraged to construct dynamic anomaly maps for user-friendly visualization of post-spillover events, such as our recent network-scientific visualizations of spatio-temporal COVID-19 contagion patterns across the US[67]. Moreover, the extreme size of the CM graphs calls for algorithmic and modeling innovations we will investigate as part of our research agenda, such as seamlessly integrating graph subsampling or dimensionality reduction techniques to the analytics pipeline. We will partner with computer vision experts affiliated to the University of Rochester Goergen Institute for Data Science (GIDS, see attached Letter of Collaboration from institute director Mujdat Cetin), to also explore the exciting prospect of integrating geo-tagged multimodal data such as video and imagery in our studies, for instance as nodal attributes embedded to the CMs.

Working Group 3 Activities: *Recruitment and diversity:* We will recruit 6 students from the GIDS-run master's program in data science, which attracts students from multidisciplinary backgrounds and significant computational proficiency to contribute in the aforementioned pilot research activities. *The CSI Leadership Team and broader UR community are committed to providing research training and educational experiences for those traditionally under-represented in STEM, including women and underrepresented minority students.* Our team has a solid track-record in actively recruiting and supervising such students at the undergraduate, masters and doctoral level, with demonstrated efforts towards broadening participation in STEM. As examples of the commitment to diversifying the field, co-PI Mateos serves as founding faculty advisor to the UR's SACNAS chapter and regularly organizes diversity, equity and inclusion panels to highlight challenges and successes of UR's Hispanic and Latino STEM community. We plan to prioritize engaging >50% women and minority students in the research and working group activities proposed here. *Coursework:* UR students will be on the existing health and bio track with an opportunity to take classes in immunology, microbiology and virology programs as well as data ethics at the University of Rochester Medical Center (URMC) as well as the college of engineering. Through our development grant, we plan to establish a special MS track on 'zoonotic surveillance' in order to train and mentor the students to lead center level activities. *Seminar and workshop series:* The students will be part of the large cohort of all the participating institutions and participate in the CSI monthly seminar series. The co-PIs (Mateos and Hoque) will conduct two workshops (and invite additional faculty members from UR as guest speakers) with hands-on lessons in mobility and human behavior modeling. *Hackathon:* The students will be encouraged to submit CSI projects in the yearly hackathon, Dandyhacks https://dandyhacks.net/), organized every fall by the University of Rochester

Computer Science Department and work with the dataset from Facebook. Additionally, co-PIs Mateos and Hoque will work with the organizers of Dandyhacks to release some of the public dataset from Facebook for other teams to explore, model, and mine the data.

Data visualization meetups at the University of Rochester's VISTA Collaboratory: To get acquainted with the different data resources at our disposal, over the first 3 months of the project the Rochester team (along with Facebook and EHA collaborators via Zoom) will hold bi-weekly team meetings with the MSc students at the UR's VISTA Collaboratory. This is a 1,000 square-feet visualization lab equipped with an interactive (20 ft. wide x 8 ft. tall) tiled-display wall, that renders massive data sets in real time, giving team members the ability to visualize and analyze complex data instantaneously and collaboratively (**Fig. 4)**. As we dissect what dataset components are central for the aforementioned pilot activities, our graduate students will develop customized data visualization tools, maps and dashboards to facilitate downstream analyses. Updates and progress reports will be shared in these meetings, giving the students a unique opportunity to develop their communication skills. The whole team will provide feedback and share ideas, making this a truly collaborative activity. Moving forward, the VISTA Collaborative offers a unique resource that we will fruitfully leverage as we scale up to full center operation.

Fig. 4. UR's Vista Collaborative will serve as a spillover intelligence "situation room" for training UR and CSI exchange students on analysis of real-time epidemiologically relevant datasets.

Partners, Initial Working Group 3 Members, and Roles: University of Rochester (co-PIs Hoque and Mateos): coordination of working group activities, development and testing of computational tools for early detection of post-spillover signatures, dissemination of research products and educational materials, recruiting and mentoring of master students, liaisons to GIDS. Facebook Data for Good (OP Pompe and Maps for Good team members Iyer and Citron): data ethics, quantification of privacy protections, data scientific support for Activity Space and Colocation Maps, mathematical epidemiology expertise for data and model interpretation. EHA (SP Mendelsohn and Ross): identification of target spatial locations in Southeast Asia, epidemiological interpretation of anomaly detection analyses and validation of detected early pandemic fingerprints.

Scaling center operations: First, our working group intensive 3-day workshop is designed to lead to new partnerships and synergies for expansion of Aim 3 activities. Second, we will expand our pilot anomaly detection—mobility data project to new geographies by adding new data on georeferenced clinics, wildlife markets and other relevant locations by engaging with our in-country network of partners. Third, we will test new streams of predictive data, e.g. mobile phone datasets, via expanded partnerships with public and private stakeholders to access and ingest these data. We will expand our policy engagement via EHA's *Alliance* and existing connections with WHO, FAO, the World Bank and others to develop behavioral risk interventions in the regions and populations we identify as high risk for zoonotic diseases.

Associated Risks and Mitigation Plans

We envision two primary risks associated with our proposed work 1) data access and privacy violations, and 2) biosafety concerns inherent with emerging zoonoses research. For data related risks, we have already established a strong collaborative relationship with FB's Data for Good team in developing this proposal and already have access to all the relevant datasets for our pilots. Data privacy concerns are taken seriously by our group, including deidentification of any personal identifying information following strict protocols on using human subject data from

FB and our already approved IRBs as part of EHA's NIH-funded work. We will amend or apply for additional IRBs as needed. For biosafety related risks, there will be no active sample collection under our proposed NSF project. We have already secured appropriate approvals (IRB, IACUCs, sampling permits) that outline PPE use and biosafety monitoring plans for our NIH-funded work. Our lateral flow assay development will be undertaken in laboratories designate at the appropriate Biosafety Level following US regulations (USU and other partner labs to be confirmed upon award).

Results from Prior NSF Support

Kevin J. Olival has not had prior NSF support, but is co-PI on NIH-funded "EID-SEARCH" (NIAID-CREID U01AI151797, 2020-25 PI Daszak). Previously co-PI on "Understanding the risk of bat coronavirus emergence" (NIAID R01 AI110964, 2014-2019, PI Daszak). ***Intellectual merit*:** Identified >50 SARSr-CoVs in bats, showed evidence of ability to infect human cells, humanized mouse models, and people in China, and used evolutionary and mammalian biodiversity patterns to analyze spillover risk for use in public health control. Produced 18 peer-reviewed papers [21,35,68-80], including two papers in *Nature* [81,82], and a review in *Cell* [83], and others submitted. ***Broader impacts*:** 2 PhD and 6 MS students trained; disease risk and potential management strategies shared with US agencies, WHO, OIE, FAO, and the public.

Ehsan Hoque: Project IIS-1750380. PI: Hoque. Period (2018-23). Amount: $511,453 "CAREER: A collaboration coach with effective intervention strategies to optimize group performance". ***Intellectual merit:*** Developing and validating the efficacy of automated mediation strategies in online meetings. ***Broader Impacts:*** >6 research papers[84-89]. **Project NRT-DESE-1449828.** PI: Hoque (2015-21). Amount: $2,965,341.00 "Graduate Training in Data-Enabled Research into Human Behavior and its Cognitive and Neural Mechanisms". ***Intellectual merit:*** Chartering a new traineeship program to train Ph.D. students at UR to advance discoveries at the intersection of computer science (cs), brain and cognitive sciences (bcs), and neuroscience. ***Broader Impacts:*** Supported over 50 PhD students in cs and bcs.

Gonzalo Mateos: **Project: CCF-1750428**. PI: Gonzalo Mateos Buckstein. Period: 4/1/18 - 3/31/23. Amount: $407,944. Title: CAREER: Inferring Graph Structure via Spectral Representations of Network Processes. ***Intellectual Merit:*** Using graph signals to estimate underlying network topology through innovative convex optimization approaches operating in the graph spectral domain. ***Broader Impacts:*** We prepared a feature article on identifying network structure via GSP[90], presented tutorials in EUSIPCO'19, CAMSAP'19 an SSP'20, organized the 2019 IEEE SPS Summer School on Network- and Data-driven Learning. Research products are available in[67,90-97].

Emily Mendenhall: National Science Foundation Doctoral Dissertation Improvement Grant, $10,000; Stories at the Border of Mind and Body: Mexican Immigrant Women's Narratives of Distress and Diabetes. **Intellectual merit:** Led to crucial insights studying syndemics - the interactions of social, environmental, psychological, and biological factors that are synergies of epidemics. Papers in The Lancet[30], Medical Anthropology Quarterly[98-100], and as a full-length book[101]. **Broader Impacts**: Understanding diabetes, trauma, and broader socio-political factors linked with infectious diseases in India, Kenya, South Africa, and Ethiopia.

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Project Management Plan

We have assembled a diverse team of experts in computational, biological, engineering, and social/behavioral research who share common interest in developing new approaches to advance *spillover intelligence* and prevent pandemics for a common good. Our multidisciplinary team will lead our proposed development project activities and build the foundations to grow our project in to the future *Center for Spillover Intelligence (CSI)*. Our team includes 5 primary institutions: EcoHealth Alliance (EHA), University of Rochester (UR), Georgetown University (GU), Uniformed Services University (USU), and Facebook's Data for Good (FB). All of these partnerships and collaborations are new, with the exception of previous collaborative work between EHA and USU. As such, we detail the plan below to facilitate the smooth communication, management, and implementation of our proposed project activities.

Specific roles of the PI, co-PIs, senior personnel, and consultants

Our project will be led by EHA as the prime organization (PI, Dr. Kevin Olival) and co-managed in conjunction with coPIs Ross (EHA), Hoque and Mateos (UR), and Mendenhall (GU). PI Olival will be responsible for all contractual obligations, including establishing subawards with UR and GU to support coPI salary, student support, and travel funds – as is detailed in our budget and budget justification. A parttime Program Coordinator at EHA will help support management and communications. USU will participate as an unfunded collaborator, as they are a government-supported institution. **Our Leadership Team (PI, coPIs, Senior Personnel)** includes a mixture of established scientists and early career leaders with diverse subject matter expertise. PI Olival will work closely with coPIs and other members of the Leadership Team to coordinate research and training activities, including with Senior Personnel (SP) Broder (serological assay development), Laing (molecular virology and serological expert), and Mendelsohn (data analytics and behavioral survey analysis); and subject matter expertise from Other Personnel (OP) Busa (biotechnology and assay development consultant) and Pompe (Facebook Data for Good liaison and data ethics). As communication is paramount for the success of our project and building new scientific collaborations, we will meet every month for 1-hour virtual meetings, and every 6 months in person (an annual Leadership Team meeting and workshop events) over the course of our project. We have budgeted for these in person meetings, as well as for 3 coPIs to accompany PI Olival to an in-person project close-out meeting with NSF.

Project management and coordination across organizations and disciplines

Our project is structured to facilitate meaningful cross-disciplinary engagement during the 18-month period of performance, via multidisciplinary pilot research projects, three working groups co-chaired by members of our Leadership Team with complementary subject matter expertise, 3-day workshops for each aim (for 12-15 people, including budget for 8 to travel from out of state), monthly project management calls and yearly dedicated Leadership Team in-person meetings, and cross-institution/cross-disciplinary student training. Our development level project was built to scale to a Center-level award (the Center for Spillover Intelligence, CSI) including the future addition of other Working Groups focused on priority research gaps yet-to-be identified, a Science and Policy Advisory Board for high level oversight and to liaise with other US and international institutions, and in transitioning our development Leadership Team (PI, coPIs and SP) into a more formal Steering Committee for Center-level oversight.

Coordination mechanisms for cross-organization and cross-disciplinary integration

We outline a detailed 18-month plan of activities to build and strengthen connections among our **5 partner institutions**, and break down disciplinary barriers that have hindered innovation and progress towards pandemic prevention. Our Leadership Team (to later grow in to a more formal CSI Steering Committee) will together oversee the project's overall goals, and ensure that the 3 working groups are aligned and sharing information. The Leadership Team will meet virtually via video conference once each month, and every six months in person.

Each of our 3 **working groups** will be led by two members of our steering committee, who will help identify and develop the full working group member list by the first 6 months of the project: Aim 1: coPI Mendenhall (GU) and SP Mendelsohn (EHA), Aim 2: coPI Ross (EHA) and SP Liaing (USU), Aim 3: coPI Hoque and coPI Mateos (RU). Each working group will be co-facilitated by one of our graduate student trainees, to develop skills in scientific program management, networking, and coordination and establish students as an additional layer of cross-team communication and collaboration.

Each of the three working groups will organize and host a **3-day intensive workshop** that will aim to identify existing gaps and synergies in each research area of our Grand Challenge and establish priorities for future Center development. We have budgeted for ~12 people to attend each workshop in person, with the aim of having 3 experts representing each of the following scientific domains: computer science, engineering, socio-behavioral, and biological. Facilities will be provided for ~12 more experts to join in virtually for a hybrid meeting. We will limit the total size of each workshop to 25 participants to enable effective discussions, breakout sessions brainstorming, and synthesis. Each workshop will culminate with a peer-reviewed, open access publication, and all materials and outputs from the workshop posted online.

Collaboration with existing disease surveillance and pandemic prevention initiatives: For Aim 2, we will leverage serological data collected under EHA's Emerging Infectious Disease Southeast Asia Research Hub (EID-SEARCH) project (2020-2025, PI Daszak, coPI Olival), one of 10 NIH-funded Center for Research in Emerging Infectious Diseases (https://creid-network.org). EID-SEARCH's partners include USU, Duke NUS, Chulalongkorn University in Thailand, and various clinics and institutions in Malaysia, and the project is currently screening large numbers of human and wildlife samples (>10K/yr) for various coronavirus, filovirus, and paramyxovirus antibodies. EHA additionally manages several biosurveillance projects funded by the US Department of Defense, Defence Threat Reduction Agency (DTRA) including one using multiplex serological assays to screen people and livestock for Henipavirus and Filovirus serology in Malaysia, that we will also collaborate with for Aim 2 model development.

Metrics of success and planning for Phase II (Center-level)

Center activity prioritization: Each Working Group will aim to define key gaps in their research area, research goals to address those gaps, and prioritization of research, implementation, and educational activities for the Phase II center to pursue. We will be successful if each group is able to prepare for publication a synthesis of these topics.

Advisory board development: Through recruitment of experts for workshops and partnerships within pilot activities, we will identify members of a Science and Policy Advisory board that would oversee the Phase II Center, as well as potential members of the Center Steering Committee beyond the Phase I leadership team. We will be successful if we have a fully-assembled team for prepared to initiate Phase II.

Pilot project success and scalability: Each working group will engage in two pilot projects aimed at facilitating larger-scale studies and surveillance of zoonotic intelligence via small-scale studies or methods development. We be successful if half of pilot projects result in success enable a Phase II study as part of research activity. Submitting peer-reviewed publications is another metric of success during Phase I.

Student and curriculum development: Our pilot phase will include seminars and student participation in workshops. We will be successful if these activities result in the development of a curriculum for a zoonotic intelligence MS track in the UR data science program, and the recruitment of our undergraduate and MS students into graduate programs in the field. The number of students that take part in and complete our semester-long Research Fellowships and 3-week Research Exchanges will be a metric of success.

Overview

The spillover and spread of wildlife-origin zoonotic viruses can have tragic societal and economic impacts, as is evidenced by the ongoing COVID-19 pandemic. Most emerging zoonoses are transmitted across species boundaries, or "spill over", when livestock or human populations increase contact with wildlife via changing ecological and behavioral risk factors. Despite significant scientific advances in understanding spillover in the last ~15 years, fundamental knowledge and implementation gaps stymie our ability to preempt and prevent future disease emergence. For example, prior to 2019 EcoHealth Alliance (EHA) and colleagues demonstrated that SARS-related coronaviruses in China pose a clear and present danger for a future human outbreak, but surveillance and research on these potential pathogens, their hosts, and their interface with humans was not conducted across a wide enough geography nor at a temporal or spatial scale needed for disease mitigation. Our grand challenge: How can we capture actionable data about the risk of zoonotic spillover (and early spread) at the scale and speed required to prevent outbreaks? In this planning proposal, we bring together a diverse team of established and emerging leaders from EHA, University of Rochester, Georgetown University, Uniformed Services University, and Facebook's Data for Good to build a collaborative research and training network to confront this grant challenge – designed to scale up to a future *Center for Spillover Intelligence (CSI)*. Our cross-disciplinary project will integrate computer scientists, engineers, and data scientists with emerging disease and social-behavioral researchers to accelerate the collection and interpretation of data to inform where, how, and what zoonotic viruses are poised to emerge.

Intellectual Merit

The primary intellectual merit of our project is in developing and piloting innovative approaches to large-scale spillover intelligence across previously siloed scientific domains. We will identify gaps and solutions for step-level changes in zoonotic spillover prediction via three multidisciplinary working groups that will engage in pilot projects, organize workshops, and engage students in immersive training. Pilot research projects will serve as a test bed for applying new theoretical frameworks to advance spillover intelligence. In Aim 1 we will transform decades-old anthropological approaches and pilot collecting large samples of quantitative and qualitative behavioral data via a unique public-private partnership with Facebook, and use image recognition and artificial intelligence to quantify and map human-wildlife contact. In Aim 2 we will advance rapid serological testing by developing rapid bioinformatics pipelines to process and interpret rich data from multiplex serological surveillance, and develop the first field-forward lateral-flow assay for Nipah virus antibody detection. In Aim 3 we will use cutting edge theoretical advances in network science, anomaly detection, and machine learning to detect changes in human mobility patterns that may portent spillover (e.g., increase congregation around wildlife markets) and that signal early epidemic spread after the point of spillover.

Broader impacts

We envision that our proposed project, and a future CSI, will transform our understanding of zoonotic spillover risk for public health via global scale data collection of survey, multimedia, and mobility data. All outputs from our 3 Working Group activities, including workshop materials, will be made publicly available, and published as both peer-reviewed manuscripts and popular press articles for wider dissemination. This development grant will initiate an interdisciplinary program to train a new generation of graduate and undergraduate students understanding medical anthropology, disease surveillance, and data and network science so as to become future leaders in spillover intelligence. It will include support for both semester-long research stipends to work on our pilot research projects and hands-on training via 3-week cross-institution research exchanges for 20 Master's level graduate students. Students from across all our institutions will participate in workshops, hackathons, and a monthly seminar series.

Multidisciplinary advances to scale up zoonotic spillover intelligence

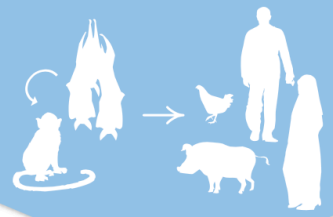
- The **Center for Spillover Intelligence** will engage leaders from diverse scientific disciplines coming together to transform the ways we think about, monitor, and respond to global viral threats.
- We will **accelerate and scale** actionable disease surveillance **to disrupt the future spillover and spread of zoonotic viruses**

Partner Institutions

EcoHealth Alliance · USU Uniformed Services University · University of Rochester · Georgetown University

Working Groups: Scalable Pilot Research and Planning Workshops

Aim 1: Human-animal contact



Piloting large-scale contact surveys with social media and image recognition

Aim 2: Disease exposure detection

Scaling serological surveillance via rapid bioinformatics and field-forward assays

Aim 3: Rapid outbreak identification

Anomaly detection to identify outbreaks in large-scale movement and network data

Interdisciplinary Synthesis

Pre-spillover intelligence → **Spillover intelligence** → **Post-spillover early detection**

Train the next generation of spillover intelligence researchers and leaders with hands-on research exchanges, seminar series, and workshop participation

Prepare to scale to Phase II with research prioritization workshops, partner and advisor recruitment, pilot evaluation, and curriculum development

From:	Kevin Olival on behalf of Kevin Olival <olival@ecohealthalliance.org>
To:	M. Ehsan Hoque; Mateos Buckstein, Gonzalo; Emily Mendenhall; Peter Daszak; Noam Ross; Emma Mendelsohn; Eric Laing; Chris Broder
Cc:	Kevin James Olival; Luke Hamel; Robin Breen; Aleksei Avery Chmura; Hongying Li
Subject:	NSF PIPP final versions submitted, and single powerpoint slide
Date:	Friday, October 1, 2021 5:18:36 PM
Attachments:	NSF_PIPP_ResearchDescription_v14.1.pdf ATT00002.bin Project Management Plan_v3.pdf ATT00004.bin Project Summary_v3.pdf ATT00006.bin PIPP_VisionSlide_Olival_1Oct2021.pptx ATT00008.bin
Importance:	High

Dear NSF PIPP Proposal Colleagues,

Our NSF PIPP proposal was successfully submitted via grants.gov about an hour ago! Please see my email below to NSF, as it was requested that all applicants submit single powerpoint slide of our project's vision via email right after we submit (Luke caught this just a couple hours before it was due, and we whipped the slide together).

Also attached for your reference are the **final Project Description, Project Summary,** and **Project Management Plan** that we submitted.

Please note in my message below that there seems to be a problem with grants.gov relaying our proposal over to research.gov (formerly Fastlane to get to NSF). Seems there is a backlog in the system (probably too many submissions), so we're hoping for positive news from research.gov that it clears (as of now still pending).

Thank you all for your hard work and collaboration on this, finger's crossed the submission full clears and we win this! Huge thanks to the EHA team: Noam, Emma, Luke, Aleksei, Hongying, and Robin for a huge team effort to get this done on our end.

Best regards,
Kevin

PROJECT DESCRIPTION

Introduction and Background

The spillover and spread of novel, zoonotic (animal origin) pathogens can have tragic societal and economic impacts, as is evidenced by the ongoing COVID-19 pandemic. The emergence of SARS-CoV-2 (the virus that causes COVID-19) is the latest in a series of viral emergence events that have accelerated in recent decades. In the last year, as the world continues to respond and adapt to COVID-19, we have seen several deadly zoonotic viruses re-emerge in the human population, including Ebola and Marburg in Guinea[1,2], Nipah virus in India[3], Rift Valley Fever virus in Kenya[4], and novel strains of influenza in China [REF]. Most emerging infectious diseases (EIDs) originate in wildlife reservoir hosts and are transmitted across species boundaries, or ("spill over"), when livestock or human populations increase contact with wildlife through factors including land use change, agricultural intensification, and the wildlife trade[5-7]. Zoonotic spillover is a dynamic process determined by many steps including 'pre-emergence' viral evolution, diversification, and transmission among wildlife hosts; 'human exposure' via direct or indirect contact between humans, livestock, and wildlife; 'post-exposure' limits to cell entry, host range, human-to-human transmission; and population-level virus amplification and spread. Once efficiently transmitted, EIDs spread rapidly through globalized travel and trade networks, threatening countries with high population growth, urbanization and international trade (**Fig 1**).

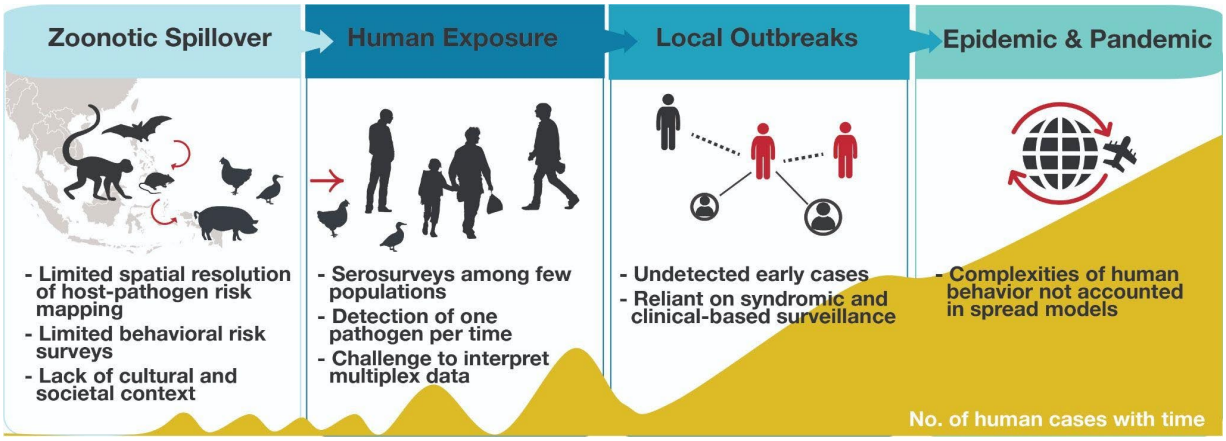


Fig 1. Four stages of zoonotic disease emergence and spread, with gaps in knowledge at each stage. Our proposed project will develop and pilot novel, multidisciplinary and technological advances to address critical gaps in the first three stages of emergence, when pandemic prevention is possible.

Novel human EIDs are typically only discovered when they cause severe or unusual symptoms, when astute clinicians are linked with laboratories capable of pathogen discovery research, and after they have been transmitted from person to person (after which control becomes more difficult).[7,8] Emerging human viruses characterized to date are a small sample of hundreds of thousands of predicted zoonotic viruses in nature but undiscovered due to limited surveillance capacity globally[9]. New research has upended the paradigm that zoonotic disease spillover is a rare event. A combination of more advanced molecular and serological assays, improved laboratory capacity around the world, and targeted surveillance of "high risk" human populations found evidence of frequent zoonotic virus transmission and infection, even in the absence of notable disease outbreaks. Often these spillover events lead to "dead end" infections in people or limited "stuttering chains" of transmission, a phenomenon referred to as "viral chatter"[10]. Community-based serological surveys from our group and others have illuminated relatively frequent and previously-unknown instances of zoonotic virus spillover including Henipaviruses

in bat hunters from Cameroon[11], SARS-related CoVs in rural communities living near caves in China (pre-2019), and Ebola virus exposure in DRC[12] and even Southeast Asia[13].

Innovative and multidisciplinary approaches are needed to disrupt the emergence of diseases at the "viral chatter" stage and further upstream at the human-animal interface to prevent epidemics and pandemics before they are out of control. While we have made significant scientific advances over the last ~15 years in understanding where and through what mechanisms novel zoonotic viruses spill over into the human population[5,14-17], there **are still fundamental knowledge and implementation gaps that stymie our ability to preempt and prevent future disease emergence events**.

Our Grand Challenge Problem

Prior to 2019, EcoHealth Alliance (EHA) and our partners demonstrated that SARS-related coronaviruses (SARSr-CoVs) in China pose a clear and present danger for a future human outbreak. We conducted extensive viral discovery in wildlife from China and Southeast Asia, phylogenetic analysis, and host-range experiments to show that certain bat species harbor a diverse assemblage of SARSr-CoVs[18,19], as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics[20-23]. We conducted behavioral risk factor surveys and serological sampling from human populations living close to caves and found serological evidence of SARSr-CoV spillover in people exposed to wildlife[24]. Building on this body of knowledge, we estimate bat-origin SARSr-CoVs infect 50,000 people in Southeast Asia each year[25]. While confidence intervals around this median estimate are large, our sensitivity analyses identify key area for future research to improve these spillover risk models: quantification and better contextual understanding of human-bat contact, the role of wildlife farming and livestock hosts, scaling up human serological surveys, and better data on sero-dynamics following novel SARSr-CoV exposure.[25]

Despite this strong body of evidence for SARSr-CoV spillover risk prior to the emergence of SARS-CoV-2, surveillance was not conducted across a wide enough geography, nor at a temporal or spatial scale to inform actionable disease mitigation. Despite large-scale efforts to strengthen zoonotic virus early warning systems, like the 10-year, 30 country USAID PREDICT program[26], led by a global consortium including EHA, current methods and systems cannot capture and disseminate enough data quickly enough to enable prevention measures at national or global scales. This is especially so for multiple and novel viral threats, and in large countries with heterogeneous human populations, diverse wildlife fauna, and known ecological and societal risk factors for emergence. Approaches to massively scale up zoonotic spillover surveillance are needed.

Our grand challenge problem: "How can we capture actionable data about the risk of zoonotic spillover and spread at the scale and speed required to prevent outbreaks?"
How can we leverage tools developed by computer scientists, data engineers, and technology companies to collect data from large, diverse populations and geographies to advance zoonotic spillover intelligence? How do we incorporate new insights from medical anthropology, sociology, wildlife biology, disease ecology, molecular virology, biomedical assay design, and data science to pioneer accelerate data collection and interpretation for zoonotic disease prediction and prevention? We propose to build a collaborative research and training network to confront this challenge, that will scale up to a future *Center for Spillover Intelligence (CSI)*. Our network will deeply integrate computer scientists, engineers, and data scientists with experts in emerging disease and social-behavioral research. New insights from social, behavioral, and anthropological research make it clear that the emergence and future trajectory of human epidemics require that we recognize potential biological risks alongside risks posed by cultural and societal factors[27-29]. Our cross-disciplinary, multi-institution project will combine innovative

research activities with training and education programs to facilitate to scale-up the collection and interpretation of data to inform where, how, and what zoonotic viruses are poised to emerge – a field we collectively refer to as *spillover intelligence*. Our inclusion of social-behavioral researchers, data ethicists, and inter-governmental EID policymakers will ensure that our aims and approaches are culturally and locally relevant, and align with our ultimate goal of improving global health.

Our Vision: We envision a world where diffuse data streams can be combined and interpreted in near real-time to disrupt the spillover and spread of future zoonotic viruses. We envision diverse leaders and scientific disciplines coming together to advance spillover intelligence and transform the ways we think about, monitor, and respond to global viral threats.

Our Team

We have assembled a multidisciplinary team of experts to lead our development project and to create and grow the Center for Spillover Intelligence (CSI). Our **Leadership Team (PI, coPIs, Senior Personnel)** includes established scientists and early career leaders working across the fields of emerging infectious diseases, computer and data science, engineering, mathematical modeling, virology, technology and biotechnology, data ethics, social science, and medical anthropology. Our project will be led by EHA (PI Olival) building off the foundational pandemic prediction work our institution has developed over the last decade, and include to new partnerships and collaborations. Thse include with computer scientists and engineers from University of Rochester (UR) and behavioral anthropologists from Georgetown University (GU). We will develop and pilot new technologies for field biosurveillance and data interpretation with Uniformed Services University (USU). Our leadership team is ethnically, culturally, and geographically diverse, including US-based researchers who originate from and/or currently conduct their primary research in global EID hotspot regions[5,14], e.g. South Asia (Hoque); Latin America (Mateos); Africa (Mendenhall); and Southeast Asia and the Pacific (Olival). Our geographic and disciplinary diversity, including long-standing international collaborators from each region, make our team well-equipped to address spillover at a global scale, while incorporating the nuance of local context.

PI Olival (EHA) is a disease ecologist and evolutionary biologist with a primary interest in developing novel analyses to target zoonoses surveillance and viral discovery. He currently leads two regional biosurveillance projects in Southeast and Western Asia, and recently served as Modeling & Analytics Coordinator for the USAID PREDICT project[26] overseeing a multi-institution team developing pandemic prediction strategies using public and project-specific data from 30 countries[5,15,30-35]. **coPI Mendenhall** (GU) is a medical anthropologist and Professor at the Edmund A. Walsh School of Foreign Service. Mendenhall works at the intersection of social, psychological, and biological lives, attempting to understand what makes people sick, why, and where. Mendenhall is currently a PI of NIH Fogarty International Center grant “Soweto Syndemics”, the first ever study to evaluate a syndemic from the ground up. **coPI Hoque** (UR) is an Associate Professor of Computer Science working at the intersection of Human-centric Computing (HCC) and Artificial Intelligence with a focus on multimodal machine learning with applications in health and wellness. Hoque is currently a PI of NSF NRT grant, and received NSF CAREER and CRII grant. Hoque has been identified as an ‘emerging leader of health and medicine’ at the National Academy of Medicine (NAM). **coPI Mateos** (UR) is Assoc. Professor of Electrical and Computer Engineering and a Data Science Fellow, his research focuses on algorithmic foundations, analysis, application of (graph) signal processing tools to the study of large-scale and dynamic networks. He has pioneered foundational work in network topology inference, online learning from graph streams, and interpretable representation learning for unstructured data. **coPI Ross** (EHA) is Principal Scientist for Computational Research. His research includes model development to interpret complex spatio-temporal disease surveillance

data in heterogeneous populations, real-time community and hospital testing, and simulating and forecasting epidemic dynamics over populations, space, and networks. He is PI on awards from DHS Science & Technology Directorate and the Defense Threats Research Agency (DTRA) for designing disease forecasting systems. **SP Mendelsohn** (EHA) is a data scientist specializing in the development and application of machine-learning models to draw insights into the occurrence and spread of emerging infectious diseases. With a focus on reproducible research methods and data management, she has been the lead analyst on data driven disease forecasting and behavioral survey-based projects characterizing animal-human contact patterns. **SP Daszak** (EHA) is an EID expert who has studied the process of disease emergence for decades and pioneered groundbreaking work in "hotspot" risk mapping to target surveillance. **SP Broder and Laing** (USU) are experts in the design of multiplex serological assays, molecular virology, and implementation of field-based human and wildlife biosurveillance strategies. **OP Busa** is a biotechnology consultant with a specialty in developing lateral flow assays and optical readers. **OP Pompe** is the Research Manager for Facebook Data for Good, who specializes in applying human mobility datasets from social media feeds and data privacy and ethical issues.

Intellectual Merit

The primary intellectual merit of our proposed project is in **developing and piloting innovative approaches to rapid and large-scale spillover intelligence across previously siloed scientific domains** including biology, computer science, electrical engineering, and social-behavioral research. We apply new theoretical advances and convergent research to address our grand challenge of capturing actionable data about the risk of zoonotic spillover and early spread at a scale and speed required to prevent outbreaks. We will identify gaps and solutions for step-level changes in zoonotic spillover prediction via three multidisciplinary working groups that will engage in pilot projects, organize workshops, and engage students in immersive training. Our pilot research projects will serve as a test bed for applying new theoretical frameworks to advance spillover intelligence test scalability in future work. For example, in Aim 1 we will transform decades-old anthropological approaches and pilot cost-effective methods to collect large samples of quantitative and qualitative behavioral data via unique public-private partnership with Facebook; and use image recognition and artificial intelligence to quantify and map human-wildlife contact globally. In Aim 2 we will develop rapid bioinformatics pipelines to process and interpret rich data from multiplex serological surveillance; and develop the first field-forward lateral-flow assay for Nipah virus antibody detection. In Aim 3 will use advances in network science, anomaly detection, and machine learning to detect changes in human mobility patterns that may portent spillover (e.g. increase congregation around wildlife markets) and that signal early epidemic spread after the point of spillover.

Broader impacts

This proposal could potentially transform our understanding of zoonotic spillover risks using survey, multimedia, and mobility data on a global scale, including aggregated and anonymized data from ~3B active Facebook users. Insights and intellectual outputs from our working group activities will have direct benefits to public health by informing disease mitigation strategies for pandemic prevention. We will synthesize our three working group outputs and prepare a special joint publication for the National Academies of Science's Forum on Microbial Threats for wide distribution, and write popular press articles to accompany our peer-reviewed manuscripts. EHA's *Alliance* includes a network of over 70 partner institutions in 30+ countries, presents a unique opportunity to work with local stakeholders in emerging virus 'hotspots' and validate models using data sets obtained through our long-standing collaboration with government partners including human health, agriculture, and environmental sectors.

We will train a new generation of graduate and undergraduate students in understanding medical anthropology, disease surveillance, and data and network science so as to become future leaders in spillover intelligence. Will do so via a unique partnership between the academic (UR, GU, USU), non-profit (EHA), and private (FB) sectors, allowing students to apply lessons learned from classroom settings to the real-world. Students will participate in project workshops, hackathons, monthly seminar series, and have access to state-of-the-art visualization and computing facilities. Our recruitment will focus on including >50% women and underrepresented minorities in science and engineering. This planning grant will be a path towards developing an interdisciplinary curriculum, including cross-training courses across institutions, and hands-on experience via internships and research exchanges inf. Through the planning grant, a new MS data science track on zoonotic surveillance will be established at UR, the first step in our plans to expand our Education and Training plan should we move towards a Center-level award in the future.

Education and Training Plan

Our Leadership Team will serve as mentors for our **CSI Student Training Program** that will offer a fellowship stipend to cross-train 20 Master's level graduate students via semester-long projects associated with proposed pilot research activities (3-4 students per project) and 3-week research exchanges between our institutions (UR, GU, EHA, USU, 4-8 students from each). The research exchange builds off EHA's successful NSF-funded EcoHealthNet training program (2011-21) and will take place in either the Summer of 2022 or 2023. We will link with currently-funded international programs at our respective institutions (e.g. Gui^{2}de in Nairobi, Kenya run by GU; and the NIH funded CREID research pilot program with EHA's EID-SEARCH project in Southeast Asia) to support students who desire to gain international, hands-on research experience as part of their research exchange. The focus of each research exchange will be on cross-disciplinary training, hands-on experience working with experts outside students area of study, and big-picture strategic thinking in pandemic prevention. Students will be drawn from respective departments across our institutions; Students with a computational background and with interests in interdisciplinary work will be recruited from UR's cross-department Goergen Institute for Data Science, Anthropology and International Affairs students from GU, and Emerging Disease and Virology students from USU. As EHA will recruit Ecology, Evolutionary Biology, and Environmental sciences students from universities where we have active partnerships, including Columbia University's E3B department where PI Olival and SP Daszak serve as adjunct faculty. We will explore specific mentorship opportunities with data scientists from Facebook. If awarded, we will additionally apply for funds through NSF's Research Experiences for Undergraduates (REU) program, with the goal of supporting summer stipend for 1 undergraduate per institution or the maximum allowable. We will host a joint monthly virtual seminar series (2 semesters long) open to graduate and undergraduate students from our respective institutions, with dedicated student "lab meetings" before each seminar. Two CSI students from diverse disciplines will lead each lab meeting discussion based on a deep read of relevant publications that each speaker will distribute in advance. The first semester of the seminar series will be led by our Leadership Team and Working Group experts, and the second semester will be for CSI Graduate students to present results or current challenges related to their pilot research projects. We hope to encourage these students to pursue Ph.D. studies. If we are successful at establishing a center through our planning grant, we should have access to a trained cohort of students with appropriate multidisciplinary skillset.

Project Overview

Our 18-month research agenda aims to understand how new methods and technologies can improve the efficiency, intensity, speed, and scope of zoonotic disease surveillance. **We have structured our development grant around three primary research aims** that will guide our interdisciplinary research and training activities. We will establish a working group composed of

multi-disciplinary experts for each primary aim that will be co-led by members of our Leadership Team. We will develop pilot research projects that capture different parts of the disease spillover process focused on specific geographies, and lead 3-day intensive workshops, monthly student seminars, and student training activities that cut across our inter-linked research aims.

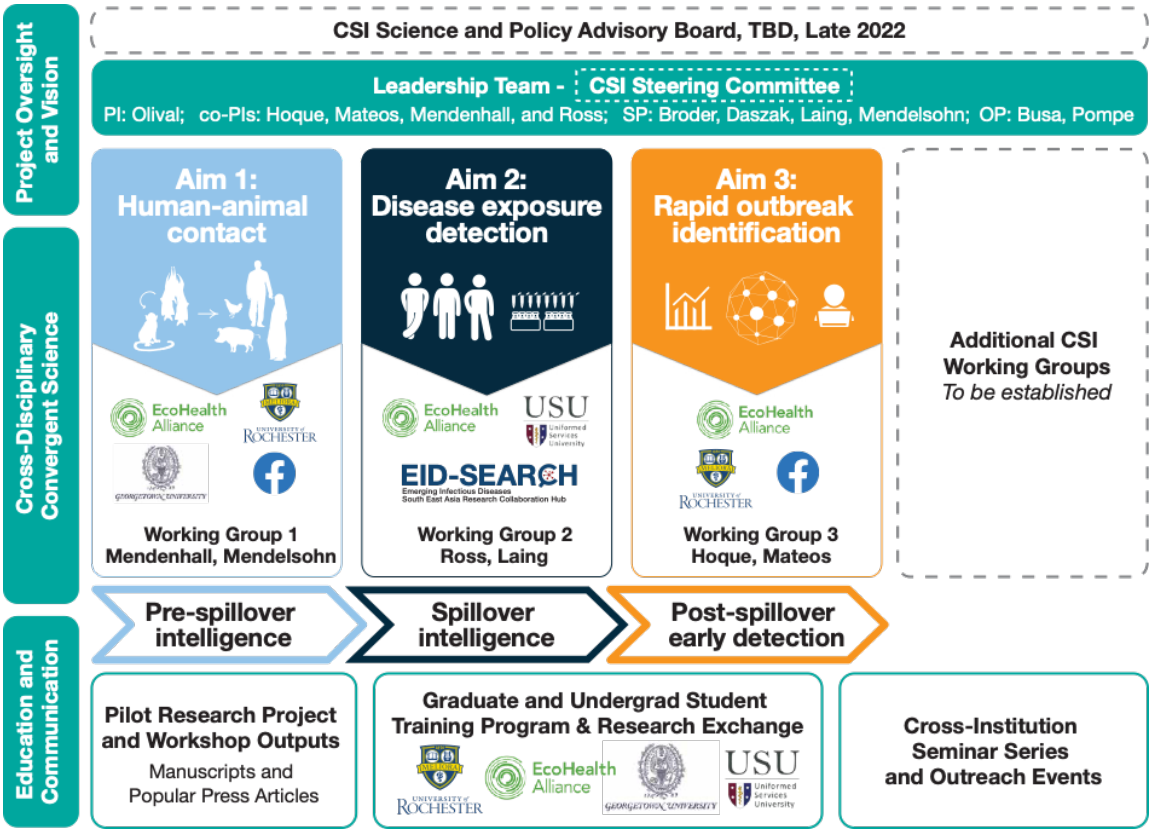


Fig 2. Project overview schematic showing our project management; three primary aims with graphical representation of pilot research activities to scale up and advance spillover intelligence; institutional partners leading activities for each aim including designated working group leads, and student education and communication activities, including pilot research projects, graduate student research exchanges, seminars, and other outreach activities. Our development-level project is built to scale to a Center level award (the Center for Spillover Intelligence, CSI) as noted with the dashed outline boxes, including the future addition of other CSI Working Groups, a Science and Policy Advisory Board, and transitioning our current Leadership Team (PI, coPIs and SP) into a more formal Steering Committee for oversight.

Aim 1: Measuring human-animal contact at scale

Interactions among humans, wildlife, and livestock create interfaces for pathogen spillover and are proximate risk factors for the emergence of all directly-transmitted zoonotic diseases.[14,36] Human-animal contact is commonplace around the world, with many factors mediating the type and frequency of contact, including cultural practices, environmental conditions, food security, and occupational demands.[36] In regions of the world considered prone to spillover--forested tropical landscapes with high biodiversity and recent land-use changes--understanding human-animal contact is crucial to pandemic prevention.[5] To measure these interactions and characterize associated behaviors, beliefs, and knowledge, researchers often implement resource-intensive surveys in key communities.[37,38] While crucial to understanding human-animal contact dynamics, on-the-ground surveys cannot yield scalable data that is comparable

across populations. Often, in place of surveys, researchers conducting analyses at broad scales rely on coarse estimates of contact such as the joint density of human and animal populations. **Our working group will uniquely combine social scientists, disease ecologists, and computational scientists to increase the scale at which we can directly measure human-animal contact.** We will develop and implement two pilot research projects. The first will use novel, social-media-based survey approaches to reach larger populations at higher frequencies while maintaining the cultural sensitivity and relevance of on-the-ground surveys. Working collaboratively with cross-disciplinary researchers, we will design surveys to understand social behaviors, cultural perceptions, and occupational hazards that may pose zoonotic spillover risk to individuals and communities. We will target geographies based on our existing spatial risk modeling work that maps infectious disease spillover potential and the ecological distributions of key reservoir host species.[5,15,25] Second, we will conduct pilot research on the use of social-media-based image recognition algorithms to identify locations of human contact with key species known to host high pathogen diversity. This pilot will include a collaboration effort to test the efficacy of such technology for understanding taxon-specific contact patterns globally, and to bring multidisciplinary context to meaningfully interpret the outputs (including species validation with taxonomic experts). If successful, will use these data on frequency of animal contact from image data to identify locations for additional survey data collection.

Aim 1 Pilot Research Projects: We will develop two pilot projects to assess the potential of 1a) social-media dispensed surveys and 1b) passive surveillance using artificial intelligence for image recognition to capture information about human-animal contact. We will target populations at wildland interfaces where contact with wildlife is likely high but internet penetration may be mixed.

1a) Social-media dispensed surveys to quantify and contextualize human-animal contact
We will pilot social-media dispensed, large scale population surveys about perceptions of and interactions with animals. We will optimize the design of these surveys for mobile platforms to facilitate higher response rates, and will also explore the possibility of longitudinal sampling (of either individuals or populations) to understand changes in conditions and responses over time. Survey questions will be based on a combination of EHA's past experience designing on-the-ground human-animal contact surveys[37,39,40] and ideas generated from our proposed working group activities involving anthropologists, data scientists, and disease ecologists who have worked extensively in the geographic and societal contexts in which we focus our work. We will design surveys that account for populations and individuals holding different beliefs, carrying different histories, and living in different ecologies. We will survey demographically and geographically diverse populations to capture direct and indirect animal-contact patterns and behaviors, and to capture the role that international, state, and local policy plays in individuals' lives. Further, we will aim to identify particular hesitancies among individuals to interact with technologies or other public health interventions.

We will explore multiple online platforms at the start of our project for survey administration. Given our unique public-private partnership with Facebook for many components of this project, using the Facebook platform to disseminate our pilot survey is likely to be a strong option, especially given Facebook's global reach (nearly half of the world's population, ~3 billion users), which introduces opportunities for digital interaction across class, gender, ethnicity, and cultural divides. We will leverage Facebook's extensive experience designing and administering surveys, especially for mobile devices, both directly on its platform (e.g. Yale Climate Change opinion survey) or off-platform (e.g. Carnegie Mellon University COVID-19 surveys. Facebook also has extensive privacy infrastructure and data-review policies to ensure anonymity and data security.

We will also explore creating games that users will be encouraged to play online to enable us to learn more about how they think about host species, viral threats, environment, and health/disease. For example, users may be given a list of 30 images and will be asked to put them into categories and describe what each category means. Adapting these "pile sorting" anthropological methods[41] to an online interface and for spillover risk will be a novel application with potential for a massive scale-up of data around cultural and conceptual dimensions of zoonotic disease risk. Data generated from this exercise will generate a more ethnographic understanding of people's experiences and thought processes, which tend to be locally mediated. We understand that there are geographic and demographic gaps in internet and social media use, and will incorporate bias corrections into our study design. We will initially focus on Southeast Asia, a region with a high likelihood of future zoonotic disease emergence and where EHA has good coverage of on-the-ground surveillance programs to ground truth online data collection.[5]

1b. Image recognition of zoonotic disease host species

We will pilot image-based surveillance of Facebook and Instagram feeds to attempt to identify human contact with key zoonotic disease host species and to explore the limits of taxonomic and behavioral recognition in images. We will work with Facebook Data for Good (see **Letter of Collaboration**) to adapt and refine their existing machine vision algorithms to target key taxa and to assess the efficacy of different approaches, working within strict guidelines and ethical standards for data de-identification. While data will be de-identified, we will photo meta-data in to not only quantify the location of animal contact in addition to species nature of contact. There are multiple image libraries that may serve as AI training datasets, including iNaturalist, the American Society of Mammologists, and Integrated Digitized Biocollections (iDigBio). We will iteratively implement and test algorithms starting with broad taxonomic groups (e.g., bats) and will explore the extent to which our approaches can identify finer taxonomic resolutions down to the genus or species level. In addition to taxa identification, we will explore image recognition of features that suggest specific types of human-animal contact, such as cages or market settings. We will use text and image metadata and explore using hashtags associated with images to boost the ability of our algorithms to identify key taxa.

This work will involve collaboration between machine learning scientists, biologists/ecologists, and social scientists. We will follow all data anonymity and privacy guidelines from Facebook and will also discuss data handling ethics in our working group. We will assess the ability of passive surveillance with image recognition to to target surveys to populations that have higher frequency of contact with wildlife, and ultimately to target biological surveillance of human and animal populations. We will consider both the accuracy of the image recognition algorithms as well as implications for survey distributions, results interpretation, and ethical data practices. We will also compare the spatial distribution of human-animal contact patterns derived from image recognition with previously generated spillover risk maps based on joint occurrences of animal and human distributions (e.g., SARS-related CoV spillover risk from Sanchez et al. 2021 preprint).[25]

Working Group 1 Activities: Our working group will convene 12-15 experts from the social sciences, disease ecology, and computational sciences for an intensive, 3 day workshop. We will structure the workshop to focus first on data ethics in human behavioral surveying and image recognition surveillance and secondly on survey design and analysis planning, with specific attention to accounting for gaps or biases in survey distributions. We will center the workshop on data collection that addressses long term social, ecological, and economic realities to understand how people perceive, experience, and interact with the risk of and repercussions from spillover events, rather than technological "quick fixes." We will also address experiences and challenges in engaging longtime ethnographers and local leaders to understand what's at stake for communities engaging with online and field surveillance programs. Our working group will help mentor students part of the CSI Student Training Program, to facilitate information sharing and idea generation among undergraduate and master-level students. Working group 1 will also contribute to the CSI Seminar Series with cross-disciplinary topics such AI image recognition (lead by a data scientist and bat biologist) and human-behavioral survey design (lead by an epidemiologist and medical anthropologist).

Partners, Initial Working Group 1 Members, and Roles: EHA (SP Mendelsohn): survey design and implementation, image recognition algorithm development and testing, novel data analyses. Facebook Data for Good (OP Pompe): survey implementation, image recognition algorithm development and testing, data ethics. University of Rochester (coPI Hoque): Image recognition algorithm development and testing. Georgetown University (coPI Mendenhall): Survey design with focus on ethnography and anthropology; online free listing/pile sorting game design; help lead expansion of WG to include emerging international leaders in social-behavioral science (e.g. Dr. Edna Bosire from Kenya). We will invite additional leaders from our extensive funded collaborations to be part of the Working Group for Aim 1.

Aim 2: Detecting zoonotic disease exposure at scale

Animal-human contact is the necessary condition for spillover of potential pandemic, directly-transmitted viruses. While a small fraction of such encounters result in exposure or infection, such exposures are frequent given the total sum of encounters. Some viruses such as Lassa spill over thousands of times per year[43]. Most spillover infections are never reported and rarely spread widely, and a very rare subset become widespread in the human population[42]. In areas of frequent spillover, the eco-evolutionary dynamics of "stuttering chains" of infection are what give rise to epidemics and pandemics[44]. Identifying populations where frequent exposure occurs is critical for deploying preventative measures and following viruses upstream to their wildlife hosts. Yet even large-scale surveillance projects cover too small populations and produce far too few detections to determine the most exposed geographic, demographic, or occupational groups at an actionable scale. This is for two reasons: field and laboratory cost of surveilling populations interacting with wildlife interface, and low detection rates of widely-used molecular PCR or metagenomic approaches.

We aim to drastically increase the scale at which populations can be monitored for exposure to animal-origin viruses by developing and piloting novel tools and analyses needed to implement large-scale serological surveys. While metagenomic methods have been favored for their ability to capture genetic sequences of previously unknown viruses, serological methods can be used to detect exposure to known and unknown viruses using multiplex assays tailored with a mixture of sensitivities and specificities[45]. Serological tools can detect antibodies for months or years after viral infection, rather than the limited window of days or weeks using molecular methods, and thus can provide a much richer profile of populations where pandemics may emerge[46]. In this planning period, we will begin pilots to test the feasibility of two complementary approaches

to enabling serological screening at large scales: 2a) the creation of rapid bioinformatics pipelines to interpret high-dimensional serological data from large streams of surveillance data, and 2b) the development of rapid field serological tests for wildlife-origin diseases.

Aim 2 Pilot Research Projects

2a. Rapid bioinformatics for high-throughput, multiplex serology

Members of our team (SP Laing, Broder) have developed multiplex serology tests that can be used to screen for exposure for many species and variants of filo-, henipa-, and coronaviruses simultaneously, and screening pipelines that can process samples at the scale of tens of thousands of samples. However, these tests pose challenges for interpretation, as high-dimensional results from multiplex bead or chip assays frequently contain signals of cross-reaction and autocorrelation (**Fig 3**). Through cross-disciplinary research between molecular virologists, serologists, mathematical modelers and data scientists on our team, we will create models to interpret this high-dimensional data and integrate them into a rapid bioinformatic pipeline that identifies individuals and populations to known viruses as well as flagging serological signals indicative of novel viruses. We will make use of data collected under our ongoing NIH and DTRA funded biosurveillance activities (Laing, Broder, Ross, Olival, Daszak) to develop detailed reaction/cross reaction profiles of a broad panel of zoonotic viruses to serological tests. We will build mechanistic, hierarchical Bayesian models that allow us to determine whether serological "profiles" are more likely to represent antibodies of known viruses, mixtures of multiple known viruses, or previously uncharacterized viruses. We will extend techniques of antigenic mapping,[47] using serological profiles to estimate phenotypic similarity of new viruses to known viral families, determine the correlation with phylogenetic relatedness, and use these measures to screen new viruses for pandemic potential. Using simulation experiments, we will test reduced-form, simple models against fully structured models so as to develop statistically robust procedures to interpret these data without high-performance computation.

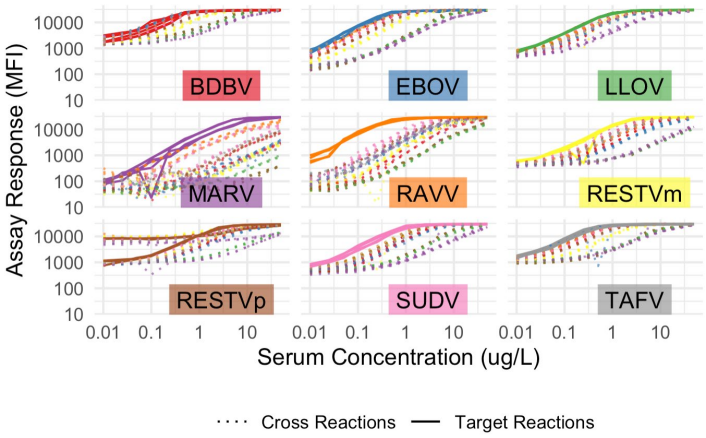


Fig 3*.* Preliminary data: Reaction-cross reaction plots of multiplex Luminex assay response to antibodies for closely-related filoviruses.

Scaling center operations: This pilot project will produce methods and a proof-of-concept pipeline for high-throughput analyses. We will leverage EHA's Emerging Infectious Disease – Southeast Asia Research Hub (EID-SEARCH) project, a NIH-funded Center for Research in Emerging Infectious Diseases (PI Daszak) who's partners include USU, Duke NUS, Chulalongkorn University in Thailand, and various clinics and institutions in Malaysia – currently screening large numbers of human samples (>10K/yr) for various coronavirus, filovirus, and paramyxovirus antibodies. In the future the CSI will implement surveillance studies screening these sample streams using multiplex serology, and utilize the analytical and bioinformatic tools we develop in the next 18 months. As part of our planning activities, we will establish protocols and approvals for this work.

2b. Development of a field-ready semi-quantitative lateral flow assays for an emerging zoonotic virus (Nipah virus)

Lateral flow assays (LFAs) are low-cost tests where reagents printed on cellulose strips react with a sample that flows via diffusion. LFAs are capable of providing quantitative results (in proportion to antibody titer) when produced via high-quality manufacturing and with carefully designed and calibrated optical scans[48]. LFAs can be used to provide diagnostics at low cost at the point of care and by consumers. Lack of commercial-scale applications has prevented LFAs from being used for serological testing for novel or emerging disease. To our knowledge, no studies have used LFAs for zoonotic virus serosurveillance in the field.

We will develop a proof-of-concept LFA for Nipah Virus (NiV). NiV has a high mortality rate and is considered to have pandemic potential that is ranked by CEPI as one of 7 high pandemic risk pathogens[49]. The range of NiV's primary wildlife hosts (*Pteropus spp,* fruit bats) covers South and Southeast Asia but human cases have only been detected in Malaysia, Bangladesh, and the Philippines. Spillovers occur regularly in Bangladesh, but are rarely reported in large portions of the country[50] and reporting is strongly associated with health care accessibility[51]. Broad-scale serological monitoring has the potential to identify populations from which pandemic strains may emerge. Through previous work, members of our working group (Laing, Broder) have developed multiplex serological assays for NiV[52] and the soluble proteins and reagents required for an LFA. We have also added experience in engineering, quality control and commercialization of LFAs (Busa, industry consultant) to our working group. For the pilot, we will engage with commercial manufacturers to create and test a prototype Nipah LFA and a protocol for smartphone-based optical reading of quantitative responses.

Scaling center operations: The pilot will create a prototype LFAs. As part of full center operations under CSI, we will aim to scale up production and test LFAs efficacy using control sera and field deployment as part of EID-SEARCH. We will additionally assess feasibility and cost of deployment, and compare performance against current laboratory-based assays.

Working Group 2 Activities: In addition to the two pilots above, working group members will contribute to the our CSI monthly seminar series, presenting on topics including high-dimensional data models, field serology, serological assay development, laboratory product design, and public-private partnerships and commercialization. We will conduct two online workshops on serological data analysis, jointly with undergraduate, masters, and doctoral students at USU and UR, leveraging partnerships with field researchers currently working in serological surveillance. Our working group will lead a 3-day intensive workshop, where we will recruit additional participants with expertise in serological assay design and surveillance, immunology, emerging zoonoses, and focus discussion on key barriers to scaling serological surveillance for known and unknown zoonotic viruses.

Partners, Initial Working Group 2 Members, and Roles: EHA (coPI Ross): Model and pipeline design, software optimization. Uniformed Services University (SP Liaing, SP Broder): data and model interpretation, reagent manufacture, assay design. SP Busa: device manufacturing, quality control, industry outreach and partnership development. We will recruit additional participants from our partner network engaged in serological field studies as well as industry representatives to be part of the Working Group for Aim 2.

Aim 3: Rapidly Identifying Anomalous Health Events

In early stages of epidemic spread, novel contact and mobility-induced behavioral data as well as communication patterns may provide signals of health-seeking activity indicative of an outbreak cluster. We contend that these signals may be detectable in large-scale data streams of population mobility and contact, or in disease reporting available through telecommunications and health care providers. Timely detection requires approaches that can manage data at scale,

analyze high-dimensional, spatio-temporally correlated streaming data in (pseudo) real time, and distinguish between known signals (e.g., typical influenza-related activity) and novel spillover-related signatures that we wish to unveil, characterize, and explain in order to trigger the necessary public policy responses.

This desideratum calls for fundamental methodological and computational advances, to go along with epidemiologically-relevant data sources that describe how humans are moving and interacting across physical space, with unprecedented temporal resolution and geographical coverage. Traditional flow-based mobility datasets used to parameterize epidemiological models have been aggregated and formatted in the form of origin-destination (OD) matrices. While valuable towards understanding population flows between regions of interest, OD matrices lack information on how much time a traveler spends in any given location. Naturally, when it comes to epidemiological studies the duration of time spent in potentially risky geographical areas is key towards deciphering actionable contact and exposure patterns[53]. Moreover, recent studies have shown that flow counts between pairs of geographical areas can be loosely correlated with, and not representative of, epidemiology-relevant mixing patterns[54]. Instead, these couplings among subpopulations are measured by effective contact rates, meaning how often people from each region come into contact in a way that is sufficient to transmit a disease. To address these challenges, our consortium will partner with the Facebook Data for Good team and leverage two recently assembled global-scale datasets - Activity Space Maps and Colocation Maps[53,54].

Our ambitious goal in this aim is to markedly advance the surveillance infrastructure available to predict anomalous health-related events before a major outbreak occurs. This differs from existing approaches that have focused on measuring the causal impact of mobility-oriented interventions. While undoubtedly a multi-faceted endeavor, in this planning grant we will take initial, but significant steps with an emphasis on network-centric, computational analyses of dynamic mobility and interaction data at a global scale. By developing the appropriate statistical tools, we will support the ultimate goal of characterizing early pandemic “fingerprints” or signatures that can be extracted from these global datasets

Aim 3 Pilot Research Projects: Working closely with Facebook Data for Good (see Letter of Collaboration from Alex Pompe), this working group will pilot data-driven research activities to explore the feasibility of extracting early (post spillover) signals of epidemic onset from Activity Space Maps and Colocation Maps. Our multi-disciplinary working group brings together leading experts in systems engineering, AI for healthcare, data science, mathematical epidemiology, graph signal and information processing, privacy-preserving mobility data generation and management, human behavioral modeling, and environmental science. We believe the current team, consisting of a wide array expertise and partnership, will instigate the cross-pollination of ideas leading to center-level activities while making significant inroads towards addressing our grand challenge. We propose two specific pilot projects:

3a. Spatio-temporal monitoring of spillover-induced activity anomalies in Southeast Asia
Activity Space Maps (ASMs) are a novel global-scale movement and mobility data set which describes how people distribute their time through geographic space.[53] They are designed to complement existing digitally-collected mobility datasets by quantifying the amount of time that people spend in different locations. Accordingly, this information is valuable for estimating the duration of contact with the environment and the potential risk of exposure to disease -- something critical especially early after spillover. More germane to the theme of this aim, it also offers the unique possibility of developing and testing sequential change-point detection algorithms to monitor multiple spatial grid cells of interest (informed, for instance, by outcomes of the image-recognition effort under Aim 1). We will target areas known to include health care

facilities in pursuit of abnormal trends of (increased) activity. Moreover, using ASMs we will examine flow and temporal activity anomalies around expert-annotated wildlife and wet market locations. Said events are expected if there are human infections, or significant associated changes in behavior around these sites early after spillover. Our initial efforts during this planning grant will be focused on Southeast Asia, which on top of its epidemiological relevance is one of the regions where Facebook ASMs have the most coverage. We will target Indonesia, where EHA has extensive on-the-ground efforts tracing networks of wildlife trade as well as market sites.[55] We will also tap into the resources stemming from EHA's Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH) to identify clinics and market sites of interest in Thailand and Malaysia. Some key challenges we will address include accounting for the implicit biases in data collection associated with the construction of ASMs,[53] and their subsequent effects on the statistical algorithms that we will develop. Our Working Group will address additional challenges of data interpretation of anomaly detection analyses with input from emerging disease experts and epidemiologists. To calibrate our models, we will bring to bear recent activity data before and after the SARS-CoV-2 outbreak in those locations, in addition to historical influenza seasonal trends extending further back in time. With the prospect of new ASM data being officially released in the last quarter of 2021, *we are in a privileged position to be the first multi-disciplinary team that leverages this invaluable global-scale resource to advance our understanding of zoonotic spillover risk and its immediate effect on human mobility.*

3b. Online network change-point detection in dynamic, global-scale Colocation Maps
Activity data are complemented by *Colocation Maps (CMs),* which estimate how often people from different regions worldwide are colocated. In particular, for a pair of geographic regions *i* and *j* (e.g., counties in the US), CMs estimate the probability that a randomly chosen person from *i* and a randomly chosen person from *j* are simultaneously located in the same place during a randomly chosen minute in a given week.[54] In other words, CMs are global-scale, dynamic network datasets with colocation probabilities encoded as edge weights. Thus, they are well suited to parametrize metapopulation models of disease spread,[56] or to measure temporal changes in interactions between people from different regions.[57] To monitor said changes as part of our second pilot activity in this aim, we will build on our recent advances in online (i.e., sequential) algorithms for anomaly and change-point detection from network streams[58,59]; see also.[60-62] Our approach starkly contrasts with most existing graph change-point detection approaches, which either rely on extensive computation,[63] or require storing and processing the entire observed CM time series.[64] We will prototype and test a lightweight online change-point detection algorithm with quantifiable performance guarantees, that is also explainable since it relies on interpretable node embeddings.[58,65] The latter feature will be leveraged to construct dynamic anomaly maps for user-friendly visualization of post-spillover events, such as our recent network-scientific visualizations of spatio-temporal COVID-19 contagion patterns across the US.[66] Moreover, the extreme size of the CM graphs calls for algorithmic and modeling innovations we will investigate as part of our research agenda, such as seamlessly integrating graph subsampling or dimensionality reduction techniques to the analytics pipeline. We will partner with computer vision experts affiliated to the University of Rochester Goergen Institute for Data Science (GIDS, see attached Letter of Collaboration from institute director Mujdat Cetin), to also explore the exciting prospect of integrating geo-tagged multimodal data such as video and imagery in our studies, for instance as nodal attributes embedded to the CMs.

Working Group 3 Activities: *Recruitment and diversity:* We will recruit 6 students from the GIDS-run master's program in data science, which attracts students from multidisciplinary backgrounds and significant computational proficiency to contribute in the aforementioned pilot research activities. *The CSI leadership and broader community are committed to providing research training and educational experiences for those traditionally under-represented in STEM, including women and underrepresented minority students.* The PI team has a solid track-record in actively recruiting and supervising such students at the undergraduate, masters and doctoral level, with demonstrated efforts towards broadening participation in STEM. As examples of the commitment to diversifying the field, co-PI Mateos serves as founding faculty advisor to the UR's SACNAS chapter and regularly organizes diversity, equity and inclusion panels to highlight challenges and successes of UR's Hispanic and Latino STEM community. We plan to prioritize engaging women and minority students in the research and working group activities proposed here. *Coursework:* These students will be on the existing health and bio track with an opportunity to take classes in immunology, microbiology and virology programs as well as data ethics at the University of Rochester Medical Center (URMC) as well as the college of engineering. Through the planning grant, we plan to establish a special MS track on 'zoonotic surveillance' in order to train and mentor the students to lead center level activities. *Seminar and workshop series:* The students will be part of the large cohort of all the participating institutions and participate in the CSI monthly seminar series. The co-PIs (Mateos and Hoque) will conduct two workshops (and invite additional faculty members from UR as guest speakers) with hands-on lessons in mobility and human behavior modeling. *Hackathon:* The students will be encouraged to participate in the yearly hackathon, Dandyhacks https://dandyhacks.net/), organized every fall by the University of Rochester Computer Science Department and work with the dataset from Facebook. Additionally, co-PIs Mateos and Hoque will work with the organizers of dandyhacks to release some of the public dataset from Facebook for other teams to explore, model and mine the data. *Data visualization meetups at the University of Rochester's VISTA Collaboratory*: To get acquainted with the different data resources at our disposal, over the first 3 months of the project the Rochester team (along with Facebook and EHA collaborators via Zoom) will hold bi-weekly team meetings with the MSc students at the UR's VISTA Collaboratory. This is a 1,000 square-feet visualization lab equipped with an interactive (20 ft. wide x 8 ft. tall) tiled-display wall, that renders massive data sets in real time, giving team members the ability to visualize and analyze complex data instantaneously and collaboratively; see Fig. 4. As we dissect what dataset components are central for the aforementioned pilot activities, our graduate students will develop customized data visualization tools, maps and dashboards to facilitate downstream analyses. Updates and progress reports will be shared in these meetings, giving the students a unique opportunity to develop their communication skills. The whole team will provide feedback and share ideas, making this a truly collaborative activity. Moving forward, the VISTA Collaborative offers a unique resource that we will fruitfully leverage as we scale up to full center operation.

Fig. 4. UR's Vista Collaborative will serve as a spillover intelligence "situation room" for training UR and CSI exchange students on analysis of real-time epidemiologically relevant datasets.

Partners, Initial Working Group 3 Members, and Roles: University of Rochester (co-PIs Hoque and Mateos): coordination of working group activities, development and testing of computational tools for early detection of post-spillover signatures, dissemination of research products and educational materials, recruiting and mentoring of master students, liaisons to

GIDS. Facebook Data for Good (OP Pompe and Maps for Good team members Iyer and Citron): data ethics, quantification of privacy protections, data scientific support for Activity Space and Colocation Maps, mathematical epidemiology expertise for data and model interpretation. EHA (SP Mendelsohn and Ross): identification of target spatial locations in Southeast Asia, epidemiological interpretation of anomaly detection analyses and validation of detected early pandemic fingerprints.

Scaling operations: Our programs and pilot research are designed to scale. First, we aim to expand on successful pilots by expanding the geographies of our work, engaging with more in-country partners. Second, we test the development of each of these new streams of predictive data, we plan to develop partnerships with local, national, and global agencies to ingest these data, expand into programs to develop behavioral interventions and testing in response to identifying regions and populations at high risk of contracting zoonotic diseases.

Working groups are really about bringing our project to scale, our intensive 3-day workshop will also lead to new partnerships and synergies for expansion.

Results from Prior NSF Support

Kevin J. Olival has not had prior NSF support, but is co-PI on NIH-funded "EID-SEARCH" (NIAID-CREID U01AI151797, 2020-25 PI Daszak). Previously co-PI on "Understanding the risk of bat coronavirus emergence" (NIAID R01 AI110964, 2014-2019, PI Daszak). ***Intellectual merit*:** Identified >50 SARSr-CoVs in bats, showed evidence of ability to infect human cells, humanized mouse models, and people in China, and used evolutionary and mammalian biodiversity patterns to analyze spillover risk for use in public health control. Produced 18 peer-reviewed papers [20,34,67-79], including two papers in *Nature* [80,81], and a review in *Cell* [82], and others submitted. ***Broader impacts*:** 2 PhD and 6 MS students trained; disease risk and potential management strategies shared with US agencies, WHO, OIE, FAO, and the public.

Ehsan Hoque: Project IIS-1750380. PI: Hoque. Period (2018-23). Amount: $511,453 "CAREER: A collaboration coach with effective intervention strategies to optimize group performance". ***Intellectual merit:*** Developing and validating the efficacy of automated mediation strategies in online meetings. ***Broader Impacts:*** >6 research papers[83-88]. **Project NRT-DESE-1449828.** PI: Hoque (2015-21). Amount: $2,965,341.00 "Graduate Training in Data-Enabled Research into Human Behavior and its Cognitive and Neural Mechanisms". ***Intellectual merit:*** Chartering a new traineeship program to train Ph.D. students at the University of Rochester to advance discoveries at the intersection of computer science (cs), brain and cognitive sciences (bcs), and neuroscience. ***Broader Impacts:*** Supported over 50 PhD students in cs and bcs, seveal won interdisciplinary research positions at R1 institutes.

Gonzalo Mateos (a) Project: CCF-1750428. PI: Gonzalo Mateos Buckstein. Period: 4/1/18 - 3/31/23. Amount: $407,944. (b) Title: CAREER: Inferring Graph Structure via Spectral Representations of Network Processes. (c) ***Intellectual Merit:*** We investigate how to use information available from graph signals to estimate the underlying network topology through innovative convex optimization approaches operating in the graph spectral domain. ***Broader Impacts:*** We prepared a feature article on identifying network structure via GSP,[89] presented tutorials in EUSIPCO'19, CAMSAP'19 an SSP'20, organized the 2019 IEEE SPS Summer School on Network- and Data-driven Learning. (d) Research products are available in.[66,89-96]

Emily Mendenhall (a) National Science Foundation Doctoral Dissertation Improvement Grant, $10,000; Stories at the Border of Mind and Body: Mexican Immigrant Women's Narratives of Distress and Diabetes. **Intellectual merit:** Led to crucial insights studying syndemics - the interactions of social, environmental, psychological, and biological factors that are synergies of epidemics. Papers in The Lancet[29], Medical Anthropology Quarterly[97-99], and as a full-length

book[100]. **Broader Impacts**: Understanding diabetes, trauma, and broader socio-political factors with infectious diseases in India, Kenya, South Africa, and Ethiopia.

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PROJECT DESCRIPTION

Introduction and Background

The spillover and spread of novel, zoonotic pathogens can have tragic societal and economic impacts, as is evidenced by the ongoing COVID-19 pandemic. Unfortunately, the emergence of SARS-CoV-2 (the virus that causes COVID-19) is just the latest in a series of viral emergence events that have been accelerating over the last several decades. In just the last year, as the world continues to respond and adapt to COVID-19, we have seen several deadly zoonotic (animal origin) viruses re-emerge in the human population, including Ebola and Marburg in Guinea[1,2], Nipah virus in India[3], Rift Valley Fever virus in Kenya[4], and novel strains of influenza in China [REF]. Most emerging infectious diseases (EIDs) originate in wildlife reservoir hosts and are transmitted across species boundaries, or ("spill over"), when livestock or human populations increase contact with wildlife through factors including land use change, agricultural intensification, and the wildlife trade[5-7]. Zoonotic spillover is a dynamic process determined by many steps including 'pre-emergence' viral evolution, diversification, and transmission among wildlife hosts; 'human exposure' via direct or indirect contact between humans, livestock, and wildlife; 'post-exposure' limits to cell entry, host range, human-to-human transmission; and population level virus amplification and spread. Once efficiently transmitted, EIDs can spread rapidly through globalized travel and trade networks, threatening countries with high population growth, urbanization and international trade (**Fig 1**).

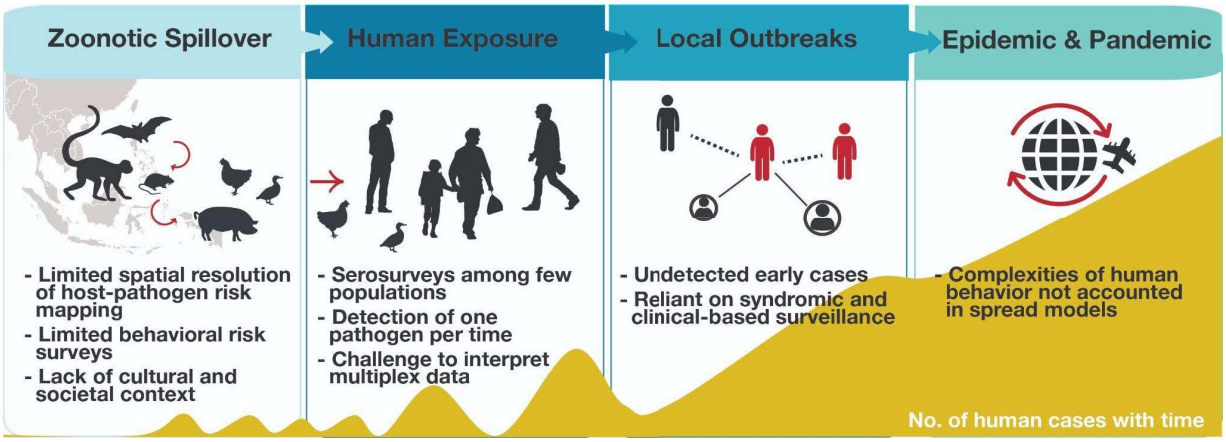

Fig 1. Four stages of zoonotic disease emergence and spread, with gaps in knowledge at each stage. Our proposed project will develop and pilot novel, multidisciplinary and technological advances to address critical gaps in the first three stages of emergence, when pandemic prevention is possible.

Novel human EIDs are typically only discovered when they cause severe or unusual symptoms, when astute clinicians are linked with laboratories capable of pathogen discovery research, and after they have been transmitted from person to person (in which case control options become more difficult).[7,8] The emerging human viruses characterized to date likely represent just the tip of the iceberg given the hundreds of thousands of predicted zoonotic viruses that exist in nature and limited surveillance capacity globally[9]. New research has disrupted the paradigm that zoonotic disease spillover is a rare event. A combination of more advanced molecular and serological assays, improved laboratory capacity around the world, and targeted surveillance of "high risk" human populations have led to evidence of zoonotic virus transmission and infection, even in the absence of notable disease outbreaks. Often these spillover events lead to "dead end" infections in people or limited 'stuttering' chains of transmission, a phenomenon referred to as "viral chatter"[10]. Community-based serological surveys from our group and others have illuminated relatively frequent and previously-unknown instances of zoonotic virus spillover

including Henipaviruses in bat hunters from Cameroon[11], SARS-related CoVs in rural communities living near caves in China (pre-2019), and Ebola virus exposure in DRC[12] and even Southeast Asia[13]

It is during these early stages of emergence, i.e. "viral chatter" with limited human transmission and even further upstream to include pre-emergence risk analyses at the human-animal interface, where innovative convergent research approaches are most needed for pandemic prevention. While we have made significant scientific advances over the last ~15 years in understanding where and through what mechanisms novel zoonotic viruses spill over into the human population[5,14-17], there **are still fundamental knowledge (and implementation) gaps that stymie our ability to preempt and prevent future disease emergence events**.

Our Grand Challenge Problem

Prior to 2019, EcoHealth Alliance (EHA) and colleagues demonstrated that SARS-related coronaviruses (SARSr-CoVs) in China pose a clear and present danger for a future human outbreak. We conducted extensive viral discovery in wildlife from China and Southeast Asia, phylogenetic analysis, and host-range experiments to show that certain bat species harbor a diverse assemblage of SARSr-CoVs[18,19], as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics[20-23]. We conducted behavioral risk factor surveys and serological sampling from human populations living close to caves and found serological evidence of SARSr-CoV spillover in people exposed to wildlife[24]. Building on this body of knowledge, our recent models suggest that bat-origin SARSr-CoVs may be infecting 400,000 (median 50,000) people in Southeast Asia each year[25]. While confidence intervals around these estimates are large, our sensitivity analyses importantly identified key area for future research to improve these spillover risk models - notably, quantification and better contextual understanding of human-bat contact, the role of wildlife farming and livestock hosts, scaling up human serological surveys, and better data on sero-dynamics following novel SARSr-CoV exposure.[25]

Despite this strong body of evidence for SARSr-CoV spillover risk (prior to the emergence of SARS-CoV-2), surveillance and research on these potential pathogens, their hosts, and their interface with humans was not conducted across a wide enough geography, nor at a temporal or spatial scale to inform actionable disease mitigation. Even for large-scale efforts to strengthen zoonotic virus early warning systems, like the 10-year, 30 country USAID PREDICT program[26], led by a global consortium including EHA, it's nearly impossible to capture enough data and disseminate that quickly enough to lead to widespread prevention measures at a national scale using existing methods – especially when considering multiple potential viral threats at once. This is especially true for large countries with heterogeneous human populations, diverse wildlife fauna, and known ecological and societal risk factors for emergence. Approaches to massively scale up zoonotic spillover surveillance are needed.

Our grand challenge problem: "How can we capture actionable data about the risk of zoonotic spillover (and early spread) at the scale and speed required to prevent outbreaks?" Can we leverage tools developed by computer scientists, data engineers, and technology companies to collect data from diverse populations and geographies to advance zoonotic spillover intelligence to a global scale? Can we incorporate new insights from medical anthropology, sociology, wildlife biology, disease ecology, molecular virology, biomedical assay design, and data science and use these to pioneer new methods in data collection and interpretation for zoonotic disease prediction and prevention? In this development proposal, we propose to build a collaborative research and training network to confront this challenge, designed at the start to scale up to a future *Center for Spillover Intelligence (CSI).* Our network will deeply integrate computer scientists, engineers, and data scientists using machine learning and other novel technological approaches together with experts in emerging disease and social-

behavioral research. New insights from social, behavioral, and anthropological research make it clear that the emergence and future trajectory of human epidemics require that we recognize potential biological risks alongside risks posed by cultural and societal factors[27-29]. Our cross-disciplinary, multi-institution project will combine synergistic research activities with training and education programs to facilitate new innovations to scale-up the collection and interpretation of data to inform where, how, and what zoonotic viruses are poised to emerge – a field we collectively refer to as *Spillover Intelligence*. Our inclusion of social-behavioral researchers, data ethicists, and inter-governmental EID policymakers will ensure that our aims and approaches are culturally and locally relevant, and align with our ultimate goal of improving global health.

Our Vision: We envision a world where diffuse data streams can be combined and interpreted in near real-time to disrupt the spillover and spread of future zoonotic viruses. We envision diverse leaders and scientific disciplines coming together to advance spillover intelligence and transform the ways we think about, monitor, and respond to global viral threats.

Our Team

We have assembled a multidisciplinary team of experts to lead our development project and to create and grow the Center for Spillover Intelligence (CSI). Our **Leadership Team (PI, coPIs, Senior Personnel)** includes a mixture of established scientists and early career leaders working across the fields of: emerging infectious diseases, computer and data science, engineering, mathematical modeling, virology, technology and biotechnology, data ethics, social science, and medical anthropology. Our project will be led by EHA (PI Olival) building off the foundational pandemic prediction work our institution has developed over the last decade, but expanding to new partnerships and collaborations we have not previously been able to explore – including with computer scientists and engineers from University of Rochester (UR) and behavioral anthropologists from Georgetown University (GU). We will develop and pilot new technologies for field biosurveillance and data interpretation with Uniformed Services University (USU). Our Leadership Team is ethnically, culturally, and geographically diverse, including US-based researchers who originate from and/or currently conduct their primary research in global EID hotspot regions[5,14], e.g. South Asia (Hoque); Latin America (Mateos); Africa (Mendenhall); and Southeast Asia and the Pacific (Olival). Our geographic and disciplinary diversity, including long-standing international collaborators from each region make our team well equipped to address spillover at a global scale, while understanding the importance of local context.

PI Olival (EHA) is a disease ecologist and evolutionary biologist with a primary interest in developing novel analyses to target zoonoses surveillance and viral discovery. He currently leads two regional biosurveillance projects in Southeast and Western Asia, and recently served as Modeling & Analytics Coordinator for the USAID PREDICT project[26] overseeing a multi-institution team developing pandemic prediction strategies using public and project-specific data from 30 countries[5,15,30-35]. **coPI Mendenhall** (GU) is a medical anthropologist and Professor at the Edmund A. Walsh School of Foreign Service. Mendenhall works at the intersection of social, psychological, and biological lives, attempting to understand what makes people sick, why, and where. Mendenhall is currently a PI of NIH Fogarty International Center grant "Soweto Syndemics", the first ever study to evaluate a syndemic from the ground up. **coPI Hoque** (UR) is an Associate Professor of Computer Science working at the intersection of Human-centric Computing (HCC) and Artificial Intelligence with a focus on multimodal machine learning with applications in health and wellness. Hoque is currently a PI of NSF NRT grant, and received NSF CAREER and CRII grant. Hoque has been identified as an 'emerging leader of health and medicine' at the National Academy of Medicine (NAM). **coPI Mateos** (UR) is Assoc. Professor of Electrical and Computer Engineering and a Data Science Fellow, his research focuses on algorithmic foundations, analysis, application of (graph) signal processing tools to the study of large-scale and dynamic networks. He has pioneered foundational work in network topology

inference, online learning from graph streams, and interpretable representation learning for unstructured data. **coPI Ross** (EHA) is Principal Scientist for Computational Research. His research includes model development to interpret complex spatio-temporal disease surveillance data in heterogeneous populations, including from serological surveys, real-time community and hospital testing, and simulating and forecasting epidemic dynamics over populations, space, and networks. He is PI on awards from DHS Science & Technology Directorate and the Defense Threats Research Agency (DTRA) for designing disease forecasting systems. **SP Mendelsohn** (EHA) is a research data scientist specializing in the development and application of machine-learning models to draw insights into the occurrence and spread of emerging infectious diseases. With a focus on reproducible research methods and data management, she has been the lead analyst on data driven disease forecasting and behavioral survey-based projects characterizing animal-human contact patterns. **SP Daszak** (EHA) is an EID expert who has studied the process of disease emergence for decades and pioneered groundbreaking work in "hotspot" risk mapping to target surveillance. **SP Broder and Laing** (USU) are experts in the design of multiplex serological assays, molecular virology, and implementation of field-based human and wildlife biosurveillance strategies. **OP Busa** is a biotechnology consultant with a specialty in developing lateral flow assays and optical readers. **OP Pompe** is the Research Manager for Facebook Data for Good, who specializes in applying human mobility datasets from social media feeds and data privacy and ethical issues.

Intellectual Merit

The primary intellectual merit of our proposed project is in **developing and piloting innovative approaches to pandemic prediction across previously siloed scientific domains** including biology, computer science, electrical engineering, and social-behavioral research. We apply new theoretical advances and convergent research to address our grand challenge of capturing actionable data about the risk of zoonotic spillover and early spread at a scale and speed required to prevent outbreaks. We will identify existing gaps and next-generation solutions for step-level changes in zoonotic spillover prediction via multi-day intensive workshops with thought-leaders organized by each of our three multidisciplinary Working Groups. Our pilot research projects will serve as a test bed for new theoretical frameworks to advance spillover intelligence. For example, in Aim 1 we will transform decades-old anthropological approaches and pilot a cost-effective, scale-up of quantitative and qualitative behavioral data collection through a unique public-private partnership Facebook; and use image recognition and artificial intelligence to quantify and map human-wildlife contact globally. In Aim 2 we will develop hierarchical Bayesian models to interpret rich, multiplex serology datasets and test these on real surveillance data as part of EHA's NIH-funded work; and develop the first field-forward lateral-flow assay for Nipah virus antibody detection. In Aim 3 will use cutting edge theoretical advances in network science, anomaly detection, and machine learning to detect changes in human mobility patterns that may portent spillover (e.g. increase congregation around wildlife markets) or signal early epidemic spread after the point of spillover.

Broader impacts

This proposal could potentially transform our understanding of zoonotic spillover risks using survey, multimedia, and mobility data on a global scale, including aggregated and anonymized data from ~3B active Facebook users. Insights and intellectual outputs from our working group activities will have direct benefits to public health by informing disease mitigation strategies for pandemic prevention. We will synthesize our three working group outputs and prepare a special joint publication for the National Academies of Science's Forum on Microbial Threats for wide distribution, and write popular press articles to accompany our peer-reviewed manuscripts. EHA's *Alliance,* includes a network of over 70 partner institutions in 30+ countries, presents a unique opportunity to work with local stakeholders in emerging virus 'hotspots' and validate

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models using data sets obtained through our long-standing collaboration with government partners including human health, agriculture, and environmental sectors.
Our education mission consists of multiple workshops, hackathons, monthly seminar series, and access to state-of-the-art visualization facilities. We will begin to train a new generation of graduate and undergraduate students who will be prepared to harness the burgeoning power of data science to make novel inroads into understanding human-animal contact, serological surveillance, and aggregated mobility data and their interaction. Our proposal builds on a unique partnership between the academic (UR, GU, USU), non-profit (EHA), and private sector (FB), allowing students to apply lessons learned from classroom settings to the real-world, and our recruitment will focus on including >50% women and underrepresented minorities in science and engineering. This planning grant will be a path towards structuring interdisciplinary education, cross-training courses across institutions, followed by hands-on experience via internships and research exchanges – with options for international work via existing programs at our institutions. Through the planning grant, a new MS data science track on 'zoonotic surveillance' will be established at UR, the first step in our plans to expand our Education and Training plan should we move towards a Center-level award in the future.

Education and Training Plan
Our Leadership Team will serve as mentors for our **CSI Student Training Program** that will offer a fellowship stipend to cross-train 20 Master's level graduate students via semester-long projects associated with proposed pilot research activities (3-4 students per project) and 3-week research exchanges between our institutions (UR, GU, EHA, USU, 4-8 students from each). The research exchange builds off EHA's successful NSF-funded EcoHealthNet training program (2011-21) and will take place in either the Summer of 2022 or 2023. We will link with currently-funded international programs at our respective institutions (e.g. Gui^{2}de in Nairobi, Kenya run by GU; and the NIH funded CREID research pilot program with EHA's EID-SEARCH project in Southeast Asia) to support students who desire to gain international, hands-on research experience as part of their research exchange. The focus of each research exchange will be on cross-disciplinary training to think 'big' about the future of pandemic prediction and ensure the next generation of spillover intelligence researchers gains valuable hands-on experience working with experts outside their area of study. Students will be drawn from respective departments across our institutions, i.e. students with a computational background and with interests in interdisciplinary work will be recruited from UR's cross-department Goergen Institute for Data Science, Anthropology and International Affairs students from GU, and Emerging Disease and Virology students from USU. As EHA is a non-profit and does not have an active student body, we will recruit Ecology, Evolutionary Biology, and Environmental sciences students from universities in the NYC area, including Columbia University's E3B department where PI Olival, coPI Ross and SP Daszak serve as adjunct faculty. We will explore specific mentorship opportunities with data scientists from Facebook. If awarded, we will additionally apply for funds through NSF's Research Experiences for Undergraduates (REU) program, with the goal of supporting summer stipend for 1 undergraduate per institution or the maximum allowable. We will host a joint monthly virtual seminar series (2 semesters long) open to graduate and undergraduate students from our respective institutions, with a dedicated student "lab meeting" before each seminar. Two CSI students from diverse disciplines will lead each lab meeting discussion based on a deep read of relevant publications that each speaker will distribute in advance. We envision the first semester of the seminar series will be led by our Leadership Team and Working Group experts, and the second semester will be for CSI Graduate students to present results or current challenges related to their pilot research projects. We hope to encourage these students to pursue Ph.D. studies. If we are successful at establishing a center through our planning grant, we should have access to a trained cohort of students with appropriate multidisciplinary skillset.

Project Overview

Our 18-month research agenda aims to understand how new methods and technologies can improve the efficiency, intensity, speed, and scope of zoonotic disease surveillance. **We have structured our development grant around three primary research aims** that will guide our interdisciplinary research and training activities. We will establish a working group composed of multi-disciplinary experts for each primary aim that will be co-led by members of our Leadership Team. We will develop pilot research projects that capture different parts of the disease spillover process focused on specific geographies, lead 3-day intensive workshops, monthly student seminars, and student training activities that cut across our inter-linked research aims.

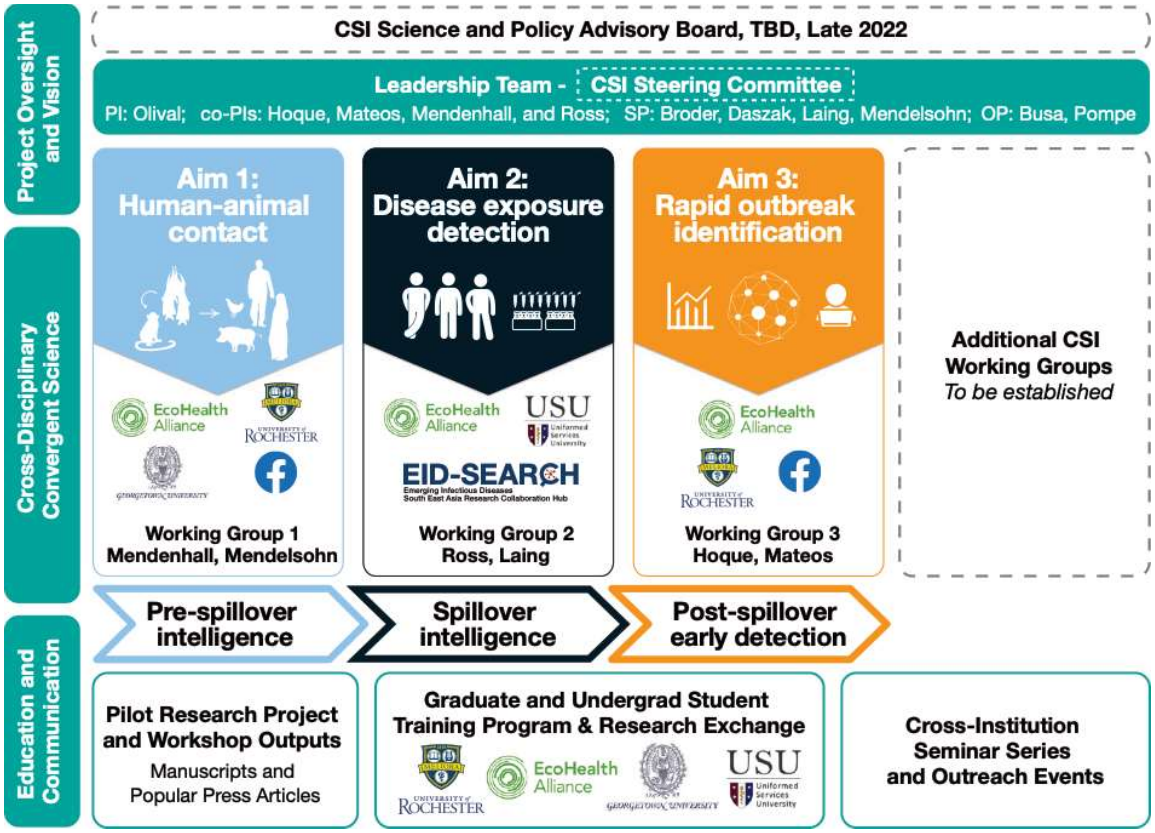

Fig 2. Project overview schematic showing our project management; three primary aims with graphical representation of pilot research activities to scale up and advance spillover intelligence; institutional partners leading activities for each aim including designated working group leads, and student education and communication activities, including pilot research projects, graduate student research exchanges, seminars, and other outreach activities. Our development level project was built to scale to a Center level award (the Center for Spillover Intelligence, CSI) as noted with the dashed outline boxes, including the future addition of other CSI Working Groups, a Science and Policy Advisory Board, and transitioning our current Leadership Team (PI, coPIs and SP) into a more formal Steering Committee for oversight.

Aim 1: Measuring human-animal contact at scale

Interactions among humans, wildlife, and livestock create interfaces for pathogen spillover and are proximate risk factors for the emergence of all directly-transmitted zoonotic diseases.[14,36] Human-animal contact is commonplace around the world, with many factors mediating the type and frequency of contact, including cultural practices, environmental conditions, food security, and occupational demands.[36] In regions of the world considered prone to spillover--forested tropical landscapes with high biodiversity and recent land-use changes--understanding human-

animal contact is crucial to pandemic prevention.[5] To measure these interactions and characterize associated behaviors, beliefs, and knowledge, researchers often implement resource-intensive surveys in key communities.[37,38] While crucial to understanding human-animal contact dynamics, on-the-ground surveys cannot yield scalable data that is comparable across populations. Often, in place of surveys, researchers conducting analyses at broad scales rely on coarse estimates of contact such as the joint density of human and animal populations. **Our working group will uniquely combine social scientists, disease ecologists, and computational scientists to increase the scale at which we can directly measure human-animal contact.** We will develop and implement two pilot research projects. The first will use novel, social-media-based survey approaches to reach larger populations at higher frequencies while maintaining the cultural sensitivity and relevance of on-the-ground surveys. Working collaboratively with cross-disciplinary researchers, we will design surveys to understand social behaviors, cultural perceptions, and occupational hazards that may pose zoonotic spillover risk to individuals and communities. We will target geographies based on our existing spatial risk modeling work that maps infectious disease spillover potential and the ecological distributions of key reservoir host species.[5,15,25] Second, we will conduct pilot research on the use of social-media-based image recognition algorithms to identify locations of human contact with key species known to host high pathogen diversity. This pilot will include a collaboration effort to test the efficacy of such technology for understanding taxon-specific contact patterns globally, and to bring multidisciplinary context to meaningfully interpret the outputs (including species validation with taxonomic experts). If successful, will use these data on frequency of animal contact from image data to identify locations for additional survey data collection.

Aim 1 Pilot Research Projects: We will develop two pilot projects to assess the potential of 1a) social-media dispensed surveys and 1b) passive surveillance using artificial intelligence for image recognition to capture information about human-animal contact. We will target populations at wildland interfaces where contact with wildlife is likely high but internet penetration may be mixed.

1a. Social-media dispensed surveys to quantify and contextualize human-animal contact
We will pilot social-media dispensed, large scale population surveys about perceptions of and interactions with animals. We will optimize the design of these surveys for mobile platforms to facilitate higher response rates, and will also explore the possibility of longitudinal sampling respondents (either individuals or populations) to understand changes in conditions and responses over time. Survey questions will be based on a combination of EHA's past experience designing on-the-ground human-animal contact surveys[37,39,40] and ideas generated from our proposed working group activities involving anthropologists, data scientists, and disease ecologists who have worked extensively in the geographic and societal contexts in which we focus our work. We will aim to design surveys that account for populations and individuals holding different beliefs, carrying different histories, and living in different ecologies. We will attempt to survey demographically and geographically diverse populations to capture direct and indirect animal-contact patterns and behaviors, and will attempt to capture the role that international, state, and local policy plays in individuals' lives. Further, we will aim to recognize particular hesitancies among individuals to interact with certain technologies or other public health interventions.

We will explore various online platforms at the start of our project for survey administration. Given our unique public-private partnership with Facebook for many components of this project, using the Facebook platform to disseminate our pilot survey is likely to be a strong option, especially given Facebook's global reach (nearly half of the world's population, ~3 billion users), which introduces opportunities for digital interaction across class, gender, ethnicity, and cultural

divides. Facebook has extensive experience administering surveys, either directly on its platform (e.g. Yale Climate Change opinion survey) or off-platform (e.g. Carnegie Mellon University COVID-19 surveys) that we will leverage. Facebook also has extensive privacy infrastructure and data-review policies to ensure anonymity and data security.

We will also explore creating games that users will be encouraged to play online to enable us to learn more about how they think about host species, viral threats, environment, and health/disease. For example, users may be given a list of 30 images and will be asked to put them into categories and describe what each category means. Adapting these "pile sorting" anthropological methods[41] to an online interface and for spillover risk, will be a novel application with potential for a massive scale-up of data around cultural and conceptual dimensions of zoonotic disease risk. Data generated from this exercise will generate a more ethnographic understanding of people's experiences and thought processes, which tend to be locally mediated. We understand that there are geographic and demographic gaps in internet and social media use, and will incorporate bias corrections into our study design. We will initially focus on Southeast Asia, a region with a high likelihood of future zoonotic disease emergence and where EHA has good coverage of on-the-ground surveillance programs to ground truth online data collection.[5]

1b. Image recognition of zoonotic disease host species

We will pilot image-based surveillance of Facebook and Instagram feeds to attempt to identify human contact with key zoonotic disease host species and to explore the limits of taxonomic and behavioral recognition in images. We will work with Facebook Data for Good (see **Letter of Collaboration**) to adapt and refine their existing machine vision algorithms to target key taxa and to assess the efficacy of different approaches, working within strict guidelines and ethical standards for data de-identification. While data will be de-identified, we will only use photos with full location meta-data, allowing us to not only quantify the type of species and contact, but where it occurred. There are multiple image libraries that may serve as AI training datasets, including iNaturalist, the American Society of Mammologists, and Integrated Digitized Biocollections (iDigBio). We will iteratively implement and test algorithms starting with broad taxonomic groups (e.g., bats) and will explore the extent to which our approaches can identify finer taxonomic resolutions down to the genus or species level. In addition to taxa identification, we will explore image recognition of features that may suggest specific types of human-animal contact, such as cages or market settings. Further, we will use text and image metadata and explore using hashtags associated with images to boost the ability of our algorithms to identify key taxa.

This work will involve collaboration between machine learning scientists, biologists/ecologists, and social scientists. We will follow all data anonymity and privacy guidelines from Facebook and will also discuss data handling ethics in our working group. We will assess the ability of passive surveillance with image recognition to be used to target surveys to populations that may have higher frequency of contact with wildlife, and ultimately to target biological surveillance of human and animal populations. We will consider both the accuracy of the image recognition algorithms as well as implications for survey distributions, results interpretation, and ethical data practices. We will also compare the spatial distribution of human-animal contact patterns derived from image recognition with previously generated spillover risk maps based on joint occurrences of animal and human distributions (e.g., SARS-related CoV spillover risk from Sanchez et al. 2021 preprint).[25]

Working Group 1 Activities: Our working group will convene 12-15 experts from the social sciences, disease ecology, and computational sciences for an intensive, 3 day workshop. We will structure the workshop to focus first on data ethics in human behavioral surveying and image recognition surveillance and secondly on survey design and analysis planning, with specific attention to accounting for gaps or biases in survey distributions. We will center the workshop around the understanding that technological quick fixes are never enough and that data collection must address long term social, ecological, and economic realities to understand how people perceive, experience, and interact with the risk of and repercussions from spillover events. We will also address experiences and challenges in engaging longtime ethnographers and local leaders to understand what's at stake for communities engaging with online and field surveillance programs. Our working group will help mentor students part of the CSI Student Training Program, to facilitate information sharing and idea generation among undergraduate and master-level students. Working group 1 will also contribute to the CSI Seminar Series with cross-disciplinary topics such AI image recognition (lead by a data scientist and bat biologist) and human-behavioral survey design (lead by an epidemiologist and medical anthropologist).

Partners, Initial Working Group 1 Members, and Roles: EHA (SP Mendelsohn): survey design and implementation, image recognition algorithm development and testing, novel data analyses. Facebook Data for Good (OP Pompe): survey implementation, image recognition algorithm development and testing, data ethics. University of Rochester (coPI Hoque): Image recognition algorithm development and testing. Georgetown University (coPI Mendenhall): Survey design with focus on ethnography and anthropology; online free listing/pile sorting game design; help lead expansion of WG to include emerging international leaders in social-behavioral science (e.g. Dr. Edna Bosire from Kenya). We will invite additional leaders from our extensive funded collaborations to be part of the Working Group for Aim 1.

Aim 2: Detecting zoonotic disease exposure at scale

Animal-human contact is the necessary condition for spillover of potential pandemic, directly-transmitted viruses. While a small fraction of such encounters result in actual exposure or infection, such exposures are frequent given the total sum of encounters. Most such infections are never reported and rarely spread widely, and only a very rare subset become widespread in the human population[42]. Other viruses such as Lassa spill over thousands of times per year[43]. In areas of frequent spillover, the eco-evolutionary dynamics of "stuttering chains" of infection are what give rise to epidemics and pandemics[44]. Identifying populations where frequent exposure occurs is critical for deploying preventative measures and following viruses upstream to their wildlife hosts. Yet even large-scale surveillance projects cover too small populations and produce far too few detections to determine the most exposed geographic, demographic, or occupational groups at an actionable scale [REF, showing PREDICT sampling efficiency]. This is for two reasons: the field and laboratory cost of surveilling populations interacting with wildlife interface and, and the low detection rates of the widely-used, molecular PCR or metagenomic approaches.

We aim to drastically increase the scale at which populations can be monitored for exposure to animal-origin viruses by developing and piloting novel tools and analyses needed to implement large-scale serological surveys. While metagenomic methods have been favored for their ability to capture genetic sequences of previously unknown viruses, serological methods can be used to detect exposure to known and unknown viruses using multiplex assays tailored with a mixture of sensitivities and specificities[45]. Serological tools can detect antibodies for months or years after viral infection, rather than the limited window of days or weeks using molecular methods, and thus can provide a much richer profile of populations where pandemics may emerge[46]. In this planning period, we will begin pilots to test the feasibility of two complementary approaches

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Our work under PREDICT is relevant here, working with local community leaders to distribute results and the 'Living Safetly with bats book'. But how do we scale this? One idea is through a network of local leaders across the landscape, like a "Community One Health Volunteer" network.

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to enabling serological screening at large scales: 2a) the creation of rapid bioinformatics pipelines to interpret high-dimensional serological data from large streams of surveillance data, and 2b) the development of rapid field serological tests for wildlife-origin diseases.

Aim 2 Pilot Research Projects

2a. Rapid bioinformatics for high-throughput, multiplex serology

Members of our team (SP Laing, Broder) have developed multiplex serology tests that can be used to screen for exposure for many species and variants of filo-, henipa-, and coronaviruses simultaneously, and screening pipelines that can process samples at the scale of tens of thousands of samples. However, these tests pose challenges for interpretation, as high-dimensional results from multiplex bead or chip assays frequently contain signals of cross-reaction and autocorrelation (**Fig 3**). Through cross-disciplinary research between molecular virologists, serologists, mathematical modelers and data scientists on our team, we will create models to interpret this high-dimensional data and integrate them into a rapid bioinformatic pipeline that identifies individuals and populations to known viruses as well as flagging serological signals indicative of novel viruses. We will make use of data collected under our ongoing NIH and DTRA funded biosurveillance activities (Laing, Broder, Ross, Olival, Daszak) to develop detailed reaction/cross reaction profiles of a broad panel of zoonotic viruses to serological tests. We will build mechanistic, hierarchical Bayesian models that allow us to determine whether serological "profiles" are more likely to represent antibodies of known viruses, mixtures of multiple known viruses, or previously uncharacterized viruses. We will extend techniques of antigenic mapping,[47] using serological profiles to estimate phenotypic similarity of new viruses to known viral families, determine the correlation with phylogenetic relatedness, and use these measures to screen new viruses for pandemic potential. Using simulation experiments, we will test reduced-form, simple models against fully structured models so as to develop statistically robust procedures to interpret these data without high-performance computation.

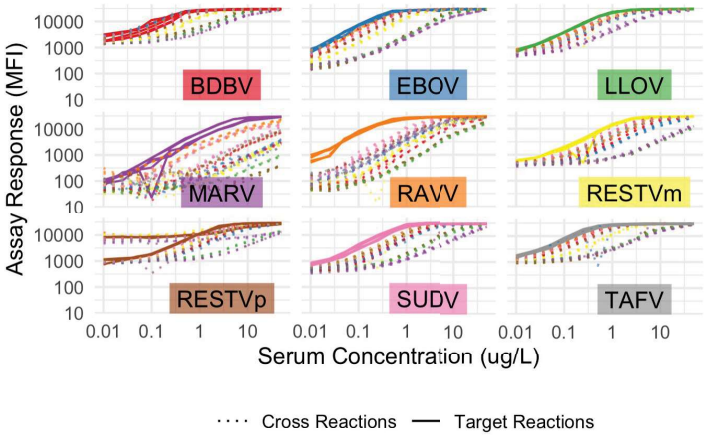

Fig 3*.* Preliminary data: Reaction-cross reaction plots of multiplex Luminex assay response to antibodies for closely-related filoviruses.

Scaling center operations: This pilot project will produce methods and a proof-of-concept pipeline for high-throughput analyses. We will leverage EHA's Emerging Infectious Disease – Southeast Asia Research Hub (EID-SEARCH) project, a NIH-funded Center for Research in Emerging Infectious Diseases (PI Daszak) who's partners include USU, Duke NUS, Chulalongkorn University in Thailand, and various clinics and institutions in Malaysia – currently screening large numbers of human samples (>10K/yr) for various coronavirus, filovirus, and paramyxovirus antibodies. In the future the CSI will implement surveillance studies screening these sample streams using multiplex serology, and utilize the analytical and bioinformatic tools we develop in the next 18 months. As part of our planning activities, we will establish protocols and approvals for this work.

2b. Development of a field-ready semi-quantitative lateral flow assays for an emerging zoonotic viruses (Nipah virus)

Lateral flow assays (LFAs), are low-cost tests where reagents printed on cellulose strips react with a sample that flows via diffusion. LFAs are capable of providing quantitative results (in proportion to antibody titer) using a combination of high-quality manufacturing and carefully designed, calibrated optical scans[48]. LFAs can be used to provide diagnostics at low cost at the point of care and by consumers. Lack of commercial-scale applications has prevented LFAs from being used for serological testing for novel or emerging disease. To our knowledge, no studies have used LFAs for zoonotic virus serosurveillance in the field.

We will develop a proof-of-concept LFA for Nipah Virus (NiV). NiV has a high mortality rate and is considered to have pandemic potential that is ranked by CEPI as one of 7 high pandemic risk pathogens[49]. The range of NiV's primary wildlife hosts (*Pteropus spp,* fruit bats) cover South and Southeast Asia but human cases have only been detected in Malaysia, Bangladesh, and the Philippines. Spillovers occur regularly in Bangladesh, but are rarely reported in large portions of the country[50] and reporting is strongly associated with health care accessibility[51]. Broad-scale serological monitoring has the potential to identify populations from which pandemic strains may emerge. Through previous work, members of our working group (Laing, Broder) have developed multiplex serological assays for NiV[52] and the soluble proteins and reagents required for an LFA. We have also added experience in engineering, quality control and commercialization of LFAs (Busa, industry consultant) to our working group. For the pilot, we will engage with commercial manufacturers to create and test a prototype Nipah LFA and a protocol for smartphone-based optical reading of quantitative responses.

Scaling center operations: The pilot will create a prototype LFAs. As part of full center operations under CSI, we will aim to scale up production and test LFAs efficacy using control sera and field deployment as part of EID-SEARCH. We will additionally assess feasibility and cost of deployment, and compare performance against current laboratory-based assays.

Working Group 2 Activities: In addition to the two pilots above, working group members will contribute to the our CSI monthly seminar series, presenting on topics including high-dimensional data models, field serology, serological assay development, laboratory product design, and public-private partnerships and commercialization. We will conduct two online workshops on serological data analysis, jointly with undergraduate, masters, and doctoral students at USU and UR, leveraging partnerships with field researchers currently working in serological surveillance. Our working group will lead a 3-day intensive workshop, where we will recruit additional participants with expertise in serological assay design and surveillance, immunology, emerging zoonoses, and focus discussion on key barriers to scaling serological surveillance for known and unknown zoonotic viruses.

Partners, Initial Working Group 2 Members, and Roles: EHA (coPI Ross): Model and pipeline design, software optimization. Uniformed Services University (SP Liaing, SP Broder): data and model interpretation, reagent manufacture, assay design. SP Busa: device manufacturing, quality control, industry outreach and partnership development. We will recruit additional participants from our partner network engaged in serological field studies as well as industry representatives to be part of the Working Group for Aim 2.

Aim 3: Rapidly Identifying Anomalous Health Events

In early stages of epidemic spread, novel contact and mobility-induced behavioral data as well as communication patterns may provide a signal of e.g., health-seeking activity indicative of an outbreak cluster. We contend that these signals may be detectable in large-scale data streams of population mobility and contact, or disease reporting available through telecommunications and health care providers. Timely detection requires approaches that can manage data at scale,

analyze high-dimensional, spatio-temporally correlated streaming data in (pseudo) real time, and distinguish between known signals (e.g., typical influenza-related activity) and novel spillover-related signatures that we wish to unveil, characterize, and explain in order to trigger the necessary public policy responses.

This desideratum calls for fundamental methodological and computational advances, to go along with epidemiologically-relevant data sources that describe how humans are moving and interacting across physical space, with unprecedented temporal resolution and geographical coverage. Traditional flow-based mobility datasets used to parameterize epidemiological models have been aggregated and formatted in the form of origin-destination (OD) matrices. While valuable towards understanding population flows between regions of interest, OD matrices lack information on how much time a traveler spends in any given location. Naturally, when it comes to epidemiological studies the duration of time spent in potentially risky geographical areas is key towards deciphering actionable contact and exposure patterns[53]. Moreover, recent studies have shown that flow counts between pairs of geographical areas can be loosely correlated with, and not representative of, epidemiology-relevant mixing patterns[54]. Instead, these couplings among subpopulations are measured by effective contact rates, meaning how often people from each region come into contact in a way that is sufficient to transmit a disease. To address these challenges, our consortium will partner with the Facebook Data for Good team and leverage two recently assembled global-scale datasets - Activity Space Maps and Colocation Maps[53,54].

Different from existing approaches that have focused on measuring the causal impact of mobility-oriented interventions, **our ambitious goal in this aim is to markedly advance the surveillance infrastructure available to predict anomalous health-related events before a major outbreak occurs**. While undoubtedly a multi-faceted endeavor, in this planning grant we will take initial, but significant steps with an emphasis on network-centric, computational analyses of dynamic mobility and interaction data at a global scale. By developing the appropriate statistical tools, we will support the ultimate goal of characterizing early pandemic "fingerprints" or signatures that can be extracted from these global datasets.

Aim 3 Pilot Research Projects: Working closely with Facebook Data for Good (see Letter of Collaboration from Alex Pompe), this working group will pilot data-driven research activities to explore the feasibility of extracting early (post spillover) signals of epidemic onset from Activity Space Maps and Colocation Maps. Our multi-disciplinary working group brings together leading experts in systems engineering, AI for healthcare, data science, mathematical epidemiology, graph signal and information processing, privacy-preserving mobility data generation and management, human behavioral modeling, and environmental science. We believe the current team, consisting of a wide array expertise and partnership, will instigate the cross-pollination of ideas leading to center-level activities while making significant inroads towards addressing our grand challenge. We propose two specific pilot projects:

3a. Spatio-temporal monitoring of spillover-induced activity anomalies in Southeast Asia
Activity Space Maps (ASMs) are a novel global-scale movement and mobility data set which describes how people distribute their time through geographic space.[53] They are designed to complement existing digitally-collected mobility datasets by quantifying the amount of time that people spend in different locations. Accordingly, this information is valuable for estimating the duration of contact with the environment and the potential risk of exposure to disease -- something critical especially early after spillover. More germane to the theme of this aim, it also offers the unique possibility of developing and testing sequential change-point detection algorithms to monitor multiple spatial grid cells of interest (informed, for instance, by outcomes

of the image-recognition effort under Aim 1). We will target areas known to include health care facilities in pursuit of abnormal trends of (increased) activity. Moreover, using ASMs we will examine flow and temporal activity anomalies around expert-annotated wildlife and wet market locations. Said events are expected if there are human infections, or significant associated changes in behavior around these sites early after spillover. Our initial efforts during this planning grant will be focused on Southeast Asia, which on top of its epidemiological relevance is one of the regions where Facebook ASMs have the most coverage. We will target Indonesia, where EHA has extensive on-the-ground efforts tracing networks of wildlife trade as well as market sites.[55] We will also tap into the resources stemming from EHA's Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH) to identify clinics and market sites of interest in Thailand and Malaysia. Some key challenges we will address include accounting for the implicit biases in data collection associated with the construction of ASMs,[53] and their subsequent effects on the statistical algorithms that we will develop. Our Working Group will address additional challenges of data interpretation of anomaly detection analyses with input from emerging disease experts and epidemiologists. To calibrate our models, we will bring to bear recent activity data before and after the SARS-CoV-2 outbreak in those locations, in addition to historical influenza seasonal trends that go further back in time. With the exciting prospect of ASM data being officially released in the last quarter of 2021, *we are in a privileged position to be the first multi-disciplinary team that leverages this invaluable global-scale resource to advance our understanding of zoonotic spillover risk and its immediate effect on human mobility.*

3b. Online network change-point detection in dynamic, global-scale Colocation Maps
Activity data are complemented by *Colocation Maps (CMs),* which estimate how often people from different regions worldwide are colocated. In particular, for a pair of geographic regions *i* and *j* (e.g., counties in the US), CMs estimate the probability that a randomly chosen person from *i* and a randomly chosen person from *j* are simultaneously located in the same place during a randomly chosen minute in a given week.[54] In other words, CMs are global-scale, dynamic network datasets with colocation probabilities encoded as edge weights. Thus, they are well suited to parametrize metapopulation models of disease spread,[56] or to measure temporal changes in interactions between people from different regions.[57] To monitor said changes as part of our second pilot activity in this aim, we will build on our recent advances in online (i.e., sequential) algorithms for anomaly and change-point detection from network streams[58,59]; see also.[60-62] The envisioned statistical methods are in stark contrast with most existing graph change-point detection approaches, which either rely on extensive computation,[63] or they would require storing and processing the entire observed CM time series.[64] We will prototype and test a lightweight online change-point detection algorithm with quantifiable performance guarantees, that is also explainable since it relies on interpretable node embeddings.[58,65] The latter desirable feature will be leveraged to construct dynamic anomaly maps for user-friendly visualization of post-spillover events; see also our recent network-scientific visualizations of spatio-temporal COVID-19 contagion patterns across the US.[66] Moreover, the sheer-size of the CM graphs calls for algorithmic and modeling innovations we will investigate as part of our research agenda, e.g., seamlessly integrating graph subsampling or dimensionality reduction techniques to the analytics pipeline. We will team up with computer vision experts affiliated to the University of Rochester Goergen Institute for Data Science (GIDS, see attached Letter of Collaboration from institute director Mujdat Cetin), to also explore the exciting prospect of integrating geo-tagged multimodal data such as video and imagery in our studies, for instance as nodal attributes embedded to the CMs.

Working Group 3 Activities: *Recruitment and diversity:* We will recruit 6 students from the GIDS-run master's program in data science, which attracts students from multidisciplinary backgrounds and significant computational proficiency to contribute in the aforementioned pilot research activities. *The CSI leadership and broader community are committed to providing research training and educational experiences for those traditionally under-represented in STEM, including women and underrepresented minority students.* The PI team has a solid track-record in actively recruiting and supervising such students at the undergraduate, masters and doctoral level, with demonstrated efforts towards broadening participation in STEM. As examples of the commitment to diversifying the field, co-PI Mateos serves as founding faculty advisor to the UR's SACNAS chapter and regularly organizes diversity, equity and inclusion panels to highlight challenges and successes of UR's Hispanic and Latino STEM community. We plan to prioritize engaging women and minority students in the research and working group activities proposed here. *Coursework:* These students will be on the existing health and bio track with an opportunity to take classes in immunology, microbiology and virology programs as well as data ethics at the University of Rochester Medical Center (URMC) as well as the college of engineering. Through the planning grant, we plan to establish a special MS track on 'zoonotic surveillance' in order to train and mentor the students to lead center level activities. *Seminar and workshop series:* The students will be part of the large cohort of all the participating institutions and participate in the CSI monthly seminar series. The co-PIs (Mateos and Hoque) will conduct two workshops (and invite additional faculty members from UR as guest speakers) with hands-on lessons in mobility and human behavior modeling. *Hackathon:* The students will be encouraged to participate in the yearly hackathon, Dandyhacks https://dandyhacks.net/), organized every fall by the University of Rochester Computer Science Department and work with the dataset from Facebook. Additionally, co-PIs Mateos and Hoque will work with the organizers of Dandyhacks to release some of the public dataset from Facebook for other teams to explore, model and mine the data. *Data visualization meetups at the University of Rochester's VISTA Collaboratory*: To get acquainted with the different data resources at our disposal, over the first 3 months of the project the Rochester team (along with Facebook and EHA collaborators via Zoom) will hold bi-weekly team meetings with the MSc students at the UR's VISTA Collaboratory. This is a 1,000 square-feet visualization lab equipped with an interactive (20 ft. wide x 8 ft. tall) tiled-display wall, that renders massive data sets in real time, giving team members the ability to visualize and analyze complex data instantaneously and collaboratively; see Fig. 4. As we dissect what dataset components are central for the aforementioned pilot activities, our graduate students will develop customized data visualization tools, maps and dashboards to facilitate downstream analyses. Updates and progress reports will be shared in these meetings, giving the students a unique opportunity to develop their communication skills. The whole team will provide feedback and share ideas, making this a truly collaborative activity. Moving forward, the VISTA Collaborative offers a unique resource that we will fruitfully leverage as we scale up to full center operation.

Fig. 4. UR's Vista Collaborative will serve as a spillover intelligence "situation room" for training UR and CSI exchange students on analysis of real-time epidemiologically relevant datasets.

Partners, Initial Working Group 3 Members, and Roles: University of Rochester (co-PIs Hoque and Mateos): coordination of working group activities, development and testing of computational tools for early detection of post-spillover signatures, dissemination of research products and educational materials, recruiting and mentoring of master students, liaisons to

GIDS. Facebook Data for Good (OP Pompe and Maps for Good team members Iyer and Citron): data ethics, quantification of privacy protections, data scientific support for Activity Space and Colocation Maps, mathematical epidemiology expertise for data and model interpretation. EHA (SP Mendelsohn and Ross): identification of target spatial locations in Southeast Asia, epidemiological interpretation of anomaly detection analyses and validation of detected early pandemic fingerprints.

Scaling operations: Our programs and pilot research are designed to scale. First, we aim to expand on successful pilots by expanding the geographies of our work, engaging with more in-country partners. Second, we test the development of each of these new streams of predictive data, we plan to develop partnerships with local, national, and global agencies to ingest these data, expand into programs to develop behavioral interventions and testing in response to identifying regions and populations at high risk of contracting zoonotic diseases.

Working groups are really about bringing our project to scale, our intensive 3-day workshop will also lead to new partnerships and synergies for expansion.

Results from Prior NSF Support

Kevin J. Olival has not had prior NSF support, but is co-PI on NIH-funded "EID-SEARCH" (NIAID-CREID U01AI151797, 2020-25 PI Daszak). Previously co-PI on "Understanding the risk of bat coronavirus emergence" (NIAID R01 AI110964, 2014-2019, PI Daszak). ***Intellectual merit*:** Identified >50 SARSr-CoVs in bats, showed evidence of ability to infect human cells, humanized mouse models, and people in China, and used evolutionary and mammalian biodiversity patterns to analyze spillover risk for use in public health control. Produced 18 peer-reviewed papers [20,34,67-79], including two papers in *Nature* [80,81], and a review in *Cell* [82], and others submitted. ***Broader impacts*:** 2 PhD and 6 MS students trained; disease risk and potential management strategies shared with US agencies, WHO, OIE, FAO, and the public.

Ehsan Hoque: Project IIS-1750380. PI: Hoque. Period (2018-23). Amount: $511,453 "CAREER: A collaboration coach with effective intervention strategies to optimize group performance". ***Intellectual merit:*** Developing and validating the efficacy of automated mediation strategies in online meetings. ***Broader Impacts:*** >6 research papers[83-88]. **Project NRT-DESE-1449828.** PI: Hoque (2015-21). Amount: $2,965,341.00 "Graduate Training in Data-Enabled Research into Human Behavior and its Cognitive and Neural Mechanisms". ***Intellectual merit:*** Chartering a new traineeship program to train Ph.D. students at the University of Rochester to advance discoveries at the intersection of computer science (cs), brain and cognitive sciences (bcs), and neuroscience. ***Broader Impacts:*** Supported over 50 PhD students in cs and bcs, seveal won interdisciplinary research positions at R1 institutes.

Gonzalo Mateos (a) Project: CCF-1750428. PI: Gonzalo Mateos Buckstein. Period: 4/1/18 - 3/31/23. Amount: $407,944. (b) Title: CAREER: Inferring Graph Structure via Spectral Representations of Network Processes. (c) ***Intellectual Merit:*** We investigate how to use information available from graph signals to estimate the underlying network topology through innovative convex optimization approaches operating in the graph spectral domain. ***Broader Impacts:*** We prepared a feature article on identifying network structure via GSP,[89] presented tutorials in EUSIPCO'19, CAMSAP'19 an SSP'20, organized the 2019 IEEE SPS Summer School on Network- and Data-driven Learning. (d) Research products are available in.[66,89-96]

Emily Mendenhall (a) National Science Foundation Doctoral Dissertation Improvement Grant, $10,000; Stories at the Border of Mind and Body: Mexican Immigrant Women's Narratives of Distress and Diabetes. **Intellectual merit:** Led to crucial insights studying syndemics - the interactions of social, environmental, psychological, and biological factors that are synergies of epidemics. Papers in The Lancet[29], Medical Anthropology Quarterly[97-99], and as a full-length

book[100]. **Broader Impacts**: Understanding diabetes, trauma, and broader socio-political factors with infectious diseases in India, Kenya, South Africa, and Ethiopia.

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PROJECT DESCRIPTION

Introduction and Background

The spillover and spread of novel, zoonotic pathogens can have tragic societal and economic impacts, as is evidenced by the ongoing COVID-19 pandemic. Unfortunately, the emergence of SARS-CoV-2 (the virus that causes COVID-19) is just the latest in a series of viral emergence events that have been accelerating over the last several decades. In just the last year, as the world continues to respond and adapt to COVID-19, we have seen several deadly zoonotic (animal origin) viruses re-emerge in the human population, including Ebola and Marburg in Guinea[1,2], Nipah virus in India[3], Rift Valley Fever virus in Kenya[4], and novel strains of influenza in China [REF]. Most emerging infectious diseases (EIDs) originate in wildlife reservoir hosts and are transmitted across species boundaries, or ("spill over"), when livestock or human populations increase contact with wildlife through factors including land use change, agricultural intensification, and the wildlife trade[5-7]. Zoonotic spillover is a dynamic process determined by many steps including 'pre-emergence' viral evolution, diversification, and transmission among wildlife hosts; 'human exposure' via direct or indirect contact between humans, livestock, and wildlife; 'post-exposure' limits to cell entry, host range, human-to-human transmission; and population level virus amplification and spread. Once efficiently transmitted, EIDs can spread rapidly through globalized travel and trade networks, threatening countries with high population growth, urbanization and international trade (**Fig 1**).

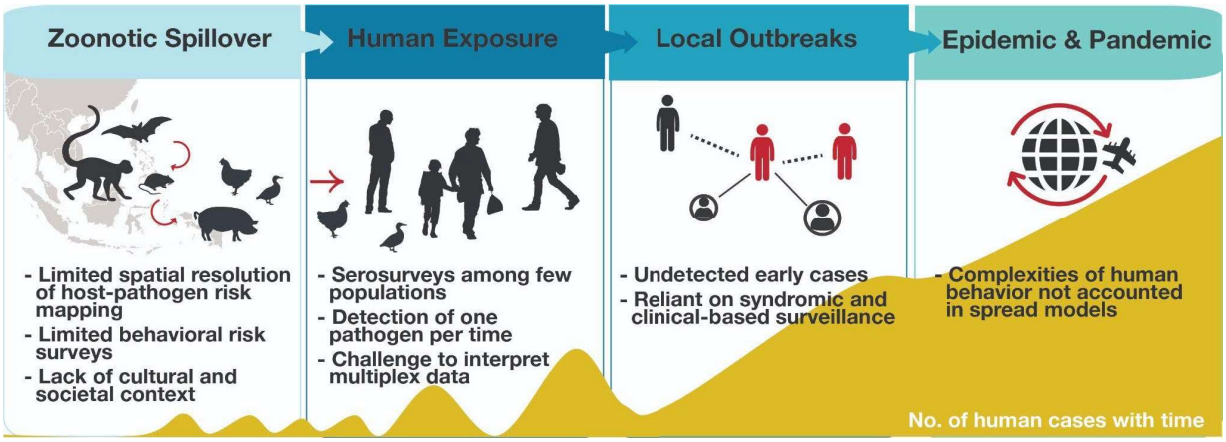

Fig 1. Four stages of zoonotic disease emergence and spread, with gaps in knowledge at each stage. Our proposed project will develop and pilot novel, multidisciplinary and technological advances to address critical gaps in the first three stages of emergence, when pandemic prevention is possible.

Novel human EIDs are typically only discovered when they cause severe or unusual symptoms, when astute clinicians are linked with laboratories capable of pathogen discovery research, and after they have been transmitted from person to person (in which case control options become more difficult).[7,8] The emerging human viruses characterized to date likely represent just the tip of the iceberg given the hundreds of thousands of predicted zoonotic viruses that exist in nature and limited surveillance capacity globally[9]. New research has disrupted the paradigm that zoonotic disease spillover is a rare event. A combination of more advanced molecular and serological assays, improved laboratory capacity around the world, and targeted surveillance of "high risk" human populations have led to evidence of zoonotic virus transmission and infection, even in the absence of notable disease outbreaks. Often these spillover events lead to "dead end" infections in people or limited 'stuttering' chains of transmission, a phenomenon referred to as "viral chatter"[10]. Community-based serological surveys from our group and others have illuminated relatively frequent and previously-unknown instances of zoonotic virus spillover

including Henipaviruses in bat hunters from Cameroon[11], SARS-related CoVs in rural communities living near caves in China (pre-2019), and Ebola virus exposure in DRC[12] and even Southeast Asia[13]

It is during these early stages of emergence, i.e. "viral chatter" with limited human transmission and even further upstream to include pre-emergence risk analyses at the human-animal interface, where innovative convergent research approaches are most needed for pandemic prevention. While we have made significant scientific advances over the last ~15 years in understanding where and through what mechanisms novel zoonotic viruses spill over into the human population[5,14-17], there **are still fundamental knowledge (and implementation) gaps that stymie our ability to preempt and prevent future disease emergence events**.

Our Grand Challenge Problem

Prior to 2019, EcoHealth Alliance (EHA) and colleagues demonstrated that SARS-related coronaviruses (SARSr-CoVs) in China pose a clear and present danger for a future human outbreak. We conducted extensive viral discovery in wildlife from China and Southeast Asia, phylogenetic analysis, and host-range experiments to show that certain bat species harbor a diverse assemblage of SARSr-CoVs[18,19], as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics[20-23]. We conducted behavioral risk factor surveys and serological sampling from human populations living close to caves and found serological evidence of SARSr-CoV spillover in people exposed to wildlife[24]. Building on this body of knowledge, our recent models suggest that bat-origin SARSr-CoVs may be infecting 400,000 (median 50,000) people in Southeast Asia each year[25]. While confidence intervals around these estimates are large, our sensitivity analyses importantly identified key area for future research to improve these spillover risk models - notably, quantification and better contextual understanding of human-bat contact, the role of wildlife farming and livestock hosts, scaling up human serological surveys, and better data on sero-dynamics following novel SARSr-CoV exposure.[25]

Despite this strong body of evidence for SARSr-CoV spillover risk (prior to the emergence of SARS-CoV-2), surveillance and research on these potential pathogens, their hosts, and their interface with humans was not conducted across a wide enough geography, nor at a temporal or spatial scale to inform actionable disease mitigation. Even for large-scale efforts to strengthen zoonotic virus early warning systems, like the 10-year, 30 country USAID PREDICT program[26], led by a global consortium including EHA, it's nearly impossible to capture enough data and disseminate that quickly enough to lead to widespread prevention measures at a national scale using existing methods – especially when considering multiple potential viral threats at once. This is especially true for large countries with heterogeneous human populations, diverse wildlife fauna, and known ecological and societal risk factors for emergence. Approaches to massively scale up zoonotic spillover surveillance are needed.

Our grand challenge problem: "How can we capture actionable data about the risk of zoonotic spillover (and early spread) at the scale and speed required to prevent outbreaks?" Can we leverage tools developed by computer scientists, data engineers, and technology companies to collect data from diverse populations and geographies to advance zoonotic spillover intelligence to a global scale? Can we incorporate new insights from medical anthropology, sociology, wildlife biology, disease ecology, molecular virology, biomedical assay design, and data science and use these to pioneer new methods in data collection and interpretation for zoonotic disease prediction and prevention? In this development proposal, we propose to build a collaborative research and training network to confront this challenge, designed at the start to scale up to a future *Center for Spillover Intelligence (CSI).* Our network will deeply integrate computer scientists, engineers, and data scientists using machine learning and other novel technological approaches together with experts in emerging disease and social-

behavioral research. New insights from social, behavioral, and anthropological research make it clear that the emergence and future trajectory of human epidemics require that we recognize potential biological risks alongside risks posed by cultural and societal factors[27-29]. Our cross-disciplinary, multi-institution project will combine synergistic research activities with training and education programs to facilitate new innovations to scale-up the collection and interpretation of data to inform where, how, and what zoonotic viruses are poised to emerge – a field we collectively refer to as *Spillover Intelligence*. Our inclusion of social-behavioral researchers, data ethicists, and inter-governmental EID policymakers will ensure that our aims and approaches are culturally and locally relevant, and align with our ultimate goal of improving global health.

Our Vision: We envision a world where diffuse data streams can be combined and interpreted in near real-time to disrupt the spillover and spread of future zoonotic viruses. We envision diverse leaders and scientific disciplines coming together to advance spillover intelligence and transform the ways we think about, monitor, and respond to global viral threats.

Our Team

We have assembled a multidisciplinary team of experts to lead our development project and to create and grow the Center for Spillover Intelligence (CSI). Our **Leadership Team (PI, coPIs, Senior Personnel)** includes a mixture of established scientists and early career leaders working across the fields of: emerging infectious diseases, computer and data science, engineering, mathematical modeling, virology, technology and biotechnology, data ethics, social science, and medical anthropology. Our project will be led by EHA (PI Olival) building off the foundational pandemic prediction work our institution has developed over the last decade, but expanding to new partnerships and collaborations we have not previously been able to explore – including with computer scientists and engineers from University of Rochester (UR) and behavioral anthropologists from Georgetown University (GU). We will develop and pilot new technologies for field biosurveillance and data interpretation with Uniformed Services University (USU). Our Leadership Team is ethnically, culturally, and geographically diverse, including US-based researchers who originate from and/or currently conduct their primary research in global EID hotspot regions[5,14], e.g. South Asia (Hoque); Latin America (Mateos); Africa (Mendenhall); and Southeast Asia and the Pacific (Olival). Our geographic and disciplinary diversity, including long-standing international collaborators from each region make our team well equipped to address spillover at a global scale, while understanding the importance of local context.

PI Olival (EHA) is a disease ecologist and evolutionary biologist with a primary interest in developing novel analyses to target zoonoses surveillance and viral discovery. He currently leads two regional biosurveillance projects in Southeast and Western Asia, and recently served as Modeling & Analytics Coordinator for the USAID PREDICT project[26] overseeing a multi-institution team developing pandemic prediction strategies using public and project-specific data from 30 countries[5,15,30-35]. **coPI Mendenhall** (GU) is a medical anthropologist and Professor at the Edmund A. Walsh School of Foreign Service. Mendenhall works at the intersection of social, psychological, and biological lives, attempting to understand what makes people sick, why, and where. Mendenhall is currently a PI of NIH Fogarty International Center grant "Soweto Syndemics", the first ever study to evaluate a syndemic from the ground up. **coPI Hoque** (UR) is an Associate Professor of Computer Science working at the intersection of Human-centric Computing (HCC) and Artificial Intelligence with a focus on multimodal machine learning with applications in health and wellness. Hoque is currently a PI of NSF NRT grant, and received NSF CAREER and CRII grant. Hoque has been identified as an 'emerging leader of health and medicine' at the National Academy of Medicine (NAM). **coPI Mateos** (UR) is Assoc. Professor of Electrical and Computer Engineering and a Data Science Fellow, his research focuses on algorithmic foundations, analysis, application of (graph) signal processing tools to the study of large-scale and dynamic networks. He has pioneered foundational work in network topology

inference, online learning from graph streams, and interpretable representation learning for unstructured data. **coPI Ross** (EHA) is Principal Scientist for Computational Research. His research includes model development to interpret complex spatio-temporal disease surveillance data in heterogeneous populations, including from serological surveys, real-time community and hospital testing, and simulating and forecasting epidemic dynamics over populations, space, and networks. He is PI on awards from DHS Science & Technology Directorate and the Defense Threats Research Agency (DTRA) for designing disease forecasting systems. **SP Mendelsohn** (EHA) is a research data scientist specializing in the development and application of machine-learning models to draw insights into the occurrence and spread of emerging infectious diseases. With a focus on reproducible research methods and data management, she has been the lead analyst on data driven disease forecasting and behavioral survey-based projects characterizing animal-human contact patterns. **SP Daszak** (EHA) is an EID expert who has studied the process of disease emergence for decades and pioneered groundbreaking work in "hotspot" risk mapping to target surveillance. **SP Broder and Laing** (USU) are experts in the design of multiplex serological assays, molecular virology, and implementation of field-based human and wildlife biosurveillance strategies. **OP Busa** is a biotechnology consultant with a specialty in developing lateral flow assays and optical readers. **OP Pompe** is the Research Manager for Facebook Data for Good, who specializes in applying human mobility datasets from social media feeds and data privacy and ethical issues.

Intellectual Merit

The primary intellectual merit of our proposed project is in **developing and piloting innovative approaches to pandemic prediction across previously siloed scientific domains** including biology, computer science, electrical engineering, and social-behavioral research. We apply new theoretical advances and convergent research to address our grand challenge of capturing actionable data about the risk of zoonotic spillover and early spread at a scale and speed required to prevent outbreaks. We will identify existing gaps and next-generation solutions for step-level changes in zoonotic spillover prediction via multi-day intensive workshops with thought-leaders organized by each of our three multidisciplinary Working Groups. Our pilot research projects will serve as a test bed for new theoretical frameworks to advance spillover intelligence. For example, in Aim 1 we will transform decades-old anthropological approaches and pilot a cost-effective, scale-up of quantitative and qualitative behavioral data collection through a unique public-private partnership Facebook; and use image recognition and artificial intelligence to quantify and map human-wildlife contact globally. In Aim 2 we will develop hierarchical Bayesian models to interpret rich, multiplex serology datasets and test these on real surveillance data as part of EHA's NIH-funded work; and develop the first field-forward lateral-flow assay for Nipah virus antibody detection. In Aim 3 will use cutting edge theoretical advances in network science, anomaly detection, and machine learning to detect changes in human mobility patterns that may portent spillover (e.g. increase congregation around wildlife markets) or signal early epidemic spread after the point of spillover.

Broader impacts

This proposal could potentially transform our understanding of zoonotic spillover risks using survey, multimedia, and mobility data on a global scale, including aggregated and anonymized data from ~3B active Facebook users. Insights and intellectual outputs from our working group activities will have direct benefits to public health by informing disease mitigation strategies for pandemic prevention. We will synthesize our three working group outputs and prepare a special joint publication for the National Academies of Science's Forum on Microbial Threats for wide distribution, and write popular press articles to accompany our peer-reviewed manuscripts. EHA's *Alliance,* includes a network of over 70 partner institutions in 30+ countries, presents a unique opportunity to work with local stakeholders in emerging virus 'hotspots' and validate

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models using data sets obtained through our long-standing collaboration with government partners including human health, agriculture, and environmental sectors.
Our education mission consists of multiple workshops, hackathons, monthly seminar series, and access to state-of-the-art visualization facilities. We will begin to train a new generation of graduate and undergraduate students who will be prepared to harness the burgeoning power of data science to make novel inroads into understanding human-animal contact, serological surveillance, and aggregated mobility data and their interaction. Our proposal builds on a unique partnership between the academic (UR, GU, USU), non-profit (EHA), and private sector (FB), allowing students to apply lessons learned from classroom settings to the real-world, and our recruitment will focus on including >50% women and underrepresented minorities in science and engineering. This planning grant will be a path towards structuring interdisciplinary education, cross-training courses across institutions, followed by hands-on experience via internships and research exchanges – with options for international work via existing programs at our institutions. Through the planning grant, a new MS data science track on 'zoonotic surveillance' will be established at UR, the first step in our plans to expand our Education and Training plan should we move towards a Center-level award in the future.

Education and Training Plan
Our Leadership Team will serve as mentors for our **CSI Student Training Program** that will offer a fellowship stipend to cross-train 20 Master's level graduate students via semester-long projects associated with proposed pilot research activities (3-4 students per project) and 3-week research exchanges between our institutions (UR, GU, EHA, USU, 4-8 students from each). The research exchange builds off EHA's successful NSF-funded EcoHealthNet training program (2011-21) and will take place in either the Summer of 2022 or 2023. We will link with currently-funded international programs at our respective institutions (e.g. Gui^{2}de in Nairobi, Kenya run by GU; and the NIH funded CREID research pilot program with EHA's EID-SEARCH project in Southeast Asia) to support students who desire to gain international, hands-on research experience as part of their research exchange. The focus of each research exchange will be on cross-disciplinary training to think 'big' about the future of pandemic prediction and ensure the next generation of spillover intelligence researchers gains valuable hands-on experience working with experts outside their area of study. Students will be drawn from respective departments across our institutions, i.e. students with a computational background and with interests in interdisciplinary work will be recruited from UR's cross-department Goergen Institute for Data Science, Anthropology and International Affairs students from GU, and Emerging Disease and Virology students from USU. As EHA is a non-profit and does not have an active student body, we will recruit Ecology, Evolutionary Biology, and Environmental sciences students from universities in the NYC area, including Columbia University's E3B department where PI Olival, coPI Ross and SP Daszak serve as adjunct faculty. We will explore specific mentorship opportunities with data scientists from Facebook. If awarded, we will additionally apply for funds through NSF's Research Experiences for Undergraduates (REU) program, with the goal of supporting summer stipend for 1 undergraduate per institution or the maximum allowable. We will host a joint monthly virtual seminar series (2 semesters long) open to graduate and undergraduate students from our respective institutions, with a dedicated student "lab meeting" before each seminar. Two CSI students from diverse disciplines will lead each lab meeting discussion based on a deep read of relevant publications that each speaker will distribute in advance. We envision the first semester of the seminar series will be led by our Leadership Team and Working Group experts, and the second semester will be for CSI Graduate students to present results or current challenges related to their pilot research projects. We hope to encourage these students to pursue Ph.D. studies. If we are successful at establishing a center through our planning grant, we should have access to a trained cohort of students with appropriate multidisciplinary skillset.

Project Overview

Our 18-month research agenda aims to understand how new methods and technologies can improve the efficiency, intensity, speed, and scope of zoonotic disease surveillance. **We have structured our development grant around three primary research aims** that will guide our interdisciplinary research and training activities. We will establish a working group composed of multi-disciplinary experts for each primary aim that will be co-led by members of our Leadership Team. We will develop pilot research projects that capture different parts of the disease spillover process focused on specific geographies, lead 3-day intensive workshops, monthly student seminars, and student training activities that cut across our inter-linked research aims.

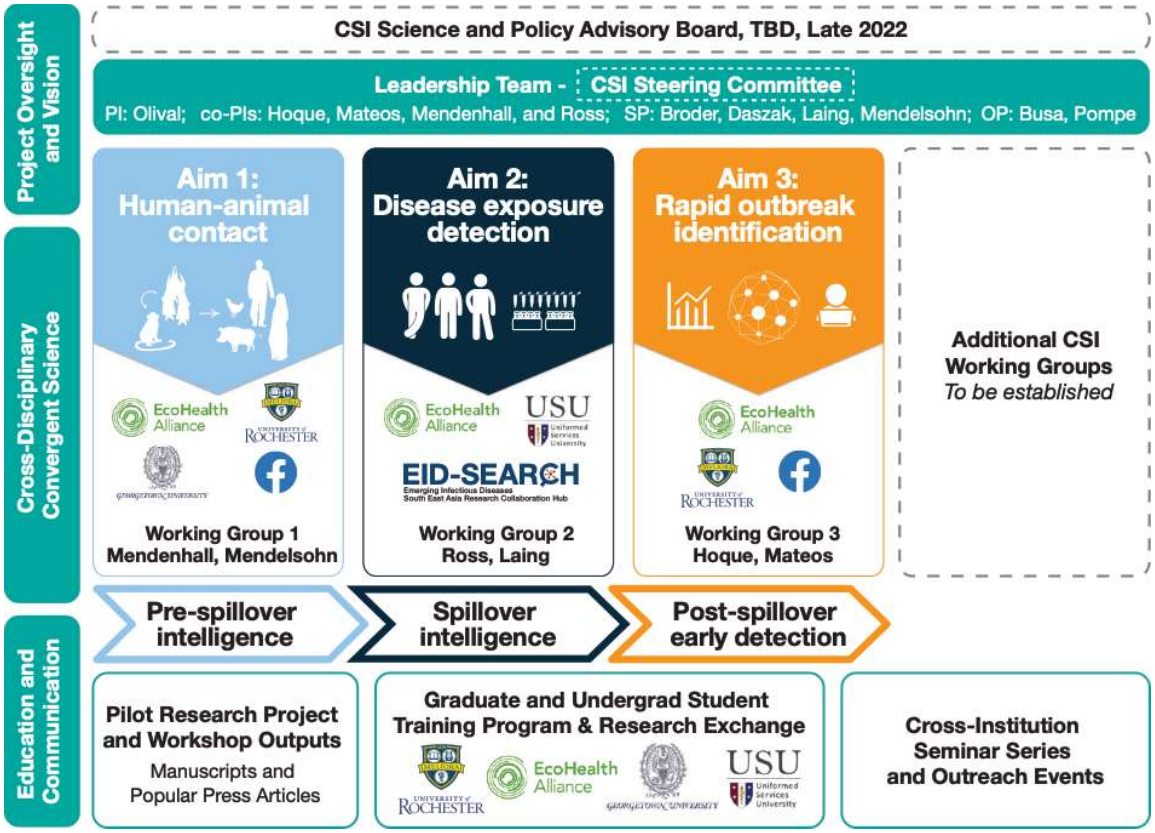

Fig 2. Project overview schematic showing our project management; three primary aims with graphical representation of pilot research activities to scale up and advance spillover intelligence; institutional partners leading activities for each aim including designated working group leads, and student education and communication activities, including pilot research projects, graduate student research exchanges, seminars, and other outreach activities. Our development level project was built to scale to a Center level award (the Center for Spillover Intelligence, CSI) as noted with the dashed outline boxes, including the future addition of other CSI Working Groups, a Science and Policy Advisory Board, and transitioning our current Leadership Team (PI, coPIs and SP) into a more formal Steering Committee for oversight.

Aim 1: Measuring human-animal contact at scale

Interactions among humans, wildlife, and livestock create interfaces for pathogen spillover and are proximate risk factors for the emergence of all directly-transmitted zoonotic diseases.[14,36] Human-animal contact is commonplace around the world, with many factors mediating the type and frequency of contact, including cultural practices, environmental conditions, food security, and occupational demands.[36] In regions of the world considered prone to spillover--forested tropical landscapes with high biodiversity and recent land-use changes--understanding human-

animal contact is crucial to pandemic prevention.[5] To measure these interactions and characterize associated behaviors, beliefs, and knowledge, researchers often implement resource-intensive surveys in key communities.[37,38] While crucial to understanding human-animal contact dynamics, on-the-ground surveys cannot yield scalable data that is comparable across populations. Often, in place of surveys, researchers conducting analyses at broad scales rely on coarse estimates of contact such as the joint density of human and animal populations. **Our working group will uniquely combine social scientists, disease ecologists, and computational scientists to increase the scale at which we can directly measure human-animal contact.** We will develop and implement two pilot research projects. The first will use novel, social-media-based survey approaches to reach larger populations at higher frequencies while maintaining the cultural sensitivity and relevance of on-the-ground surveys. Working collaboratively with cross-disciplinary researchers, we will design surveys to understand social behaviors, cultural perceptions, and occupational hazards that may pose zoonotic spillover risk to individuals and communities. We will target geographies based on our existing spatial risk modeling work that maps infectious disease spillover potential and the ecological distributions of key reservoir host species.[5,15,25] Second, we will conduct pilot research on the use of social-media-based image recognition algorithms to identify locations of human contact with key species known to host high pathogen diversity. This pilot will include a collaboration effort to test the efficacy of such technology for understanding taxon-specific contact patterns globally, and to bring multidisciplinary context to meaningfully interpret the outputs (including species validation with taxonomic experts). If successful, will use these data on frequency of animal contact from image data to identify locations for additional survey data collection.

Aim 1 Pilot Research Projects: We will develop two pilot projects to assess the potential of 1a) social-media dispensed surveys and 1b) passive surveillance using artificial intelligence for image recognition to capture information about human-animal contact. We will target populations at wildland interfaces where contact with wildlife is likely high but internet penetration may be mixed.

1a) Social-media dispensed surveys to quantify and contextualize human-animal contact
We will pilot social-media dispensed, large scale population surveys about perceptions of and interactions with animals. We will optimize the design of these surveys for mobile platforms to facilitate higher response rates, and will also explore the possibility of longitudinal sampling respondents (either individuals or populations) to understand changes in conditions and responses over time. Survey questions will be based on a combination of EHA's past experience designing on-the-ground human-animal contact surveys[37,39,40] and ideas generated from our proposed working group activities involving anthropologists, data scientists, and disease ecologists who have worked extensively in the geographic and societal contexts in which we focus our work. We will aim to design surveys that account for populations and individuals holding different beliefs, carrying different histories, and living in different ecologies. We will attempt to survey demographically and geographically diverse populations to capture direct and indirect animal-contact patterns and behaviors, and will attempt to capture the role that international, state, and local policy plays in individuals' lives. Further, we will aim to recognize particular hesitancies among individuals to interact with certain technologies or other public health interventions.

We will explore various online platforms at the start of our project for survey administration. Given our unique public-private partnership with Facebook for many components of this project, using the Facebook platform to disseminate our pilot survey is likely to be a strong option, especially given Facebook's global reach (nearly half of the world's population, ~3 billion users), which introduces opportunities for digital interaction across class, gender, ethnicity, and cultural

divides. Facebook has extensive experience administering surveys, either directly on its platform (e.g. Yale Climate Change opinion survey) or off-platform (e.g. Carnegie Mellon University COVID-19 surveys) that we will leverage. Facebook also has extensive privacy infrastructure and data-review policies to ensure anonymity and data security.

We will also explore creating games that users will be encouraged to play online to enable us to learn more about how they think about host species, viral threats, environment, and health/disease. For example, users may be given a list of 30 images and will be asked to put them into categories and describe what each category means. Adapting these "pile sorting" anthropological methods[41] to an online interface and for spillover risk, will be a novel application with potential for a massive scale-up of data around cultural and conceptual dimensions of zoonotic disease risk. Data generated from this exercise will generate a more ethnographic understanding of people's experiences and thought processes, which tend to be locally mediated. We understand that there are geographic and demographic gaps in internet and social media use, and will incorporate bias corrections into our study design. We will initially focus on Southeast Asia, a region with a high likelihood of future zoonotic disease emergence and where EHA has good coverage of on-the-ground surveillance programs to ground truth online data collection.[5]

1b. Image recognition of zoonotic disease host species

We will pilot image-based surveillance of Facebook and Instagram feeds to attempt to identify human contact with key zoonotic disease host species and to explore the limits of taxonomic and behavioral recognition in images. We will work with Facebook Data for Good (see **Letter of Collaboration**) to adapt and refine their existing machine vision algorithms to target key taxa and to assess the efficacy of different approaches, working within strict guidelines and ethical standards for data de-identification. While data will be de-identified, we will only use photos with full location meta-data, allowing us to not only quantify the type of species and contact, but where it occurred. There are multiple image libraries that may serve as AI training datasets, including iNaturalist, the American Society of Mammologists, and Integrated Digitized Biocollections (iDigBio). We will iteratively implement and test algorithms starting with broad taxonomic groups (e.g., bats) and will explore the extent to which our approaches can identify finer taxonomic resolutions down to the genus or species level. In addition to taxa identification, we will explore image recognition of features that may suggest specific types of human-animal contact, such as cages or market settings. Further, we will use text and image metadata and explore using hashtags associated with images to boost the ability of our algorithms to identify key taxa.

This work will involve collaboration between machine learning scientists, biologists/ecologists, and social scientists. We will follow all data anonymity and privacy guidelines from Facebook and will also discuss data handling ethics in our working group. We will assess the ability of passive surveillance with image recognition to be used to target surveys to populations that may have higher frequency of contact with wildlife, and ultimately to target biological surveillance of human and animal populations. We will consider both the accuracy of the image recognition algorithms as well as implications for survey distributions, results interpretation, and ethical data practices. We will also compare the spatial distribution of human-animal contact patterns derived from image recognition with previously generated spillover risk maps based on joint occurrences of animal and human distributions (e.g., SARS-related CoV spillover risk from Sanchez et al. 2021 preprint).[25]

Working Group 1 Activities: Our working group will convene 12-15 experts from the social sciences, disease ecology, and computational sciences for an intensive, 3 day workshop. We will structure the workshop to focus first on data ethics in human behavioral surveying and image recognition surveillance and secondly on survey design and analysis planning, with specific attention to accounting for gaps or biases in survey distributions. We will center the workshop around the understanding that technological quick fixes are never enough and that data collection must address long term social, ecological, and economic realities to understand how people perceive, experience, and interact with the risk of and repercussions from spillover events. We will also address experiences and challenges in engaging longtime ethnographers and local leaders to understand what's at stake for communities engaging with online and field surveillance programs. Our working group will help mentor students part of the CSI Student Training Program, to facilitate information sharing and idea generation among undergraduate and master-level students. Working group 1 will also contribute to the CSI Seminar Series with cross-disciplinary topics such AI image recognition (lead by a data scientist and bat biologist) and human-behavioral survey design (lead by an epidemiologist and medical anthropologist).

Partners, Initial Working Group 1 Members, and Roles: EHA (SP Mendelsohn): survey design and implementation, image recognition algorithm development and testing, novel data analyses. Facebook Data for Good (OP Pompe): survey implementation, image recognition algorithm development and testing, data ethics. University of Rochester (coPI Hoque): Image recognition algorithm development and testing. Georgetown University (coPI Mendenhall): Survey design with focus on ethnography and anthropology; online free listing/pile sorting game design; help lead expansion of WG to include emerging international leaders in social-behavioral science (e.g. Dr. Edna Bosire from Kenya). We will invite additional leaders from our extensive funded collaborations to be part of the Working Group for Aim 1.

Aim 2: Detecting zoonotic disease exposure at scale

Animal-human contact is the necessary condition for spillover of potential pandemic, directly-transmitted viruses. While a small fraction of such encounters result in actual exposure or infection, such exposures are frequent given the total sum of encounters. Most such infections are never reported and rarely spread widely, and only a very rare subset become widespread in the human population[42]. Other viruses such as Lassa spill over thousands of times per year[43]. In areas of frequent spillover, the eco-evolutionary dynamics of "stuttering chains" of infection are what give rise to epidemics and pandemics[44]. Identifying populations where frequent exposure occurs is critical for deploying preventative measures and following viruses upstream to their wildlife hosts. Yet even large-scale surveillance projects cover too small populations and produce far too few detections to determine the most exposed geographic, demographic, or occupational groups at an actionable scale [REF, showing PREDICT sampling efficiency]. This is for two reasons: the field and laboratory cost of surveilling populations interacting with wildlife interface and, and the low detection rates of the widely-used, molecular PCR or metagenomic approaches.

We aim to drastically increase the scale at which populations can be monitored for exposure to animal-origin viruses by developing and piloting novel tools and analyses needed to implement large-scale serological surveys. While metagenomic methods have been favored for their ability to capture genetic sequences of previously unknown viruses, serological methods can be used to detect exposure to known and unknown viruses using multiplex assays tailored with a mixture of sensitivities and specificities[45]. Serological tools can detect antibodies for months or years after viral infection, rather than the limited window of days or weeks using molecular methods, and thus can provide a much richer profile of populations where pandemics may emerge[46]. In this planning period, we will begin pilots to test the feasibility of two complementary approaches

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Our work under PREDICT is relevant here, working with local community leaders to distribute results and the 'Living Safetly with bats book'. But how do we scale this? One idea is through a network of local leaders across the landscape, like a "Community One Health Volunteer" network.

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to enabling serological screening at large scales: 2a) the creation of rapid bioinformatics pipelines to interpret high-dimensional serological data from large streams of surveillance data, and 2b) the development of rapid field serological tests for wildlife-origin diseases.

Aim 2 Pilot Research Projects

2a. Rapid bioinformatics for high-throughput, multiplex serology

Members of our team (SP Laing, Broder) have developed multiplex serology tests that can be used to screen for exposure for many species and variants of filo-, henipa-, and coronaviruses simultaneously, and screening pipelines that can process samples at the scale of tens of thousands of samples. However, these tests pose challenges for interpretation, as high-dimensional results from multiplex bead or chip assays frequently contain signals of cross-reaction and autocorrelation (**Fig 3**). Through cross-disciplinary research between molecular virologists, serologists, mathematical modelers and data scientists on our team, we will create models to interpret this high-dimensional data and integrate them into a rapid bioinformatic pipeline that identifies individuals and populations to known viruses as well as flagging serological signals indicative of novel viruses. We will make use of data collected under our ongoing NIH and DTRA funded biosurveillance activities (Laing, Broder, Ross, Olival, Daszak) to develop detailed reaction/cross reaction profiles of a broad panel of zoonotic viruses to serological tests. We will build mechanistic, hierarchical Bayesian models that allow us to determine whether serological "profiles" are more likely to represent antibodies of known viruses, mixtures of multiple known viruses, or previously uncharacterized viruses. We will extend techniques of antigenic mapping,[47] using serological profiles to estimate phenotypic similarity of new viruses to known viral families, determine the correlation with phylogenetic relatedness, and use these measures to screen new viruses for pandemic potential. Using simulation experiments, we will test reduced-form, simple models against fully structured models so as to develop statistically robust procedures to interpret these data without high-performance computation.

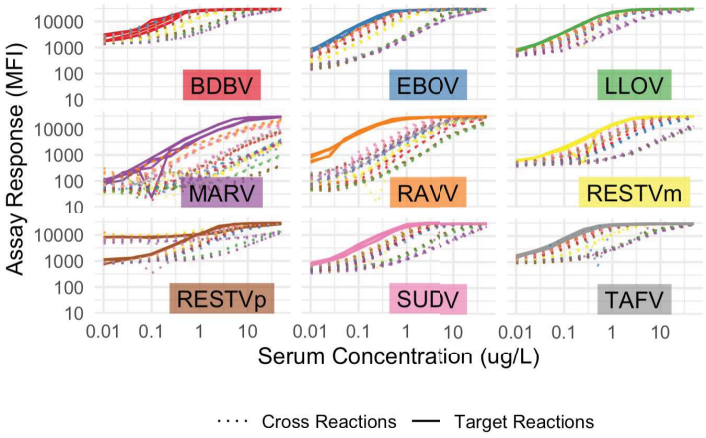

Fig 3*.* Preliminary data: Reaction-cross reaction plots of multiplex Luminex assay response to antibodies for closely-related filoviruses.

Scaling center operations: This pilot project will produce methods and a proof-of-concept pipeline for high-throughput analyses. We will leverage EHA's Emerging Infectious Disease – Southeast Asia Research Hub (EID-SEARCH) project, a NIH-funded Center for Research in Emerging Infectious Diseases (PI Daszak) who's partners include USU, Duke NUS, Chulalongkorn University in Thailand, and various clinics and institutions in Malaysia – currently screening large numbers of human samples (>10K/yr) for various coronavirus, filovirus, and paramyxovirus antibodies. In the future the CSI will implement surveillance studies screening these sample streams using multiplex serology, and utilize the analytical and bioinformatic tools we develop in the next 18 months. As part of our planning activities, we will establish protocols and approvals for this work.

2b. Development of a field-ready semi-quantitative lateral flow assays for an emerging zoonotic viruses (Nipah virus)

Lateral flow assays (LFAs), are low-cost tests where reagents printed on cellulose strips react with a sample that flows via diffusion. LFAs are capable of providing quantitative results (in proportion to antibody titer) using a combination of high-quality manufacturing and carefully designed, calibrated optical scans[48]. LFAs can be used to provide diagnostics at low cost at the point of care and by consumers. Lack of commercial-scale applications has prevented LFAs from being used for serological testing for novel or emerging disease. To our knowledge, no studies have used LFAs for zoonotic virus serosurveillance in the field.

We will develop a proof-of-concept LFA for Nipah Virus (NiV). NiV has a high mortality rate and is considered to have pandemic potential that is ranked by CEPI as one of 7 high pandemic risk pathogens[49]. The range of NiV's primary wildlife hosts (*Pteropus spp,* fruit bats) cover South and Southeast Asia but human cases have only been detected in Malaysia, Bangladesh, and the Philippines. Spillovers occur regularly in Bangladesh, but are rarely reported in large portions of the country[50] and reporting is strongly associated with health care accessibility[51]. Broad-scale serological monitoring has the potential to identify populations from which pandemic strains may emerge. Through previous work, members of our working group (Laing, Broder) have developed multiplex serological assays for NiV[52] and the soluble proteins and reagents required for an LFA. We have also added experience in engineering, quality control and commercialization of LFAs (Busa, industry consultant) to our working group. For the pilot, we will engage with commercial manufacturers to create and test a prototype Nipah LFA and a protocol for smartphone-based optical reading of quantitative responses.

Scaling center operations: The pilot will create a prototype LFAs. As part of full center operations under CSI, we will aim to scale up production and test LFAs efficacy using control sera and field deployment as part of EID-SEARCH. We will additionally assess feasibility and cost of deployment, and compare performance against current laboratory-based assays.

Working Group 2 Activities: In addition to the two pilots above, working group members will contribute to the our CSI monthly seminar series, presenting on topics including high-dimensional data models, field serology, serological assay development, laboratory product design, and public-private partnerships and commercialization. We will conduct two online workshops on serological data analysis, jointly with undergraduate, masters, and doctoral students at USU and UR, leveraging partnerships with field researchers currently working in serological surveillance. Our working group will lead a 3-day intensive workshop, where we will recruit additional participants with expertise in serological assay design and surveillance, immunology, emerging zoonoses, and focus discussion on key barriers to scaling serological surveillance for known and unknown zoonotic viruses.

Partners, Initial Working Group 2 Members, and Roles: EHA (coPI Ross): Model and pipeline design, software optimization. Uniformed Services University (SP Liaing, SP Broder): data and model interpretation, reagent manufacture, assay design. SP Busa: device manufacturing, quality control, industry outreach and partnership development. We will recruit additional participants from our partner network engaged in serological field studies as well as industry representatives to be part of the Working Group for Aim 2.

Aim 3: Rapidly Identifying Anomalous Health Events

In early stages of epidemic spread, novel contact and mobility-induced behavioral data as well as communication patterns may provide a signal of e.g., health-seeking activity indicative of an outbreak cluster. We contend that these signals may be detectable in large-scale data streams of population mobility and contact, or disease reporting available through telecommunications and health care providers. Timely detection requires approaches that can manage data at scale,

analyze high-dimensional, spatio-temporally correlated streaming data in (pseudo) real time, and distinguish between known signals (e.g., typical influenza-related activity) and novel spillover-related signatures that we wish to unveil, characterize, and explain in order to trigger the necessary public policy responses.

This desideratum calls for fundamental methodological and computational advances, to go along with epidemiologically-relevant data sources that describe how humans are moving and interacting across physical space, with unprecedented temporal resolution and geographical coverage. Traditional flow-based mobility datasets used to parameterize epidemiological models have been aggregated and formatted in the form of origin-destination (OD) matrices. While valuable towards understanding population flows between regions of interest, OD matrices lack information on how much time a traveler spends in any given location. Naturally, when it comes to epidemiological studies the duration of time spent in potentially risky geographical areas is key towards deciphering actionable contact and exposure patterns[53]. Moreover, recent studies have shown that flow counts between pairs of geographical areas can be loosely correlated with, and not representative of, epidemiology-relevant mixing patterns[54]. Instead, these couplings among subpopulations are measured by effective contact rates, meaning how often people from each region come into contact in a way that is sufficient to transmit a disease. To address these challenges, our consortium will partner with the Facebook Data for Good team and leverage two recently assembled global-scale datasets - Activity Space Maps and Colocation Maps[53,54].

Different from existing approaches that have focused on measuring the causal impact of mobility-oriented interventions, **our ambitious goal in this aim is to markedly advance the surveillance infrastructure available to predict anomalous health-related events before a major outbreak occurs**. While undoubtedly a multi-faceted endeavor, in this planning grant we will take initial, but significant steps with an emphasis on network-centric, computational analyses of dynamic mobility and interaction data at a global scale. By developing the appropriate statistical tools, we will support the ultimate goal of characterizing early pandemic "fingerprints" or signatures that can be extracted from these global datasets.

Aim 3 Pilot Research Projects: Working closely with Facebook Data for Good (see Letter of Collaboration from Alex Pompe), this working group will pilot data-driven research activities to explore the feasibility of extracting early (post spillover) signals of epidemic onset from Activity Space Maps and Colocation Maps. Our multi-disciplinary working group brings together leading experts in systems engineering, AI for healthcare, data science, mathematical epidemiology, graph signal and information processing, privacy-preserving mobility data generation and management, human behavioral modeling, and environmental science. We believe the current team, consisting of a wide array expertise and partnership, will instigate the cross-pollination of ideas leading to center-level activities while making significant inroads towards addressing our grand challenge. We propose two specific pilot projects:

3a. Spatio-temporal monitoring of spillover-induced activity anomalies in Southeast Asia
Activity Space Maps (ASMs) are a novel global-scale movement and mobility data set which describes how people distribute their time through geographic space.[53] They are designed to complement existing digitally-collected mobility datasets by quantifying the amount of time that people spend in different locations. Accordingly, this information is valuable for estimating the duration of contact with the environment and the potential risk of exposure to disease -- something critical especially early after spillover. More germane to the theme of this aim, it also offers the unique possibility of developing and testing sequential change-point detection algorithms to monitor multiple spatial grid cells of interest (informed, for instance, by outcomes

of the image-recognition effort under Aim 1). We will target areas known to include health care facilities in pursuit of abnormal trends of (increased) activity. Moreover, using ASMs we will examine flow and temporal activity anomalies around expert-annotated wildlife and wet market locations. Said events are expected if there are human infections, or significant associated changes in behavior around these sites early after spillover. Our initial efforts during this planning grant will be focused on Southeast Asia, which on top of its epidemiological relevance is one of the regions where Facebook ASMs have the most coverage. We will target Indonesia, where EHA has extensive on-the-ground efforts tracing networks of wildlife trade as well as market sites.[55] We will also tap into the resources stemming from EHA's Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH) to identify clinics and market sites of interest in Thailand and Malaysia. Some key challenges we will address include accounting for the implicit biases in data collection associated with the construction of ASMs,[53] and their subsequent effects on the statistical algorithms that we will develop. Our Working Group will address additional challenges of data interpretation of anomaly detection analyses with input from emerging disease experts and epidemiologists. To calibrate our models, we will bring to bear recent activity data before and after the SARS-CoV-2 outbreak in those locations, in addition to historical influenza seasonal trends that go further back in time. With the exciting prospect of ASM data being officially released in the last quarter of 2021, *we are in a privileged position to be the first multi-disciplinary team that leverages this invaluable global-scale resource to advance our understanding of zoonotic spillover risk and its immediate effect on human mobility.*

3b. Online network change-point detection in dynamic, global-scale Colocation Maps
Activity data are complemented by *Colocation Maps (CMs),* which estimate how often people from different regions worldwide are colocated. In particular, for a pair of geographic regions *i* and *j* (e.g., counties in the US), CMs estimate the probability that a randomly chosen person from *i* and a randomly chosen person from *j* are simultaneously located in the same place during a randomly chosen minute in a given week.[54] In other words, CMs are global-scale, dynamic network datasets with colocation probabilities encoded as edge weights. Thus, they are well suited to parametrize metapopulation models of disease spread,[56] or to measure temporal changes in interactions between people from different regions.[57] To monitor said changes as part of our second pilot activity in this aim, we will build on our recent advances in online (i.e., sequential) algorithms for anomaly and change-point detection from network streams[58,59]; see also.[60-62] The envisioned statistical methods are in stark contrast with most existing graph change-point detection approaches, which either rely on extensive computation,[63] or they would require storing and processing the entire observed CM time series.[64] We will prototype and test a lightweight online change-point detection algorithm with quantifiable performance guarantees, that is also explainable since it relies on interpretable node embeddings; see e.g., *Hamilton_Book*.[58,65] The latter desirable feature will be leveraged to construct dynamic anomaly maps for user-friendly visualization of post-spillover events; see also our recent network-scientific visualizations of spatio-temporal COVID-19 contagion patterns across the US.[66] Moreover, the sheer-size of the CM graphs calls for algorithmic and modeling innovations we will investigate as part of our research agenda, e.g., seamlessly integrating graph subsampling or dimensionality reduction techniques to the analytics pipeline. We will team up with computer vision experts affiliated to the University of Rochester Goergen Institute for Data Science (GIDS, see attached Letter of Collaboration from institute director Mujdat Cetin), to also explore the exciting prospect of integrating geo-tagged multimodal data such as video and imagery in our studies, for instance as nodal attributes embedded to the CMs.

Working Group 3 Activities: *Recruitment and diversity:* We will recruit 6 students from the GIDS-run master's program in data science, which attracts students from multidisciplinary backgrounds and significant computational proficiency to contribute in the aforementioned pilot research activities. *The CSI leadership and broader community are committed to providing research training and educational experiences for those traditionally under-represented in STEM, including women and underrepresented minority students.* The PI team has a solid track-record in actively recruiting and supervising such students at the undergraduate, masters and doctoral level, with demonstrated efforts towards broadening participation in STEM. As examples of the commitment to diversifying the field, co-PI Mateos serves as founding faculty advisor to the UR's SACNAS chapter and regularly organizes diversity, equity and inclusion panels to highlight challenges and successes of UR's Hispanic and Latino STEM community. We plan to prioritize engaging women and minority students in the research and working group activities proposed here. *Coursework:* These students will be on the existing health and bio track with an opportunity to take classes in immunology, microbiology and virology programs as well as data ethics at the University of Rochester Medical Center (URMC) as well as the college of engineering. Through the planning grant, we plan to establish a special MS track on 'zoonotic surveillance' in order to train and mentor the students to lead center level activities. *Seminar and workshop series:* The students will be part of the large cohort of all the participating institutions and participate in the CSI monthly seminar series. The co-PIs (Mateos and Hoque) will conduct two workshops (and invite additional faculty members from UR as guest speakers) with hands-on lessons in mobility and human behavior modeling. *Hackathon:* The students will be encouraged to participate in the yearly hackathon, Dandyhacks https://dandyhacks.net/), organized every fall by the University of Rochester Computer Science Department and work with the dataset from Facebook. Additionally, co-PIs Mateos and Hoque will work with the organizers of dandyhacks to release some of the public dataset from Facebook for other teams to explore, model and mine the data. *Data visualization meetups at the University of Rochester's VISTA Collaboratory*: To get acquainted with the different data resources at our disposal, over the first 3 months of the project the Rochester team (along with Facebook and EHA collaborators via Zoom) will hold bi-weekly team meetings with the MSc students at the UR's VISTA Collaboratory. This is a 1,000 square-feet visualization lab equipped with an interactive (20 ft. wide x 8 ft. tall) tiled-display wall, that renders massive data sets in real time, giving team members the ability to visualize and analyze complex data instantaneously and collaboratively; see Fig. 4. As we dissect what dataset components are central for the aforementioned pilot activities, our graduate students will develop customized data visualization tools, maps and dashboards to facilitate downstream analyses. Updates and progress reports will be shared in these meetings, giving the students a unique opportunity to develop their communication skills. The whole team will provide feedback and share ideas, making this a truly collaborative activity. Moving forward, the VISTA Collaborative offers a unique resource that we will fruitfully leverage as we scale up to full center operation.

Fig. 4. UR's Vista Collaborative will serve as a spillover intelligence "situation room" for training UR and CSI exchange students on analysis of real-time epidemiologically relevant datasets.

Partners, Initial Working Group 3 Members, and Roles: University of Rochester (co-PIs Hoque and Mateos): coordination of working group activities, development and testing of computational tools for early detection of post-spillover signatures, dissemination of research products and educational materials, recruiting and mentoring of master students, liaisons to

GIDS. Facebook Data for Good (OP Pompe and Maps for Good team members Iyer and Citron): data ethics, quantification of privacy protections, data scientific support for Activity Space and Colocation Maps, mathematical epidemiology expertise for data and model interpretation. EHA (SP Mendelsohn and Ross): identification of target spatial locations in Southeast Asia, epidemiological interpretation of anomaly detection analyses and validation of detected early pandemic fingerprints.

Scaling operations: Our programs and pilot research are designed to scale. First, we aim to expand on successful pilots by expanding the geographies of our work, engaging with more in-country partners. Second, we test the development of each of these new streams of predictive data, we plan to develop partnerships with local, national, and global agencies to ingest these data, expand into programs to develop behavioral interventions and testing in response to identifying regions and populations at high risk of contracting zoonotic diseases.

Working groups are really about bringing our project to scale, our intensive 3-day workshop will also lead to new partnerships and synergies for expansion.

Results from Prior NSF Support

Kevin J. Olival has not had prior NSF support, but is co-PI on NIH-funded "EID-SEARCH" (NIAID-CREID U01AI151797, 2020-25 PI Daszak). Previously co-PI on "Understanding the risk of bat coronavirus emergence" (NIAID R01 AI110964, 2014-2019, PI Daszak). ***Intellectual merit*:** Identified >50 SARSr-CoVs in bats, showed evidence of ability to infect human cells, humanized mouse models, and people in China, and used evolutionary and mammalian biodiversity patterns to analyze spillover risk for use in public health control. Produced 18 peer-reviewed papers [20,34,67-79], including two papers in *Nature* [80,81], and a review in *Cell* [82], and others submitted. ***Broader impacts*:** 2 PhD and 6 MS students trained; disease risk and potential management strategies shared with US agencies, WHO, OIE, FAO, and the public.

Ehsan Hoque: Project IIS-1750380. PI: Hoque. Period (2018-23). Amount: $511,453 "CAREER: A collaboration coach with effective intervention strategies to optimize group performance". ***Intellectual merit:*** Developing and validating the efficacy of automated mediation strategies in online meetings. ***Broader Impacts:*** >6 research papers[83-88]. **Project NRT-DESE-1449828.** PI: Hoque (2015-21). Amount: $2,965,341.00 "Graduate Training in Data-Enabled Research into Human Behavior and its Cognitive and Neural Mechanisms". ***Intellectual merit:*** Chartering a new traineeship program to train Ph.D. students at the University of Rochester to advance discoveries at the intersection of computer science (cs), brain and cognitive sciences (bcs), and neuroscience. ***Broader Impacts:*** Supported over 50 PhD students in cs and bcs, seveal won interdisciplinary research positions at R1 institutes.

Gonzalo Mateos (a) Project: CCF-1750428. PI: Gonzalo Mateos Buckstein. Period: 4/1/18 - 3/31/23. Amount: $407,944. (b) Title: CAREER: Inferring Graph Structure via Spectral Representations of Network Processes. (c) ***Intellectual Merit:*** We investigate how to use information available from graph signals to estimate the underlying network topology through innovative convex optimization approaches operating in the graph spectral domain. ***Broader Impacts:*** We prepared a feature article on identifying network structure via GSP,[89] presented tutorials in EUSIPCO'19, CAMSAP'19 an SSP'20, organized the 2019 IEEE SPS Summer School on Network- and Data-driven Learning. (d) Research products are available in.[66,89-96]

Emily Mendenhall (a) National Science Foundation Doctoral Dissertation Improvement Grant, $10,000; Stories at the Border of Mind and Body: Mexican Immigrant Women's Narratives of Distress and Diabetes. **Intellectual merit:** Led to crucial insights studying syndemics - the interactions of social, environmental, psychological, and biological factors that are synergies of epidemics. Papers in The Lancet[29], Medical Anthropology Quarterly[97-99], and as a full-length

book[100]. **Broader Impacts**: Understanding diabetes, trauma, and broader socio-political factors with infectious diseases in India, Kenya, South Africa, and Ethiopia.

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From: Noam Ross on behalf of Noam Ross <ross@ecohealthalliance.org>
To: Kevin Olival
Cc: M. Ehsan Hoque; Mateos Buckstein, Gonzalo; Peter Daszak; Emily Mendenhall; Emma Mendelsohn; Eric Laing; Chris Broder; Luke Hamel; Robin Breen; Aleksei Avery Chmura; Hongying Li; Alison Andre
Subject: Re: [EXT] Penultimate draft of NSF PIPP Research Description to submit today (Oct 1))
Date: Friday, October 1, 2021 1:14:07 PM
Attachments: NSF_PIPP_ResearchDescription_v12.1_NR-2021-10-01 1310.docx

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My edits to the main research description attached.
--

Dr. Noam Ross
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EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.

On Fri, Oct 1, 2021 at 12:01 PM Kevin Olival <olival@ecohealthalliance.org> wrote:

> Dear all,
>
> Project Summary draft attached. Please let me know ASAP if you have any specific edits, and track changes.
>
> Cheers,
> Kevin
>
> **Kevin J. Olival, PhD**
> *Vice President for Research*
>
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>
> *EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation*
>
> > On Oct 1, 2021, at 10:30 AM, M. Ehsan Hoque <mehoque@cs.rochester.edu> wrote:
> >
> > Glad to see the proposal coming along. I have a long day ahead with back to back meetings. :-(
> >
> > Do you have the summary page written up yet? I would be happy to take a quick look at it.
> >
> > Best,
> > Ehsan
> >
> > On Fri, Oct 1, 2021 at 9:42 AM Kevin Olival <olival@ecohealthalliance.org> wrote:
> > > Thanks Gonzalo (cc'ing all for awareness). Will include your edits and also try and add a short paragraph on "Associated Risks and Mitigation Plans".
> >
> >
> >
> > **Kevin J. Olival, PhD**
> > *Vice President for Research*
> >
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EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation

On Oct 1, 2021, at 9:11 AM, Mateos Buckstein, Gonzalo <gmateosb@ur.rochester.edu> wrote:

Hi Kevin,

Amazing work, this reads really well. Attached are just some suggestions to fix a couple minor typos.

Something else I just caught on the solicitation (just before Section III - Award Information)

Several bullet points are listed under "Investigations that are outside the scope of this PIPP announcement include:" and one of them is:

- Projects that do not discuss associated risks and mitigation plans.

I do not think we have touched upon this explicitly in the proposal. Probably too late now, but if somebody finds a way to incorporate a paragraph that should be super.

Best,
Gonzalo

From: Kevin Olival <olival@ecohealthalliance.org>
Sent: Friday, October 1, 2021 4:07 AM
To: Peter Daszak <daszak@ecohealthalliance.org>; Noam Ross <ross@ecohealthalliance.org>; Emily Mendenhall <em1061@georgetown.edu>; Hoque, Mohammed <mehoque@cs.rochester.edu>; Mateos Buckstein, Gonzalo <gmateosb@ur.rochester.edu>; Emma Mendelsohn <mendelsohn@ecohealthalliance.org>; Eric Laing <eric.laing@usuhs.edu>; Chris Broder <christopher.broder@usuhs.edu>
Cc: Luke Hamel <hamel@ecohealthalliance.org>; Robin Breen <breen@ecohealthalliance.org>; Aleksei Avery Chmura <chmura@ecohealthalliance.org>; Hongying Li <li@ecohealthalliance.org>; Alison Andre <andre@ecohealthalliance.org>
Subject: [EXT] Penultimate draft of NSF PIPP Research Description to submit today (Oct 1))

Dear all,

Here is the cleaned up, penultimate version of our NSF PIPP Proposal Research Description (15 page main doc).

Please review this morning if possible and send back to me with tracked changes before 12:30pm today so we can incorporate edits and get this uploaded in the system before 5pm!

Attached also is the cleaned up Data Management Plan. I still need to clean up 1 page Summary and Project Management (2 pages) later this morning, but for now have a eye twitch and need to get some zzzzzzs.

Cheers,
Kevin

Kevin J. Olival, PhD
Vice President for Research

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EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation

<NSF_PIPP_ResearchDescription_v12_GM.docx>

PROJECT DESCRIPTION

Introduction and Background

The spillover and spread of novel, zoonotic pathogens can have tragic societal and economic impacts, as is evidenced by the ongoing COVID-19 pandemic. Unfortunately, the emergence of SARS-CoV-2 (the virus that causes COVID-19) is just the latest in a series of viral emergence events that have been accelerating over the last several decades. In just the last year, as the world continues to respond and adapt to COVID-19, we have seen several deadly zoonotic (animal origin) viruses re-emerge in the human population, including Ebola and Marburg in Guinea[1,2], Nipah virus in India[3], Rift Valley Fever virus in Kenya[4], and novel strains of influenza in China [REF]. Most emerging infectious diseases (EIDs) originate in wildlife reservoir hosts and are transmitted across species boundaries, or ("spill over"), when livestock or human populations increase contact with wildlife through factors including land use change, agricultural intensification, and the wildlife trade[5-7]. Zoonotic spillover is a dynamic process determined by many steps including 'pre-emergence' viral evolution, diversification, and transmission among wildlife hosts; 'human exposure' via direct or indirect contact between humans, livestock, and wildlife; 'post-exposure' limits to cell entry, host range, human-to-human transmission; and population level virus amplification and spread. Once efficiently transmitted, EIDs can spread rapidly through globalized travel and trade networks, threatening countries with high population growth, urbanization and international trade (**Fig 1**).

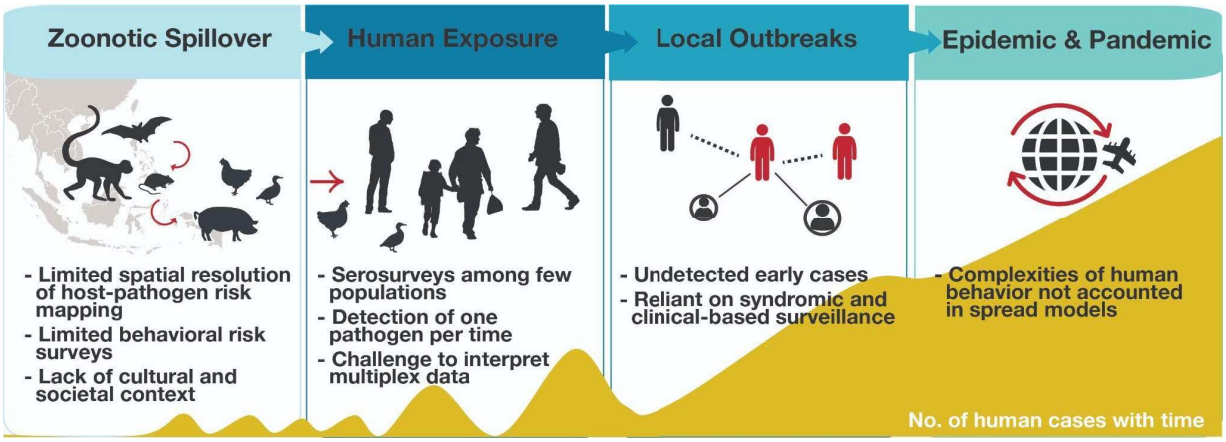

Fig 1. Four stages of zoonotic disease emergence and spread, with gaps in knowledge at each stage. Our proposed project will develop and pilot novel, multidisciplinary and technological advances to address critical gaps in the first three stages of emergence, when pandemic prevention is possible.

Novel human EIDs are typically only discovered when they cause severe or unusual symptoms, when astute clinicians are linked with laboratories capable of pathogen discovery research, and after they have been transmitted from person to person (in which case control options become more difficult).[7,8] The emerging human viruses characterized to date likely represent just the tip of the iceberg given the hundreds of thousands of predicted zoonotic viruses that exist in nature and limited surveillance capacity globally[9]. New research has disrupted the paradigm that zoonotic disease spillover is a rare event. A combination of more advanced molecular and serological assays, improved laboratory capacity around the world, and targeted surveillance of "high risk" human populations have led to evidence of zoonotic virus transmission and infection, even in the absence of notable disease outbreaks. Often these spillover events lead to "dead end" infections in people or limited 'stuttering' chains of transmission, a phenomenon referred to as "viral chatter"[10]. Community-based serological surveys from our group and others have illuminated relatively frequent and previously-unknown instances of zoonotic virus spillover

including Henipaviruses in bat hunters from Cameroon[11], SARS-related CoVs in rural communities living near caves in China (pre-2019), and Ebola virus exposure in DRC[12] and even Southeast Asia[13]

It is during these early stages of emergence, i.e. "viral chatter" with limited human transmission and even further upstream to include pre-emergence risk analyses at the human-animal interface, where innovative convergent research approaches are most needed for pandemic prevention. While we have made significant scientific advances over the last ~15 years in understanding where and through what mechanisms novel zoonotic viruses spill over into the human population[5,14-17], there **are still fundamental knowledge (and implementation) gaps that stymie our ability to preempt and prevent future disease emergence events**.

Our Grand Challenge Problem

Prior to 2019, EcoHealth Alliance (EHA) and colleagues demonstrated that SARS-related coronaviruses (SARSr-CoVs) in China pose a clear and present danger for a future human outbreak. We conducted extensive viral discovery in wildlife from China and Southeast Asia, phylogenetic analysis, and host-range experiments to show that certain bat species harbor a diverse assemblage of SARSr-CoVs[18,19], as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics[20-23]. We conducted behavioral risk factor surveys and serological sampling from human populations living close to caves and found serological evidence of SARSr-CoV spillover in people exposed to wildlife[24]. Building on this body of knowledge, our recent models suggest that bat-origin SARSr-CoVs may be infecting 400,000 (median 50,000) people in Southeast Asia each year[25]. While confidence intervals around these estimates are large, our sensitivity analyses importantly identified key area for future research to improve these spillover risk models - notably, quantification and better contextual understanding of human-bat contact, the role of wildlife farming and livestock hosts, scaling up human serological surveys, and better data on sero-dynamics following novel SARSr-CoV exposure.[25]

Despite this strong body of evidence for SARSr-CoV spillover risk (prior to the emergence of SARS-CoV-2), surveillance and research on these potential pathogens, their hosts, and their interface with humans was not conducted across a wide enough geography, nor at a temporal or spatial scale to inform actionable disease mitigation. Even for large-scale efforts to strengthen zoonotic virus early warning systems, like the 10-year, 30 country USAID PREDICT program[26], led by a global consortium including EHA, it's nearly impossible to capture enough data and disseminate that quickly enough to lead to widespread prevention measures at a national scale using existing methods – especially when considering multiple potential viral threats at once. This is especially true for large countries with heterogeneous human populations, diverse wildlife fauna, and known ecological and societal risk factors for emergence. Approaches to massively scale up zoonotic spillover surveillance are needed.

Our grand challenge problem: "How can we capture actionable data about the risk of zoonotic spillover (and early spread) at the scale and speed required to prevent outbreaks?" Can we leverage tools developed by computer scientists, data engineers, and technology companies to collect data from diverse populations and geographies to advance zoonotic spillover intelligence to a global scale? Can we incorporate new insights from medical anthropology, sociology, wildlife biology, disease ecology, molecular virology, biomedical assay design, and data science and use these to pioneer new methods in data collection and interpretation for zoonotic disease prediction and prevention? In this development proposal, we propose to build a collaborative research and training network to confront this challenge, designed at the start to scale up to a future *Center for Spillover Intelligence (CSI).* Our network will deeply integrate computer scientists, engineers, and data scientists using machine learning and other novel technological approaches together with experts in emerging disease and social-

behavioral research. New insights from social, behavioral, and anthropological research make it clear that the emergence and future trajectory of human epidemics require that we recognize potential biological risks alongside risks posed by cultural and societal factors[27-29]. Our cross-disciplinary, multi-institution project will combine synergistic research activities with training and education programs to facilitate new innovations to scale-up the collection and interpretation of data to inform where, how, and what zoonotic viruses are poised to emerge – a field we collectively refer to as *Spillover Intelligence*. Our inclusion of social-behavioral researchers, data ethicists, and inter-governmental EID policymakers will ensure that our aims and approaches are culturally and locally relevant, and align with our ultimate goal of improving global health.

Our Vision: We envision a world where diffuse data streams can be combined and interpreted in near real-time to disrupt the spillover and spread of future zoonotic viruses. We envision diverse leaders and scientific disciplines coming together to advance spillover intelligence and transform the ways we think about, monitor, and respond to global viral threats.

Our Team

We have assembled a multidisciplinary team of experts to lead our development project and to create and grow the Center for Spillover Intelligence (CSI). Our **Leadership Team (PI, coPIs, Senior Personnel)** includes a mixture of established scientists and early career leaders working across the fields of: emerging infectious diseases, computer and data science, engineering, mathematical modeling, virology, technology and biotechnology, data ethics, social science, and medical anthropology. Our project will be led by EHA (PI Olival) building off the foundational pandemic prediction work our institution has developed over the last decade, but expanding to new partnerships and collaborations we have not previously been able to explore – including with computer scientists and engineers from University of Rochester (UR) and behavioral anthropologists from Georgetown University (GU). We will develop and pilot new technologies for field biosurveillance and data interpretation with Uniformed Services University (USU). Our Leadership Team is ethnically, culturally, and geographically diverse, including US-based researchers who originate from and/or currently conduct their primary research in global EID hotspot regions[5,14], e.g. South Asia (Hoque); Latin America (Mateos); Africa (Mendenhall); and Southeast Asia and the Pacific (Olival). Our geographic and disciplinary diversity, including long-standing international collaborators from each region make our team well equipped to address spillover at a global scale, while understanding the importance of local context.

PI Olival (EHA) is a disease ecologist and evolutionary biologist with a primary interest in developing novel analyses to target zoonoses surveillance and viral discovery. He currently leads two regional biosurveillance projects in Southeast and Western Asia, and recently served as Modeling & Analytics Coordinator for the USAID PREDICT project[26] overseeing a multi-institution team developing pandemic prediction strategies using public and project-specific data from 30 countries[5,15,30-35]. **coPI Mendenhall** (GU) is a medical anthropologist and Professor at the Edmund A. Walsh School of Foreign Service. Mendenhall works at the intersection of social, psychological, and biological lives, attempting to understand what makes people sick, why, and where. Mendenhall is currently a PI of NIH Fogarty International Center grant "Soweto Syndemics", the first ever study to evaluate a syndemic from the ground up. **coPI Hoque** (UR) is an Associate Professor of Computer Science working at the intersection of Human-centric Computing (HCC) and Artificial Intelligence with a focus on multimodal machine learning with applications in health and wellness. Hoque is currently a PI of NSF NRT grant, and received NSF CAREER and CRII grant. Hoque has been identified as an 'emerging leader of health and medicine' at the National Academy of Medicine (NAM). **coPI Mateos** (UR) is Assoc. Professor of Electrical and Computer Engineering and a Data Science Fellow, his research focuses on algorithmic foundations, analysis, application of (graph) signal processing tools to the study of large-scale and dynamic networks. He has pioneered foundational work in network topology

inference, online learning from graph streams, and interpretable representation learning for unstructured data. **coPI Ross** (EHA) is Principal Scientist for Computational Research. His research includes model development to interpret complex spatio-temporal disease surveillance data in heterogeneous populations, including from serological surveys, real-time community and hospital testing, and simulating and forecasting epidemic dynamics over populations, space, and networks. He is PI on awards from DHS Science & Technology Directorate and the Defense Threats Research Agency (DTRA) for designing disease forecasting systems. **SP Mendelsohn** (EHA) is a research data scientist specializing in the development and application of machine-learning models to draw insights into the occurrence and spread of emerging infectious diseases. With a focus on reproducible research methods and data management, she has been the lead analyst on data driven disease forecasting and behavioral survey-based projects characterizing animal-human contact patterns. **SP Daszak** (EHA) is an EID expert who has studied the process of disease emergence for decades and pioneered groundbreaking work in "hotspot" risk mapping to target surveillance. **SP Broder and Laing** (USU) are experts in the design of multiplex serological assays, molecular virology, and implementation of field-based human and wildlife biosurveillance strategies. **OP Busa** is a biotechnology consultant with a specialty in developing lateral flow assays and optical readers. **OP Pompe** is the Research Manager for Facebook Data for Good, who specializes in applying human mobility datasets from social media feeds and data privacy and ethical issues.

Intellectual Merit

The primary intellectual merit of our proposed project is in **developing and piloting innovative approaches to pandemic prediction across previously siloed scientific domains** including biology, computer science, electrical engineering, and social-behavioral research. We apply new theoretical advances and convergent research to address our grand challenge of capturing actionable data about the risk of zoonotic spillover and early spread at a scale and speed required to prevent outbreaks. We will identify existing gaps and next-generation solutions for step-level changes in zoonotic spillover prediction via multi-day intensive workshops with thought-leaders organized by each of our three multidisciplinary Working Groups. Our pilot research projects will serve as a test bed for new theoretical frameworks to advance spillover intelligence. For example, in Aim 1 we will transform decades-old anthropological approaches and pilot a cost-effective, scale-up of quantitative and qualitative behavioral data collection through a unique public-private partnership Facebook; and use image recognition and artificial intelligence to quantify and map human-wildlife contact globally. In Aim 2 we will develop hierarchical Bayesian models to interpret rich, multiplex serology datasets and test these on real surveillance data as part of EHA's NIH-funded work; and develop the first field-forward lateral-flow assay for Nipah virus antibody detection. In Aim 3 will use cutting edge theoretical advances in network science, anomaly detection, and machine learning to detect changes in human mobility patterns that may portent spillover (e.g. increase congregation around wildlife markets) or signal early epidemic spread after the point of spillover.

Broader impacts

This proposal could potentially transform our understanding of zoonotic spillover risks using survey, multimedia, and mobility data on a global scale, including aggregated and anonymized data from ~3B active Facebook users. Insights and intellectual outputs from our working group activities will have direct benefits to public health by informing disease mitigation strategies for pandemic prevention. We will synthesize our three working group outputs and prepare a special joint publication for the National Academies of Science's Forum on Microbial Threats for wide distribution, and write popular press articles to accompany our peer-reviewed manuscripts. EHA's *Alliance,* includes a network of over 70 partner institutions in 30+ countries, presents a unique opportunity to work with local stakeholders in emerging virus 'hotspots' and validate

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models using data sets obtained through our long-standing collaboration with government partners including human health, agriculture, and environmental sectors.
Our education mission consists of multiple workshops, hackathons, monthly seminar series, and access to state-of-the-art visualization facilities. We will begin to train a new generation of graduate and undergraduate students who will be prepared to harness the burgeoning power of data science to make novel inroads into understanding human-animal contact, serological surveillance, and aggregated mobility data and their interaction. Our proposal builds on a unique partnership between the academic (UR, GU, USU), non-profit (EHA), and private sector (FB), allowing students to apply lessons learned from classroom settings to the real-world, and our recruitment will focus on including >50% women and underrepresented minorities in science and engineering. This planning grant will be a path towards structuring interdisciplinary education, cross-training courses across institutions, followed by hands-on experience via internships and research exchanges – with options for international work via existing programs at our institutions. Through the planning grant, a new MS data science track on 'zoonotic surveillance' will be established at UR, the first step in our plans to expand our Education and Training plan should we move towards a Center-level award in the future.

Education and Training Plan
Our Leadership Team will serve as mentors for our **CSI Student Training Program** that will offer a fellowship stipend to cross-train 20 Master's level graduate students via semester-long projects associated with proposed pilot research activities (3-4 students per project) and 3-week research exchanges between our institutions (UR, GU, EHA, USU, 4-8 students from each). The research exchange builds off EHA's successful NSF-funded EcoHealthNet training program (2011-21) and will take place in either the Summer of 2022 or 2023. We will link with currently-funded international programs at our respective institutions (e.g. Gui^{2}de in Nairobi, Kenya run by GU; and the NIH funded CREID research pilot program with EHA's EID-SEARCH project in Southeast Asia) to support students who desire to gain international, hands-on research experience as part of their research exchange. The focus of each research exchange will be on cross-disciplinary training to think 'big' about the future of pandemic prediction and ensure the next generation of spillover intelligence researchers gains valuable hands-on experience working with experts outside their area of study. Students will be drawn from respective departments across our institutions, i.e. students with a computational background and with interests in interdisciplinary work will be recruited from UR's cross-department Goergen Institute for Data Science, Anthropology and International Affairs students from GU, and Emerging Disease and Virology students from USU. As EHA is a non-profit and does not have an active student body, we will recruit Ecology, Evolutionary Biology, and Environmental sciences students from universities in the NYC area, including Columbia University's E3B department where PI Olival, coPI Ross and SP Daszak serve as adjunct faculty. We will explore specific mentorship opportunities with data scientists from Facebook. If awarded, we will additionally apply for funds through NSF's Research Experiences for Undergraduates (REU) program, with the goal of supporting summer stipend for 1 undergraduate per institution or the maximum allowable. We will host a joint monthly virtual seminar series (2 semesters long) open to graduate and undergraduate students from our respective institutions, with a dedicated student "lab meeting" before each seminar. Two CSI students from diverse disciplines will lead each lab meeting discussion based on a deep read of relevant publications that each speaker will distribute in advance. We envision the first semester of the seminar series will be led by our Leadership Team and Working Group experts, and the second semester will be for CSI Graduate students to present results or current challenges related to their pilot research projects. We hope to encourage these students to pursue Ph.D. studies. If we are successful at establishing a center through our planning grant, we should have access to a trained cohort of students with appropriate multidisciplinary skillset.

Project Overview

Our 18-month research agenda aims to understand how new methods and technologies can improve the efficiency, intensity, speed, and scope of zoonotic disease surveillance. **We have structured our development grant around three primary research aims** that will guide our interdisciplinary research and training activities. We will establish a working group composed of multi-disciplinary experts for each primary aim that will be co-led by members of our Leadership Team. We will develop pilot research projects that capture different parts of the disease spillover process focused on specific geographies, lead 3-day intensive workshops, monthly student seminars, and student training activities that cut across our inter-linked research aims.

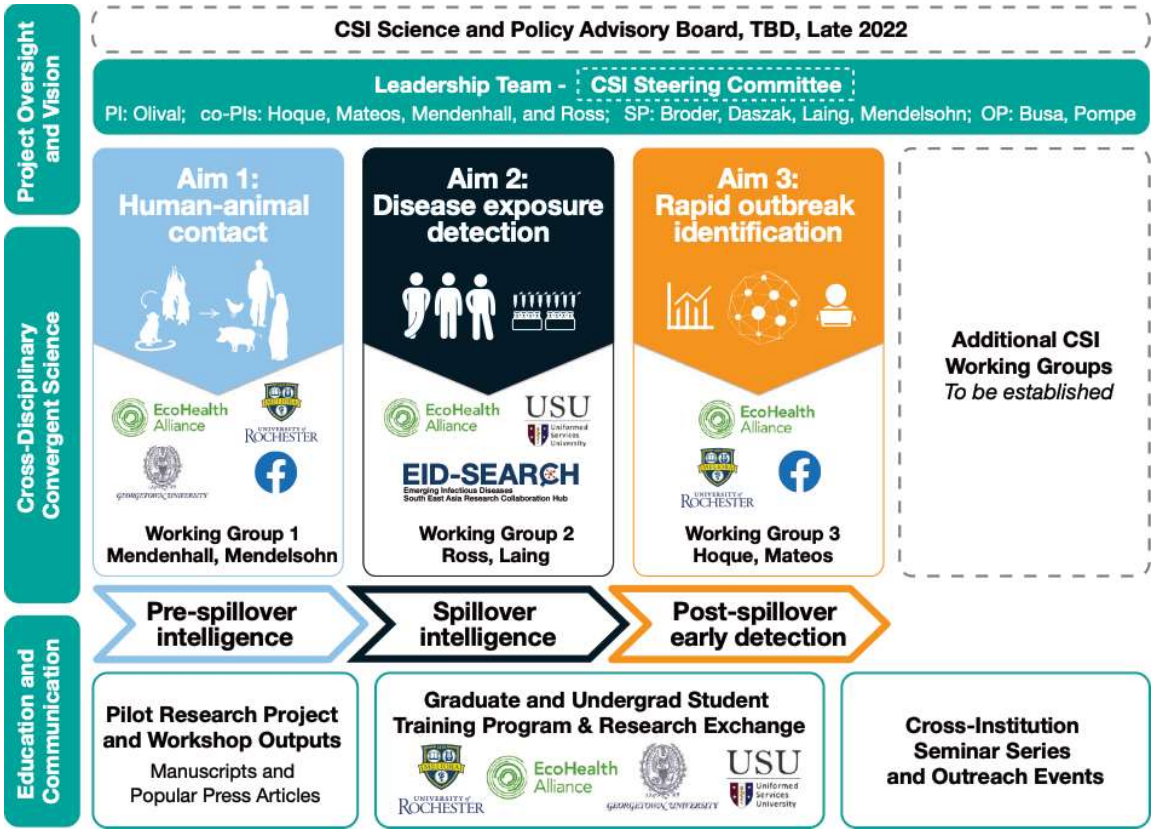

Fig 2. Project overview schematic showing our project management; three primary aims with graphical representation of pilot research activities to scale up and advance spillover intelligence; institutional partners leading activities for each aim including designated working group leads, and student education and communication activities, including pilot research projects, graduate student research exchanges, seminars, and other outreach activities. Our development level project was built to scale to a Center level award (the Center for Spillover Intelligence, CSI) as noted with the dashed outline boxes, including the future addition of other CSI Working Groups, a Science and Policy Advisory Board, and transitioning our current Leadership Team (PI, coPIs and SP) into a more formal Steering Committee for oversight.

Aim 1: Measuring human-animal contact at scale

Interactions among humans, wildlife, and livestock create interfaces for pathogen spillover and are proximate risk factors for the emergence of all directly-transmitted zoonotic diseases.[14,36] Human-animal contact is commonplace around the world, with many factors mediating the type and frequency of contact, including cultural practices, environmental conditions, food security, and occupational demands.[36] In regions of the world considered prone to spillover--forested tropical landscapes with high biodiversity and recent land-use changes--understanding human-

animal contact is crucial to pandemic prevention.[5] To measure these interactions and characterize associated behaviors, beliefs, and knowledge, researchers often implement resource-intensive surveys in key communities.[37,38] While crucial to understanding human-animal contact dynamics, on-the-ground surveys cannot yield scalable data that is comparable across populations. Often, in place of surveys, researchers conducting analyses at broad scales rely on coarse estimates of contact such as the joint density of human and animal populations. **Our working group will uniquely combine social scientists, disease ecologists, and computational scientists to increase the scale at which we can directly measure human-animal contact.** We will develop and implement two pilot research projects. The first will use novel, social-media-based survey approaches to reach larger populations at higher frequencies while maintaining the cultural sensitivity and relevance of on-the-ground surveys. Working collaboratively with cross-disciplinary researchers, we will design surveys to understand social behaviors, cultural perceptions, and occupational hazards that may pose zoonotic spillover risk to individuals and communities. We will target geographies based on our existing spatial risk modeling work that maps infectious disease spillover potential and the ecological distributions of key reservoir host species.[5,15,25] Second, we will conduct pilot research on the use of social-media-based image recognition algorithms to identify locations of human contact with key species known to host high pathogen diversity. This pilot will include a collaboration effort to test the efficacy of such technology for understanding taxon-specific contact patterns globally, and to bring multidisciplinary context to meaningfully interpret the outputs (including species validation with taxonomic experts). If successful, will use these data on frequency of animal contact from image data to identify locations for additional survey data collection.

Aim 1 Pilot Research Projects: We will develop two pilot projects to assess the potential of 1a) social-media dispensed surveys and 1b) passive surveillance using artificial intelligence for image recognition to capture information about human-animal contact. We will target populations at wildland interfaces where contact with wildlife is likely high but internet penetration may be mixed.

1a. Social-media dispensed surveys to quantify and contextualize human-animal contact
We will pilot social-media dispensed, large scale population surveys about perceptions of and interactions with animals. We will optimize the design of these surveys for mobile platforms to facilitate higher response rates, and will also explore the possibility of longitudinal sampling respondents (either individuals or populations) to understand changes in conditions and responses over time. Survey questions will be based on a combination of EHA's past experience designing on-the-ground human-animal contact surveys[37,39,40] and ideas generated from our proposed working group activities involving anthropologists, data scientists, and disease ecologists who have worked extensively in the geographic and societal contexts in which we focus our work. We will aim to design surveys that account for populations and individuals holding different beliefs, carrying different histories, and living in different ecologies. We will attempt to survey demographically and geographically diverse populations to capture direct and indirect animal-contact patterns and behaviors, and will attempt to capture the role that international, state, and local policy plays in individuals' lives. Further, we will aim to recognize particular hesitancies among individuals to interact with certain technologies or other public health interventions.

We will explore various online platforms at the start of our project for survey administration. Given our unique public-private partnership with Facebook for many components of this project, using the Facebook platform to disseminate our pilot survey is likely to be a strong option, especially given Facebook's global reach (nearly half of the world's population, ~3 billion users), which introduces opportunities for digital interaction across class, gender, ethnicity, and cultural

divides. Facebook has extensive experience administering surveys, either directly on its platform (e.g. Yale Climate Change opinion survey) or off-platform (e.g. Carnegie Mellon University COVID-19 surveys) that we will leverage. Facebook also has extensive privacy infrastructure and data-review policies to ensure anonymity and data security.

We will also explore creating games that users will be encouraged to play online to enable us to learn more about how they think about host species, viral threats, environment, and health/disease. For example, users may be given a list of 30 images and will be asked to put them into categories and describe what each category means. Adapting these "pile sorting" anthropological methods[41] to an online interface and for spillover risk, will be a novel application with potential for a massive scale-up of data around cultural and conceptual dimensions of zoonotic disease risk. Data generated from this exercise will generate a more ethnographic understanding of people's experiences and thought processes, which tend to be locally mediated. We understand that there are geographic and demographic gaps in internet and social media use, and will incorporate bias corrections into our study design. We will initially focus on Southeast Asia, a region with a high likelihood of future zoonotic disease emergence and where EHA has good coverage of on-the-ground surveillance programs to ground truth online data collection[5], and expand to new geographies as we develop Center-level activities.

1b. Image recognition of zoonotic disease host species

We will pilot image-based surveillance of Facebook and Instagram feeds to attempt to identify human contact with key zoonotic disease host species and to explore the limits of taxonomic and behavioral recognition in images. We will work with Facebook Data for Good (see **Letter of Collaboration**) to adapt and refine their existing machine vision algorithms to target key taxa and to assess the efficacy of different approaches, working within strict guidelines and ethical standards for data de-identification. While data will be de-identified, we will only use photos with full location meta-data, allowing us to not only quantify the type of species and contact, but where it occurred. There are multiple image libraries that may serve as AI training datasets, including iNaturalist, the American Society of Mammologists, and Integrated Digitized Biocollections (iDigBio). We will iteratively implement and test algorithms starting with broad taxonomic groups (e.g., bats) and will explore the extent to which our approaches can identify finer taxonomic resolutions down to the genus or species level. In addition to taxa identification, we will explore image recognition of features that may suggest specific types of human-animal contact, such as cages or market settings. Further, we will use text and image metadata and explore using hashtags associated with images to boost the ability of our algorithms to identify key taxa.

This work will involve collaboration between machine learning scientists, biologists/ecologists, and social scientists. We will follow all data anonymity and privacy guidelines from Facebook and will also discuss data handling ethics in our working group. We will assess the ability of passive surveillance with image recognition to be used to target surveys to populations that may have higher frequency of contact with wildlife, and ultimately to target biological surveillance of human and animal populations. We will consider both the accuracy of the image recognition algorithms as well as implications for survey distributions, results interpretation, and ethical data practices. We will also compare the spatial distribution of human-animal contact patterns derived from image recognition with previously generated spillover risk maps based on joint occurrences of animal and human distributions (e.g., SARS-related CoV spillover risk from Sanchez et al. 2021 preprint).[25]

Working Group 1 Activities: Our working group will convene 12-15 experts from the social sciences, disease ecology, and computational sciences for an intensive, 3 day workshop. We will structure the workshop to focus first on data ethics in human behavioral surveying and image recognition surveillance and secondly on survey design and analysis planning, with specific attention to accounting for gaps or biases in survey distributions. We will center the workshop around the understanding that technological quick fixes are never enough and that data collection must address long term social, ecological, and economic realities to understand how people perceive, experience, and interact with the risk of and repercussions from spillover events. We will also address experiences and challenges in engaging longtime ethnographers and local leaders to understand what's at stake for communities engaging with online and field surveillance programs. Our working group will help mentor students part of the CSI Student Training Program, to facilitate information sharing and idea generation among undergraduate and master-level students. Working group 1 will also contribute to the CSI Seminar Series with cross-disciplinary topics such AI image recognition (lead by a data scientist and bat biologist) and human-behavioral survey design (lead by an epidemiologist and medical anthropologist).

Partners, Initial Working Group 1 Members, and Roles: EHA (SP Mendelsohn): survey design and implementation, image recognition algorithm development and testing, novel data analyses. Facebook Data for Good (OP Pompe): survey implementation, image recognition algorithm development and testing, data ethics. University of Rochester (coPI Hoque): Image recognition algorithm development and testing. Georgetown University (coPI Mendenhall): Survey design with focus on ethnography and anthropology; online free listing/pile sorting game design; help lead expansion of WG to include emerging international leaders in social-behavioral science (e.g. Dr. Edna Bosire from Kenya). We will invite additional leaders from our extensive funded collaborations to be part of the Working Group for Aim 1.

Aim 2: Detecting zoonotic disease exposure at scale

Animal-human contact is the necessary condition for spillover of potential pandemic, directly-transmitted viruses. While a small fraction of such encounters result in actual exposure or infection, such exposures are frequent given the total sum of encounters. Most such infections are never reported and rarely spread widely, and only a very rare subset become widespread in the human population[42]. Other viruses such as Lassa spill over thousands of times per year[43]. In areas of frequent spillover, the eco-evolutionary dynamics of "stuttering chains" of infection are what give rise to epidemics and pandemics[44]. Identifying populations where frequent exposure occurs is critical for deploying preventative measures and following viruses upstream to their wildlife hosts. Yet even large-scale surveillance projects cover too small populations and produce far too few detections to determine the most exposed geographic, demographic, or occupational groups at an actionable scale [REF, showing PREDICT sampling efficiency]. This is for two reasons: the field and laboratory cost of surveilling populations interacting with wildlife interface and, and the low detection rates of the widely-used, molecular PCR or metagenomic approaches.

We aim to drastically increase the scale at which populations can be monitored for exposure to animal-origin viruses by developing and piloting novel tools and analyses needed to implement large-scale serological surveys. While metagenomic methods have been favored for their ability to capture genetic sequences of previously unknown viruses, serological methods can be used to detect exposure to known and unknown viruses using multiplex assays tailored with a mixture of sensitivities and specificities[45]. Serological tools can detect antibodies for months or years after viral infection, rather than the limited window of days or weeks using molecular methods, and thus can provide a much richer profile of populations where pandemics may emerge[46]. In this planning period, we will begin pilots to test the feasibility of two complementary approaches

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Our work under PREDICT is relevant here, working with local community leaders to distribute results and the 'Living Safetly with bats book'. But how do we scale this? One idea is through a network of local leaders across the landscape, like a "Community One Health Volunteer" network.

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to enabling serological screening at large scales: 2a) the creation of rapid bioinformatics pipelines to interpret high-dimensional serological data from large streams of surveillance data, and 2b) the development of rapid field serological tests for wildlife-origin diseases.

Aim 2 Pilot Research Projects

2a. Rapid bioinformatics for high-throughput, multiplex serology

Members of our team (SP Laing, Broder) have developed multiplex serology tests that can be used to screen for exposure for many species and variants of filo-, henipa-, and coronaviruses simultaneously, and screening pipelines that can process samples at the scale of tens of thousands of samples. However, these tests pose challenges for interpretation, as high-dimensional results from multiplex bead or chip assays frequently contain signals of cross-reaction and autocorrelation (**Fig 3**). Through cross-disciplinary research between molecular virologists, serologists, mathematical modelers and data scientists on our team, we will create models to interpret this high-dimensional data and integrate them into a rapid bioinformatic pipeline that identifies individuals and populations to known viruses as well as flagging serological signals indicative of novel viruses. We will make use of data collected under our ongoing NIH and DTRA funded biosurveillance activities (Laing, Broder, Ross, Olival, Daszak) to develop detailed reaction/cross reaction profiles of a broad panel of zoonotic viruses to serological tests. We will build mechanistic, hierarchical Bayesian models that allow us to determine whether serological "profiles" are more likely to represent antibodies of known viruses, mixtures of multiple known viruses, or previously uncharacterized viruses. We will extend techniques of antigenic mapping,[47] using serological profiles to estimate phenotypic similarity of new viruses to known viral families, determine the correlation with phylogenetic relatedness, and use these measures to screen new viruses for pandemic potential. Using simulation experiments, we will test reduced-form, simple models against fully structured models so as to develop statistically robust procedures to interpret these data without high-performance computation.

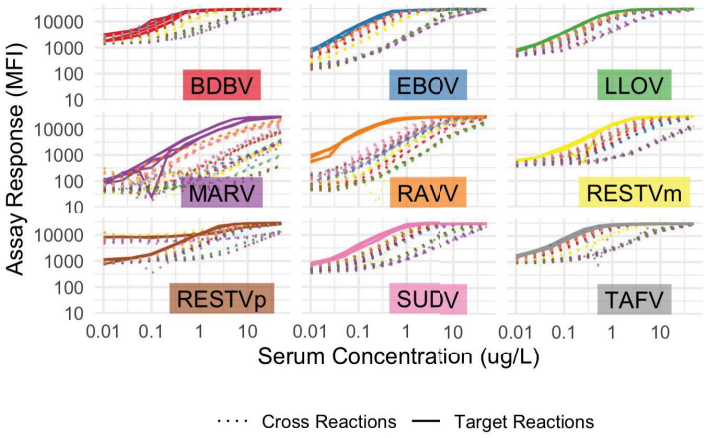

Fig 3*.* Preliminary data: Reaction-cross reaction plots of multiplex Luminex assay response to antibodies for closely-related filoviruses.

Scaling center operations: This pilot project will produce methods and a proof-of-concept pipeline for high-throughput analyses. We will leverage EHA's Emerging Infectious Disease – Southeast Asia Research Hub (EID-SEARCH) project, a NIH-funded Center for Research in Emerging Infectious Diseases (PI Daszak) who's partners include USU, Duke NUS, Chulalongkorn University in Thailand, and various clinics and institutions in Malaysia – currently screening large numbers of human samples (>10K/yr) for various coronavirus, filovirus, and paramyxovirus antibodies. In the future the CSI will implement surveillance studies screening these sample streams using multiplex serology, and utilize the analytical and bioinformatic tools we develop in the next 18 months. As part of our planning activities, we will establish protocols and approvals for this work.

2b. Development of a field-ready semi-quantitative lateral flow assays for an emerging zoonotic viruses (Nipah virus)

Lateral flow assays (LFAs), are low-cost tests where reagents printed on cellulose strips react with a sample that flows via diffusion. LFAs are capable of providing quantitative results (in proportion to antibody titer) using a combination of high-quality manufacturing and carefully designed, calibrated optical scans[48]. LFAs can be used to provide diagnostics at low cost at the point of care and by consumers. Lack of commercial-scale applications has prevented LFAs from being used for serological testing for novel or emerging disease. To our knowledge, no studies have used LFAs for zoonotic virus serosurveillance in the field.

We will develop a proof-of-concept LFA for Nipah Virus (NiV). NiV has a high mortality rate and is considered to have pandemic potential that is ranked by CEPI as one of 7 high pandemic risk pathogens[49]. The range of NiV's primary wildlife hosts (*Pteropus spp,* fruit bats) cover South and Southeast Asia but human cases have only been detected in Malaysia, Bangladesh, and the Philippines. Spillovers occur regularly in Bangladesh, but are rarely reported in large portions of the country[50] and reporting is strongly associated with health care accessibility[51]. Broad-scale serological monitoring has the potential to identify populations from which pandemic strains may emerge. Through previous work, members of our working group (Laing, Broder) have developed multiplex serological assays for NiV[52] and the soluble proteins and reagents required for an LFA. We have also added experience in engineering, quality control and commercialization of LFAs (Busa, industry consultant) to our working group. For the pilot, we will engage with commercial manufacturers to create and test a prototype Nipah LFA and a protocol for smartphone-based optical reading of quantitative responses.

Scaling center operations: The pilot will create a prototype LFAs. As part of full center operations under CSI, we will aim to scale up production and test LFAs efficacy using control sera and field deployment as part of EID-SEARCH. We will additionally assess feasibility and cost of deployment, and compare performance against current laboratory-based assays.

Working Group 2 Activities: In addition to the two pilots above, working group members will contribute to the our CSI monthly seminar series, presenting on topics including high-dimensional data models, field serology, serological assay development, laboratory product design, and public-private partnerships and commercialization. We will conduct two online workshops on serological data analysis, jointly with undergraduate, masters, and doctoral students at USU and UR, leveraging partnerships with field researchers currently working in serological surveillance. Our working group will lead a 3-day intensive workshop, where we will recruit additional participants with expertise in serological assay design and surveillance, immunology, emerging zoonoses, and focus discussion on key barriers to scaling serological surveillance for known and unknown zoonotic viruses.

Partners, Initial Working Group 2 Members, and Roles: EHA (coPI Ross): Model and pipeline design, software optimization. Uniformed Services University (SP Liaing, SP Broder): data and model interpretation, reagent manufacture, assay design. SP Busa: device manufacturing, quality control, industry outreach and partnership development. We will recruit additional participants from our partner network engaged in serological field studies as well as industry representatives to be part of the Working Group for Aim 2.

Aim 3: Rapidly Identifying Anomalous Health Events

In early stages of epidemic spread, novel contact and mobility-induced behavioral data as well as communication patterns may provide a signal of e.g., health-seeking activity indicative of an outbreak cluster. We contend that these signals may be detectable in large-scale data streams of population mobility and contact, or disease reporting available through telecommunications and health care providers. Timely detection requires approaches that can manage data at scale,

analyze high-dimensional, spatio-temporally correlated streaming data in (pseudo) real time, and distinguish between known signals (e.g., typical influenza-related activity) and novel spillover-related signatures that we wish to unveil, characterize, and explain in order to trigger the necessary public policy responses.

This desideratum calls for fundamental methodological and computational advances, to go along with epidemiologically-relevant data sources that describe how humans are moving and interacting across physical space, with unprecedented temporal resolution and geographical coverage. Traditional flow-based mobility datasets used to parameterize epidemiological models have been aggregated and formatted in the form of origin-destination (OD) matrices. While valuable towards understanding population flows between regions of interest, OD matrices lack information on how much time a traveler spends in any given location. Naturally, when it comes to epidemiological studies the duration of time spent in potentially risky geographical areas is key towards deciphering actionable contact and exposure patterns[53]. Moreover, recent studies have shown that flow counts between pairs of geographical areas can be loosely correlated with, and not representative of, epidemiology-relevant mixing patterns[54]. Instead, these couplings among subpopulations are measured by effective contact rates, meaning how often people from each region come into contact in a way that is sufficient to transmit a disease. To address these challenges, our consortium will partner with the Facebook Data for Good team and leverage two recently assembled global-scale datasets - Activity Space Maps and Colocation Maps[53,54].

Different from existing approaches that have focused on measuring the causal impact of mobility-oriented interventions, **our ambitious goal in this aim is to markedly advance the surveillance infrastructure available to predict anomalous health-related events before a major outbreak occurs**. While undoubtedly a multi-faceted endeavor, in this planning grant we will take initial, but significant steps with an emphasis on network-centric, computational analyses of dynamic mobility and interaction data at a global scale. By developing the appropriate statistical tools, we will support the ultimate goal of characterizing early pandemic "fingerprints" or signatures that can be extracted from these global datasets.

Aim 3 Pilot Research Projects: Working closely with Facebook Data for Good (see Letter of Collaboration from Alex Pompe), this working group will pilot data-driven research activities to explore the feasibility of extracting early (post spillover) signals of epidemic onset from Activity Space Maps and Colocation Maps. Our multi-disciplinary working group brings together leading experts in systems engineering, AI for healthcare, data science, mathematical epidemiology, graph signal and information processing, privacy-preserving mobility data generation and management, human behavioral modeling, and environmental science. We believe the current team, consisting of a wide array expertise and partnership, will instigate the cross-pollination of ideas leading to center-level activities while making significant inroads towards addressing our grand challenge. We propose two specific pilot projects:

3a. Spatio-temporal monitoring of spillover-induced activity anomalies in Southeast Asia
Activity Space Maps (ASMs) are a novel global-scale movement and mobility data set which describes how people distribute their time through geographic space.[53] They are designed to complement existing digitally-collected mobility datasets by quantifying the amount of time that people spend in different locations. Accordingly, this information is valuable for estimating the duration of contact with the environment and the potential risk of exposure to disease -- something critical especially early after spillover. More germane to the theme of this aim, it also offers the unique possibility of developing and testing sequential change-point detection algorithms to monitor multiple spatial grid cells of interest (informed, for instance, by outcomes

of the image-recognition effort under Aim 1). We will target areas known to include health care facilities in pursuit of abnormal trends of (increased) activity. Moreover, using ASMs we will examine flow and temporal activity anomalies around expert-annotated wildlife and wet market locations. Said events are expected if there are human infections, or significant associated changes in behavior around these sites early after spillover. Our initial efforts during this planning grant will be focused on Southeast Asia, which on top of its epidemiological relevance is one of the regions where Facebook ASMs have the most coverage. We will target Indonesia, where EHA has extensive on-the-ground efforts tracing networks of wildlife trade as well as market sites.[55] We will also tap into the resources stemming from EHA's Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH) to identify clinics and market sites of interest in Thailand and Malaysia. Some key challenges we will address include accounting for the implicit biases in data collection associated with the construction of ASMs,[53] and their subsequent effects on the statistical algorithms that we will develop. Our Working Group will address additional challenges of data interpretation of anomaly detection analyses with input from emerging disease experts and epidemiologists. To calibrate our models, we will bring to bear recent activity data before and after the SARS-CoV-2 outbreak in those locations, in addition to historical influenza seasonal trends that go further back in time. With the exciting prospect of ASM data being officially released in the last quarter of 2021, *we are in a privileged position to be the first multi-disciplinary team that leverages this invaluable global-scale resource to advance our understanding of zoonotic spillover risk and its immediate effect on human mobility.*

3b. Online network change-point detection in dynamic, global-scale Colocation Maps

Activity data are complemented by *Colocation Maps (CMs),* which estimate how often people from different regions worldwide are colocated. In particular, for a pair of geographic regions *i* and *j* (e.g., counties in the US), CMs estimate the probability that a randomly chosen person from *i* and a randomly chosen person from *j* are simultaneously located in the same place during a randomly chosen minute in a given week.[54] In other words, CMs are global-scale, dynamic network datasets with colocation probabilities encoded as edge weights. Thus, they are well suited to parametrize metapopulation models of disease spread,[56] or to measure temporal changes in interactions between people from different regions.[57] To monitor said changes as part of our second pilot activity in this aim, we will build on our recent advances in online (i.e., sequential) algorithms for anomaly and change-point detection from network streams[58,59]; see also.[60-62] The envisioned statistical methods are in stark contrast with most existing graph change-point detection approaches, which either rely on extensive computation,[63] or they would require storing and processing the entire observed CM time series.[64] We will prototype and test a lightweight online change-point detection algorithm with quantifiable performance guarantees, that is also explainable since it relies on interpretable node embeddings.[58,65] The latter desirable feature will be leveraged to construct dynamic anomaly maps for user-friendly visualization of post-spillover events; see also our recent network-scientific visualizations of spatio-temporal COVID-19 contagion patterns across the US.[66] Moreover, the sheer-size of the CM graphs calls for algorithmic and modeling innovations we will investigate as part of our research agenda, e.g., seamlessly integrating graph subsampling or dimensionality reduction techniques to the analytics pipeline. We will team up with computer vision experts affiliated to the University of Rochester Goergen Institute for Data Science (GIDS, see attached Letter of Collaboration from institute director Mujdat Cetin), to also explore the exciting prospect of integrating geo-tagged multimodal data such as video and imagery in our studies, for instance as nodal attributes embedded to the CMs.

Working Group 3 Activities: *Recruitment and diversity:* We will recruit 6 students from the GIDS-run master's program in data science, which attracts students from multidisciplinary backgrounds and significant computational proficiency to contribute in the aforementioned pilot research activities. *The CSI leadership and broader community are committed to providing research training and educational experiences for those traditionally under-represented in STEM, including women and underrepresented minority students.* The PI team has a solid track-record in actively recruiting and supervising such students at the undergraduate, masters and doctoral level, with demonstrated efforts towards broadening participation in STEM. As examples of the commitment to diversifying the field, co-PI Mateos serves as founding faculty advisor to the UR's SACNAS chapter and regularly organizes diversity, equity and inclusion panels to highlight challenges and successes of UR's Hispanic and Latino STEM community. We plan to prioritize engaging women and minority students in the research and working group activities proposed here. *Coursework:* These students will be on the existing health and bio track with an opportunity to take classes in immunology, microbiology and virology programs as well as data ethics at the University of Rochester Medical Center (URMC) as well as the college of engineering. Through the planning grant, we plan to establish a special MS track on 'zoonotic surveillance' in order to train and mentor the students to lead center level activities. *Seminar and workshop series:* The students will be part of the large cohort of all the participating institutions and participate in the CSI monthly seminar series. The co-PIs (Mateos and Hoque) will conduct two workshops (and invite additional faculty members from UR as guest speakers) with hands-on lessons in mobility and human behavior modeling. *Hackathon:* The students will be encouraged to participate in the yearly hackathon, Dandyhacks https://dandyhacks.net/), organized every fall by the University of Rochester Computer Science Department and work with the dataset from Facebook. Additionally, co-PIs Mateos and Hoque will work with the organizers of Dandyhacks to release some of the public dataset from Facebook for other teams to explore, model and mine the data. *Data visualization meetups at the University of Rochester's VISTA Collaboratory*: To get acquainted with the different data resources at our disposal, over the first 3 months of the project the Rochester team (along with Facebook and EHA collaborators via Zoom) will hold bi-weekly team meetings with the MSc students at the UR's VISTA Collaboratory. This is a 1,000 square-feet visualization lab equipped with an interactive (20 ft. wide x 8 ft. tall) tiled-display wall, that renders massive data sets in real time, giving team members the ability to visualize and analyze complex data instantaneously and collaboratively; see Fig. 4. As we dissect what dataset components are central for the aforementioned pilot activities, our graduate students will develop customized data visualization tools, maps and dashboards to facilitate downstream analyses. Updates and progress reports will be shared in these meetings, giving the students a unique opportunity to develop their communication skills. The whole team will provide feedback and share ideas, making this a truly collaborative activity. Moving forward, the VISTA Collaborative offers a unique resource that we will fruitfully leverage as we scale up to full center operation.

Fig. 4. UR's Vista Collaborative will serve as a spillover intelligence "situation room" for training UR and CSI exchange students on analysis of real-time epidemiologically relevant datasets.

Partners, Initial Working Group 3 Members, and Roles: University of Rochester (co-PIs Hoque and Mateos): coordination of working group activities, development and testing of computational tools for early detection of post-spillover signatures, dissemination of research products and educational materials, recruiting and mentoring of master students, liaisons to

GIDS. Facebook Data for Good (OP Pompe and Maps for Good team members Iyer and Citron): data ethics, quantification of privacy protections, data scientific support for Activity Space and Colocation Maps, mathematical epidemiology expertise for data and model interpretation. EHA (SP Mendelsohn and Ross): identification of target spatial locations in Southeast Asia, epidemiological interpretation of anomaly detection analyses and validation of detected early pandemic fingerprints.

Scaling center operations: First, our working group intensive 3-day workshop are designed to lead to new partnerships and synergies for expansion of Aim 3 activities. Second, we will expand our pilot anomaly detection-- mobility data project to new geographies by including new data on georeferenced clinics, wildlife markets and other relevant locations by engaging with our in-country network of partners. Third, we test new streams of predictive data, e.g. mobile phone datasets, via expanded partnerships with public and private stakeholders to access and ingest these data. We will expand our policy engagement via EHA's *Alliance* and existing connections with WHO, FAO, the World Bank and others to develop behavioral risk interventions in the regions and populations we identify as high risk for zoonotic diseases.

Associated Risks and Mitigation Plans: We envision two primary risks associated with our proposed work 1) data access and privacy violations and 2) biosafety concerns inherent with emerging zoonoses research. For data related risks, we have already established a strong collaborative relationship with FB's Data for Good team in developing this proposal and have access to all the relevant datasets for our pilots. Data privacy concerns are taken seriously by our group, including deidentification of any personal identifying information, and we will adhere to strict protocols on using human subject data as outlined in our already approved IRBs as part of NIH-funded work. For biosafety related risks, there will be no active sample collection under our proposed NSF project, although we will use data leveraged from EHA's NIH-funded research. We have already secured appropriate approvals (IRB, IACUCs) that outline safety and PPE use and have established biosafety monitoring plans for this work. Our lateral flow assay development will be undertaken in laboratories designate at the appropriate Biosafety Level following US regulations (partner labs to be confrimed upon award).

Results from Prior NSF Support

Kevin J. Olival has not had prior NSF support, but is co-PI on NIH-funded "EID-SEARCH" (NIAID-CREID U01AI151797, 2020-25 PI Daszak). Previously co-PI on "Understanding the risk of bat coronavirus emergence" (NIAID R01 AI110964, 2014-2019, PI Daszak). ***Intellectual merit*:** Identified >50 SARSr-CoVs in bats, showed evidence of ability to infect human cells, humanized mouse models, and people in China, and used evolutionary and mammalian biodiversity patterns to analyze spillover risk for use in public health control. Produced 18 peer-reviewed papers [20,34,67-79], including two papers in *Nature* [80,81], and a review in *Cell* [82], and others submitted. ***Broader impacts*:** 2 PhD and 6 MS students trained; disease risk and potential management strategies shared with US agencies, WHO, OIE, FAO, and the public.

Ehsan Hoque: Project IIS-1750380. PI: Hoque. Period (2018-23). Amount: $511,453 "CAREER: A collaboration coach with effective intervention strategies to optimize group performance". ***Intellectual merit:*** Developing and validating the efficacy of automated mediation strategies in online meetings. ***Broader Impacts:*** >6 research papers[83-88]. **Project NRT-DESE-1449828.** PI: Hoque (2015-21). Amount: $2,965,341.00 "Graduate Training in Data-Enabled Research into Human Behavior and its Cognitive and Neural Mechanisms". ***Intellectual merit:*** Chartering a new traineeship program to train Ph.D. students at the University of Rochester to advance discoveries at the intersection of computer science (cs),

brain and cognitive sciences (bcs), and neuroscience. ***Broader Impacts:*** Supported over 50 PhD students in cs and bcs, seveal won interdisciplinary research positions at R1 institutes.

Gonzalo Mateos (a) Project: CCF-1750428. PI: Gonzalo Mateos Buckstein. Period: 4/1/18 - 3/31/23. Amount: $407,944. (b) Title: CAREER: Inferring Graph Structure via Spectral Representations of Network Processes. (c) ***Intellectual Merit:*** We investigate how to use information available from graph signals to estimate the underlying network topology through innovative convex optimization approaches operating in the graph spectral domain. ***Broader Impacts:*** We prepared a feature article on identifying network structure via GSP,[89] presented tutorials in EUSIPCO'19, CAMSAP'19 an SSP'20, organized the 2019 IEEE SPS Summer School on Network- and Data-driven Learning. (d) Research products are available in.[66,89-96]

Emily Mendenhall (a) National Science Foundation Doctoral Dissertation Improvement Grant, $10,000; Stories at the Border of Mind and Body: Mexican Immigrant Women's Narratives of Distress and Diabetes. **Intellectual merit:** Led to crucial insights studying syndemics - the interactions of social, environmental, psychological, and biological factors that are synergies of epidemics. Papers in The Lancet[29], Medical Anthropology Quarterly[97-99], and as a full-length book[100]. **Broader Impacts**: Understanding diabetes, trauma, and broader socio-political factors with infectious diseases in India, Kenya, South Africa, and Ethiopia.

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From:	Kevin Olival on behalf of Kevin Olival <olival@ecohealthalliance.org>
To:	Mateos Buckstein, Gonzalo; Peter Daszak; Noam Ross; Emily Mendenhall
Cc:	M. Ehsan Hoque; Emma Mendelsohn; Eric Laing; Chris Broder; Luke Hamel; Robin Breen; Aleksei Avery Chmura; Hongying Li; Alison Andre
Subject:	Re: [EXT] Penultimate draft of NSF PIPP Research Description to submit today (Oct 1))
Date:	Friday, October 1, 2021 10:35:59 AM
Attachments:	NSF_PIPP_ResearchDescription_v12_GM_KJO.docx ATT00002.bin

Dear ALL,

See page 15 on the attached. I accepted Gonzalo's minor edits and revised the scaling operations section on p15 and added in a "Associated Risks and Mitigation Plans" section… but now we're a bit more over length, so need to cut back more. How does this new section look?? Missing any big risks?

Associated Risks and Mitigation Plans: We envision two primary risks associated with our proposed work 1) data access and privacy violations, and 2) biosafety concerns inherent with emerging zoonoses research. For data related risks, we have already established a strong collaborative relationship with FB's Data for Good team in developing this proposal and already have access to all the relevant datasets for our pilots. Data privacy concerns are taken seriously by our group, including deidentification of any personal identifying information following strict protocols on using human subject data from FB and our already approved IRBs as part of EHA's NIH-funded work. We will amend or apply for additional IRBs as needed. For biosafety related risks, there will be no active sample collection under our proposed NSF project. We have already secured appropriate approvals (IRB, IACUCs, sampling permits) that outline PPE use and biosafety monitoring plans for our NIH-funded work. Our lateral flow assay development will be undertaken in laboratories designate at the appropriate Biosafety Level following US regulations (USU and other partner labs to be confrimed upon award).

PROJECT DESCRIPTION

Introduction and Background

The spillover and spread of novel, zoonotic pathogens can have tragic societal and economic impacts, as is evidenced by the ongoing COVID-19 pandemic. Unfortunately, the emergence of SARS-CoV-2 (the virus that causes COVID-19) is just the latest in a series of viral emergence events that have been accelerating over the last several decades. In just the last year, as the world continues to respond and adapt to COVID-19, we have seen several deadly zoonotic (animal origin) viruses re-emerge in the human population, including Ebola and Marburg in Guinea[1,2], Nipah virus in India[3], Rift Valley Fever virus in Kenya[4], and novel strains of influenza in China [REF]. Most emerging infectious diseases (EIDs) originate in wildlife reservoir hosts and are transmitted across species boundaries, or ("spill over"), when livestock or human populations increase contact with wildlife through factors including land use change, agricultural intensification, and the wildlife trade[5-7]. Zoonotic spillover is a dynamic process determined by many steps including 'pre-emergence' viral evolution, diversification, and transmission among wildlife hosts; 'human exposure' via direct or indirect contact between humans, livestock, and wildlife; 'post-exposure' limits to cell entry, host range, human-to-human transmission; and population level virus amplification and spread. Once efficiently transmitted, EIDs can spread rapidly through globalized travel and trade networks, threatening countries with high population growth, urbanization and international trade (**Fig 1**).

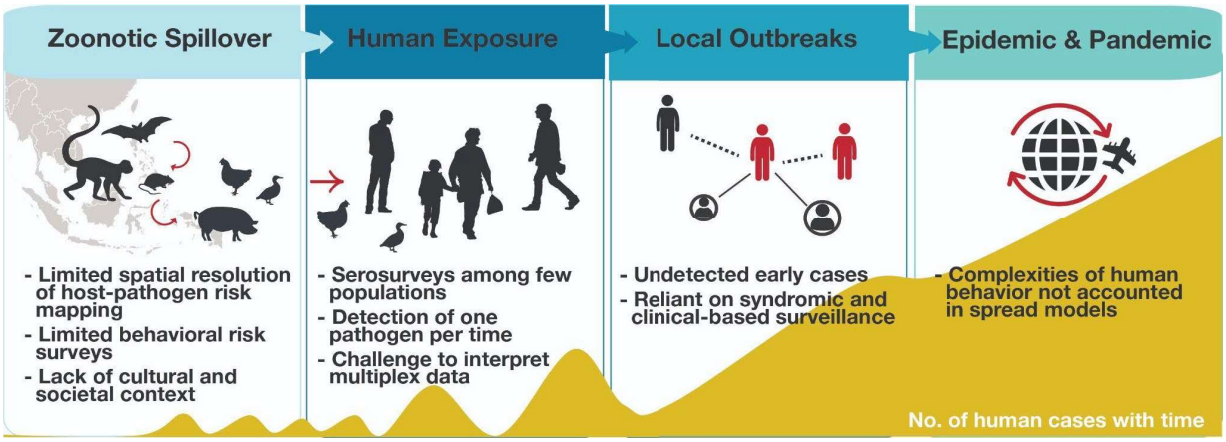

Fig 1. Four stages of zoonotic disease emergence and spread, with gaps in knowledge at each stage. Our proposed project will develop and pilot novel, multidisciplinary and technological advances to address critical gaps in the first three stages of emergence, when pandemic prevention is possible.

Novel human EIDs are typically only discovered when they cause severe or unusual symptoms, when astute clinicians are linked with laboratories capable of pathogen discovery research, and after they have been transmitted from person to person (in which case control options become more difficult).[7,8] The emerging human viruses characterized to date likely represent just the tip of the iceberg given the hundreds of thousands of predicted zoonotic viruses that exist in nature and limited surveillance capacity globally[9]. New research has disrupted the paradigm that zoonotic disease spillover is a rare event. A combination of more advanced molecular and serological assays, improved laboratory capacity around the world, and targeted surveillance of "high risk" human populations have led to evidence of zoonotic virus transmission and infection, even in the absence of notable disease outbreaks. Often these spillover events lead to "dead end" infections in people or limited 'stuttering' chains of transmission, a phenomenon referred to as "viral chatter"[10]. Community-based serological surveys from our group and others have illuminated relatively frequent and previously-unknown instances of zoonotic virus spillover

including Henipaviruses in bat hunters from Cameroon[11], SARS-related CoVs in rural communities living near caves in China (pre-2019), and Ebola virus exposure in DRC[12] and even Southeast Asia[13]

It is during these early stages of emergence, i.e. "viral chatter" with limited human transmission and even further upstream to include pre-emergence risk analyses at the human-animal interface, where innovative convergent research approaches are most needed for pandemic prevention. While we have made significant scientific advances over the last ~15 years in understanding where and through what mechanisms novel zoonotic viruses spill over into the human population[5,14-17], there **are still fundamental knowledge (and implementation) gaps that stymie our ability to preempt and prevent future disease emergence events**.

Our Grand Challenge Problem

Prior to 2019, EcoHealth Alliance (EHA) and colleagues demonstrated that SARS-related coronaviruses (SARSr-CoVs) in China pose a clear and present danger for a future human outbreak. We conducted extensive viral discovery in wildlife from China and Southeast Asia, phylogenetic analysis, and host-range experiments to show that certain bat species harbor a diverse assemblage of SARSr-CoVs[18,19], as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics[20-23]. We conducted behavioral risk factor surveys and serological sampling from human populations living close to caves and found serological evidence of SARSr-CoV spillover in people exposed to wildlife[24]. Building on this body of knowledge, our recent models suggest that bat-origin SARSr-CoVs may be infecting 400,000 (median 50,000) people in Southeast Asia each year[25]. While confidence intervals around these estimates are large, our sensitivity analyses importantly identified key area for future research to improve these spillover risk models - notably, quantification and better contextual understanding of human-bat contact, the role of wildlife farming and livestock hosts, scaling up human serological surveys, and better data on sero-dynamics following novel SARSr-CoV exposure.[25]

Despite this strong body of evidence for SARSr-CoV spillover risk (prior to the emergence of SARS-CoV-2), surveillance and research on these potential pathogens, their hosts, and their interface with humans was not conducted across a wide enough geography, nor at a temporal or spatial scale to inform actionable disease mitigation. Even for large-scale efforts to strengthen zoonotic virus early warning systems, like the 10-year, 30 country USAID PREDICT program[26], led by a global consortium including EHA, it's nearly impossible to capture enough data and disseminate that quickly enough to lead to widespread prevention measures at a national scale using existing methods – especially when considering multiple potential viral threats at once. This is especially true for large countries with heterogeneous human populations, diverse wildlife fauna, and known ecological and societal risk factors for emergence. Approaches to massively scale up zoonotic spillover surveillance are needed.

Our grand challenge problem: "How can we capture actionable data about the risk of zoonotic spillover (and early spread) at the scale and speed required to prevent outbreaks?" Can we leverage tools developed by computer scientists, data engineers, and technology companies to collect data from diverse populations and geographies to advance zoonotic spillover intelligence to a global scale? Can we incorporate new insights from medical anthropology, sociology, wildlife biology, disease ecology, molecular virology, biomedical assay design, and data science and use these to pioneer new methods in data collection and interpretation for zoonotic disease prediction and prevention? In this development proposal, we propose to build a collaborative research and training network to confront this challenge, designed at the start to scale up to a future *Center for Spillover Intelligence (CSI).* Our network will deeply integrate computer scientists, engineers, and data scientists using machine learning and other novel technological approaches together with experts in emerging disease and social-

behavioral research. New insights from social, behavioral, and anthropological research make it clear that the emergence and future trajectory of human epidemics require that we recognize potential biological risks alongside risks posed by cultural and societal factors[27-29]. Our cross-disciplinary, multi-institution project will combine synergistic research activities with training and education programs to facilitate new innovations to scale-up the collection and interpretation of data to inform where, how, and what zoonotic viruses are poised to emerge – a field we collectively refer to as *Spillover Intelligence*. Our inclusion of social-behavioral researchers, data ethicists, and inter-governmental EID policymakers will ensure that our aims and approaches are culturally and locally relevant, and align with our ultimate goal of improving global health.

Our Vision: We envision a world where diffuse data streams can be combined and interpreted in near real-time to disrupt the spillover and spread of future zoonotic viruses. We envision diverse leaders and scientific disciplines coming together to advance spillover intelligence and transform the ways we think about, monitor, and respond to global viral threats.

Our Team

We have assembled a multidisciplinary team of experts to lead our development project and to create and grow the Center for Spillover Intelligence (CSI). Our **Leadership Team (PI, coPIs, Senior Personnel)** includes a mixture of established scientists and early career leaders working across the fields of: emerging infectious diseases, computer and data science, engineering, mathematical modeling, virology, technology and biotechnology, data ethics, social science, and medical anthropology. Our project will be led by EHA (PI Olival) building off the foundational pandemic prediction work our institution has developed over the last decade, but expanding to new partnerships and collaborations we have not previously been able to explore – including with computer scientists and engineers from University of Rochester (UR) and behavioral anthropologists from Georgetown University (GU). We will develop and pilot new technologies for field biosurveillance and data interpretation with Uniformed Services University (USU). Our Leadership Team is ethnically, culturally, and geographically diverse, including US-based researchers who originate from and/or currently conduct their primary research in global EID hotspot regions[5,14], e.g. South Asia (Hoque); Latin America (Mateos); Africa (Mendenhall); and Southeast Asia and the Pacific (Olival). Our geographic and disciplinary diversity, including long-standing international collaborators from each region make our team well equipped to address spillover at a global scale, while understanding the importance of local context.

PI Olival (EHA) is a disease ecologist and evolutionary biologist with a primary interest in developing novel analyses to target zoonoses surveillance and viral discovery. He currently leads two regional biosurveillance projects in Southeast and Western Asia, and recently served as Modeling & Analytics Coordinator for the USAID PREDICT project[26] overseeing a multi-institution team developing pandemic prediction strategies using public and project-specific data from 30 countries[5,15,30-35]. **coPI Mendenhall** (GU) is a medical anthropologist and Professor at the Edmund A. Walsh School of Foreign Service. Mendenhall works at the intersection of social, psychological, and biological lives, attempting to understand what makes people sick, why, and where. Mendenhall is currently a PI of NIH Fogarty International Center grant "Soweto Syndemics", the first ever study to evaluate a syndemic from the ground up. **coPI Hoque** (UR) is an Associate Professor of Computer Science working at the intersection of Human-centric Computing (HCC) and Artificial Intelligence with a focus on multimodal machine learning with applications in health and wellness. Hoque is currently a PI of NSF NRT grant, and received NSF CAREER and CRII grant. Hoque has been identified as an 'emerging leader of health and medicine' at the National Academy of Medicine (NAM). **coPI Mateos** (UR) is Assoc. Professor of Electrical and Computer Engineering and a Data Science Fellow, his research focuses on algorithmic foundations, analysis, application of (graph) signal processing tools to the study of large-scale and dynamic networks. He has pioneered foundational work in network topology

inference, online learning from graph streams, and interpretable representation learning for unstructured data. **coPI Ross** (EHA) is Principal Scientist for Computational Research. His research includes model development to interpret complex spatio-temporal disease surveillance data in heterogeneous populations, including from serological surveys, real-time community and hospital testing, and simulating and forecasting epidemic dynamics over populations, space, and networks. He is PI on awards from DHS Science & Technology Directorate and the Defense Threats Research Agency (DTRA) for designing disease forecasting systems. **SP Mendelsohn** (EHA) is a research data scientist specializing in the development and application of machine-learning models to draw insights into the occurrence and spread of emerging infectious diseases. With a focus on reproducible research methods and data management, she has been the lead analyst on data driven disease forecasting and behavioral survey-based projects characterizing animal-human contact patterns. **SP Daszak** (EHA) is an EID expert who has studied the process of disease emergence for decades and pioneered groundbreaking work in "hotspot" risk mapping to target surveillance. **SP Broder and Laing** (USU) are experts in the design of multiplex serological assays, molecular virology, and implementation of field-based human and wildlife biosurveillance strategies. **OP Busa** is a biotechnology consultant with a specialty in developing lateral flow assays and optical readers. **OP Pompe** is the Research Manager for Facebook Data for Good, who specializes in applying human mobility datasets from social media feeds and data privacy and ethical issues.

Intellectual Merit

The primary intellectual merit of our proposed project is in **developing and piloting innovative approaches to pandemic prediction across previously siloed scientific domains** including biology, computer science, electrical engineering, and social-behavioral research. We apply new theoretical advances and convergent research to address our grand challenge of capturing actionable data about the risk of zoonotic spillover and early spread at a scale and speed required to prevent outbreaks. We will identify existing gaps and next-generation solutions for step-level changes in zoonotic spillover prediction via multi-day intensive workshops with thought-leaders organized by each of our three multidisciplinary Working Groups. Our pilot research projects will serve as a test bed for new theoretical frameworks to advance spillover intelligence. For example, in Aim 1 we will transform decades-old anthropological approaches and pilot a cost-effective, scale-up of quantitative and qualitative behavioral data collection through a unique public-private partnership Facebook; and use image recognition and artificial intelligence to quantify and map human-wildlife contact globally. In Aim 2 we will develop hierarchical Bayesian models to interpret rich, multiplex serology datasets and test these on real surveillance data as part of EHA's NIH-funded work; and develop the first field-forward lateral-flow assay for Nipah virus antibody detection. In Aim 3 will use cutting edge theoretical advances in network science, anomaly detection, and machine learning to detect changes in human mobility patterns that may portent spillover (e.g. increase congregation around wildlife markets) or signal early epidemic spread after the point of spillover.

Broader impacts

This proposal could potentially transform our understanding of zoonotic spillover risks using survey, multimedia, and mobility data on a global scale, including aggregated and anonymized data from ~3B active Facebook users. Insights and intellectual outputs from our working group activities will have direct benefits to public health by informing disease mitigation strategies for pandemic prevention. We will synthesize our three working group outputs and prepare a special joint publication for the National Academies of Science's Forum on Microbial Threats for wide distribution, and write popular press articles to accompany our peer-reviewed manuscripts. EHA's *Alliance,* includes a network of over 70 partner institutions in 30+ countries, presents a unique opportunity to work with local stakeholders in emerging virus 'hotspots' and validate

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models using data sets obtained through our long-standing collaboration with government partners including human health, agriculture, and environmental sectors.
Our education mission consists of multiple workshops, hackathons, monthly seminar series, and access to state-of-the-art visualization facilities. We will begin to train a new generation of graduate and undergraduate students who will be prepared to harness the burgeoning power of data science to make novel inroads into understanding human-animal contact, serological surveillance, and aggregated mobility data and their interaction. Our proposal builds on a unique partnership between the academic (UR, GU, USU), non-profit (EHA), and private sector (FB), allowing students to apply lessons learned from classroom settings to the real-world, and our recruitment will focus on including >50% women and underrepresented minorities in science and engineering. This planning grant will be a path towards structuring interdisciplinary education, cross-training courses across institutions, followed by hands-on experience via internships and research exchanges – with options for international work via existing programs at our institutions. Through the planning grant, a new MS data science track on 'zoonotic surveillance' will be established at UR, the first step in our plans to expand our Education and Training plan should we move towards a Center-level award in the future.

Education and Training Plan
Our Leadership Team will serve as mentors for our **CSI Student Training Program** that will offer a fellowship stipend to cross-train 20 Master's level graduate students via semester-long projects associated with proposed pilot research activities (3-4 students per project) and 3-week research exchanges between our institutions (UR, GU, EHA, USU, 4-8 students from each). The research exchange builds off EHA's successful NSF-funded EcoHealthNet training program (2011-21) and will take place in either the Summer of 2022 or 2023. We will link with currently-funded international programs at our respective institutions (e.g. Gui^{2}de in Nairobi, Kenya run by GU; and the NIH funded CREID research pilot program with EHA's EID-SEARCH project in Southeast Asia) to support students who desire to gain international, hands-on research experience as part of their research exchange. The focus of each research exchange will be on cross-disciplinary training to think 'big' about the future of pandemic prediction and ensure the next generation of spillover intelligence researchers gains valuable hands-on experience working with experts outside their area of study. Students will be drawn from respective departments across our institutions, i.e. students with a computational background and with interests in interdisciplinary work will be recruited from UR's cross-department Goergen Institute for Data Science, Anthropology and International Affairs students from GU, and Emerging Disease and Virology students from USU. As EHA is a non-profit and does not have an active student body, we will recruit Ecology, Evolutionary Biology, and Environmental sciences students from universities in the NYC area, including Columbia University's E3B department where PI Olival, coPI Ross and SP Daszak serve as adjunct faculty. We will explore specific mentorship opportunities with data scientists from Facebook. If awarded, we will additionally apply for funds through NSF's Research Experiences for Undergraduates (REU) program, with the goal of supporting summer stipend for 1 undergraduate per institution or the maximum allowable. We will host a joint monthly virtual seminar series (2 semesters long) open to graduate and undergraduate students from our respective institutions, with a dedicated student "lab meeting" before each seminar. Two CSI students from diverse disciplines will lead each lab meeting discussion based on a deep read of relevant publications that each speaker will distribute in advance. We envision the first semester of the seminar series will be led by our Leadership Team and Working Group experts, and the second semester will be for CSI Graduate students to present results or current challenges related to their pilot research projects. We hope to encourage these students to pursue Ph.D. studies. If we are successful at establishing a center through our planning grant, we should have access to a trained cohort of students with appropriate multidisciplinary skillset.

Project Overview

Our 18-month research agenda aims to understand how new methods and technologies can improve the efficiency, intensity, speed, and scope of zoonotic disease surveillance. **We have structured our development grant around three primary research aims** that will guide our interdisciplinary research and training activities. We will establish a working group composed of multi-disciplinary experts for each primary aim that will be co-led by members of our Leadership Team. We will develop pilot research projects that capture different parts of the disease spillover process focused on specific geographies, lead 3-day intensive workshops, monthly student seminars, and student training activities that cut across our inter-linked research aims.

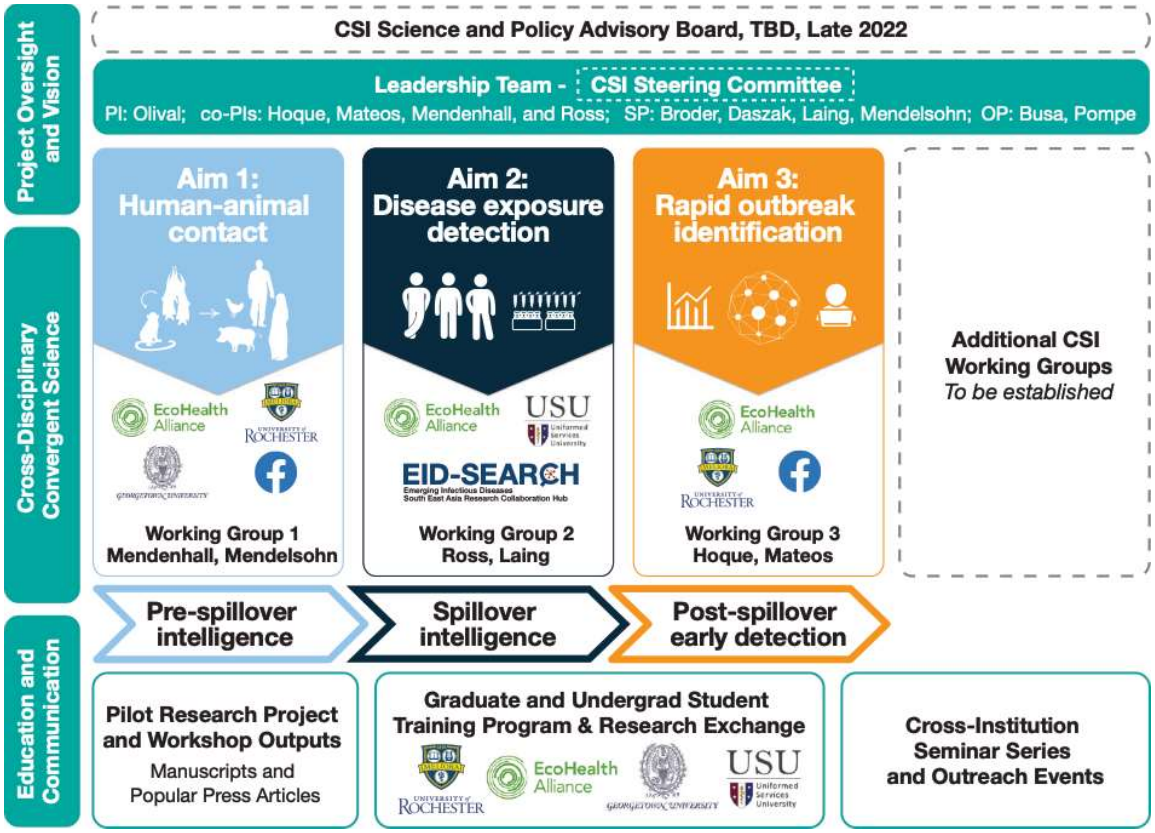

Fig 2. Project overview schematic showing our project management; three primary aims with graphical representation of pilot research activities to scale up and advance spillover intelligence; institutional partners leading activities for each aim including designated working group leads, and student education and communication activities, including pilot research projects, graduate student research exchanges, seminars, and other outreach activities. Our development level project was built to scale to a Center level award (the Center for Spillover Intelligence, CSI) as noted with the dashed outline boxes, including the future addition of other CSI Working Groups, a Science and Policy Advisory Board, and transitioning our current Leadership Team (PI, coPIs and SP) into a more formal Steering Committee for oversight.

Aim 1: Measuring human-animal contact at scale

Interactions among humans, wildlife, and livestock create interfaces for pathogen spillover and are proximate risk factors for the emergence of all directly-transmitted zoonotic diseases.[14,36] Human-animal contact is commonplace around the world, with many factors mediating the type and frequency of contact, including cultural practices, environmental conditions, food security, and occupational demands.[36] In regions of the world considered prone to spillover--forested tropical landscapes with high biodiversity and recent land-use changes--understanding human-

animal contact is crucial to pandemic prevention.[5] To measure these interactions and characterize associated behaviors, beliefs, and knowledge, researchers often implement resource-intensive surveys in key communities.[37,38] While crucial to understanding human-animal contact dynamics, on-the-ground surveys cannot yield scalable data that is comparable across populations. Often, in place of surveys, researchers conducting analyses at broad scales rely on coarse estimates of contact such as the joint density of human and animal populations. **Our working group will uniquely combine social scientists, disease ecologists, and computational scientists to increase the scale at which we can directly measure human-animal contact.** We will develop and implement two pilot research projects. The first will use novel, social-media-based survey approaches to reach larger populations at higher frequencies while maintaining the cultural sensitivity and relevance of on-the-ground surveys. Working collaboratively with cross-disciplinary researchers, we will design surveys to understand social behaviors, cultural perceptions, and occupational hazards that may pose zoonotic spillover risk to individuals and communities. We will target geographies based on our existing spatial risk modeling work that maps infectious disease spillover potential and the ecological distributions of key reservoir host species.[5,15,25] Second, we will conduct pilot research on the use of social-media-based image recognition algorithms to identify locations of human contact with key species known to host high pathogen diversity. This pilot will include a collaboration effort to test the efficacy of such technology for understanding taxon-specific contact patterns globally, and to bring multidisciplinary context to meaningfully interpret the outputs (including species validation with taxonomic experts). If successful, will use these data on frequency of animal contact from image data to identify locations for additional survey data collection.

Aim 1 Pilot Research Projects: We will develop two pilot projects to assess the potential of 1a) social-media dispensed surveys and 1b) passive surveillance using artificial intelligence for image recognition to capture information about human-animal contact. We will target populations at wildland interfaces where contact with wildlife is likely high but internet penetration may be mixed.

1a) Social-media dispensed surveys to quantify and contextualize human-animal contact
We will pilot social-media dispensed, large scale population surveys about perceptions of and interactions with animals. We will optimize the design of these surveys for mobile platforms to facilitate higher response rates, and will also explore the possibility of longitudinal sampling respondents (either individuals or populations) to understand changes in conditions and responses over time. Survey questions will be based on a combination of EHA's past experience designing on-the-ground human-animal contact surveys[37,39,40] and ideas generated from our proposed working group activities involving anthropologists, data scientists, and disease ecologists who have worked extensively in the geographic and societal contexts in which we focus our work. We will aim to design surveys that account for populations and individuals holding different beliefs, carrying different histories, and living in different ecologies. We will attempt to survey demographically and geographically diverse populations to capture direct and indirect animal-contact patterns and behaviors, and will attempt to capture the role that international, state, and local policy plays in individuals' lives. Further, we will aim to recognize particular hesitancies among individuals to interact with certain technologies or other public health interventions.

We will explore various online platforms at the start of our project for survey administration. Given our unique public-private partnership with Facebook for many components of this project, using the Facebook platform to disseminate our pilot survey is likely to be a strong option, especially given Facebook's global reach (nearly half of the world's population, ~3 billion users), which introduces opportunities for digital interaction across class, gender, ethnicity, and cultural

divides. Facebook has extensive experience administering surveys, either directly on its platform (e.g. Yale Climate Change opinion survey) or off-platform (e.g. Carnegie Mellon University COVID-19 surveys) that we will leverage. Facebook also has extensive privacy infrastructure and data-review policies to ensure anonymity and data security.

We will also explore creating games that users will be encouraged to play online to enable us to learn more about how they think about host species, viral threats, environment, and health/disease. For example, users may be given a list of 30 images and will be asked to put them into categories and describe what each category means. Adapting these "pile sorting" anthropological methods[41] to an online interface and for spillover risk, will be a novel application with potential for a massive scale-up of data around cultural and conceptual dimensions of zoonotic disease risk. Data generated from this exercise will generate a more ethnographic understanding of people's experiences and thought processes, which tend to be locally mediated. We understand that there are geographic and demographic gaps in internet and social media use, and will incorporate bias corrections into our study design. We will initially focus on Southeast Asia, a region with a high likelihood of future zoonotic disease emergence and where EHA has good coverage of on-the-ground surveillance programs to ground truth online data collection.[5]

1b. Image recognition of zoonotic disease host species

We will pilot image-based surveillance of Facebook and Instagram feeds to attempt to identify human contact with key zoonotic disease host species and to explore the limits of taxonomic and behavioral recognition in images. We will work with Facebook Data for Good (see **Letter of Collaboration**) to adapt and refine their existing machine vision algorithms to target key taxa and to assess the efficacy of different approaches, working within strict guidelines and ethical standards for data de-identification. While data will be de-identified, we will only use photos with full location meta-data, allowing us to not only quantify the type of species and contact, but where it occurred. There are multiple image libraries that may serve as AI training datasets, including iNaturalist, the American Society of Mammologists, and Integrated Digitized Biocollections (iDigBio). We will iteratively implement and test algorithms starting with broad taxonomic groups (e.g., bats) and will explore the extent to which our approaches can identify finer taxonomic resolutions down to the genus or species level. In addition to taxa identification, we will explore image recognition of features that may suggest specific types of human-animal contact, such as cages or market settings. Further, we will use text and image metadata and explore using hashtags associated with images to boost the ability of our algorithms to identify key taxa.

This work will involve collaboration between machine learning scientists, biologists/ecologists, and social scientists. We will follow all data anonymity and privacy guidelines from Facebook and will also discuss data handling ethics in our working group. We will assess the ability of passive surveillance with image recognition to be used to target surveys to populations that may have higher frequency of contact with wildlife, and ultimately to target biological surveillance of human and animal populations. We will consider both the accuracy of the image recognition algorithms as well as implications for survey distributions, results interpretation, and ethical data practices. We will also compare the spatial distribution of human-animal contact patterns derived from image recognition with previously generated spillover risk maps based on joint occurrences of animal and human distributions (e.g., SARS-related CoV spillover risk from Sanchez et al. 2021 preprint).[25]

Working Group 1 Activities: Our working group will convene 12-15 experts from the social sciences, disease ecology, and computational sciences for an intensive, 3 day workshop. We will structure the workshop to focus first on data ethics in human behavioral surveying and image recognition surveillance and secondly on survey design and analysis planning, with specific attention to accounting for gaps or biases in survey distributions. We will center the workshop around the understanding that technological quick fixes are never enough and that data collection must address long term social, ecological, and economic realities to understand how people perceive, experience, and interact with the risk of and repercussions from spillover events. We will also address experiences and challenges in engaging longtime ethnographers and local leaders to understand what's at stake for communities engaging with online and field surveillance programs. Our working group will help mentor students part of the CSI Student Training Program, to facilitate information sharing and idea generation among undergraduate and master-level students. Working group 1 will also contribute to the CSI Seminar Series with cross-disciplinary topics such AI image recognition (lead by a data scientist and bat biologist) and human-behavioral survey design (lead by an epidemiologist and medical anthropologist).

Partners, Initial Working Group 1 Members, and Roles: EHA (SP Mendelsohn): survey design and implementation, image recognition algorithm development and testing, novel data analyses. Facebook Data for Good (OP Pompe): survey implementation, image recognition algorithm development and testing, data ethics. University of Rochester (coPI Hoque): Image recognition algorithm development and testing. Georgetown University (coPI Mendenhall): Survey design with focus on ethnography and anthropology; online free listing/pile sorting game design; help lead expansion of WG to include emerging international leaders in social-behavioral science (e.g. Dr. Edna Bosire from Kenya). We will invite additional leaders from our extensive funded collaborations to be part of the Working Group for Aim 1.

Aim 2: Detecting zoonotic disease exposure at scale

Animal-human contact is the necessary condition for spillover of potential pandemic, directly-transmitted viruses. While a small fraction of such encounters result in actual exposure or infection, such exposures are frequent given the total sum of encounters. Most such infections are never reported and rarely spread widely, and only a very rare subset become widespread in the human population[42]. Other viruses such as Lassa spill over thousands of times per year[43]. In areas of frequent spillover, the eco-evolutionary dynamics of "stuttering chains" of infection are what give rise to epidemics and pandemics[44]. Identifying populations where frequent exposure occurs is critical for deploying preventative measures and following viruses upstream to their wildlife hosts. Yet even large-scale surveillance projects cover too small populations and produce far too few detections to determine the most exposed geographic, demographic, or occupational groups at an actionable scale [REF, showing PREDICT sampling efficiency]. This is for two reasons: the field and laboratory cost of surveilling populations interacting with wildlife interface and, and the low detection rates of the widely-used, molecular PCR or metagenomic approaches.

We aim to drastically increase the scale at which populations can be monitored for exposure to animal-origin viruses by developing and piloting novel tools and analyses needed to implement large-scale serological surveys. While metagenomic methods have been favored for their ability to capture genetic sequences of previously unknown viruses, serological methods can be used to detect exposure to known and unknown viruses using multiplex assays tailored with a mixture of sensitivities and specificities[45]. Serological tools can detect antibodies for months or years after viral infection, rather than the limited window of days or weeks using molecular methods, and thus can provide a much richer profile of populations where pandemics may emerge[46]. In this planning period, we will begin pilots to test the feasibility of two complementary approaches

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Our work under PREDICT is relevant here, working with local community leaders to distribute results and the 'Living Safetly with bats book'. But how do we scale this? One idea is through a network of local leaders across the landscape, like a "Community One Health Volunteer" network.

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to enabling serological screening at large scales: 2a) the creation of rapid bioinformatics pipelines to interpret high-dimensional serological data from large streams of surveillance data, and 2b) the development of rapid field serological tests for wildlife-origin diseases.

Aim 2 Pilot Research Projects

2a. Rapid bioinformatics for high-throughput, multiplex serology

Members of our team (SP Laing, Broder) have developed multiplex serology tests that can be used to screen for exposure for many species and variants of filo-, henipa-, and coronaviruses simultaneously, and screening pipelines that can process samples at the scale of tens of thousands of samples. However, these tests pose challenges for interpretation, as high-dimensional results from multiplex bead or chip assays frequently contain signals of cross-reaction and autocorrelation (**Fig 3**). Through cross-disciplinary research between molecular virologists, serologists, mathematical modelers and data scientists on our team, we will create models to interpret this high-dimensional data and integrate them into a rapid bioinformatic pipeline that identifies individuals and populations to known viruses as well as flagging serological signals indicative of novel viruses. We will make use of data collected under our ongoing NIH and DTRA funded biosurveillance activities (Laing, Broder, Ross, Olival, Daszak) to develop detailed reaction/cross reaction profiles of a broad panel of zoonotic viruses to serological tests. We will build mechanistic, hierarchical Bayesian models that allow us to determine whether serological "profiles" are more likely to represent antibodies of known viruses, mixtures of multiple known viruses, or previously uncharacterized viruses. We will extend techniques of antigenic mapping,[47] using serological profiles to estimate phenotypic similarity of new viruses to known viral families, determine the correlation with phylogenetic relatedness, and use these measures to screen new viruses for pandemic potential. Using simulation experiments, we will test reduced-form, simple models against fully structured models so as to develop statistically robust procedures to interpret these data without high-performance computation.

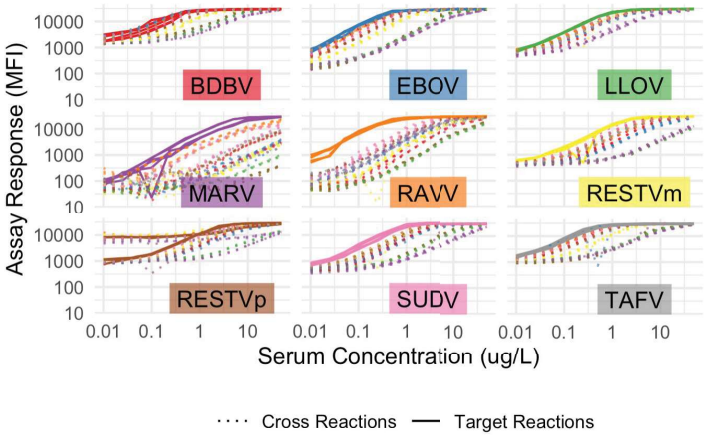

Fig 3*.* Preliminary data: Reaction-cross reaction plots of multiplex Luminex assay response to antibodies for closely-related filoviruses.

Scaling center operations: This pilot project will produce methods and a proof-of-concept pipeline for high-throughput analyses. We will leverage EHA's Emerging Infectious Disease – Southeast Asia Research Hub (EID-SEARCH) project, a NIH-funded Center for Research in Emerging Infectious Diseases (PI Daszak) who's partners include USU, Duke NUS, Chulalongkorn University in Thailand, and various clinics and institutions in Malaysia – currently screening large numbers of human samples (>10K/yr) for various coronavirus, filovirus, and paramyxovirus antibodies. In the future the CSI will implement surveillance studies screening these sample streams using multiplex serology, and utilize the analytical and bioinformatic tools we develop in the next 18 months. As part of our planning activities, we will establish protocols and approvals for this work.

2b. Development of a field-ready semi-quantitative lateral flow assays for an emerging zoonotic viruses (Nipah virus)

Lateral flow assays (LFAs), are low-cost tests where reagents printed on cellulose strips react with a sample that flows via diffusion. LFAs are capable of providing quantitative results (in proportion to antibody titer) using a combination of high-quality manufacturing and carefully designed, calibrated optical scans[48]. LFAs can be used to provide diagnostics at low cost at the point of care and by consumers. Lack of commercial-scale applications has prevented LFAs from being used for serological testing for novel or emerging disease. To our knowledge, no studies have used LFAs for zoonotic virus serosurveillance in the field.

We will develop a proof-of-concept LFA for Nipah Virus (NiV). NiV has a high mortality rate and is considered to have pandemic potential that is ranked by CEPI as one of 7 high pandemic risk pathogens[49]. The range of NiV's primary wildlife hosts (*Pteropus spp,* fruit bats) cover South and Southeast Asia but human cases have only been detected in Malaysia, Bangladesh, and the Philippines. Spillovers occur regularly in Bangladesh, but are rarely reported in large portions of the country[50] and reporting is strongly associated with health care accessibility[51]. Broad-scale serological monitoring has the potential to identify populations from which pandemic strains may emerge. Through previous work, members of our working group (Laing, Broder) have developed multiplex serological assays for NiV[52] and the soluble proteins and reagents required for an LFA. We have also added experience in engineering, quality control and commercialization of LFAs (Busa, industry consultant) to our working group. For the pilot, we will engage with commercial manufacturers to create and test a prototype Nipah LFA and a protocol for smartphone-based optical reading of quantitative responses.

Scaling center operations: The pilot will create a prototype LFAs. As part of full center operations under CSI, we will aim to scale up production and test LFAs efficacy using control sera and field deployment as part of EID-SEARCH. We will additionally assess feasibility and cost of deployment, and compare performance against current laboratory-based assays.

Working Group 2 Activities: In addition to the two pilots above, working group members will contribute to the our CSI monthly seminar series, presenting on topics including high-dimensional data models, field serology, serological assay development, laboratory product design, and public-private partnerships and commercialization. We will conduct two online workshops on serological data analysis, jointly with undergraduate, masters, and doctoral students at USU and UR, leveraging partnerships with field researchers currently working in serological surveillance. Our working group will lead a 3-day intensive workshop, where we will recruit additional participants with expertise in serological assay design and surveillance, immunology, emerging zoonoses, and focus discussion on key barriers to scaling serological surveillance for known and unknown zoonotic viruses.

Partners, Initial Working Group 2 Members, and Roles: EHA (coPI Ross): Model and pipeline design, software optimization. Uniformed Services University (SP Liaing, SP Broder): data and model interpretation, reagent manufacture, assay design. SP Busa: device manufacturing, quality control, industry outreach and partnership development. We will recruit additional participants from our partner network engaged in serological field studies as well as industry representatives to be part of the Working Group for Aim 2.

Aim 3: Rapidly Identifying Anomalous Health Events

In early stages of epidemic spread, novel contact and mobility-induced behavioral data as well as communication patterns may provide a signal of e.g., health-seeking activity indicative of an outbreak cluster. We contend that these signals may be detectable in large-scale data streams of population mobility and contact, or disease reporting available through telecommunications and health care providers. Timely detection requires approaches that can manage data at scale,

analyze high-dimensional, spatio-temporally correlated streaming data in (pseudo) real time, and distinguish between known signals (e.g., typical influenza-related activity) and novel spillover-related signatures that we wish to unveil, characterize, and explain in order to trigger the necessary public policy responses.

This desideratum calls for fundamental methodological and computational advances, to go along with epidemiologically-relevant data sources that describe how humans are moving and interacting across physical space, with unprecedented temporal resolution and geographical coverage. Traditional flow-based mobility datasets used to parameterize epidemiological models have been aggregated and formatted in the form of origin-destination (OD) matrices. While valuable towards understanding population flows between regions of interest, OD matrices lack information on how much time a traveler spends in any given location. Naturally, when it comes to epidemiological studies the duration of time spent in potentially risky geographical areas is key towards deciphering actionable contact and exposure patterns[53]. Moreover, recent studies have shown that flow counts between pairs of geographical areas can be loosely correlated with, and not representative of, epidemiology-relevant mixing patterns[54]. Instead, these couplings among subpopulations are measured by effective contact rates, meaning how often people from each region come into contact in a way that is sufficient to transmit a disease. To address these challenges, our consortium will partner with the Facebook Data for Good team and leverage two recently assembled global-scale datasets - Activity Space Maps and Colocation Maps[53,54].

Different from existing approaches that have focused on measuring the causal impact of mobility-oriented interventions, **our ambitious goal in this aim is to markedly advance the surveillance infrastructure available to predict anomalous health-related events before a major outbreak occurs**. While undoubtedly a multi-faceted endeavor, in this planning grant we will take initial, but significant steps with an emphasis on network-centric, computational analyses of dynamic mobility and interaction data at a global scale. By developing the appropriate statistical tools, we will support the ultimate goal of characterizing early pandemic "fingerprints" or signatures that can be extracted from these global datasets.

Aim 3 Pilot Research Projects: Working closely with Facebook Data for Good (see Letter of Collaboration from Alex Pompe), this working group will pilot data-driven research activities to explore the feasibility of extracting early (post spillover) signals of epidemic onset from Activity Space Maps and Colocation Maps. Our multi-disciplinary working group brings together leading experts in systems engineering, AI for healthcare, data science, mathematical epidemiology, graph signal and information processing, privacy-preserving mobility data generation and management, human behavioral modeling, and environmental science. We believe the current team, consisting of a wide array expertise and partnership, will instigate the cross-pollination of ideas leading to center-level activities while making significant inroads towards addressing our grand challenge. We propose two specific pilot projects:

3a. Spatio-temporal monitoring of spillover-induced activity anomalies in Southeast Asia
Activity Space Maps (ASMs) are a novel global-scale movement and mobility data set which describes how people distribute their time through geographic space.[53] They are designed to complement existing digitally-collected mobility datasets by quantifying the amount of time that people spend in different locations. Accordingly, this information is valuable for estimating the duration of contact with the environment and the potential risk of exposure to disease -- something critical especially early after spillover. More germane to the theme of this aim, it also offers the unique possibility of developing and testing sequential change-point detection algorithms to monitor multiple spatial grid cells of interest (informed, for instance, by outcomes

of the image-recognition effort under Aim 1). We will target areas known to include health care facilities in pursuit of abnormal trends of (increased) activity. Moreover, using ASMs we will examine flow and temporal activity anomalies around expert-annotated wildlife and wet market locations. Said events are expected if there are human infections, or significant associated changes in behavior around these sites early after spillover. Our initial efforts during this planning grant will be focused on Southeast Asia, which on top of its epidemiological relevance is one of the regions where Facebook ASMs have the most coverage. We will target Indonesia, where EHA has extensive on-the-ground efforts tracing networks of wildlife trade as well as market sites.[55] We will also tap into the resources stemming from EHA's Emerging Infectious Diseases – South East Asia Research Collaboration Hub (EID-SEARCH) to identify clinics and market sites of interest in Thailand and Malaysia. Some key challenges we will address include accounting for the implicit biases in data collection associated with the construction of ASMs,[53] and their subsequent effects on the statistical algorithms that we will develop. Our Working Group will address additional challenges of data interpretation of anomaly detection analyses with input from emerging disease experts and epidemiologists. To calibrate our models, we will bring to bear recent activity data before and after the SARS-CoV-2 outbreak in those locations, in addition to historical influenza seasonal trends that go further back in time. With the exciting prospect of ASM data being officially released in the last quarter of 2021, *we are in a privileged position to be the first multi-disciplinary team that leverages this invaluable global-scale resource to advance our understanding of zoonotic spillover risk and its immediate effect on human mobility.*

3b. Online network change-point detection in dynamic, global-scale Colocation Maps
Activity data are complemented by *Colocation Maps (CMs),* which estimate how often people from different regions worldwide are colocated. In particular, for a pair of geographic regions *i* and *j* (e.g., counties in the US), CMs estimate the probability that a randomly chosen person from *i* and a randomly chosen person from *j* are simultaneously located in the same place during a randomly chosen minute in a given week.[54] In other words, CMs are global-scale, dynamic network datasets with colocation probabilities encoded as edge weights. Thus, they are well suited to parametrize metapopulation models of disease spread,[56] or to measure temporal changes in interactions between people from different regions.[57] To monitor said changes as part of our second pilot activity in this aim, we will build on our recent advances in online (i.e., sequential) algorithms for anomaly and change-point detection from network streams[58,59]; see also.[60-62] The envisioned statistical methods are in stark contrast with most existing graph change-point detection approaches, which either rely on extensive computation,[63] or they would require storing and processing the entire observed CM time series.[64] We will prototype and test a lightweight online change-point detection algorithm with quantifiable performance guarantees, that is also explainable since it relies on interpretable node embeddings; see e.g., *Hamilton_Book*.[58,65] The latter desirable feature will be leveraged to construct dynamic anomaly maps for user-friendly visualization of post-spillover events; see also our recent network-scientific visualizations of spatio-temporal COVID-19 contagion patterns across the US.[66] Moreover, the sheer-size of the CM graphs calls for algorithmic and modeling innovations we will investigate as part of our research agenda, e.g., seamlessly integrating graph subsampling or dimensionality reduction techniques to the analytics pipeline. We will team up with computer vision experts affiliated to the University of Rochester Goergen Institute for Data Science (GIDS, see attached Letter of Collaboration from institute director Mujdat Cetin), to also explore the exciting prospect of integrating geo-tagged multimodal data such as video and imagery in our studies, for instance as nodal attributes embedded to the CMs.

Working Group 3 Activities: *Recruitment and diversity:* We will recruit 6 students from the GIDS-run master's program in data science, which attracts students from multidisciplinary backgrounds and significant computational proficiency to contribute in the aforementioned pilot research activities. *The CSI leadership and broader community are committed to providing research training and educational experiences for those traditionally under-represented in STEM, including women and underrepresented minority students.* The PI team has a solid track-record in actively recruiting and supervising such students at the undergraduate, masters and doctoral level, with demonstrated efforts towards broadening participation in STEM. As examples of the commitment to diversifying the field, co-PI Mateos serves as founding faculty advisor to the UR's SACNAS chapter and regularly organizes diversity, equity and inclusion panels to highlight challenges and successes of UR's Hispanic and Latino STEM community. We plan to prioritize engaging women and minority students in the research and working group activities proposed here. *Coursework:* These students will be on the existing health and bio track with an opportunity to take classes in immunology, microbiology and virology programs as well as data ethics at the University of Rochester Medical Center (URMC) as well as the college of engineering. Through the planning grant, we plan to establish a special MS track on 'zoonotic surveillance' in order to train and mentor the students to lead center level activities. *Seminar and workshop series:* The students will be part of the large cohort of all the participating institutions and participate in the CSI monthly seminar series. The co-PIs (Mateos and Hoque) will conduct two workshops (and invite additional faculty members from UR as guest speakers) with hands-on lessons in mobility and human behavior modeling. *Hackathon:* The students will be encouraged to participate in the yearly hackathon, Dandyhacks https://dandyhacks.net/), organized every fall by the University of Rochester Computer Science Department and work with the dataset from Facebook. Additionally, co-PIs Mateos and Hoque will work with the organizers of dandyhacks to release some of the public dataset from Facebook for other teams to explore, model and mine the data. *Data visualization meetups at the University of Rochester's VISTA Collaboratory*: To get acquainted with the different data resources at our disposal, over the first 3 months of the project the Rochester team (along with Facebook and EHA collaborators via Zoom) will hold bi-weekly team meetings with the MSc students at the UR's VISTA Collaboratory. This is a 1,000 square-feet visualization lab equipped with an interactive (20 ft. wide x 8 ft. tall) tiled-display wall, that renders massive data sets in real time, giving team members the ability to visualize and analyze complex data instantaneously and collaboratively; see Fig. 4. As we dissect what dataset components are central for the aforementioned pilot activities, our graduate students will develop customized data visualization tools, maps and dashboards to facilitate downstream analyses. Updates and progress reports will be shared in these meetings, giving the students a unique opportunity to develop their communication skills. The whole team will provide feedback and share ideas, making this a truly collaborative activity. Moving forward, the VISTA Collaborative offers a unique resource that we will fruitfully leverage as we scale up to full center operation.

Fig. 4. UR's Vista Collaborative will serve as a spillover intelligence "situation room" for training UR and CSI exchange students on analysis of real-time epidemiologically relevant datasets.

Partners, Initial Working Group 3 Members, and Roles: University of Rochester (co-PIs Hoque and Mateos): coordination of working group activities, development and testing of computational tools for early detection of post-spillover signatures, dissemination of research products and educational materials, recruiting and mentoring of master students, liaisons to

GIDS. Facebook Data for Good (OP Pompe and Maps for Good team members Iyer and Citron): data ethics, quantification of privacy protections, data scientific support for Activity Space and Colocation Maps, mathematical epidemiology expertise for data and model interpretation. EHA (SP Mendelsohn and Ross): identification of target spatial locations in Southeast Asia, epidemiological interpretation of anomaly detection analyses and validation of detected early pandemic fingerprints.

Scaling operations: Our programs and pilot research are designed to scale. First, we aim to expand on successful pilots by expanding the geographies of our work, engaging with more in-country partners. Second, we test the development of each of these new streams of predictive data, we plan to develop partnerships with local, national, and global agencies to ingest these data, expand into programs to develop behavioral interventions and testing in response to identifying regions and populations at high risk of contracting zoonotic diseases.

Working groups are really about bringing our project to scale, our intensive 3-day workshop will also lead to new partnerships and synergies for expansion.

Results from Prior NSF Support

Kevin J. Olival has not had prior NSF support, but is co-PI on NIH-funded "EID-SEARCH" (NIAID-CREID U01AI151797, 2020-25 PI Daszak). Previously co-PI on "Understanding the risk of bat coronavirus emergence" (NIAID R01 AI110964, 2014-2019, PI Daszak). ***Intellectual merit*:** Identified >50 SARSr-CoVs in bats, showed evidence of ability to infect human cells, humanized mouse models, and people in China, and used evolutionary and mammalian biodiversity patterns to analyze spillover risk for use in public health control. Produced 18 peer-reviewed papers [20,34,67-79], including two papers in *Nature* [80,81], and a review in *Cell* [82], and others submitted. ***Broader impacts*:** 2 PhD and 6 MS students trained; disease risk and potential management strategies shared with US agencies, WHO, OIE, FAO, and the public.

Ehsan Hoque: Project IIS-1750380. PI: Hoque. Period (2018-23). Amount: $511,453 "CAREER: A collaboration coach with effective intervention strategies to optimize group performance". ***Intellectual merit:*** Developing and validating the efficacy of automated mediation strategies in online meetings. ***Broader Impacts:*** >6 research papers[83-88]. **Project NRT-DESE-1449828.** PI: Hoque (2015-21). Amount: $2,965,341.00 "Graduate Training in Data-Enabled Research into Human Behavior and its Cognitive and Neural Mechanisms". ***Intellectual merit:*** Chartering a new traineeship program to train Ph.D. students at the University of Rochester to advance discoveries at the intersection of computer science (cs), brain and cognitive sciences (bcs), and neuroscience. ***Broader Impacts:*** Supported over 50 PhD students in cs and bcs, seveal won interdisciplinary research positions at R1 institutes.

Gonzalo Mateos (a) Project: CCF-1750428. PI: Gonzalo Mateos Buckstein. Period: 4/1/18 - 3/31/23. Amount: $407,944. (b) Title: CAREER: Inferring Graph Structure via Spectral Representations of Network Processes. (c) ***Intellectual Merit:*** We investigate how to use information available from graph signals to estimate the underlying network topology through innovative convex optimization approaches operating in the graph spectral domain. ***Broader Impacts:*** We prepared a feature article on identifying network structure via GSP,[89] presented tutorials in EUSIPCO'19, CAMSAP'19 an SSP'20, organized the 2019 IEEE SPS Summer School on Network- and Data-driven Learning. (d) Research products are available in.[66,89-96]

Emily Mendenhall (a) National Science Foundation Doctoral Dissertation Improvement Grant, $10,000; Stories at the Border of Mind and Body: Mexican Immigrant Women's Narratives of Distress and Diabetes. **Intellectual merit:** Led to crucial insights studying syndemics - the interactions of social, environmental, psychological, and biological factors that are synergies of epidemics. Papers in The Lancet[29], Medical Anthropology Quarterly[97-99], and as a full-length

book[100]. **Broader Impacts**: Understanding diabetes, trauma, and broader socio-political factors with infectious diseases in India, Kenya, South Africa, and Ethiopia.

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Cc:	Luke Hamel; Robin Breen; Aleksei Avery Chmura; Hongying Li; Alison Andre
Subject:	Re: Penultimate draft of NSF PIPP Research Description to submit today (Oct 1))
Date:	Friday, October 1, 2021 4:09:32 AM
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BIOGRAPHICAL SKETCH

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NAME: Jonathan H. Epstein

eRA COMMONS USER NAME: (b) (6)

POSITION TITLE: Vice President for Science and Outreach

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Brandeis University, MA	BA	1996	Biology
Tufts University, Sch. Vet. Med., Boston, MA	DVM	2002	Wildlife Med., Intl. Med.
Tufts University, Sch. Vet. Med., Boston, MA	Cert Intl Med	2002	Zoonotic Diseases
Tufts University, Medical School, Boston, MA	MPH	2002	Epidemiology
Columbia University, NY	Fellowship	2006-2009	Molecular Virology
Kingston University, UK	PhD	2017	Disease Ecology

A. Personal Statement

The goal of this research is to improve our understanding of why Nipah virus outbreaks seem to be restricted to a specific region in western Bangladesh despite the host, virus, and route of transmission being present in eastern Bangladesh. This project builds on more than 15 years of NIH-funded Nipah virus research conducted by myself and our organization in Malaysia and Bangladesh. This work will involve field surveillance of bats and people, serological, molecular, and virological testing to detect and characterize Nipah virus strains in bats, and BSL-4 animal model experiments at the NIH Rocky Mountain labs to identify phenotypic differences among NiV strains. I am well positioned to lead this work, having spent 15+ years conducting epidemiological studies of bat-origin viruses and having spent the last 10 years managing large-scale multidisciplinary surveillance in wildlife and humans in Bangladesh and four other countries for viral zoonoses, including henipaviruses under the USAID funded EPT RPEDICT project. I have trained teams and led fieldwork in Malaysia, India, Bangladesh, Saudi Arabia, Cambodia, The Philippines, China, and Liberia and conducted ecological and epidemiological studies of zoonotic viruses in bats, including Nipah, Hendra, MERS-CoV, SARS-CoV and Ebola virus. My work has led to the discovery of bats as the reservoir for SARS CoV, the identification of MERS-CoV in bats and camels in Saudi Arabia, the identification of Reston ebolavirus in bats in The Philippines and the detection of Zaire ebolavirus for the first time in bats in West Africa (Liberia). I have also conducted and coordinated data analyses from field and lab activities including identifying novel viral agents (the first bat pegivirus), and confirming that *Pteropus* fruit bats are reservoirs for henipaviruses. I spent five years working as a visiting research fellow at Columbia University's Center for Infection and Immunity conducting Nipah virus and pathogen discovery research, including molecular and serology techniques, bioinformatics, phylogenetic analyses, all of which has given me a strong understanding of the challenges of conducting viral surveillance in wildlife and the constraints of molecular and serological diagnostics. I have extensively collaborated with each of the co-investigators and key personnel on this proposal and continue to collaborate under currently active projects. Under this proposal, we bring strong partnerships and preliminary data from prior NIH R01 and K08 awards.

B. Positions and Honors

Positions and Employment

1999 Intern, Brisbane South Public Health Unit & DPI Queensland Animal Research Institute, AUS.
2002 Extern, Division of Viral and Rickettsial Diseases, CDC, Atlanta, GA
2003-09 Senior Research Scientist, EcoHealth Alliance, New York, NY.
2003 - Adjunct Clinical Associate, Tufts University Cummings School of Vet. Med., MA.
2006-10 Research Fellow, Center for Infection and Immunity, Columbia University, NY,
2009-16 Associate Vice President, Conservation Medicine Program EcoHealth Alliance, NY
Executive Director, Consortium for Conservation Medicine, EcoHealth Alliance, NY
2016- Adjunct Associate Professor, Columbia University Mailman School of Public Health, NY
2016 - Vice President for Science and Outreach**,** EcoHealth Alliance

Other Experience and Professional Memberships

2003 - Member, IUCN Veterinary / Wildlife Health Specialist Group
2004 Invited speaker, *WHO, Emerging Zoonotic Diseases Working Group* meeting
2006 Member, Delta Omega Public Health Honors Society
2010- Invited speaker, Institute of Medicine Forum on Microbial Threats, National Academies of Science
2011- Admissions Committee, Tufts University Masters in Conservation Medicine degree program
2012- Scientific Advisory Board, Lubee Bat Conservancy
2013- Science Advisor: Center for Health and the Global Environment, Harvard University
2015 - Editorial Board, O*ne Health* journal;
2016 Subject Matter Expert for USDA APHIS National Wildlife Research Center personnel review
2017 - Chief Science Advisor to Smithsonian National Museum of Natural History "Outbreak" exhibit (through 2021);
Board of Advisors, Tufts University Cummings School of Veterinary Medicine
2018 - WHO Blueprint R&D Nipah virus SME; Scientific review committee for IMED 2018
2019 - Global Health SME for Aspen Institute Congressional Delegation: Rwanda;
WHO SEARO Nipah virus prevention strategy development for South Asia (invited expert)

Honors

2002 Certificate of International Veterinary Medicine, Tufts University Sch. Vet. Med. (1st recipient)
2002 Hills award for excellence in veterinary clinical nutrition
2002 Sylvia Mainzer Award for Outstanding Achievement in Public Health
2006 Inductee & Keynote speaker, Delta Omega Honor Society for Public Health (Alpha Rho Chapter)
2007 Outstanding Alumnus award, Tufts Cummings School of Veterinary Medicine
2012 Young Alumni Achievement Award, Tufts University
2013 Named one of the ten "Distinguished Alumni" by Tufts University School of Medicine
2014 Commencement speaker**,** Tufts University Medical School Graduate Programs in Public Health

C. Contribution to Science

1. Research on the epidemiology of emerging zoonotic viruses, including Nipah virus. A range high impact emerging viruses appear to have bat origins (e.g. SARS-CoV, Ebola viruses, NIV, HeV, MERS-CoV). My research has focused on identifying bat reservoirs and the epidemiology of Nipah and Hendra virus, Ebola Reston virus, SARS-CoV, and MERS-CoV. I have trained field teams in Asia and Africa to conduct surveillance for zoonotic viruses in bats and other wildlife, as well as humans and livestock. In Malaysia, my work helped identify the agricultural drivers of NiV emergence. In Bangladesh, I helped characterize NiV infection dynamics and the ecology of its bat reservoir including patterns of habitat use as they relate to spillover to humans and livestock. In China, I led initial fieldwork that identified Horseshoe bats as the natural reservoir for SARS-like CoVs and collaborated on the identification of SL-CoVs in bats that

that use the same receptor as SARS-CoV. Under the USAID-funded Emerging Pandemic Threats: PREDICT program, I currently manage field and laboratory teams in Liberia, Malaysia, India, and Bangladesh that conduct human and wildlife surveillance for novel viruses as well as important viral zonooses such as Nipah virus, MERS-CoV, Ebola, and HPAI. We recently identified Zaire ebolavirus in a bat in Liberia, the first detection of Ebola in a bat in the region. This work has been instrumental in advancing our understanding of viral dynamics and diversity in wildlife reservoirs and how environmental change and human behavioral factors drive zoonotic virus emergence.

a. **Epstein, J.H.***, Prakash, V., Smith, CS., Daszak, P., McLaughlin, AB., Meehan, G., Field, HE., and Cunningham, AA. Evidence for Henipavirus infection in Indian *Pteropus giganteus* (Chiroptera; Pteropodidae) fruit bats 2008. ***Emer. Infect. Dis****. 20(8).* *Corr. author

b. Li, W., Shi, Z., Yu, M., Ren, W., Smith, C., **Epstein, J.H.**, Wang, H., Crameri, G., Hu, Z., Zhang, H., Zhang, J., McEachern, J., Field, H., Daszak, P., Eaton, B.T., Zhang, S. & Wang, L-F. (2005). Bats are natural reservoirs of SARS-like coronaviruses. ***Science*** 310: 676-679.

c. Ge X-Y, Li J-L, Yang X-L, Chmura AA, Zhu G, **Epstein JH**, Mazet JK, Hu B, Zhang W, Peng C, Zhang Y-J, Luo C-M, Tan B, Wang N, Zhu Y, Crameri G, Zhang S-Y, Wang L-F, Daszak P, Shi Z-L (2013). Isolation and characterization of a bat SARS-like Coronavirus that uses the ACE2 receptor. ***Nature*** 503: 535-538.

d. Ariful Islam, **Jonathan H Epstein***, Melinda K. Rostal, Shariful Islam, Md Ziaur Rahman, Md Enayet Hossain, M. Salim Uzzaman, Vincent J. Munster, Malik Peiris, Meerjady Sabrina Flora, Mahmudur Rahman, and Peter Daszak. Middle East Respiratory Syndrome Coronavirus (MERS-CoV) antibodies in dromedary camels (*Camelus dromedarius*), Bangladesh. ***Emer Infect. Dis***. 2018

2. **Identification of novel pathogens in bats and other wildlife with zoonotic potential.** My work on the ecology of viruses in bats led to my discovery of a novel flavivirus (GB virus) in the Bangladesh fruit bat *Pteropus medius*, related to the hepatitis C virus, and now classified as a *Pegivirus*. An additional 55 novel viruses, some related to Nipah, and serological evidence of filoviruses have been identified in these samples, demonstrating the importance of livestock surveillance in rural South Asia to discover potential zoonoses. Under the USAID PREDICT project, I lead work in Liberia, Bangladesh, India, and Malaysia. As a collaborative group, we have discovered more than 1200 novel viruses in 30 countries. I have as a senior technical lead since the project inception in 2009 designing SOPs for wildlife surveillance, and training dozens of scientists and government officials from wildlife and agricultural ministries in more than 30 countries, leading.

a. **Epstein, J.H.***, Quan, P.L., Briese, T., Street, C., Jabado, O., Conlan, S., Khan, S.A., Verdugo, D., Hossain, M.J., Hutchison, S.K., Egholm, M., Luby, S.P., Daszak, P., and Lipkin, W.I. (2010). Identification of GBV-D, a Novel GB-like Flavivirus from Old World Frugivorous Bats (*Pteropus giganteus*) in Bangladesh. ***PLoS Pathogens*** 6(7): e1000972. doi:10.1371/journal.ppat.1000972.

b. Quan, Phenix-Lan, Firth, C, Conte, J M, Williams, S.H., Zambrana-Torrelio, C.M., Anthony, S.J., Ellison, James A., Gilbert, A.T., Kuzmin, I.V., Niezgoda, M., Osinubi, M.O. V., Recuenco, S., Markotter, W., Breiman, R.F., Kalemba, L., Malekani, J., Lindblade, K.A., Rostal, M.K., Ojeda-Flores, R., Suzan, G., D., Lora B., Blau, D.M., Ogunkoya, A.B., Alvarez C., Danilo A., Moran, D., Ngam, S., Akaibe, D., Agwanda, B., Briese, T., **Epstein, J.H.,** Daszak, P., Rupprecht, C.E., Holmes, E.C., and Lipkin, W.I. Bats are a major natural reservoir for hepaciviruses and pegiviruses. ***PNAS*** **doi:10.1073/pnas.1303037110**

c. Anthony SJ, **Epstein JH**, Murray KA, Navarrete-Macias I, Zambrana-Torrelio CM, Solovyov A, Ojeda-Flores R, Arrigo NC, Islam A, Ali Khan S, Hosseini P, Bogich TL, Olival KJ, Sanchez-Leon MD, Karesh WB, Goldstein T, Luby SP, Morse SS, Mazet JAK, Daszak P, Lipkin WI. 2013. A strategy to estimate unknown viral diversity in mammals. ***mBio*** 4(5):e00598-13. doi:10.1128/mBio.00598-13.

d. Olival KJ, Islam A, Yu M, Anthony SJ, **Epstein JH**, Khan SA, et al. Ebola virus antibodies in fruit bats, Bangladesh. ***Emerg Infect Dis.*** 2013 Feb. http://dx.doi.org/10.3201/eid1902.120524

3. Studies of bat ecology and immunology to understand emerging zoonoses. Over the last few years, I have worked to integrate wildlife ecology and immunology into viral epidemiological studies. I have helped

pioneer the use of satellite telemetry and mark-recapture studies on bat Nipah virus reservoirs to help define geographic range of these viruses. My work has helped show that in Malaysia, Nipah virus bat reservoirs have long-range, international migratory movements that explain how viral maintenance at low incidence and drivers of viral diversity. I have integrated immunological experiments into Nipah virus studies in an effort to generate actual data about antibody persistence in bats, which has informed mechanistic models of viral dynamics. This work has enhanced our ability to understand how Nipah virus circulates in bat populations and how cohorts of juveniles contribute to viral outbreaks within bats.

a. **Epstein J.H.***, Olival KJ, Pulliam JRC, Smith C, Westrum J, Hughes T, et al. *Pteropus vampyrus*, a hunted migratory species with a multinational home-range and a need for regional management. ***J. App. Ecol***. 2009 Oct;46(5):991-1002
b. Hahn, M. B., **Epstein, J. H.***, Gurley, E. S., Islam, M. S., Luby, S. P., Daszak, P. & Patz, J. A. Roosting behaviour and habitat selection of *Pteropus giganteus* reveals potential links to Nipah virus epidemiology. ***J. App. Ecol***. doi:10.1111/1365-2664.12212 (2013).
c. **Epstein JH,** Baker ML, Zambrana-Torrelio C, Middleton D, Barr JA, et al. (2013) Duration of Maternal Antibodies against Canine Distemper Virus and Hendra Virus in Pteropid Bats. ***PLoS ONE*** 8(6): e67584. doi:10.1371/journal.pone.0067584
d. Mandl Judith N, Ahmed R, Barreiro Luis B, Daszak P, **Epstein JH**, Virgin Herbert W, et al. Reservoir Host Immune Responses to Emerging Zoonotic Viruses. (2015) ***Cell.*** 160(1):20-35. 10.1016/j.cell.2014.12.003
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From:	Madeline Salino on behalf of Madeline Salino <salino@ecohealthalliance.org>
To:	Cadhla Firth; Jon Epstein; Maryska Kaczmarek; Ariful Islam; Dr. Mohammed Ziaur Rahman; Thomas Hughes; Munster, Vincent (NIH/NIAID) [E]; De wit, Emmie (NIH/NIAID) [E]; jessica.manning@nih.gov; Amita Jain; rajesh bhatia; Broder, Christopher
Cc:	tahmina.shirin14@gmail.com
Subject:	CEPI Biosketches
Date:	Friday, September 17, 2021 4:15:10 PM
Attachments:	Epstein NIH Biosketch Example.docx

Hello, all!

I am hoping that everyone can create or update a NIH-formatted biosketch and forward it to me for inclusion in our CEPI proposal.

A recent version of Jon's biosketch is attached as an example. Section A, the personal statement, should be adjusted to be relevant to the CEPI proposal and your Nipah virus work. We've also removed Section D (funding/financial information).

For those whose biosketches we already have, from previous recent proposals, I will follow up separately in response to this email and ask that you update your biosketch as necessary. (Arif, Zia, Prof. Tahmina, Chris, and Vincent.)

Please let me know if you have any questions, and have a great weekend!
Madeline
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ANNUAL REVIEWS

Annual Review of Virology

Vaccines to Emerging Viruses: Nipah and Hendra

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Keywords

henipavirus, Hendra virus, Nipah virus, vaccine, subunit vaccine, henipavirus countermeasures

Abstract

Hendra virus (HeV) and Nipah virus (NiV) are bat-borne zoonotic paramyxoviruses identified in the mid- to late 1990s in outbreaks of severe disease in livestock and people in Australia and Malaysia, respectively. HeV repeatedly re-emerges in Australia while NiV continues to cause outbreaks in South Asia (Bangladesh and India), and these viruses have remained transboundary threats. In people and several mammalian species, HeV and NiV infections present as a severe systemic and often fatal neurologic and/or respiratory disease. NiV stands out as a potential pandemic threat because of its associated high case-fatality rates and capacity for human-to-human transmission. The development of effective vaccines, suitable for people and livestock, against HeV and NiV has been a research focus. Here, we review the progress made in NiV and HeV vaccine development, with an emphasis on those approaches that have been tested in established animal challenge models of NiV and HeV infection and disease.

INTRODUCTION

Nipah virus (NiV) and Hendra virus (HeV) are bat-borne viral zoonoses that were discovered in the mid- to late 1990s in outbreaks of severe disease in livestock and people in Australia (HeV) and Malaysia [NiV-Malaysia (NiV-M)] (1). They are the prototype members of the genus *Henipavirus* in the family *Paramyxoviridae* (2). NiV outbreaks have also been recorded in Bangladesh and India by a closely related strain, NiV-Bangladesh (NiV-B) (3). Three other henipaviruses are also recognized: Cedar virus (CedV) as an isolate and Ghana virus (GhV) and Mojiang virus (MojV) known only from sequence data (4–7). Both NiV and HeV are highly pathogenic in a broad range of mammalian hosts that are capable of infecting and causing severe disease in humans, monkeys, pigs, horses, cats, dogs, ferrets, hamsters, and guinea pigs and that span six mammalian orders including bats, although bats do not exhibit disease when infected (8–21). In contrast, CedV is nonpathogenic in well-characterized models of HeV and NiV disease including ferrets and hamsters (4, 22). The pathogenic potential of GhV and MojV is unknown.

Several species of *Pteropus* fruit bats are the natural reservoir hosts of NiV, HeV, and CedV (4, 23–27). NiV- or HeV-mediated disease has not been reported in wild or experimentally infected bats (13, 28–30). NiV and HeV infections in people and many animals manifest as severe systemic and often fatal neurologic and/or respiratory diseases (31–33). Both NiV and HeV are regarded as transboundary biological threats to both human and animal health and are classified as biosafety level 4 (BSL-4) select agents (34, 35). NiV and henipaviral diseases are included in the World Health Organization (WHO) R&D Blueprint list of priority pathogens with epidemic potential that need research attention (36). This review summarizes the important characteristics of the NiV and HeV pathogens, the modes of virus transmission, and the immunization strategies being developed against them.

Emergence and Outbreaks of Hendra and Nipah Viruses

In 1994 in the Brisbane suburb of Hendra, Australia, an outbreak of severe respiratory disease resulted in the deaths of 14 horses and their trainer, along with the nonfatal infection of 7 other horses and 1 other person. This led to the discovery of a novel paramyxovirus initially termed equine morbillivirus, now known as HeV (37–39). The first known cases of HeV in horses and a human actually occurred a few months prior, where one person became ill after assisting in the necropsies of two horses later shown to have died from HeV (40, 41). This individual experienced a relapsed fatal encephalitis caused by HeV 13 months later (42). HeV has since re-emerged in Australia 62 times with a total of 104 horse deaths (fatal or euthanized), along with 4 human fatalities of 7 cases (43). Every recorded occurrence of HeV in Australia has involved horses, all resulting in a severe or fatal disease, and all cases of human infection were acquired from virus-shedding horses (31, 44).

In 1998, an outbreak of encephalitis among pig farmers in Peninsular Malaysia occurred and a virus was isolated from samples of cerebrospinal fluid (CSF) of two patients who had died; cells infected with this virus cross-reacted with antibodies against HeV (45). Genetic studies revealed a new paramyxovirus that was closely related to HeV, and it was named Nipah after the village in Malaysia where one of the patients had lived (45). There were 265 cases of human infection with 105 fatalities in Malaysia and 11 cases and 1 fatality among abattoir workers in Singapore (46, 47). This outbreak was controlled through the culling of more than 1 million pigs, resulting in significant economic impacts to the region (48, 49).

A genetically similar but distinct strain of NiV was identified as the causative agent of fatal encephalitis in people in Bangladesh (NiV-B) (3, 50). Since 2001, nearly annual occurrences of human NiV-B infections have occurred in Bangladesh, and there have been three outbreaks in

India (51–54). The recent 2018 NiV outbreak in Kerala, India, was significant, having occurred in a new geographic region far from locations in Bangladesh and India where all prior outbreaks had occurred and with a case fatality rate of 91% (51). In 2014, an outbreak of NiV-M encephalitis occurred in the Philippines with 9 fatalities of 11 human cases of acute encephalitis and influenza-like illness or meningitis in another 6 individuals (55). Altogether, there have been over 650 cases of human NiV infection (combined ~60% fatality rate) in South Asia and Southeast Asia in five countries (54, 56).

Transmission of Hendra and Nipah Viruses

The routes of transmission of virus infection to humans from animals are different for HeV and NiV, with horses the only spillover host of HeV in Australia, while for NiV it was pigs in Malaysia and horses in the Philippines (**Figure 1**). However, human NiV infections in Bangladesh, India, and the Philippines also include bat-to-human and human-to-human transmission (57–60). Transmission routes of HeV and NiV to animals are likely urine from infected bats contaminating pastures or pigsties and/or virus-contaminated fruit spat from bats that is ingested (61, 62) (**Figure 1**). Recoverable virus is shed in the urine of experimentally infected bats and can also be detected in throat and rectal swabs (13, 28–30). Pooled urine samples from flying foxes are also routinely used to detect and isolate henipaviruses (4, 13, 23, 27, 63–65).

It was previously suggested that infected horses could transmit HeV to people during the feeding of ill animals (38). Also, the majority of all HeV-infected horse cases have involved a single animal, suggesting that HeV is not readily transmitted between horses, and multiple horse outbreaks are likely via contamination of fomites (43, 66). The transmission risk of HeV from infected horses to humans appears to be virus-contaminated fluids or tissues during examination procedures and/or the necropsy of horses (31, 67) (**Figure 1**). Indeed, all cases of human HeV infection have been associated with postmortem examination of horses or close contact with ill horses (31, 38, 42, 68). In Malaysia, it was contact with infected pigs or fresh infected pig products that was required for transmission of virus to humans (45, 69, 70) (**Figure 1**). NiV shedding in respiratory fluids of infected pigs suggested that it probably spread among farmed animals by aerosol droplets or direct contact (16, 71, 72). In Bangladesh, the transmission of NiV from bats to people has been linked to the consumption of virus-contaminated fresh date palm sap, and bats will consume sap during its collection (57, 73, 74). Domestic animals have also been linked to NiV infection in people in Bangladesh from unwell animals (cows and goats) and pigs (50, 59). Human-to-human transmission of NiV has been well documented in Bangladesh and India (52, 58–60, 75) (**Figure 1**). A study of human NiV-B cases in Bangladesh spanning 14 years reported that of 248 cases studied, one-third were caused by human-to-human transmission (56). Human-to-human transmission of NiV-M was not apparent in Malaysia (76, 77), whereas in the Philippines' NiV-M outbreak, human cases were linked to horse slaughtering and horse meat consumption or exposure to other human patients, indicating both horse-to-human and human-to-human transmission (55) (**Figure 1**). The NiV-B outbreak in Kerala had a very high rate of human-to-human transmission (22 of 23 cases) at three different hospital locations (51).

Naturally acquired NiV infections were also recorded in cats, dogs, and horses in the initial Malaysian outbreak (**Figure 1**), and serological evidence of natural NiV infection in dogs was linked to outbreak farms (11, 61, 78). In the Philippines, both dogs and cats were linked to NiV-M infection, with cats dying after eating horse meat and dogs having NiV-neutralizing antibodies (55) (**Figure 1**). In Australia, a dog was found to be seropositive for HeV and later euthanized but showed no signs of disease, and a second HeV-positive dog was identified in 2013 following exposure to blood from an infected horse (79) (**Figure 1**). Dogs are susceptible to experimental HeV infection and shed virus but show little evidence of clinical illness (80).

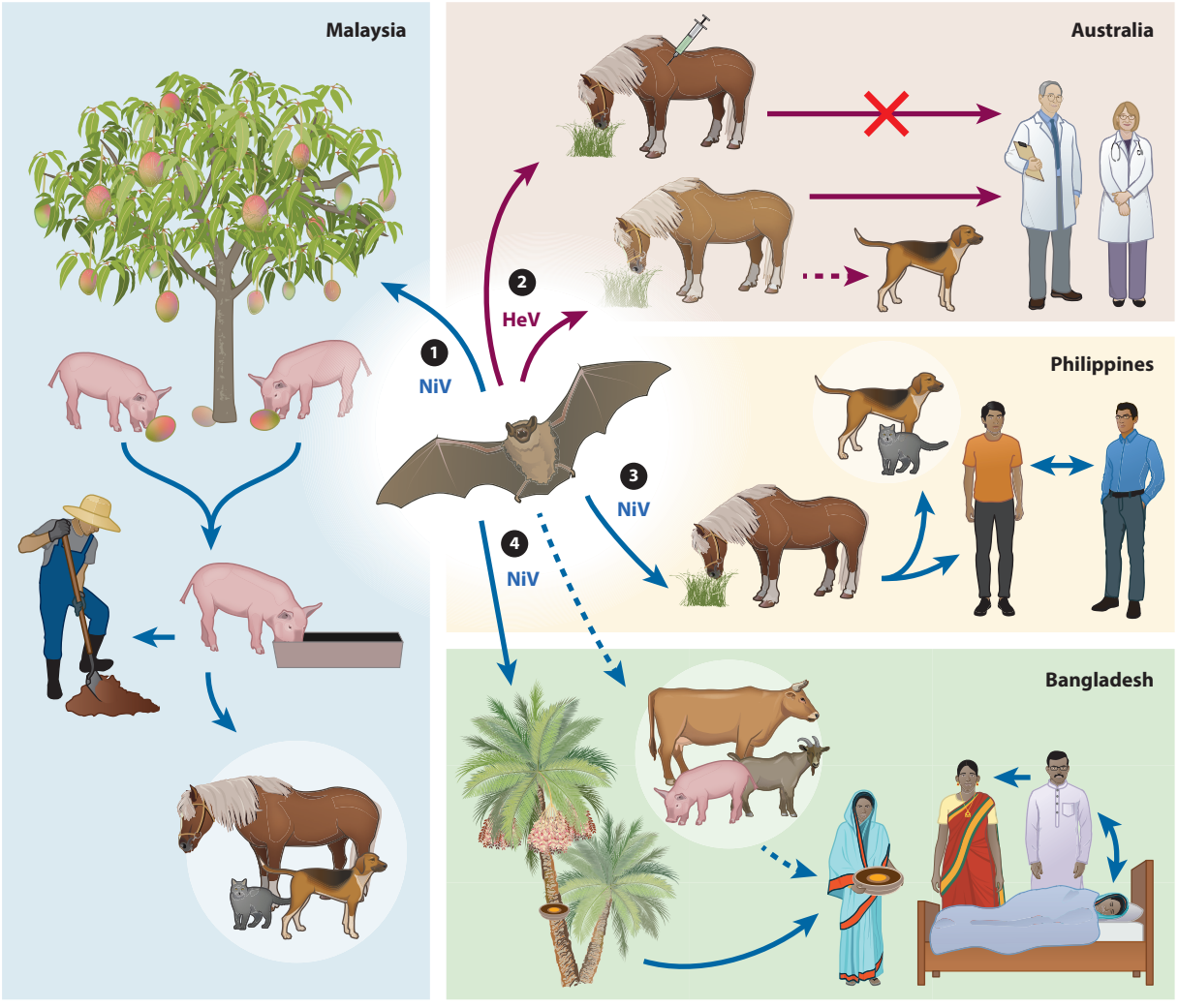

Figure 1

Nipah virus (NiV) and Hendra virus (HeV) modes of transmission in different countries. The transmission routes of NiV in Malaysia (*left*), Philippines (*middle right*), Bangladesh (*bottom right*), and HeV (*top right*) are depicted. Solid lines represent transmission that has been observed and documented, and dashed lines represent suspected transmission in natural conditions. Fruit bats are the natural reservoirs of NiV and HeV. (①) Pigs are infected by consuming partially eaten or contaminated fruit from infected bats (urine, saliva) and transmit NiV to other pigs, pig farmers, or other animals (dogs, cats, and horses) through close or direct contact. (②) Horses can be infected from grazing in contaminated pastures and transmit HeV to humans and on occasion domestic dogs through close contact. A One Health vaccine approach was developed for vaccination of horses in Australia with the dual purpose of saving horses from lethal HeV infection and preventing HeV transmission from horses to humans. (③) NiV is transmitted to humans through close contact with infected horses. NiV transmission to humans, cats, and dogs appears to have occurred following close contact with or consumption of infected horse meat. Human-to-human NiV transmission can occur through close contact. (④) Bat-to-human NiV transmission occurs through consumption of contaminated date palm sap. Human-to-human transmission can occur through close contact with infected patients. Humans may also become infected through contact with infected animals. Figure adapted with permission from Reference 171.

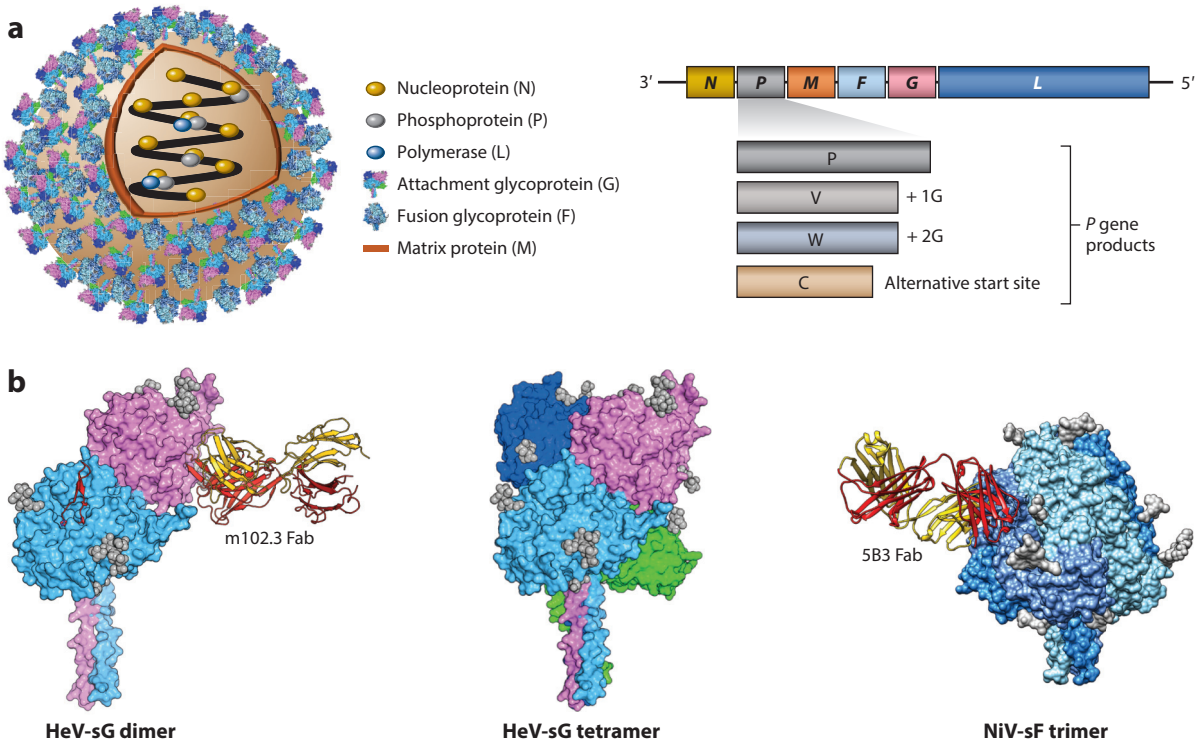

Figure 2

Henipavirus structure and genome organization and models of the G and F glycoprotein soluble ectodomains, Hendra virus (HeV-sG) and Nipah virus (NiV-sF), respectively, and their complexes with respective NiV and HeV cross-reactive neutralizing monoclonal antibodies m102.3 (anti-G) and 5B3 (anti-F). (*a*) Schematic representation of a henipavirus particle with the structural proteins depicted in different colors (*left*) and the henipavirus genome (*right*). HeV and NiV *P* genes encode 3 nonstructural proteins: The C protein is expressed from an alternative start site, and the V and W proteins are expressed following the addition of one or two G residues at the messenger RNA editing site, respectively (*right*). (*b*, *left*) HeV-sG shown as a dimer solvent-accessible surface view with one monomer (*cyan*) overlaid with the monoclonal antibody m102.3 CDR-H3 loop (*red*) at the receptor binding site, and the other monomer (*magenta*) in complex with m102.3 Fab, which has an identical heavy chain and a similar light chain, that was used in place of the m102.4 monoclonal antibody (mAb) in the structural solution of the complex (109). The HeV-sG consists of amino acids 76–604, and the structures of the two globular head domains of HeV-sG are derived from the crystal structure (103, 172). The stalk regions of each G monomer (residues 77–136) are modeled (173). The light chain of m102.3 Fab is colored in yellow, and the heavy chain is colored in red. (*b*, *middle*) The HeV-sG tetramer surface view is modeled with one dimer (*cyan* and *magenta*) in front and the other dimer (*blue* and *green*) in back. *N*-linked glycans are gray spheres. (*b*, *right*) Structural model of the NiV-sF trimer in complex with the 5B3 Fab derived from the cryo–electron microscopy structure (110). The NiV-sF consists of amino acid residues 1–494 with a FLAG tag (DYKDDDK) introduced between residues L104-V105 and a C-terminal GCN4 motif. Each monomer of NiV-sF is in a different shade of blue, 5B3 heavy chain is in red, and light chain is in gold. *N*-linked glycans are illustrated in gray.

Entry and Tropism of Nipah and Hendra Viruses

NiV and HeV are enveloped viruses containing an unsegmented, single-stranded, negative-sense RNA genome (2). **Figure 2*a*** is an illustration of the viral particle and the associated viral proteins. The genomes of HeV and NiV, and also CedV, GhV, and MojV, are considerably longer than the genomes of other paramyxoviruses, at greater than 18 kb. Henipavirus genomes encode 6 structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion glycoprotein (F), attachment glycoprotein (G), and the polymerase protein (L) (**Figure 2*a***). The N, P, and L proteins comprise the replication complex. The *P* gene undergoes RNA editing

to produce 2 additional nonstructural proteins, V and W, that are interferon (IFN) antagonists (81–84). The C protein is transcribed from a second open reading frame in the *P* gene (**Figure 2*a***). NiV has been central to understanding the V, W, P, and C protein roles in antagonizing the innate immune responses via a diverse set of mechanisms (85, 86). Recent in vivo studies with recombinant NiV variants have further defined the varying importance of these nonstructural proteins in pathogenesis, but only a lack of the V protein results in a nonlethal infection (87–89).

The henipavirus virion bears surface projections composed of the F and G glycoproteins that are anchored in the viral membrane and together mediate infection of host cells, and they are the major antigens of vaccine strategies (1) (**Figure 2*a***). The F glycoprotein facilitates membrane fusion between the virus and host cell. The G glycoprotein consists of a characteristic stalk with a globular head that engages entry receptors on host cells, leading to the fusion activation of F and virus infection. The native structure of G is a tetramer while F is a trimer, and together they are the key determinants of infection and tropism (90–92). Models of the soluble ectodomain of the HeV G (HeV-sG) as a dimer and tetramer and the soluble ectodomain of the NiV F (NiV-sF) as a trimer are shown in **Figure 2*b***. NiV and HeV utilize the host cell proteins ephrin-B2 and ephrin-B3 for entry (93–96). Ephrin-B2 and ephrin-B3 are members of a large family of ligands that bind to Eph receptors and are highly sequence conserved among mammals (97, 98). Ephrin-B2 expression is prominent in the vasculature of multiple organs, whereas ephrin-B3 is found predominantly in the nervous system (99–101). The ability of HeV and NiV to use these ephrins as receptors provided explanations of their broad host and tissue tropism (32, 33, 102). The NiV and HeV G head domain structures alone and in complex with ephrin-B2 and ephrin-B3 receptors have been determined (103–106). The structures of both the NiV and HeV F in their prefusion conformation have also been determined (107, 108). These studies have provided insights into understanding the virus entry receptors and host tropism features of the viruses on the molecular level and also facilitated further structural studies of henipavirus G and F glycoproteins in complexes with specific virus-neutralizing antibodies, providing valuable information that has aided vaccine design and choice (109, 110).

Nipah Virus and Hendra Virus Infection in Humans and Animals

Human NiV and HeV infections are generally accepted to occur via the oronasal route, and the incubation periods for both have been estimated to be 1 to 2 weeks (31, 51, 111). Acute infection in people is a systemic infection likely via hematogenous spread of the virus from the respiratory system (112). In general, HeV and NiV disease onset is characterized by fever, myalgia, shortness of breath, and cough (38, 111). Human HeV infections have resulted in both fatal respiratory or encephalitic disease and also recovery from infection (31, 38, 42, 68). The predominant clinical feature in the NiV-M outbreak in Malaysia was encephalitis, but respiratory symptoms were also common with fever, cough, and headache (47, 111, 112). The clinical presentation of NiV-B infections in Bangladesh also includes severe respiratory disease. In the 2018 NiV-B outbreak in Kerala, 83% of cases presented with acute respiratory distress syndrome (ARDS) (51, 113). Central findings of human NiV and HeV infection are a widespread endothelial cell tropism and systemic vasculitis, with prominent parenchymal cell infection in most major organs with the brain and lung significantly affected (45, 112, 114). Human NiV and HeV infections can also take a protracted course following apparent recovery, and some patients can experience late-onset encephalitis or relapsed encephalitis can occur in patients who previously recovered (42, 115). Relapsed encephalitis caused by NiV appears to result from a recrudescence of virus replication in the central nervous system (CNS), with cases presenting from a few months to as long as 11 years later (116–118). Recrudescence of virus has important implications for vaccine development.

The development of animal models of NiV and HeV infection and pathogenesis has been a major focus since the late 1990s and an essential component of vaccine development and testing. Also, the approval process of countermeasures for NiV and HeV would fall under the animal rule requirement set forth by the US Food and Drug Administration (FDA) in 2002 as an alternative licensing pathway for countermeasures against highly pathogenic agents when human efficacy studies are not feasible or ethical (119, 120). Several animal models of NiV and HeV infection have emerged that well reflect the pathogenesis seen in infected people, which includes a systemic vasculitis with both respiratory and neurological diseases. Detailed reviews of NiV and HeV infections of a variety of mammalian species have recently been published (33, 121–123). It is generally accepted that the pathogenic processes of NiV and HeV infection in the hamster, ferret, and African green monkey (AGM) best reflect the pathogenesis observed in humans, whereas the most appropriate models for livestock are the horse and pig themselves.

VACCINATION

The attachment and fusion glycoproteins of paramyxoviruses such as measles, mumps, and parainfluenza viruses are the viral antigens to which virtually all neutralizing antibodies are directed (124–126). Likewise, immunization strategies for NiV and HeV have largely targeted their G and F glycoproteins.

Passive Immunization Strategies

Early passive immunization studies in the hamster model demonstrated that polyclonal antiserums or mouse monoclonal antibodies (mAbs) to NiV F or G could provide complete protection against NiV-M or HeV when administered before and immediately after virus infection (10, 127, 128). These studies demonstrated a major role of a viral glycoprotein-specific antibody in protection.

Recombinant human antibody technology was used to generate a potent cross-neutralizing mAb against NiV and HeV (m102.4) (129, 130). The m102.4 mAb epitope maps to the ephrin receptor binding site of G and blocks virus infection (see the left side of **Figure 2*b***), and it can neutralize NiV-M, NiV-B, and HeV (8, 109). The m102.4 mAb provided complete protection from NiV-M-mediated disease in ferrets as a single 50 mg dose administered 10 h post-challenge (8). In the AGM model, m102.4 administered as two 20 mg/kg doses, intravenously, at 10 h and again on day 3, on days 1 and 3 (days 1/3), or on days 3/5, after HeV challenge [4×10^5 50% tissue culture infectious dose ($TCID_{50}$)] by intratracheal (i.t.) administration, protected 100% of treated subjects (131). All treated subjects seroconverted against HeV F glycoprotein with a rise in antibody titer over time, indicating all animals had become infected with HeV and recovered, whereas untreated control subjects succumbed to HeV disease and failed to mount a protective immune response. No clinical signs were evident at any time in the early treatment groups; although neurological symptoms were observed in subjects in the late treatment group (days 3/5), all later recovered from infection. There was no HeV antigen or virus-specific histopathology detected in the lung or brain at the conclusion of the study in any treated subject, and infectious virus could not be recovered from any tissue. A similar study evaluated m102.4 against NiV-M disease in the AGM model at several time points following virus challenge (5×10^5 PFU), including a late cohort where treatment was initiated at the onset of clinical illness (day 5) (132). All subjects became infected after challenge, and all subjects that received m102.4 survived infection and all controls succumbed to disease. Subjects in the late day 5/7 treatment group exhibited disease, but all recovered. A comparative study in AGMs using NiV-M and NiV-B [5×10^5 PFU divided by i.t. and intranasal (i.n.) administration] revealed that NiV-B caused a more aggressive disease, with a

shortened time to death and higher virus loads in tissues and fluids (133). When m102.4 was tested in this model, all subjects in the days 1/3 and days 3/5 post-infection treatment groups survived NiV-B challenge, but subjects in the days 5/7 treatment group succumbed, indicating a shorter therapeutic window in treating NiV-B infection (133). Another well-characterized, humanized mouse mAb, 5B3 (h5B3.1), that is cross-reactive to the F glycoprotein of NiV and HeV and binds a prefusion conformation epitope on F, preventing membrane fusion, was recently tested (110, 134) (**Figure 2*b***). The h5B3.1 mAb was given to ferrets in 20 mg/kg doses by intraperitoneal (i.p.) injection, at 1 to several days post-challenge, with either NiV or HeV ($\sim 5 \times 10^3$ PFU) delivered i.n. (135). All subjects that received h5B3.1 after infection were protected from disease and had increasing neutralizing antibody titers, whereas all controls died. No pathology was observed, and no infectious virus could be isolated at the study endpoint. Altogether, these studies demonstrate that passive immunization with mAbs can provide therapeutic benefit and allow the infected host an extended period to mount a protective immune response. The findings from these experiments were also important because they suggest that vaccine approaches designed to induce adequate neutralizing antibody responses to NiV and HeV should be effective.

The m102.4 mAb producing cell line was provided to the Queensland Government, Australia, to produce the mAb for compassionate use in future cases of high-risk human HeV infection. To date, 14 individuals exposed to either HeV in Australia ($n = 13$) or NiV in the United States ($n = 1$) have been given high-dose m102.4 therapy (15–20 mg/kg) by emergency use protocols, and all have remained well. In Australia, m102.4 was used in a randomized, controlled phase I study in healthy adults (136). The study included four single and one repeat dosing groups, and the m102.4 mAb was found to be safe and well tolerated, with a half-life ranging between ~16.5 and 27 days, and no observed immunogenicity was reported. Two doses of 20 mg/kg (days 1/3) were as well tolerated as a single dose. This study's findings will aid in the design of future dosing regimens of mAbs for evaluating their ability to prevent and/or treat HeV and NiV human infections.

Active Immunization Strategies

A variety of immunization strategies have been developed to prevent NiV and HeV infection including several live-recombinant virus vectors, protein subunit, and virus-like particle (VLP) approaches, and all target the virus attachment and entry steps of infection by employing the G and/or F glycoprotein antigens. Here we summarize these various vaccination countermeasure approaches to NiV and HeV infection (**Tables 1** and **2**).

Poxvirus vectored. Poxviruses have a long history as a platform for the expression of heterologous genes to study protein function and serve as vaccine candidates as a live-attenuated viral vaccine platform capable of inducing both cell-mediated and humoral immune responses (137). The F and G glycoproteins of NiV and HeV were functionally characterized using recombinant vaccinia viruses in the early 2000s (138, 139). The first NiV vaccine tested used a highly attenuated vaccinia virus strain (NYVAC) encoding either the F or G glycoproteins from NiV-M (127). Hamsters were vaccinated by subcutaneous (s.c.) injection in a prime-boost strategy with NYVAC-NiV-F or NYVAC-NiV-G, individually and in combination, and then 3 months later challenged i.p. with NiV-M. Vaccination yielded complete protection from NiV-M with no detection of viral RNA, and control subjects succumbed 7–10 days after challenge (127) (**Table 1**). Another poxvirus-based approach was examined as a vaccine for pigs using canarypox (ALVAC) vaccine vectors encoding either NiV-M F or G glycoprotein (140). A prime-boost strategy with ALVAC-NiV-F or ALVAC-NiV-G vectors was tested alone or in combination in pigs. The animals were then challenged 28 days later with NiV-M via i.n. administration. All vaccinated animals survived NiV-M challenge

Table 1 Virus vectored vaccine strategies for NiV and HeV

Approach	Name(s)	Animal model	Vaccination dose, route, schedule	Adjuvant	Challenge	Challenge dose, route, schedule	Survival	Correlate(s) of immunity/ protection	Reference
Poxvirus	NYVAC-NiV-F and/or -G	Hamster	2 doses at 1×10^7 PFU, s.c., 1 month apart	None	NiV-M	1×10^3 PFU, i.p., 3 months later	100%	NAb response, viral RNA	127
	ALVAC-NiV-F and/or -G	Pigs	2 doses at 1×10^8 PFU, i.m., 2 weeks apart	None	NiV-M	2.5×10^5 PFU, i.n., 28 days later	100%	NAb response, viral RNA, infectious virus, viral shedding, cytokine production	140
	ALVAC-HeV-F and/or -G	Hamster	2 doses at 7.4 or 5.4 log10 $CCID_{50}$, s.c., 3 weeks apart	None	HeV	1×10^3 LD_{50}, i.p., 21 days later	89% and 63%	NAb response, viral RNA, viral antigen, viral shedding	141
		Ponies	2 doses at 6 log10 $CCID_{50}$, i.m., 3 weeks apart	None	NT	NA	NA	High NAb titers	
	MVA-NiV-sG and/or MVA-NiV-G	IFNAR−/− mice	1 or 2 doses at 1×10^8 PFU, i.m., 3 weeks apart	None	NT	NA	NA	High serum IgG titers, NiV-G-specific CD8 and CD4 T cells	142

(*Continued*)

Table 1 (*Continued*)

Approach	Name(s)	Animal model	Vaccination dose, route, schedule	Adjuvant	Challenge	Challenge dose, route, schedule	Survival	Correlate(s) of immunity/ protection	Reference
VSV	VSV-NiV-F and/or -G	Mice	5×10^3 PFU, i.n. or i.m.	None	NT	NA	NA	High NAb titers	144
		Hamster	1×10^6 infectious particles, i.m.		NiV-M	1×10^5 $TCID_{50}$, i.p., 32 days later	100%	NAb response, viral RNA, viral antigen	145
	VSV-NiV-B F and/or G	Ferret	1×10^7 PFU, i.m.	None	NiV-M	5×10^3 PFU, i.n., 28 days later	100%	Serum IgG response, viral RNA, viral antigen	146
		AGM			NiV-B	5×10^5 PFU, i.t. and i.n., 28 days later		NAb response, viral RNA, viral antigen	147
	VSV-ZEBOV-GP-NiV F, G, or N	Hamster	1×10^5 PFU, i.p.	None	NiV-M	1×10^3 LD_{50}, i.p., 28 days later	100%	NAb response, viral RNA, infectious virus	148
		AGM	1×10^7 PFU, i.m.			1×10^5 $TCID_{50}$, i.t., 29 days later		NAb response, viral RNA, infectious virus, viral shedding	149
	VSV-HeV-G	Mice	1×10^5 PFU, i.m.	None	NT	NA	NA	Serum IgG, NAb response	150
AAV	AAV8 NiV.G	Mice	2×10^{10} genome particles, i.m. or 1×10^{10} genome particles, i.d.	None	NT	NA	NA	Serum IgG, NAb response	151
		Hamster	6×10^{11} genome particles, i.m.		NiV-M	1×10^4 PFU, i.p., 5 weeks later	100%	Serum IgG, NAb response, viral RNA, viral antigen	
					HeV		50%		

(*Continued*)

Table 1 (*Continued*)

Approach	Name(s)	Animal model	Vaccination dose, route, schedule	Adjuvant	Challenge	Challenge dose, route, schedule	Survival	Correlate(s) of immunity/ protection	Reference
Adenovirus	ChAdOx1 NiV-B G	Hamster	2 doses at 1×10^8 IU, i.m., 28 days apart	None	NiV-B	5.3×10^5 $TCID_{50}$, i.p., 28 days later	100%	NAb response, viral RNA, infectious virus, virus shedding	153
			1×10^8 IU, i.m.		NiV-M	6.8×10^4 $TCID_{50}$, i.p., 28 days later	100%		
					HeV	6×10^3 $TCID_{50}$, i.p., 28 days later	33%		
Measles virus	rMV-Ed-G or rMV-HL-G	Hamster	2 doses at 2×10^4 $TCID_{50}$, i.p., 3 weeks apart	None	NiV-M	1×10^3 $TCID_{50}$, i.p., 1 week later	100%	Serum IgG response	NA
	rMV-Ed-G	AGM	2 doses at 1×10^5 $TCID_{50}$, s.c., 4 weeks apart			1×10^5 $TCID_{50}$, i.p., 1 week later		Serum IgG response, viral RNA	154
Inactivated RABV	RABV-HeV-G	Mice	3 doses at 10 μg, i.m., 2 weeks apart	None	NT	NA	NA	High NAb titers, serum IgG response	150
	RABV-NiV-B G		2 doses at 10 μg, i.m., 4 weeks apart						155
RABV	RABV-NiV-F and/or -G	Mice	$1 \times 10^{6.5}$ FFU, oral	None	NT	NA	NA	Serum IgG, NAb response	156

All NiV glycoprotein vaccines employ the NiV-M strain unless otherwise indicated.

Abbreviations: AAV, adeno-associated virus; AGM, African green monkey; $CCID_{50}$, 50% cell culture infectious dose; ChAdOx1, chimpanzee adenovirus Oxford 1; F, fusion glycoprotein; FFU, focus forming units; G, attachment glycoprotein; HeV, Hendra virus; i.d., intradermal; i.m., intramuscular; i.n., intranasal; i.p., intraperitoneal; i.t., intratracheal; IFNAR, interferon receptor; IgG, immunoglobulin G; IU, infectious unit; LD_{50}, 50% lethal dose; MVA, modified vaccinia virus Ankara; NA, not applicable; NAb, neutralizing antibody; NiV, Nipah virus; NiV-B, Nipah virus Bangladesh; NiV-M, Nipah virus Malaysia; NT, not tested; PFU, plaque forming unit; RABV, rabies virus; rMV-Ed, recombinant measles virus Edmonston; rMV-HL, recombinant measles virus HL; s.c., subcutaneous; sF, F glycoprotein soluble ectodomain; sG, G glycoprotein soluble ectodomain; $TCID_{50}$, 50% tissue culture infectious dose; VSV, vesicular stomatitis virus; ZEBOV-GP, *Zaire ebolavirus* glycoprotein.

Table 2 VLP, subunit, and nucleic acid–based vaccine strategies for NiV and HeV

Approach	Name	Animal model	Immunization dose, route, schedule	Adjuvant	Challenge	Challenge dose, route, schedule	Survival	Immune correlate(s) of survival	Reference
VLPs	VLPs-NiV M/F/G	Mice	2 doses at 1.75, 3.5, 7, or 14 μg and 6 μg, s.c., 2 weeks apart	None	NT	NA	NA	High NAb titers	159
		Hamster	1 dose or 3 doses at 30 μg, i.m., 3 weeks apart	Alhydrogel/MPLA or Alhydrogel/CpG	NiV-M	1.6×10^4 PFU (3-dose trial) or 3.3×10^4 PFU (1-dose trial), i.p., 28 days later	100%	NAb response, viral RNA	160
Subunit vaccines	NiV-sG	Cat	3 doses at 100 μg, s.c., 2 weeks apart	CSIRO triple adjuvant	NiV-M	5×10^2 $TCID_{50}$, s.c., 2 months later	100%	NAb response, viral antigen, viral genome	14
	HeV-sG	Cat	3 doses at 100 μg, s.c., 2 weeks apart	CSIRO triple adjuvant	NiV-M	5×10^2 $TCID_{50}$, s.c., 2 months later	100%	NAb response, viral antigen, viral genome	14
			2 doses at 50, 25, or 5 μg, i.m., 3 weeks apart	CpG/Alhydrogel		5×10^4 $TCID_{50}$, o.n., 2 weeks later	100%	Serum IgG, NAb response, viral RNA, viral shedding, infectious virus	162
		Ferret	2 doses at 100, 20, or 4 μg, i.m., 20 days apart	CpG	HeV	5×10^3 $TCID_{50}$, o.n., 3 weeks later	100%	NAb response, viral RNA, infectious virus	163
				CpG/Alhydrogel	NiV-B	5×10^4 $TCID_{50}$, 20 days later or 12 months later	100%	viral RNA, viral antigen, infectious virus	164

(*Continued*)

Table 2 (*Continued*)

Approach	Name	Animal model	Immunization dose, route, schedule	Adjuvant	Challenge	Challenge dose, route, schedule	Survival	Immune correlate(s) of survival	Reference
		AGM	2 doses at 100, 50, or 10 μg, i.m., 3 weeks apart	CpG/Alhydrogel	NiV-M	1×10^5 $TCID_{50}$, i.t., 3 weeks later	100%	Serum IgG, NAb response, viral RNA, viral antigen, infectious virus	165
			2 doses at 100 μg, i.m., 3 weeks apart	Alhydrogel or CpG/Alhydrogel	HeV	5×10^5 PFU, i.t., 21 days later	100%	NAb response, viral RNA	166
		Horse	2 doses at 100 or 50 μg, i.m., 3 weeks apart	Zoetis	HeV	2×10^6 $TCID_{50}$, o.n., 28 or 194 days later	100%	NAb response, viral RNA, viral antigen, infectious virus	167
		Pig	2 doses of 2 mL preformulation, i.m., 3 weeks apart	Zoetis	HeV	5×10^5 PFU, i.n., 35 days later	Partial	NAb response, viral RNA, infectious virus, viral shedding	168
					NiV-M		0%		
Nucleic acid–based vaccine	HeV-sG mRNA LNP	Hamster	10 or 30 μg, i.m.	None	NiV-M	1×10^5 $TCID_{50}$, i.p., 30 days later	30% or 70%	Serum IgG, NAb response	169

All NiV glycoprotein vaccines employ the NiV-M strain.

Abbreviations: AGM, African green monkey; CSIRO, Commonwealth Scientific and Industrial Research Organisation; F, fusion glycoprotein; G, attachment glycoprotein; HeV, Hendra virus; i.m., intramuscular; i.n., intranasal; i.p., intraperitoneal; i.t., intratracheal; IgG, immunoglobulin G; LNP, lipid nanoparticle; M, matrix protein; MPLA, monophosphoryl lipid A; mRNA, messenger RNA; NA, not applicable; NAb, neutralizing antibody; NiV, Nipah virus; NiV-B, Nipah virus Bangladesh; NiV-M, Nipah virus Malaysia; NT, not tested; o.n., oronasal; PFU, plaque forming unit; s.c., subcutaneous; sG, G glycoprotein soluble ectodomain; $TCID_{50}$, 50% tissue culture infectious dose; VLP, virus-like particle.

as determined by the lack of NiV RNA and infectious virus from nasal washes, pharyngeal swabs, and a variety of sampled organs (140).

ALVAC-vectored vaccines encoding HeV F or G glycoprotein for potential use in horses were also examined (141). ALVAC-HeV-F or ALVAC-HeV-G vectors were combined and first used to vaccinate hamsters at a high or low dose of each vector, by s.c. injection, and then challenged with HeV by i.p. administration. Vaccination did not result in complete protection, with 8 out of 9 subjects in the high-dose group and 5 out of 8 subjects in the low-dose group surviving challenge. No signs of disease were noted, and viral antigen or viral RNA could not be detected in survivors. Nine ponies vaccinated using the same prime-boost regimen were able to develop high cross-neutralizing antibody titers to HeV and NiV-M at day 28 after vaccination. Although ponies were not challenged, most animals yielded titers of at least 1:32 and were considered likely protective (141).

More recently, a modified vaccinia virus Ankara (MVA) vector encoding NiV-M G glycoprotein and a soluble version of G (NiV-sG) were examined in interferon receptor α and β (IFNAR−/−) knockout mice (142) (**Table 1**). IFNAR−/− mice were immunized once with MVA-NiV-G or MVA-NiV-sG or prime-boosted. IFNAR−/− mice developed high serum immunoglobulin G (IgG) titers to NiV-G and also generated NiV-G-specific CD8 and CD4 T cells following vaccination. MVA-NiV-sG vaccination induced rapid and significantly higher amounts of NiV-G epitope-specific CD8 T cells compared with the MVA-NiV-G candidate vaccine, suggesting superior immunogenicity. Together, these immunization studies with poxvirus vectors highlight that both T cell and B cell responses play a role in an adaptive immune response to NiV and HeV. However, detailed studies on the adaptive immune responses in animal experiments with henipaviruses have been limited. Future studies evaluating the role of NiV-specific T cells will be important because two human survivors of NiV-B infection in the 2018 outbreak in Kerala showed marked elevation of activated $CD8^+$ T cells, which coincided with virus clearance (143).

Vesicular stomatitis virus vectored. Recombinant vesicular stomatitis virus (rVSV) vectors as a vaccine platform suitable for single immunization strategies to potentially meet emergency use requirements have been tested by several groups (**Table 1**). A method of using two defective VSVΔG vectors each expressing only the NiV G or F glycoprotein was devised using VSV G glycoprotein complementation that can generate replication-defective VSV vectors that could elicit NiV-neutralizing antibodies (144). Using this technique, researchers tested rVSV vaccines expressing either NiV-M F or G glycoproteins (VSV-ΔG-NiVG, VSV-ΔG-NiVF) in hamsters by intramuscular (i.m.) vaccination (145). Hamsters were then challenged 32 days later with NiV-M by i.p. administration. All vaccinated animals survived lethal infection with no clinical signs of disease. No viral RNA or viral antigen could be detected in the sampled tissues when compared with controls, and there was a lack of an anamnestic immune response in vaccinated subjects following challenge, suggesting the induction of sterilizing immunity.

Another study used rVSV-ΔG vectors expressing NiV-B F or G glycoprotein and also tested them as single-injection vaccinations in NiV-M-challenged ferrets (146). Ferrets were vaccinated i.m. with rVSV-NiV-B F or rVSV-NiV-B G complemented with VSV G or a mix of both vectors, rVSV-NiV-B F/G, that was generated as a complementing pair in the absence of VSV G and then challenged at 28 days with NiV-M by i.n. administration. All vaccinated ferrets were completely protected against NiV-M challenge. Although viral RNA was detected in blood at day 6 post-challenge in 2 of 5 animals in each group, those levels were 100 times lower than in the unvaccinated controls, and by day 21 no viral RNA was detected (146). In a second study, rVSV-NiV-B F and rVSV-NiV-B G were assessed separately and in combination in AGMs (147). Cohorts were

vaccinated with the rVSVs by i.m. injection and challenged 28 days later with NiV-B divided between the i.t. and i.n. routes (147). Complete protection was recorded from NiV-B disease with no gross pathology and no detectable NiV antigen in lung or spleen tissues. Viral RNA was detected in nasal and oral swabs of the vaccinated groups, but no viral RNA could be detected in blood samples.

Replication-competent rVSV-NiV-M F or G vectors, generated by the retention of the envelope glycoprotein from *Zaire ebolavirus* (ZEBOV-GP), which allowed virus stocks to be propagated (rVSV-ZEBOV-GP-NiVF, rVSV-ZEBOV-GP-NiVG, and rVSV-ZEBOV-GP-NiVN), have also been tested (148). These rVSVs were used to immunize hamsters by i.p. administration and were challenged 28 days later with NiV-M. All subjects vaccinated with either the NiV F or G glycoprotein encoding rVSV vectors were completely protected with no clinical disease or pathology, whereas those vaccinated with the NiV N protein were only partially protected (2 of 6 animals) with no clinical signs of disease and the other subjects succumbed to infection. The protective efficacy of the rVSV-ZEBOV-GP-NiVG was also tested in AGMs, where vaccinated subjects were challenged with NiV-M by i.t. administration 29 days later (149). All vaccinated subjects were protected from lethal challenge and showed no signs of clinical disease, no viral RNA was detected in the blood or oral and nasal swabs, and no infectious virus could be recovered. Another study using a rVSV vector expressing a codon-optimized HeV *G* gene together with an inactivated counterpart was evaluated in mice for humoral immune responses only as a comparator to a recombinant rabies virus vaccine encoding HeV G as a HeV vaccine candidate (150). Here, the live rVSV vectors induced greater levels of HeV G-specific antibodies and higher levels of HeV-neutralizing antibodies than did the recombinant rabies virus vectors (see the section titled Rabies Virus Vectored).

Adeno-associated virus and adenovirus vectored. Adeno-associated virus (AAV) vectors as a vaccine platform against infectious diseases, particularly viral pathogens, have been explored. AAV is a small, single-stranded DNA virus in the family *Parvoviridae*. Immunization of hamsters with an AAV vector expressing NiV-M G glycoprotein (AAV8 NiV.G) by i.m. injection demonstrated complete protection from a challenge of NiV-M by i.p. administration, and no signs of clinical disease were recorded (151) (**Table 1**). Neutralizing antibodies to NiV were induced, no viral RNA or viral antigen was detected in any of the sampled tissues, and there was only a moderate anamnestic response observed in a single subject, suggestive of potential sterilizing immunity. However, in a cross-protection study, AAV8 NiV.G protected only 50% of hamsters challenged with HeV.

Chimpanzee adenoviral (ChAd) vectors circumvent issues of the preexisting immunity observed with human adenovirus vectors (152). Adenoviruses are double-stranded DNA viruses in the family *Adenoviridae*. An engineered replication-deficient ChAd vector, Oxford 1 (ChAdOx1), was tested as a NiV/HeV vaccine (153). Here, ChAdOx1 encoding NiV-B (ChAdOx1 NiV-B) G glycoprotein was used to vaccinate hamsters by i.m. injection, either as a single dose or as a prime-boost protocol. Hamsters were challenged by i.p. administration with NiV-B 42 days following the booster or the single vaccination. Neutralizing antibodies were detectable, and all vaccinated hamsters were protected against lethal disease with no lung pathology, suggesting that a single dose of ChAdOx1 NiV-B was sufficient to completely protect against NiV-B. No viral RNA in the lung tissue and no viral shedding in oropharyngeal swabs could be detected, and no infectious virus could be isolated. A second cohort using a single dose of ChAdOx1 NiV-B to vaccinate hamsters was trialed, and these animals were challenged 28 days later with NiV-M or HeV. All vaccinated animals were protected from lethal NiV-M challenge, but 4 out of 6 hamsters succumbed to HeV disease between days 5 and 7 post-challenge. Neither virus shedding in oropharyngeal swabs nor

infectious virus was detected in the lung or brain tissues of NiV-M-challenged vaccinated hamsters. In contrast, infectious virus was detected in the lung tissues of 75% of the HeV-challenged vaccinated animals. The lower cross-protection observation using NiV G vaccination followed by HeV challenge was not unexpected, as it was previously shown that when the G glycoprotein (as a recombinant soluble subunit immunogen) of either HeV or NiV was used to vaccinate cats, both could completely protect against lethal NiV-M challenge, and that the HeV-sG elicited greater heterologous neutralizing antibody responses in comparison to NiV-sG (14).

Measles virus vectored. Recombinant measles virus vectors based on the HL (rMV-HL) and Edmonston (rMV-Ed) measles virus strains have also been explored in which they encoded the NiV-M G glycoprotein (rMV-HL-G and rMV-Ed-G) (154) (**Table 1**). Hamsters were immunized twice by i.p. administration of rMV-HL-G or rMV-Ed-G. All vaccinated animals produced NiV G-specific antibody titers after the booster immunization. Animals were challenged 1 week after the second immunization with NiV-M by i.p. administration. All immunized hamsters exhibited no clinical symptoms and survived challenge. The study was extended to non-human primates (NHPs), where 2 AGMs were immunized twice by s.c. injection with rMV-Ed-G. Subjects were challenged 2 weeks after the second immunization with NiV-M by i.p. administration. Here, immunization completely protected the AGMs with no observed clinical disease and no detectable pathological changes, and no viral RNA could be detected in sampled tissues. Although this was a small study, the safety profile and success of the live-attenuated measles virus vaccine suggests that a recombinant platform encoding the NiV G glycoprotein as a NiV vaccine candidate is promising and should induce a balanced and long-lasting immune response against NiV.

Rabies virus vectored. A rabies virus (RABV) SAD B19 vaccine strain, BNSP333, expressing HeV or NiV G glycoproteins has been evaluated (150, 155). Recombinant BNSP333 encoding either the wild-type or a codon-optimized HeV *G* gene, together with their inactivated counterparts, was used in mice (150) (**Table 1**). Mice were immunized by i.m. injection with a single dose of the RABV-based vectors or with 3 doses of their inactivated versions. The inactivated RABV-based vectors induced higher and more rapid HeV G-specific antibody responses and higher neutralizing antibody titers than their live counterparts. The inactivated RABV-coHeV-G induced cross-neutralizing antibodies against NiV. A similar study used the BNSP333 vector expressing NiV-B G glycoprotein (RABV-NiV-BG) (**Table 1**) and elicited NiV G-specific neutralizing antibodies (155).

Recently, the recombinant RABV Evelyn-Rokitnicki-Abelseth (ERA) strain ($rERAG_{333E}$) expressing either NiV-M F or G glycoproteins was evaluated in mice and pigs (156) (**Table 1**). This vector, $rERAG_{333E}$, serves as an oral vaccine in dogs. Here, mice were orally immunized with RABV-NiV-F or RABV-NiV-G either individually or in combination. Pigs were also immunized in a similar manner but with 2 doses of each vector either alone or in combination. RABV-NiV-F and/or RABV-NiV-G immunization induced NiV F- and G-specific IgG antibody responses and neutralizing antibodies in both mice and pigs with the combination vaccine inducing higher titers. Although not suitable for human use, the live-attenuated $rERAG_{333E}$ vector is of interest as a potential veterinary vaccine for NiV because it is already approved for use in some animals and could be adapted for emergency use to protect against NiV infection in livestock, particularly swine.

Many of these virus-vectored vaccines for NiV are promising candidates because of their established safety profiles and ease of genetic modification. Several of these virus-vectored vaccines also require no adjuvants, and some are clearly efficacious as a single immunization strategy, suitable features for emergency use circumstances. In addition, several of these platforms are able to induce both cell-mediated and humoral immune responses, which may also be desirable but as yet

are not fully explored in the development of vaccines for NiV and HeV. Although animals immunized with viral vectors encoding the NiV G glycoprotein and challenged with the homologous virus were completely protected, cross-protection studies with some of these vaccines against a HeV challenge were less effective. For example, only 50% of AAV8 NiV.G-immunized hamsters or 33% of ChAdOx1 NiV-B G glycoprotein–immunized hamsters were protected from a lethal HeV challenge (151, 153). In addition, the ALVAC-HeV-F and ALVAC-HeV-G vaccination studies showed that these vectors did not provide 100% protection in hamsters challenged with HeV, perhaps due to either a suboptimal immunization dose or the immunization route (141).

Virus-like particles. VLPs have been explored as a vaccine platform because of the resemblance of their surface structure, dimensions, and compositions to authentic virus yet are of high safety because of the lack of viral genetic material. Earlier studies revealed that the M protein of NiV was capable of orchestrating the formation and budding of NiV VLPs when expressed in cells that appeared structurally similar to authentic NiV virions, and these VLPs could also incorporate other viral proteins such as the F and G glycoproteins (157, 158). VLPs composed of NiV M, F, and G were used to vaccinate mice s.c. at weeks 0, 2, and 4 and demonstrated they could induce high neutralizing antibody titers by day 35 (159) (**Table 2**). NiV VLPs were later used in NiV-M challenge studies either alone or in combination with adjuvant, monophosphoryl lipid A (MPLA) and Alhydrogel^{TM} (15 μg/50 μg) or CpG and Alhydrogel (40 μg/50 μg) (160). Hamsters were vaccinated i.m. either as a single dose or as a 3-dose regimen and then challenged via i.p. administration of NiV-M at 28 days or 58 days, respectively. In all cohorts, 100% of the vaccinated animals survived with no clinical signs of disease and no detection of viral RNA in any of the sampled tissues, regardless of the presence of adjuvant. VLPs are thus an alternative means, with inherent safety, of producing an inactivated whole virus vaccine from an otherwise highly pathogenic virus.

Subunit vaccine. The HeV-sG subunit vaccine has been extensively evaluated in several studies. Here, a brief summary of earlier reports is made, but the focus is on studies in NHPs and livestock. Recombinant HeV-sG and NiV-sG can elicit a potent neutralizing antibody response and were first tested as vaccine immunogens in the feline model (14, 161) (**Table 2**). Both HeV-sG and NiV-sG vaccination of cats completely protected against lethal NiV-M challenge, and HeV-sG elicited greater heterologous neutralizing titers than did NiV-sG, demonstrating that a single subunit vaccine may be effective against both NiV and HeV (14). Other studies using lower doses of HeV-sG (**Table 2**) demonstrated that a pre-challenge neutralizing titer of 1:32 could protect against NiV-M (162). Additional studies in ferrets showed that low doses of HeV-sG could protect against HeV and NiV-B (163, 164) (**Table 2**). Also, a longevity study showed that vaccinated ferrets challenged with NiV-B at 14 months post-immunization, with pre-challenge neutralizing titers of 1:16 to 1:128, were also protected (164).

The HeV-sG vaccine has been extensively evaluated in AGMs (**Table 2**). In a cross-protection study, 100 μg, 50 μg, or 10 μg doses of HeV-sG in combination with Alhydrogel and CpG were administered i.m. as a prime-boost, on days 0 and 21. Pre-challenge 50% neutralization titers ranged from 1:28 to 1:379. All subjects were challenged with NiV-M by i.t. administration on day 42. All vaccinated subjects were completely protected, displaying no clinical signs of disease, and no viral RNA could be detected in blood and tissues and no infectious virus was isolated (165). Similarly, HeV-sG vaccination HeV challenge in AGMs has also been performed. Using a prime-boost regimen, AGMs were vaccinated twice, 3 weeks apart, by i.m. injection with 100 μg HeV-sG with Alhydrogel or HeV-sG with Alhydrogel and CpG, and then challenged 3 weeks later with HeV by i.t. administration (166). All vaccinated animals were completely protected from clinical

disease, and no HeV RNA or viral antigen could be detected in swabs, blood, or tissues, and notably HeV-sG formulated in only Alhydrogel protected (166).

The efficacy and inherent safety of the HeV-sG subunit led to its development as an equine vaccine to prevent HeV infection of horses and also reduce the risk of HeV transmission to people, as a One Health concept (**Figure 1**). HeV-sG, formulated in an approved equine adjuvant (Zoetis, Inc.), was evaluated in two efficacy studies; the first tested 50 μg and 100 μg doses of the same HeV-sG used in prior animal studies to vaccinate horses, and the second used 100 μg doses of HeV-sG produced in Chinese hamster ovary cells (Zoetis, Inc.). Two vaccinations were given by i.m. administration 3 weeks apart. All horses in these efficacy studies were challenged by oronasal inoculation with HeV (**Table 2**). Seven horses were challenged at 28 days and 3 horses were challenged at 194 days after the second immunization. All vaccinated horses remained clinically healthy following challenge; pre-challenge neutralization titers ranged from 1:128 to more than 4,096 in horses challenged 21 days after vaccination and only from 1:16 to 1:32 in horses challenged at 6 months. There was no gross or histologic evidence of infection in any of the vaccinated horses at study completion, and all tissues examined were negative for viral antigen, with no viral genome detected in any tissue. In 9 of 10 vaccinated horses, HeV nucleic acid was not detected in daily nasal, oral, or rectal swab samples or from blood, urine, or fecal samples collected before euthanasia, no recoverable virus was present, and no rise in antibody titer was detected in any vaccinated horse following challenge (167). The HeV-sG horse vaccine (Equivac® HeV) was launched by Zoetis, Inc., in November 2012 on a minor use permit by the regulatory authority, the Australian Pesticides and Veterinary Medicines Authority (APVMA), and is the first commercially developed and deployed vaccine against a BSL-4 agent. All vaccinated horses are microchipped, and a database is maintained. Equivac HeV received full registration by the APVMA in 2015. To date, more than 765,000 doses of Equivac HeV vaccine have been administered to more than 179,000 unique horses, and laboratory-confirmed HeV infections in horses have since occurred only in unvaccinated animals.

Studies showed HeV-sG as a NiV vaccine in the pig model (which is a non-lethal challenge model) was much less effective in comparison to results observed in the cat, ferret, NHP, and horse, and HeV-sG was only partially protective against HeV challenge and unprotective against NiV-M in the pig (168). These experiments also indicated that both humoral and cellular immune responses were required for protection of swine against NiV and HeV. Here, pigs were immunized with HeV-sG in a proprietary adjuvant (Zoetis, Inc.), and subjects were challenged with HeV or NiV via i.n. administration (**Table 2**). HeV-sG-vaccinated pigs developed neutralizing titers ranging from 1:160 to 1:320 to HeV, but only partial protection was achieved with reduced viral RNA in tissues and no recoverable virus, and there was no reduction of viral shedding in nasal washes. These HeV-sG-vaccinated pigs did not develop neutralizing antibodies to NiV-M that were considered protective (low), nor did they have measurable activation of cellular immune memory. Only a comparative group of pigs that were first orally infected (vaccinated) with NiV (and recovered) were subsequently protected against an i.n. rechallenge with NiV. This group of pigs developed protective antibody levels and cell-mediated immune memory responses (168).

Single-dose lipid nanoparticle mRNA, HeV-sG vaccine. More recently, messenger RNA (mRNA)-based vaccines have emerged as an attractive vaccine strategy because of safety, efficacy, and rapid implementation features. In a recent study, the efficacy of an mRNA vaccine approach was assessed in a NiV-M animal challenge model (169). mRNA transcripts encoding HeV-sG were complexed with lipid nanoparticles (LNPs) to generate HeV-sG mRNA LNP. Two groups of 10 hamsters were vaccinated with a single dose of HeV-sG mRNA LNP at either 10 μg or 30 μg by i.m. injection. Subjects were challenged with NiV-M by i.p. administration 30 days

post-vaccination (**Table 2**). The HeV-sG mRNA LNP was only partially protective, with 3 hamsters from the low-dose group and 7 hamsters from the high-dose group surviving challenge. Of the surviving animals, signs of clinical disease were observed in 2 low-dose group and 6 high-dose group hamsters; however, disease symptoms were gone by study termination. NiV *N* gene RNA levels in the blood and a variety of tissues in surviving hamsters were lower compared with nonsurvivors, but NiV RNA copy levels were not different compared with controls. No anti-NiV IgG or virus-neutralizing activity was detected in vaccinated animals prior to challenge; however, all post-challenge survivors were positive for anti-NiV IgG antibodies, and all survivors (in both groups) had similar neutralizing titers ranging from 1:160 to 1:640. Euthanized animals had little to undetectable neutralizing activity, highlighting the correlation of this immune response to protection. Although promising, the partial efficacy of HeV-sG mRNA LNP observed in this study suggests that further optimization of vaccination route, addition of an adjuvant, and/or a prime-boost regimen is needed.

SUMMARY AND FUTURE PERSPECTIVES

The frequency of henipavirus outbreaks and human infections is a significant global health concern. A promising passive immunization strategy has been developed using a human mAb, m102.4, shown effective in the NHP challenge model, which has also been administered numerous times to people by compassionate use protocol and has successfully completed a phase I safety trial in Australia. In addition, the Equivac HeV vaccine is available, targeting the protection of horses and also people by breaking the chain of HeV transmission to people, and is an example of a One Health approach to counter an infectious disease threat. Over the past 15 years, nearly a dozen NiV and HeV vaccine approaches have been trialed in animal challenge models, and many show promise as effective human-use vaccines. Recently, the formation of the Coalition for Epidemic Preparedness Innovations (CEPI), a global partnership between public and private organizations, was undertaken with the goals of developing vaccines against emerging infectious diseases and offering equitable access to those vaccines (170). Indeed, without the support of CEPI, the prospects of having a NiV or HeV vaccine suitable for use in people, at a deployable stage in the event of a significant outbreak, would have remained academic. Research teams can now capitalize on the large body of basic and preclinical vaccine development data on a half-dozen important emerging viral threats including NiV and, with the support of CEPI, can develop vaccine candidates for clinical use and future licensure. Several of the NiV human vaccine candidates described in this review are now supported by CEPI.

DISCLOSURE STATEMENT

C.C.B. is a US federal employee and co-inventor on US and foreign patents pertaining to soluble forms of HeV and NiV G and F glycoproteins and monoclonal antibodies against HeV and NiV whose assignee is the United States as represented by the Henry M. Jackson Foundation for the Advancement of Military Medicine (Bethesda, Maryland). The soluble forms of the HeV and NiV G glycoproteins are licensed to Zoetis, Inc., and Aurobindo Pharma USA Inc. M.A. declares no competing interests.

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Errata

From:	Broder, Christopher on behalf of Broder, Christopher <christopher.broder@usuhs.edu>
To:	Raúl Gómez Román
Cc:	epstein@ecohealthalliance.org; Epidemiology; Eileen Fairclough; Maïna L"Azou Jackson; Gabrielle Breugelmans; Eric Laing; Antony Dimitrov
Subject:	Re: CEPI RfP on Nipah epidemiology
Date:	Wednesday, August 18, 2021 12:06:28 PM
Attachments:	AA-Amaya-Published-annurev-virology-021920-113833.pdf

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hi Raúl

Sure, that's fine.

Sorry but what I meant before on the last question, was "we" Jon, myself and Eric **do not** know who could address all 3 workflows easily .

I guess my HeV point is:

If the goal of the RFP is aimed at gathering genetic data in support of vaccine development, why
is the RFP focused only on Nipah virus isolates, all of which are highly similar (92-99.9% identical), when we know now
that the Hendra G glycoprotein (only 78% identical to Nipah G) can completely protect against Nipah Malaysia and Nipah Bangladesh
in multiple animal models?

Nipah from Thailand being essentially identical to Bangladesh and all Bangladesh isolates essentially identical amongst themselves (99,9%),
and Nipah Malaysia to Bangladesh >92% identical among all proteins with G being 95.5% identical. Any vaccine using G as the immunogen
of choice delivered in a way to induce a broad polyclonal response will be effective against all the isolates of Nipah virus we know of.

Importantly, Nipah G does not provide complete protection against a Hendra virus challenge; this has been done with ChAdOx1 and AAV.
We settled on HeV-sG as the vaccine of choice in 2004, because the first test in the cat model showed it to be superior in eliciting higher cross-reactive
neut titers to Hendra and Nipah, as compared to Nipah sG

What if a new Nipah virus emerges in SEA or Oceana that is more related to Hendra?
Those Nipah G vaccines based on the Bangladesh G will likely not be as effective, whereas the Hendra G vaccine will be.

Chris

Eric will inquire with his partners in a soon to be funded DTRA project for virus surveillance in the Philippines. But we do not think there are any human
samples available. Jon has active surveillance projects in India and Bangladesh and Malaysia now,
but most of these activities target wildlife/livestock but some human sampling, and are serology based.

best wishes
Chris

On Tue, Aug 17, 2021 at 3:32 PM Raúl Gómez Román <raul.gomezroman@cepi.net> wrote:

> Hello Chris,
>
> Thank you for your email – always good to hear from you!
>
> This RfP was prepared and launched by CEPI's Epidemiology group, which is primarily responsible for its contents. I disseminated the RfP to our Task Force members as a courtesy, and I will be supporting the Epi team tangentially by reviewing applications and by ensuring that we engage external stakeholders in a coordinated fashion.
>
> On that note, you raise questions and comments along the lines of others who have replied. Would it be OK with you if your questions highlighted in yellow are logged to be followed up in CEPI's public response to questions raised? The questions would be anonymized. If you have additional questions to these, please let us know. The HeV point is an important one and I would suggest framing into a question for public follow up.
>
> Best wishes,
>
> Raúl
>
> ---
>
> **From:** Broder, Christopher <christopher.broder@usuhs.edu>
> **Sent:** Tuesday, August 17, 2021 8:43 PM
> **To:** Raúl Gómez Román <raul.gomezroman@cepi.net>; epstein@ecohealthalliance.org; Antony Dimitrov <antony.dimitrov.ctr@usuhs.edu>
> **Subject:** CEPI RfP on Nipah epidemiology
>
>
>
> hi Raúl

--
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viruses

MDPI

Review

Overview of Bat and Wildlife Coronavirus Surveillance in Africa: A Framework for Global Investigations

Marike Geldenhuys [1], Marinda Mortlock [1], Jonathan H. Epstein [1,2], Janusz T. Pawęska [1,3], Jacqueline Weyer [1,3,4] and Wanda Markotter [1,*]

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Abstract: The ongoing coronavirus disease 2019 (COVID-19) pandemic has had devastating health and socio-economic impacts. Human activities, especially at the wildlife interphase, are at the core of forces driving the emergence of new viral agents. Global surveillance activities have identified bats as the natural hosts of diverse coronaviruses, with other domestic and wildlife animal species possibly acting as intermediate or spillover hosts. The African continent is confronted by several factors that challenge prevention and response to novel disease emergences, such as high species diversity, inadequate health systems, and drastic social and ecosystem changes. We reviewed published animal coronavirus surveillance studies conducted in Africa, specifically summarizing surveillance approaches, species numbers tested, and findings. Far more surveillance has been initiated among bat populations than other wildlife and domestic animals, with nearly 26,000 bat individuals tested. Though coronaviruses have been identified from approximately 7% of the total bats tested, surveillance among other animals identified coronaviruses in less than 1%. In addition to a large undescribed diversity, sequences related to four of the seven human coronaviruses have been reported from African bats. The review highlights research gaps and the disparity in surveillance efforts between different animal groups (particularly potential spillover hosts) and concludes with proposed strategies for improved future biosurveillance.

Keywords: coronaviruses; surveillance; biosurveillance; Africa; bat; emerging; African bat coronaviruses; wildlife; domestic animals; COVID-19; HCoV-229E; HCoV-NL63; MERS-CoV; SARS-CoV; SARS-CoV 2; surveillance strategies

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1. Introduction

In the past two decades, four novel coronaviruses of public and veterinary health importance have emerged. These include the three agents originating from China; severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002 [1,2], swine acute diarrhea syndrome coronavirus (SADS-CoV) among localized pig farms in 2017 with re-emergence in 2019 [3,4], and SARS-CoV 2 towards the end of 2019 [1,4–6]. The fourth emergent coronavirus, Middle East respiratory syndrome coronavirus (MERS-CoV), emerged in the Arabian Peninsula in 2012 [7,8]. These events show that coronaviruses have the potential to spillover from natural hosts into different species and cause severe diseases with devastating consequences. Dromedary camels are considered the reservoirs of MERS-CoV,

though the original source and transmission routes from animals are still uncertain for SARS-CoV and SARS-CoV 2 [9–13], with related viruses identified in bats. Different amplification hosts are considered to be involved in all three human coronavirus (HCoV) outbreaks.

The link between bats and emerging coronaviruses was first considered in 2005 following the identification of coronaviruses related to SARS-CoV in specific Asian rhinolophid bat species [14–16]. Since then, a high diversity of coronavirus nucleic acids has been detected in bats, several of which are related to coronaviruses infecting human and domestic animals, with hundreds of unclassified sequences pending characterization. The expanding knowledge of coronavirus diversity has additionally allowed for novel insights into their evolutionary history, including linking bats as the ancestors of specific mammalian coronavirus lineages [17,18]. More specifically, bat coronaviruses with genetic similarity to known coronavirus species, such as HCoV229E and HCoVNL63, are suggested to have acted as ancestors of these human viruses from previous spillover events [19].

Biosurveillance of wildlife hosts, including bats, are one of the first steps towards understanding how viruses emerge [20,21] and include identifying viral diversity, host species, and distribution ranges. However, several factors have been implicated in spillover events, including genetic, ecologic, epidemiological, and anthropological elements [22]. Unless the underlying factors are also identified and mitigated, coronaviruses are likely to continue to emerge in the future.

The high biodiversity on the African continent supports viral species richness, which has been correlated with disease hotspot mapping and novel viral diseases that have emerged or re-emerged in Africa to date [22]. Many communities in Africa live in close contact with wildlife, domesticated animals, and livestock. Some surveillance for bat coronaviruses has been performed in Africa. A recent review by Markotter et al. [23] provides a comprehensive summary of potentially zoonotic coronaviruses reported from Africa (relatives of HCoV229E, HCoVNL63, MERS-CoV, and SARS-CoV), focusing on the distribution of the host bat species, and concluding that inferences on zoonotic potential based on the genetic relatedness is limiting. This review focuses in greater detail on the total coronavirus diversity identified among African animal species. We review published literature concerning bat species targeted, sample sizes, viral genetic diversity, and evolutionary links to specific host species. The review was also expanded to include the currently available surveillance data among non-bat wildlife and domesticated livestock as hosts of coronavirus diversity. We highlight surveillance approaches from previous studies, important findings, and gaps in current surveillance and propose a surveillance framework to guide the design of future biosurveillance studies.

2. The Importance of Viral Taxonomy

The hierarchical levels of the coronavirus taxonomy are well described [24]. There are currently four genera in the *Orthocoronavirinae* subfamily: the *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. The *Alphacoronavirus* and *Betacoronavirus* genera predominantly infect mammals and are further divided into subgenera (Figure 1A,B). Human coronaviruses group within either the *Duvinacovirus, Setracovirus, Sarbecovirus, Merbecovirus*, or *Embecovirus* subgenera (Figure 1A,B). Coronavirus genomes consist of several non-structural genes in open reading frame (ORF) 1 (encoding the replicase polyprotein pp1ab), followed by four structural genes and several accessory genes depending on the species (Figure 1C). Current classification criteria for coronaviruses (ICTV code 2019.021S) rely on comparative amino acid sequence analysis of five domains within the replicase polyprotein pp1ab: 3CLpro, NiRAN, RdRp, ZBD, and HEL1 [6,25]. Computational approaches are used to estimate genetic divergence, and thresholds are utilized as demarcation criteria at various taxonomic levels (Figure 1C,D) [24]. Moreover, only complete genomes are considered for formal taxonomic placement.

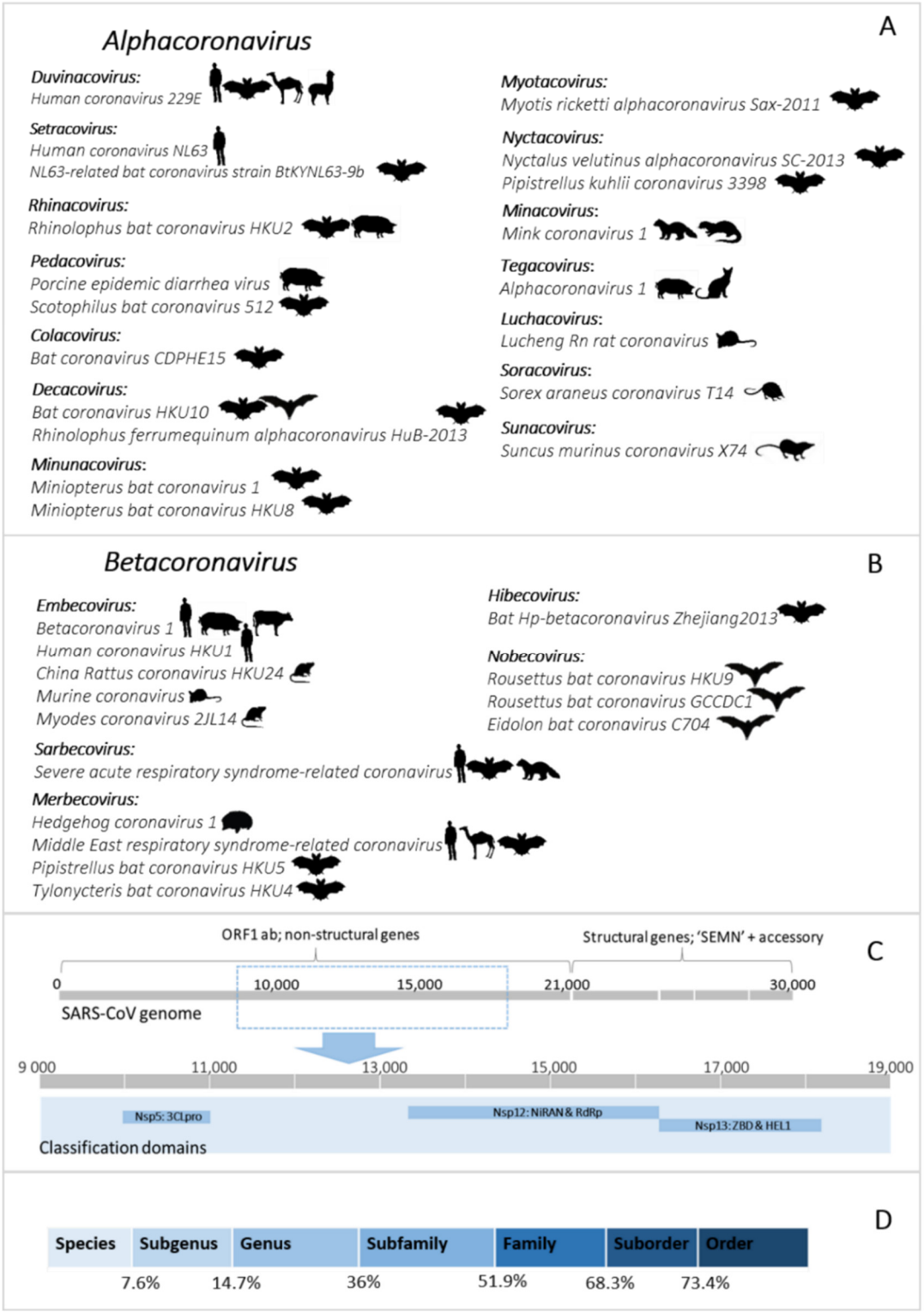


Figure 1. (**A**,**B**) Current coronavirus subgenera (bold) and species of the *Alphacoronavirus* and *Betacoronavirus* genera. The images indicate host species associated with the virus species. Figure constructed with the species listed on the 2019 Release of the ICTV Virus Taxonomy 9th Report MSL#35: (Available at https://talk.ictvonline.org/ictv-reports/ictv_9th_report/positive-sense-rna-viruses-2011/w/posrna_viruses/222/coronaviridae accessed on 12 December 2020). (**C**) Representation of the coronavirus genome (based on the reference genome NC_004718.3 SARS coronavirus Tor2) depicting the locations of important domains for classification of species (NSP5 (3CLpro), NSP12 (NiRAN and RdRp), and NSP13 (ZBD and HEL1)). (**D**) Thresholds of the taxonomic demarcation criteria [24]. Novel viruses are part of a taxonomic level if the divergence within the five concatenated replicase domains is less than the indicated amino acid percentage.

Since the initial identification of bat coronaviruses in 2005, a total of 16 formally recognized coronavirus species have been described from bats. Biosurveillance research mainly report on partial sequences of the coronavirus genome and can only be described to a limited extent by their phylogenetic grouping or similarity percentages. Sequences are considered 'related' to genetically similar sequences in a phylogenetic cluster, pending the viral diversity included in the inference. This 'related' terminology has become widely misrepresented. It is frequently used to indicate the relatedness of sequences to the closest human coronavirus (HCoV) in a phylogeny, even if these sequences may be significantly distant. For example, SARS-CoV belongs to the *Sarbecovirus* subgenus; and the *Hibecovirus* subgenus forms a sister-clade to the sarbecoviruses (Figure S2). Sequences with low similarity to sarbecoviruses, and which should be part of the hibecoviruses, have (even recently) been deemed as 'SARS-related'. Erroneous conclusions may be readily avoided by including all representative diversity of the current taxonomy in phylogenies. In this review, we will employ the convention of limiting the use of 'related' only to describe bat coronaviruses deemed sufficiently similar to known species according to demarcation criteria (e.g., MERS-related, SARS-related, 229E-related, and NL63-related). All others will be described in relation to phylogenetic clusters, using sequence similarities where possible, or indicating possible grouping within a subgenus (Figure 1A).

3. Biosurveillance Studies Based on Nucleic Acid Detection in Africa

Table 1 stipulates the selection criteria utilized to identify and classify publications included in the review. Several surveillance studies focused on bat species were identified [19,26–47], though few studies were found in regards to surveillance among other wild animals or livestock [40,48–51] (Table 1). This may be due to the 'reactive' nature of surveillance among livestock, domestic animals, and non-bat wildlife in response to outbreak events among farmed animals or human populations; such events have not been regularly reported in Africa. Global examples include studies involving farmed civets following the first SARS-CoV outbreak, surveillance in camel herds after identifying MERS-CoV and detecting SARS-CoV 2 among mink farms in Europe [9,52,53]. Coincidentally, the use of passive unbiased metagenomic next-generation sequencing among illegally smuggled pangolins identified sarbecoviruses with overall genome similarity of 85.5% to 92.4% to SARS-CoV 2 in Asia [10,54,55].

Table 1. Selection and classification criteria of studies included in the review.

Search criteria:	Google scholar searches with keywords: "bat, bats, fruit bats, insectivorous bats, animal, mammal, livestock, domestic, domesticated, wildlife, coronavirus, coronaviruses, detections, Africa, Sub-Saharan, Southern Africa, Eastern Africa, nucleic acid, molecular detection, serology, serological, surveillance, survey" were used to search for peer-reviewed publications documenting surveys for coronaviruses in mammals from Africa (mainland Africa as well as islands associated with Africa such as Madagascar, Reunion Island, Seychelles).
Selection criteria:	For a suitably thorough synopsis of the findings, publications were limited to research available until the end of December 2020 and excluded dissertations, theses, or non-peer-reviewed publications. Sequences included in phylogenetic analyses in this review also excluded sequences from dissertations, theses, or unpublished sequences on GenBank that are not linked to available publications. However, PREDICT surveillance data ('PREDICT 1 and 2 surveillance and test data') linked to a 2017 publication was accessed online from Healthmap.org [56] and included both surveillance among bats and other wildlife and livestock.
Criteria for 'primary surveillance reports':	Reports containing a description of the collection and testing of samples from animals for coronavirus surveillance. For bat surveillance, we focused on surveillance strategies using nucleic acid detection methodologies such as family-wide consensus PCR analysis or unbiased high throughput metagenomic sequencing. This includes re-testing samples from an earlier report with a different assay and reporting additional coronaviruses detected. Primary surveillance reports may contain varying levels of characterization for detected viruses. We expanded this criterion for livestock and non-bat wildlife to include both nucleic acid and serological surveillance.
Criteria for 'secondary characterization reports':	Refers specifically to studies based on a primary surveillance report that does not describe new sample collection but a detailed characterization of viral sequences identified in a previous publication or more in-depth analysis of data obtained from primary surveillance reports.

3.1. Surveillance in African Bats

Several surveillance studies focused on bats have been performed in Africa since the first reports in 2009 [26,37]. We identified 23 primary surveillance reports and four subsequent secondary characterization reports [57–60] (Table 2 and Figure 2) that included sampling in 24/54 African countries (www.un.org, accessed on 6 September 2020). Several reports originate from Kenya, Ghana, Gabon, and South Africa (Table 2, Figure 2), with limited surveillance in Morocco and Tunisia [33]. Most studies focused on one or more sites within a single country (Table S1), though few studies include once-off sampling from multiple African countries [30,33,38,45]. Anthony et al. [30] describe the PREDICT surveillance performed over a 5-year timespan in more than 20 countries, seven of which took place in Africa (with Rwanda surveillance further detailed in Nziza et al. [36]). Furthermore, nine reports identified coronaviruses while conducting broader virological surveillance [29,31,32,34–36,39,45], whereas others were coronavirus specific. Supplementary Tables S1–S4 summarize the different reports in terms of approach, species and sample numbers, nucleic-acid detection strategy, and overall findings, including when the information was omitted or not sufficiently described.

Table 2. Bat coronavirus surveillance performed in Africa, per country.

Country (3 Letter Country Code)	References [Primary Surveillance]/(Characterization Report) *
Cameroon	[30,34]
Central African Republic (CAF)	[45]
Democratic Republic of the Congo (DRC)	[30]
Egypt (EGY)	[27]
Gabon (GAB)	[30,40,45]
Ghana (GHA)	[37,44,46]
Guinea (GIN)	[39]
Kenya (KEN)	[19,26,29,(57)]
Madagascar (MDG)	[38,47]
Mauritius (MUS)	[38]
Mayotte (MYT)	[38]
Morocco (MAR)	[38]
Mozambique (MOZ)	[38]
Nigeria (NGA)	[28,41]
Republic of the Congo (COG)	[30,45]
Reunion Island (REU)	[38]
Rwanda (RWA)	[30,35,36,(60)]
Senegal (SEN)	[45]
Seychelles (SYC)	[38]
South Africa (RSA)	[32,42,43,(58)]
Tanzania (TZA)	[30,(60)]
Tunisia (TUN)	[33]
Uganda (UGA)	[30,(59,60)]
Zimbabwe (ZWE)	[31]

* References in square brackets indicate primary surveillance reports; Round brackets refer to 'secondary characterization reports'.

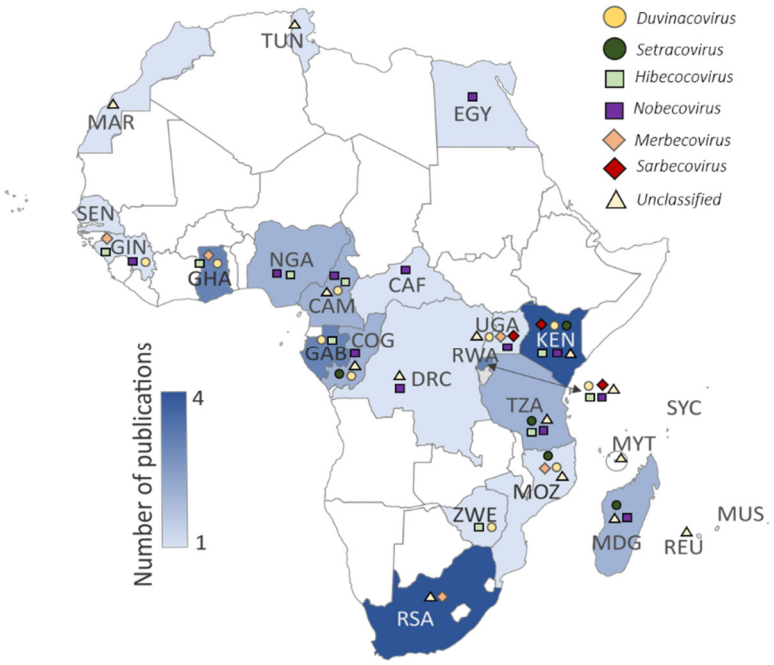


Figure 2. Published bat coronavirus surveillance studies per country (shading denoting the number of publications). Symbols in the key above the map represent different coronaviruses detected in the respective countries: Duvinacovirus as a yellow circle (HCoV229E-related viruses), Setracoronavirus as a dark green circle (HCoVNL63-related viruses), Sarbecoviruses as a red diamond (HCoV-SARS-related viruses), Merbecoviruses as an orange diamond (HCoV-MERS-related viruses), Nobecoviruses as a purple square, Hibecoviruses as a green square, and unclassified viruses as a black triangle. Further details on coronaviruses identified can be reviewed in Table S4. Three-letter ISO country code abbreviations are shown on the map.

3.1.1. Sampling Approaches and Methodologies of Bat Coronavirus Surveillance

Overall, the primary aim of most of the reports was to detect the presence of coronavirus RNA in bat species, with limited subsequent genetic characterization. Bat species and sample numbers were opportunistically sampled at roosts in mainly cross-sectional once-off sampling focused on a targeted population, region, or species. The frequency of sampling was generally poorly described (Table S1). Exceptions include reports from Madagascar, Nigeria, and Zimbabwe, where multiple sampling events (2 or more) were performed at the same roosts [28,31,47]. Figure 3 provides a graphical summary of the approaches employed by surveillance efforts for bat coronaviruses (Tables S1 and S2).

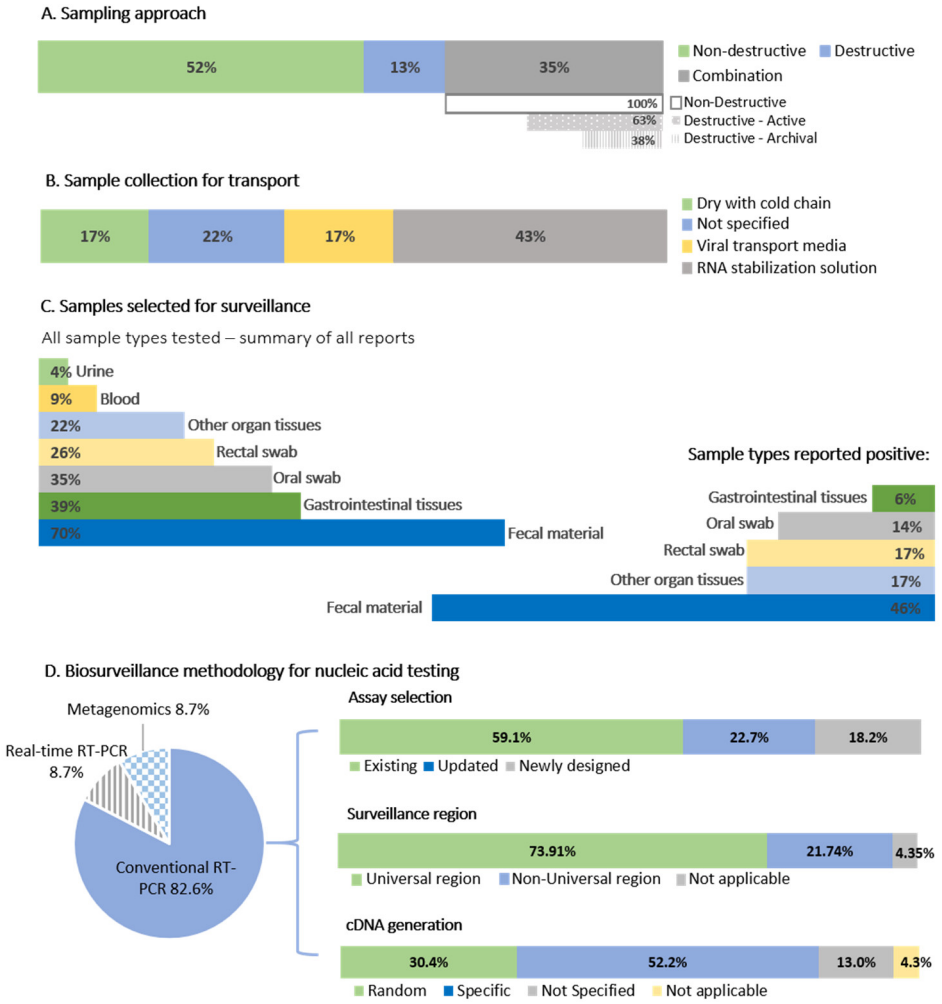


Figure 3. A summary of coronavirus sampling approaches and methodology. (**A**) The sampling approaches of the 23 primary surveillance reports. Combination studies are split into those employing new or archival destructive sampling. (**B**) Sample preservation methods. (**C**) Sample types selected for surveillance and samples testing positive. (**D**) Biosurveillance methodology for nucleic acid testing, percentage of studies using conventional, real-time, or metagenomic approaches. The conventional assays were further split into existing assays from the literature, updated exiting assays, or whether new assays were developed. The percentages of studies targeting the 'universal surveillance region' (see text for an explanation) contrast to those using different genome regions, and whether specific or random primers were chosen for cDNA preparation.

It is well established that coronaviruses display a gastrointestinal tropism in bats [61], and fecal material or other gastrointestinal sample types such as rectal swabs (non-destructive) or intestinal tissue (destructive) is the preferred sample types for surveillance (Figure 3). Sample collection was mostly non-destructive (52% of studies), including fecal material collected beneath roosting bats in caves and trees [28,29,31,33,37] or fecal material and rectal swabs from individual bats [19,26,27,30,32,34,35,37,42–44,46,47]. For this review, we are assuming fecal swabs are the same as rectal swabs. Only 13% of studies solely implemented destructive sampling (collection of organ tissues), and 35% of studies (Figure 3) combined both methodologies to collect sample material for multi-pathogen surveillance [27,30,35,41,43,45] or were tested due to availability within archival tissue banks [32,39,42]. Along with gastrointestinal samples, oral (or throat) swabs were also collected [19,26,27,30,47], but infrequently contained coronavirus RNA [19,27,30,36,39]. Due to limited reporting information provided per study, coronavirus detection among oral swabs can only be roughly estimated. Of all reports investigated, only 35% tested oral swabs (Figure 3). From these reports, 62.5% identified coronavirus RNA, representing positive

oral swabs from only 14% of studies overall (Table S1). Coronaviruses were also opportunistically detected within lung and liver tissues [27,38,45], though it is unclear what other positive individuals' organs were also tested.

The basic methodology implemented in all but two studies [32,34] involved RNA extraction of samples followed by nucleic acid detection targeting a conserved region of the genome. A region of the RNA dependent RNA polymerase (RdRp) gene within the open reading frame (ORF) 1b of the coronavirus genome (Figure 4) is mostly targeted and corresponds to approximate nucleotide position 15,200–15,600 in the coronavirus genome (using reference NC_004718.3 SARS coronavirus Tor2) (Figure 4, Table S2). Targeting of this "universal coronavirus surveillance region" enables comparison between studies, though 74% of the African bat surveillance studies utilized assays based on the region (22% either used a non-universal region or combination of both; Table S2). The addition of a nested step is generally essential for the detection of low concentration viral RNA. A small number of studies in Africa quantified viral concentrations of positive samples, obtaining as little as 50–450 RNA copies/mg fecal material for some low concentration samples, or between 323 to 1.5 × 10^8 RNA copies/g of fecal material [37,44].

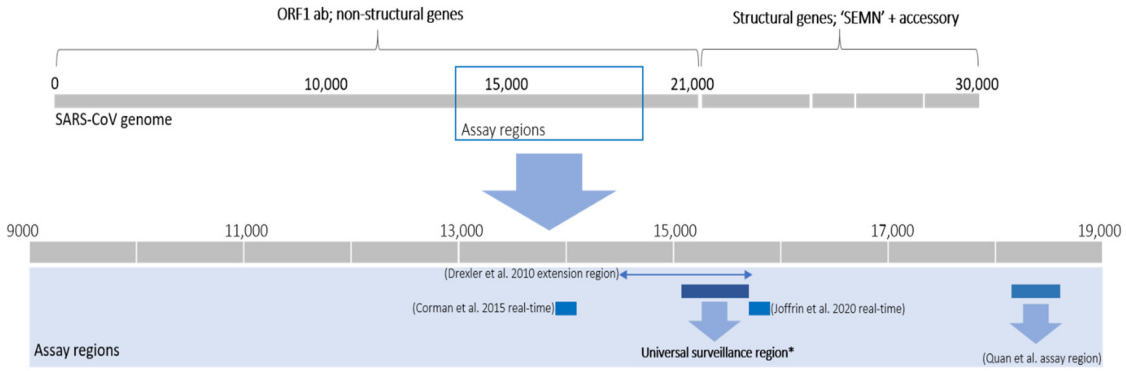


Figure 4. Representation of the coronavirus genome (based on the reference genome NC_004718.3 SARS coronavirus Tor2) depicting the assay regions. The assays corresponding to this universal region included in Tong et al. [26], de Souza Luna [62], Geldenhuys et al. [42] and Geldenhuys et al. [32] (based on primers from Woo et al. [63]), Razanajatovo et al. [47] (based on Poon et al. [14]), Shehata et al. [27], Waruhiu et al. [29] (based on Watanabe et al. [64]), Chu et al. [65], Gouilh et al., [33]. The RdRp grouping units (RGU) amplification region by Drexler et al. [66] is indicated with the line and arrows.

The majority of surveillance studies (52.2%) implemented a one-step kit approach (i.e., utilizes RNA templates in a single reaction with target-specific primers for cDNA followed directly by PCR amplification), with seven (30.4%) implementing an unbiased methodology for the preparation of cDNA with random hexamers before PCR amplification [31–33,35]. An unbiased approach is more beneficial where only limited sample material is available and multi-pathogen surveillance is done. Suitable assays were either selected from the literature (with the assay from de Souza Luna et al. [62] most frequently employed), constitute newly developed assays (included if no reference was provided for assay modifications), or were updated/modified from the literature (Table S2 and Figure 3). Assays selected from the literature were constructed using the available sequence information known at that point in time. The expanding genetic diversity of coronaviruses is high, and even though these assays target a conserved region, existing primers may be less sensitive toward the detection of more diverse viruses. For example, primers developed before the 2012 emergence of MERS-CoV might not be sufficiently sensitive to detect diverse coronaviruses from the Merbecovirus subgenus. Developing new assays or updating available primers have the added advantage of ensuring that some of the expanding sequence diversity of emerging human coronaviruses and newly detected animal coronaviruses can be incorporated; reducing the probability of highly diverse clades going undetected.

Exceptions to this 'universal CoV surveillance' region are represented mainly by the nested RT-PCR assay developed by Quan et al. [41], targeting a region downstream of the universal CoV surveillance region, corresponding to the approximate nucleotide position 18,300–18,700 (Figure 4). Sequences amplified with the assay from Quan et al. [41] cannot be directly incorporated in phylogenies using the short universal CoV surveillance region and may only be compared to viruses for which this corresponding genome region is available or with full genomes. The PREDICT surveillance described in Anthony et al. [30] and Nziza et al. [36] utilized two surveillance assays to test samples; that of Watanabe et al. [64] based on the universal region and Quan et al. [41]. In total, the Watanabe assay detected 950 coronavirus sequences compared to the 654 sequences from the Quan assay, with only a 27% overlap [30].

Overall, it is not possible to directly compare methodologies to conclude best practices for coronavirus surveillance. However, non-destructive sampling methodologies (swab collection or fecal material from underneath roosting bats) associated with a gastrointestinal origin allow for successful coronavirus identification with minimal injury to the hosts or ecosystem. Proper preservation of sample material is good practice (cold chain or using preservation media), and unbiased cDNA preparation approaches allow for the conservation of reagents and sample material. The use of appropriate assays and overlapping target regions are essential to enable comparisons between studies.

3.1.2. Summary of Sample Sizes and Bat Species Tested

The surveillance data from the 23 publications were compared to the 2019 African Chiropteran Report (comprehensive report of the current taxonomy with data based on museum records from bats collected across the continent) to determine an estimate of total bats sampled per species (Table S4; [67]). There are 13 extant bat families in Africa, with an estimated 324 species [67]. Eleven families have been included in coronavirus surveillance reports (Table 3). Several publications provided the total bats sampled within a study though may not have specified per species or country, and thus 1966 sampled bats could not be included [29,41]. The sample numbers (per species per country) were not specifically indicated in Anthony et al. [30], but total PREDICT surveillance data for the seven African-surveyed countries was accessed online from Healthmap.org and included in the analyses. We acknowledge that the data likely exceeds the sample size for the countries used for the analysis in the 2017 publication; however, we felt that including the data in our assessment greatly contributes to the total bats sampled in Africa per species—by over 10,000 individuals. Moreover, this data was also used in Tables 2 and S1–S3. Of the approximate 127 total bat species included in studies, bat coronaviruses were identified in 59. Nearly 26,000 bat individuals are estimated to have been tested for coronaviruses in African surveillance studies using one or more assays. However, this number comprises mainly pteropid and hipposiderid bats (41.8% and 33%, respectively) and varies per family. The table below highlights the need for additional surveillance in several families, such as the Vespertilionidae. These are abundant bats, and increasing the sample size tested of species in this family may provide a greater understanding of the host ecology of coronavirus species such as MERS-related viruses.

Table 3. Coronavirus detections according bat host taxonomy.

Bat Families Tested	Number of Species	Species Tested	Bat Species Positive	Number of Individuals Tested Per Family *	Positive Individuals #
Pteropodidae	44	22	14	10,851	881 (8.1%)
Hipposideridae	21	10	8	8563	257 (3%)
Molossidae	44	16	8	2144	286 (13.3%)
Miniopteridae	22	12	5	1464	120 (8.2%)
Vespertilionidae	114	37	9	918	41 (4.5%)
Rhinolophidae	38	14	9	728	68 (9.3%)
Emballonuridae	11	4	0	678	0
Nycteridae	15	6	3	299	51 (17.1%)
Rhinonycteridae	6	3	2	250	74 (29.6%)
Megadermatidae	2	2	1	25	3 (12%)
Rhinopomatidae	3	1	0	1	0
Myzopodidae	2	0	0	0	-
Cistugonidae	2	0	0	0	-
Totals	324	127	59	25,921	1779 (6.9%)

* Counts for number of individuals tested reflect individuals from publications reporting total individuals tested per species per country, or total positive individuals in reports where total sampled are not provided. These counts exclude 1966 bats tested in [29,41] from which species totals were not provided, and studies testing colony-level fecal samples [28,31].
# Approximate number of positives from Table S5.

Coronavirus RNA has been detected in nine of the eleven families sampled, excluding the Emballonuridae and Rhinopomatidae. The Rhinopomatidae represents only one tested individual; approximately 678 bats from four species in the Emballonuridae family have been investigated (*Coleura afra, Taphozous perforates*, *Taphozous mauritianus,* and *Taphozous hildegardeae*). This includes surveillance from eight countries with sample sizes varying from 1 to 172 (Tables S4 and S5). Comparatively, coronaviruses have been identified from families like the Megadermatidae, Rhinonycteridae, or Nycteridae, from which far fewer individuals were analyzed (25–299). The lack of viral detection from the Emballonuridae family could be due to insufficient sample sizes, extremely low prevalence, time of sampling, highly diverse viruses missed by consensus primers, or the absence of coronaviruses. The remaining unsampled Myzopodidae and Cistugonidae families are small (two species each), with limited distributions in Madagascar and Southern Africa, respectively.

Primary surveillance reports investigating one or two species/genera typically focus on abundant hosts that may form large populations with frequent opportunities for contact with human communities [28,31,32,34]. Studies sampling many diverse genera/species (83% of primary surveillance reports) mostly sample species opportunistically present at one or more surveillance sites (Table S3). To estimate sample sizes per species, we looked at the total and average number of individuals per species tested in these reports and specifically noted sample sizes of less than ten individuals (Table S3). For some species, below ten individuals were tested, whereas several hundred [19,27,30,36,45,47] or even thousands of individuals from other species were sampled [44,46]. It was more common for less than 100 individuals to be sampled per species, though a few reports averaged 100–150 per species [19,27,30,36,45,47]. The percentage of species within a report for which less than ten individuals were sampled ranged between 18.5 to 100% of species (Table S3). This constituted more than 50% of species sampled from 11 of the reports and likely represented opportunistically caught individuals. This could not be determined for a further four reports, as sufficient detail was not specified, or samples collected represent colony or population-level sample collection.

A guideline for optimal sample sizes per species was proposed by the meta-analysis of coronavirus surveillance in 20 countries by Anthony et al. [30], with the optimal sampling number being approximately 397 individuals. This was calculated to detect the av-

erage number of unique coronavirus groups relating to probable viral species (2.67) estimated to be present in each bat species. Their findings identified that sampling less than 154 individuals per species constituted poor returns on investment and sampling effort [30]. The percentage of species per report from which coronavirus nucleic acids were detected varied between 8.3% to 66.7% (excluding when only one species was sampled). Overall, the percentage positivity of coronaviruses per total samples ranged from below 1% to 25.7% (excluding pools) (Table S3). As expected, increasing either sample sizes or number of species tested show correlation with increased positivity percentages (Pearson's product correlation $t = 8.9289$, df = 21, $p < 0.001$ and $t = 5.4952$, df = 20, $p < 0.001$, respectively). The differences in positivity can be attributed to many factors, including the nucleic acid detection assay, the methodology for sample collection (preservation of nucleic acids), time of sampling coinciding with coronavirus excretion, species sampled, and sufficient sample numbers per species. Tables S4 and S5 highlight species commonly detected to host coronaviruses; a detailed description of 'high-risk' viruses identified from host species is described below.

3.1.3. Importance of Accurate Bat Species Identification

Correct identification of bat species is essential to conclude potential virus-host associations and estimation of host-viral distribution ranges. This is especially important for complex bat species with similar morphological markers, such as members of the Hipposideridae, Rhinolopidae, and Vespertilionidae. Since the start of coronavirus nucleic acid surveillance among bat species in Africa in 2009, several bat species have undergone species reassignments and name changes. We could not update all new species names for this review and used the taxonomy described in the 2019 African Chiropteran report [67]. However, recent changes of note are among the Hipposideridae, Rhinolophidae, Miniopteridae, and Vespertilionidae families, with additions of new genera (*Afronycteris*, *Pseudoromicia, Vansonia* (elevated to genus)) and the reassignment of species to existing and new genera [68–71]. Some of these include *Hipposideros* species reassignments to the genus *Macronycteris* and the resolution of some *Neoromicia* species with reassignments to *Laephotis, Afronycteris,* and *Pseudoromicia* genera [68,69]. Currently recognized species may be accessed at www.batnames.org (accessed 18 November 2020) [72], and new species need to be correctly correlated to geographical distributions.

We investigated the methodologies for host identification implemented by the primary surveillance reports (Table S3). No identification methodologies for bat species were stipulated in seven (30%) of the bat coronavirus surveillance studies; five (22%) report the use of keys to determine morphological identities by either field teams, veterinarians, or experienced chiroptologists; and two (9%) report the use of molecular means of species confirmation. Only nine reports (39%) describe both morphological and molecular methods to identify and confirm host species (Table S3). Molecular methods include either mitochondrial cytochrome B gene or cytochrome C oxidase subunit I sequencing [73,74]. Not only is this good practice in ensuring accurate determination of host species identity, but if deposited on public reference databases, it ensures that the records of these sequences for sampled species are expanded. However, depositing sequences of individuals lacking accurate morphological identification and failure to update taxonomic changes generally leads to confusion and incorrect host reporting. Thus, reference material on these databases must be associated with correctly identified individuals where morphological identification was conducted by highly trained individuals or experienced bat taxonomists.

3.1.4. Characterization of Bat Coronavirus Genomes and Virus Isolation Attempts

Bat coronavirus surveillance in Africa primarily focused on amplifying and sequencing short amplicon sequences and subsequent diversity determination. The majority of African bat coronaviruses are therefore unclassified and are only represented by a short-sequenced region. Further characterization of the detected coronaviruses is essential for

improved phylogenetic placement and comparisons of various genes/proteins for phenotypic analyses. Studies aiming to further characterize identified coronaviruses employed diverse methodologies (Table S2). Sequence-specific primers have been successful in extending the sequenced regions of the ORF1ab [28,47] or recovering complete coding regions of structural genes like the nucleoprotein gene [27,37]. Sequencing these regions generally involved primer-walking strategies with conventional Sanger sequencing or even high throughput sequencing platforms to overcome the length limit of conventional sequencing. The informal RdRp gene grouping units (referred to as RGU; Figure 4) developed by Drexler et al. [66] amplifies an 816 nucleotide amplicon of the RdRp gene. The pairwise distances of the translated 816 nucleotide fragments (272 amino acids) have been used to delimit different groups as a surrogate system for taxonomic placement of detected bat coronaviruses that lack complete genomes. Grouping units of alphacoronaviruses differ by 4.8% and betacoronaviruses by 5.1% [61]. These grouping units have been used as an extension assay by 22% of African bat coronavirus studies [32,35,43,44,46]. It is worth noting that these units are an unofficial estimate of possible species groupings and may be subject to revision as new diversity is detected (as evident by previous decreasing betacoronavirus thresholds from 6.3% to 5.1%) [61].

The number of bat coronaviruses that can correctly be assigned to a viral species is limited to those with available complete genomes. From African studies, there are over 1840 partial coronavirus gene sequences available among public domains (such as NCBI's GenBank), though only 13 complete genomes and 12 near-complete genomes [19,32,34,41,46,57–59]. The MERS-related *Pipistrellus* bat coronavirus from Uganda was recovered with unbiased sequence-independent high throughput sequencing on the MiSeq platform [59] and a near-complete genome of Zaria bat coronavirus from Nigeria using 454 pyrosequencing [41]. Sanger sequencing with classic primer-walking spanning the entire genome with 70 overlapping hemi-nested PCR assays was implemented to recover a MERS-related *Neoromicia* bat coronavirus from South Africa [58], with a second variant from the same host sequenced using 11 overlapping hemi-nested PCR assays on the MiSeq platform [32]. For more novel viruses, amplification of more conserved coronavirus genome segments with nested consensus degenerate primers are frequently required before being able to sequence more diverse regions with long-range PCRs [19,46,57].

The limited number of complete African bat coronavirus genomes are reflective of the challenges involved. These include the limited scope of certain studies, low viral RNA concentrations, unavailability of sufficient material, lacking related reference genomes for primer design, availability of high throughput sequencing platforms, expertise, and cost [32,37,46]. To overcome some of these constraints, such as limited availability of material, virus culturing can be attempted. However, coronaviruses are notoriously difficult to isolate in vitro, with various methodologies utilized (reviewed in Geldenhuys et al. [75]). Only bat coronaviruses closely related to SARS-CoV have thus far been successfully isolated in Vero cells because the bat viruses could use the same receptors as SARS-CoV [76,77]. This challenge and limited sample material available after nucleic acid extraction and high-biocontainment requirements are likely contributing factors to why none of the 23 primary surveillance publications or secondary characterization reports attempted cultivation of coronaviruses in cell culture (nor described attempts).

It is important to note the formats of naming conventions among bat coronavirus studies, with only some providing sufficient information on the origins of sequences (Table S2). The Coronavirus Study Group of the ICTV recommends adopting a standardized format for nomenclature that has been used for Influenza viruses and avian coronaviruses [6]. Namely, the reference to a host organism from which the viral nucleic acid was derived, the place of detection, a unique strain identifier as well as mention of the time of sampling (e.g., virus/host/location/isolate/date or as an example BtCoV/Neoromicia/RSA/UP5038/2015). This format also allows rapid identification of inter-genus viral sharing in phylogenetic trees and highlights similar clades of viruses occurring in related

species independent of geography. More importantly, this naming convention makes no inference of belonging to a particular species, as species assignments may only be performed once the requirements have been met (i.e., sequencing the genome according to species demarcations).

3.1.5. Coronavirus RNA Identified in African Bats

Global coronavirus surveillance in bats has established several generalizations, with which African studies are in agreement. Namely, bat coronaviruses generally display host specificity, which is usually evident at the genus-level [19,61,78–80]. As a result, certain viral species or even subgenera may be predominantly associated with specific host genera (e.g., rhinolophid bats and *Sarbecovirus*). This association has been observed to be independent of the geographical isolation of the bat hosts [38,81,82]. The evolution of coronaviruses has been suggested to involve a combination of two mechanisms, co-evolution between viral and host taxa and frequent cross-species transmission events [78]. Co-evolution is evident by genus-specificity and the large diversity of bat coronaviruses globally sampled, though many taxa host more than one species/group of coronaviruses [37,78]. Meta-analyses of publicly available bat coronavirus sequences confirmed long-term evolution among bats and determined that frequent cross-species transmissions occur, particularly among sympatric species, though often result in transient spillover among distantly related host taxa [19,30,78]. Such transmissions potentially create viral adaptation opportunities to new hosts and increase overall genetic diversity [83]. Uniquely for Africa, the genetic information of bat coronaviruses sharing similarity to human coronaviruses have been identified in four of the five subgenera associated with human coronaviruses—*Duvinacovirus, Setracovirus, Merbecovirus,* and *Sarbecovirus* (Figure 1A,B). Such findings suggest opportunities for transmission from bats to other animals or directly to humans may have occurred in the past. Though these viruses are still circulating among these hosts, discerning current risks of spillover is limited by available evidence.

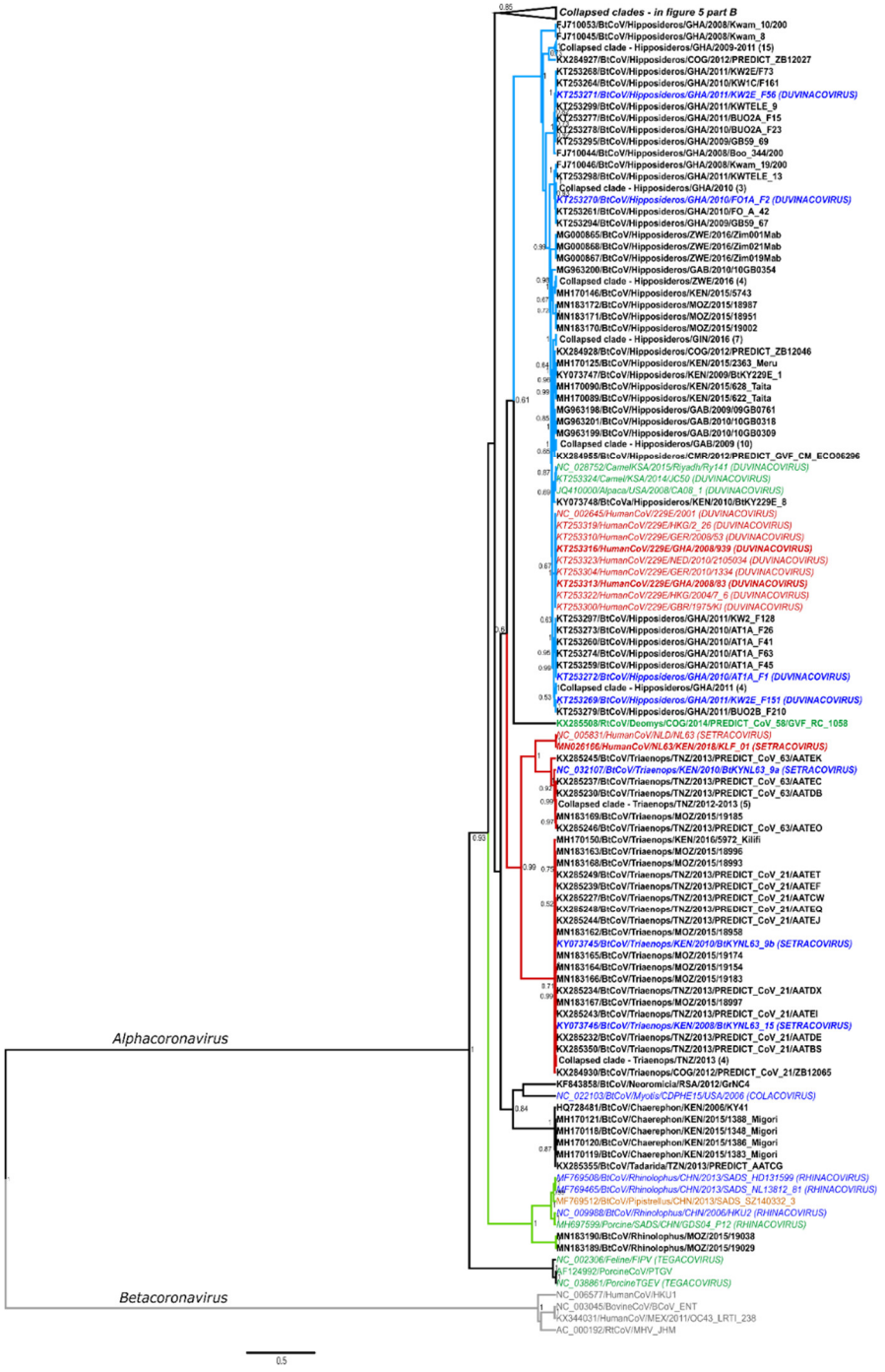
Together with highly variable mutation rates [84,85], coronaviruses are also known for recombination events, where homologous recombination between similar coronaviruses is the most likely. However, recombination between different co-infecting coronaviruses from different subgenera/genera has also been documented [86–88]. Opportunities also increase when bats are co-infected by more than one species of coronavirus. Moreover, heterologous recombination between viral families has also led to the assimilation of novel genes in certain coronaviruses [86,87]. Recombination hotspots within the spike gene have been identified for diverse coronaviruses originating from humans, domestic animals, and bats [89]. Some of the new resultant variants may have improved fitness advantages within their native or new hosts, and new recombinants may be more suited to the usage of new receptor molecules.

Phylogenies were constructed with the sequences from the 23 primary surveillance reports and secondary characterization research studies, representing the sequence diversity of African bat coronaviruses compared to formally classified species and relevant reference sequences (see Appendix A and complete phylogenies in Figures S1 and S2). The following sections summarize the information available regarding detected bat coronaviruses associated with known human coronaviruses and highlight the importance of recombination in the emergence of novel viruses. We also discuss the large diversity of unclassified and unstudied viruses in some highly abundant host species and consider possible interaction opportunities between humans and bat hosts.

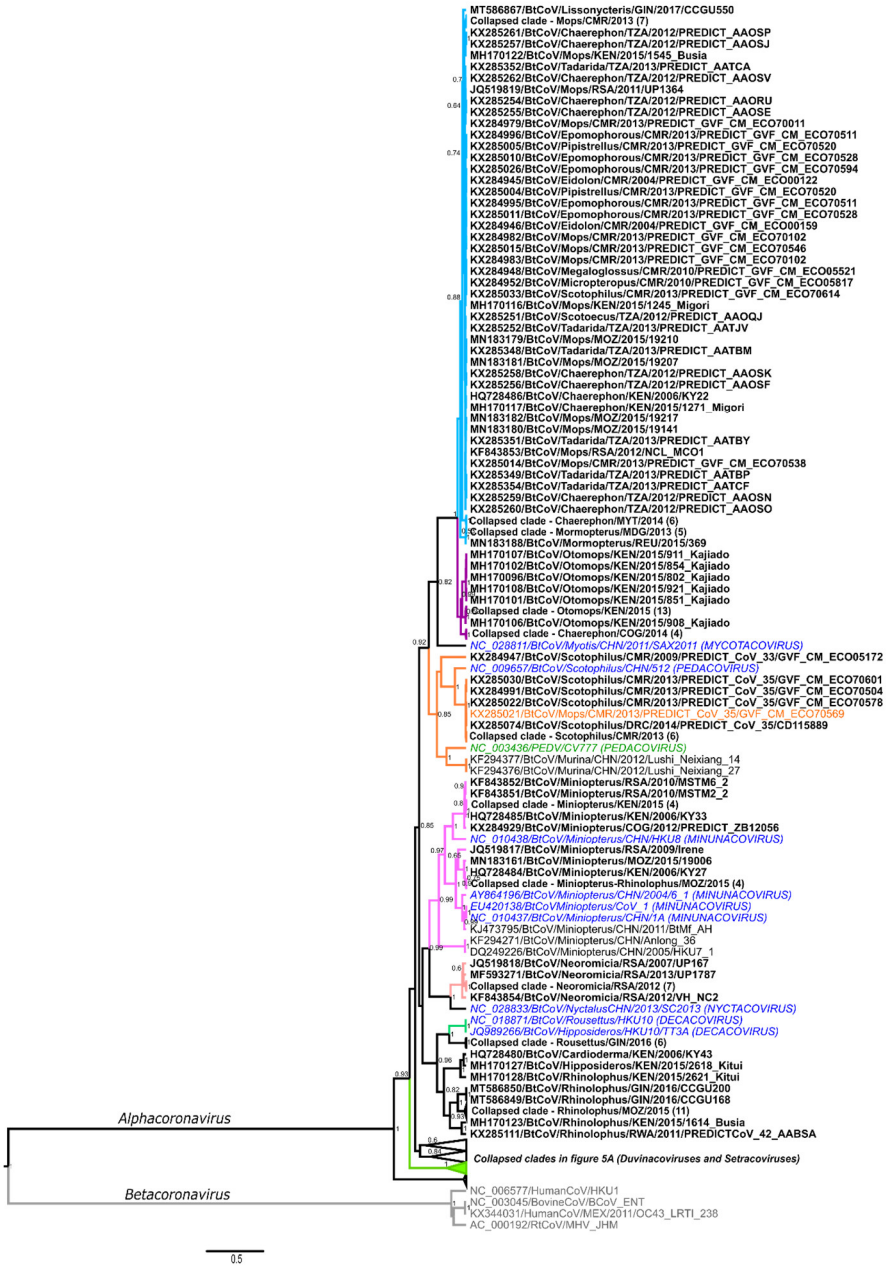
Alphacoronaviruses—*Duvinacovirus*, *Setracovirus*, and Unclassified Virus Relatives of Human Alphacoronaviruses

Several African bat coronaviruses share genetic similarity with the two human alphacoronaviruses, HCoV229E (*Duvinacovirus*) and HCoVNL63 (*Setracovirus*). As seen in Figure 5A, hipposiderid bats (genus *Hipposideros*) are associated with coronavirus se-

quences similar to HCoV229E and have been reported across a wide geographical distribution (Ghana, Kenya, Cameroon, Zimbabwe, Republic of the Congo, Rwanda, Uganda, Gabon, Mozambique, and Guinea) [19,29–31,37–39,45,46]. Due to taxonomic revisions and reassignments [69], the *Macronycteris* genus (Hipposideridae) may also be associated with duvinacoviruses (Table S5).



(**A**)



(**B**)

Figure 5. (**A**,**B**): Alphacoronavirus Bayesian phylogeny of the RdRp partial region (corresponding to approximately 15,200–15,400 nt of the coronavirus genome). Clades collapsed in A are shown in B (and vice versa). To include the maximum number of sequences, sequence lengths were trimmed to a generally useable length of 260 nucleotides. Sequences resulting in shorter lengths were omitted. Sequences in italics indicate formally recognized species (subgenera indicated in capital letters at the end of sequence names); sequences in bold originate in Africa; red highlights human viruses; green indicate non-bat animal hosts; blue/italics indicate formally recognized bat species; orange indicate viral detections from hosts not typically associated with a particular group of coronaviruses. All sequence names were edited to conform to the correct convention, with the modification of the unique sequence identifier listed last due to convenience. Only posterior probabilities of greater than 0.5 are indicated. No unpublished sequences are shown.

Full genomes of four *Hipposideros* alphacoronaviruses from Ghana were compared to current and historical isolates of HCoV229E and an alpaca coronavirus (similar to HCoV229E) from the USA [46]. Sufficient similarity was found between genomes to consider them members of the same *Human coronavirus 229E* species within the *Duvinacovirus* subgenus. The analysis suggested multiple recombination events have occurred among

genomes, including gene losses (e.g., ORF8 within human viruses) and deletions within the spike gene [46]. Several of the bat viruses with similarity to HCoV229E for which no complete genomes are available indicate that there are sequence divergences of approximately 13.5% among RdRp partial gene segments, suggesting circulation of highly diverse HCoV229E-related viruses. The scenario would suggest that HCoV229E may have originated from the large diversity of *Hipposideros* HCoV229E-related bat coronaviruses in the past 200 years (based on the current sequence diversity), with camelids (alpacas, camels, etc.) as possible intermediate hosts [46].

Similarly, several African bat sequences cluster around HCoVNL63 (Figure 5A) and originate from the genus *Triaenops* (Rhinonycteridae family). *Triaenops afer* is the only mainland Africa species currently recognized within the genus after it was split from *T. persicus*, which only occurs in the Middle East [67,90] (with *Triaenops menamena* from Madagascar). Partial and complete genomes were first reported in Kenya [19] with additional partial genomes from the Republic of the Congo, Tanzania, Mozambique, and Madagascar [30,38] (Table S4). Three full genomes were recovered from Kenyan *T. afer* bats and compared to HCoVNL63 [19]. Much like 229E-related bat viruses and HCoV229E, comparisons of the bat viruses to HCoVNL63 identified additional ORFs (ORFx) in bat viral genomes that were absent in HCoVNL63 [19]. The new species, *NL63-related bat coronavirus strain BtKYNL63-9b* (*Setracovirus*), comprised of *Triaenops* coronavirus strains, has been recognized. *Triaenops* virus 9a shares the closest similarity to HCoVNL63 with 78% overall nucleotide identity. The spike was the most divergent gene, with gene phylogenies showing the spike gene of HCoVNL63 grouping with *Hipposideros* 229E-related bat viruses detected in the same study [19]. Recombination analysis of HCoVNL63 indicates multiple breakpoints within the spike gene and suggests a history of recombination between the *Triaenops* NL63-related viruses and *Hipposideros* 229E-related viruses giving rise to the lineage of HCoVNL63 before its introduction into human populations [19]. As with HCoV229E, an intermediate host (and not bats directly) may likely have been involved in introducing progenitor HCoVNL63 viruses into the human populations. Such intermediate hosts are often domesticated livestock animals (such as camelids in the case of HCoV229E) as they have more frequent contact with people, underscoring the need for expansive surveillance within domestic animals to complement surveillance in wildlife.

Bats from the *Hipposideros, Myonycteris* and *Triaenops* genera are all small insectivorous bats and have many overlapping ecological features in terms of habitat. *Hipposideros* and *Myonycteris* primarily roost in caves, though certain species have been known to roost in rock crevices, under bridges, and in tunnels [67]. *Triaenops* have been found roosting in small trees and certain shrubs and mines and caves [91]. Moreover, bats from all three genera are sensitive to human activities that lead to habitat loss and roost disturbance [67]. The surveillance findings show that these viruses continue to circulate in these hosts, with the potential to recombine and create new variants. Establishing whether these viruses pose possible zoonotic risks is limited due to lacking evidence. In vitro studies can assist with determining permissivity or pathogenicity in different cell lines, and protein modeling can suggest the likelihood of receptor binding of bat viruses in spillover hosts. There is also a lack of nucleic or serological investigations into potential spillover animal species that overlap with the bat hosts' geographical distributions and ecological niches.

Alphacoronaviruses—Molossids and a Large Diversity of Uncharacterized Bat Coronaviruses

The diversity of bat alphacoronaviruses from Africa is high. Much of the reported sequences share genetic similarity to members of described subgenera, such as *Rhinacovirus*, *Pedacovirus*, and *Minunacovirus* (Figure 5B). Many of the other sequences represent undescribed diversity and may possibly belong to new subgenera. A large number of unclassified alphacoronaviruses have been identified from molossid bats (Figure 5B). Generally, these sequences form three clades, with sequences similar to a species of

Colacovirus detected in *Chaerephon* and *Tadarida*; a sister clade of the *Mycotacovirus* subgenus that split into an *Otomops*-specific species clade from Kenya; a predominantly *Mops*/*Chaerephon* group of alphacoronaviruses from several countries (Cameroon, Kenya, Tanzania, South Africa and the Republic of the Congo). The latter group also contains a large volume of sequences from various pteropid species (as well as a few vesper species) from Cameroon [30], making it a mixed family clade or a group of viruses frequently prone to host switching. Sequence information on these viruses largely constitutes short sequences from surveillance assays as well as a few partial genomes (HQ728486/BtCoV/*Chaerephon*/KEN/2006/KY22 and HQ728481/BtCoV/*Chaerephon*/KEN/2006/KY41) [57]. These coronaviruses were detected from molossid species such as *Chaerephon pumilus, Mops condylurus, Otomops martiensseni,* and *Tadarida aegyptiaca,* with only 16 of the 44 species from the Molossidae family having been included in surveillance studies. Of note are recent taxonomy changes among this family [72]. Moreover, as indicated in Table S4, large numbers of molossid bats tested are only specified to genus level, with nearly 171 *Chaerephon* spp., 30 *Mops* spp., and 64 *Tadarida* spp. reported. This again reiterates the need to identify hosts down to species level. These species are highly abundant with widespread distributions throughout Africa and are often encountered in urban settings. They are frequently found to be roosting in large populations (several hundred) in the rafters or roofs of buildings such as houses or public institutions like schools, universities, and libraries [67]. As a result, opportunities for contact arise between bat excreta and people (and domestic animals). Though there is no current zoonotic association with these coronaviruses, their abundance among a commonly encountered bat species, with possibly frequent exposure opportunities warrant investigation. Significant characterization of these viral groups is required to better understand this diversity and investigate the zoonotic potential of these alphacoronaviruses.

Betacoronaviruses—Merbecoviruses and Vespertilionid Bats

MERS-CoV emerged on the Arabian Peninsula in 2012 and is now considered endemic to the region due to the presence of the primary reservoir, the dromedary camel [7,92,93]. According to reports from Africa, Europe, Asia, and even South America, viruses sharing similarities to MERS-CoV (*Merbecovirus*) are associated with more than one bat host genus or family [32,43,44,59,83,94,95]. The MERS-related coronaviruses genomes currently sharing the highest similarity to human and camel MERS-CoV were detected in Africa from *Neoromicia capensis* (South Africa) and *Pipistrellus hesperidus* (Uganda) [32,58,59]. Both *Neoromicia* and *Pipistrellus* are small insectivorous bats belonging to the Vespertilionidae family, with several species reassignments occurring in 2020 [68]. Due to taxonomic rearrangements, the genera *Laephotis, Afronycteris,* and *Pseudoromicia*, necessitate inclusion into future MERS-related coronavirus surveillance due to possible intra-host sharing of coronaviruses. Sampling efforts into the previously recognized *Neoromicia* species include approximately 238 individuals and only 100 individuals among *Pipistrellus* species (Table S4), warranting intensified surveillance. According to published reports, very few individuals have been found to harbor MERS-related viruses from these bats sampled.

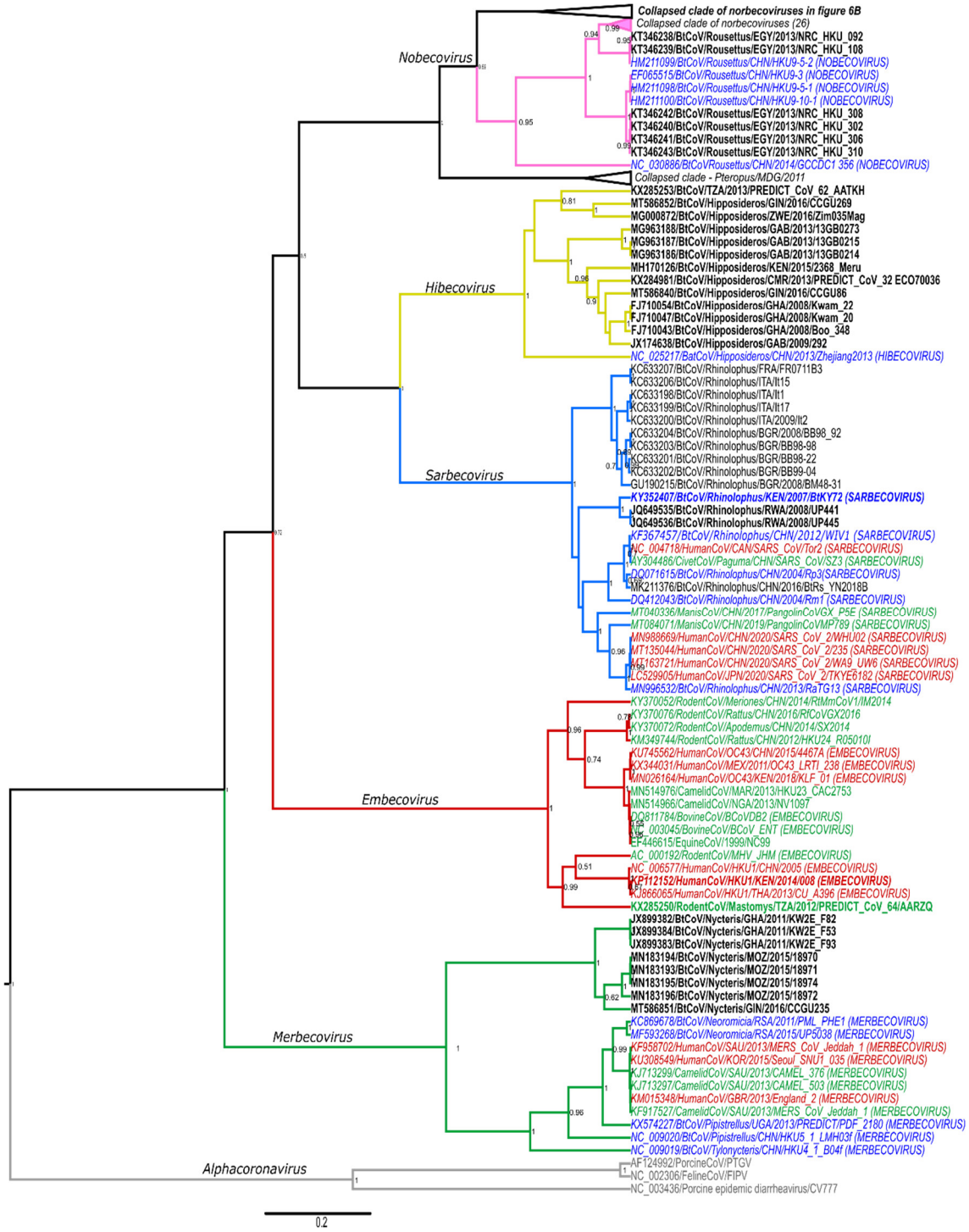
The three available viral full genomes recovered from *Neoromicia (Laephotis)* and *Pipistrellus* were used to classify the viruses as belonging to the same viral species as human and camel MERS-CoV. Within the bat-borne MERS-related viral genomes, the spike genes shared the lowest similarity to human and camel MERS-CoV spike genes (approximately 63–64% nucleotide identity) [32,58,59]. The latter viruses utilize the DPP4 (Dipeptidely peptidase 4) as an entry receptor. Using homology models based on the crystalized structure of the spike protein of the *Pipistrellus* MERS-related virus, Anthony et al. [59] determined that the bat virus spike was unlikely to utilize DPP4 due to insufficient similarities among the required residues to facilitate binding of the spike to the receptor. This was practically demonstrated when recombinant MERS-CoV particles containing the spike from the *Pipistrellus* MERS-related virus were unable to enter Vero cells (unlike wild-type

MERS-CoV) [59]. Moreover, recombination analysis also identified potential breakpoints within the spike gene for *Neoromicia (Laephotis)* MERS-related virus PML/PHE1 and *Pipistrellus* MERS-related virus PREDICT/PDF-2180 [58,59]. The data thus suggests that the identified bat-borne MERS-related viruses have not served as direct progenitors of MERS-CoV detectable in camels and humans, though whether recombination occurred in a bat host or an intermediate host is uncertain.

Depending on the species, both *N. capensis (reassigned as Laephotis capensis)* and *P. hesperidus* have widespread distributions in various parts of Africa [67,68]. *N. capensis* (*L. capensis*) is an abundant and adaptable species distributed from sub-Saharan Africa to South Africa. They typically roost under bark or rock crevices that limit roost sizes to a few individuals [96]. However, these bats have adapted to occupy increasingly available urban roost sites such as cracks in walls and the roofs of houses, which allow populations over 50 individuals to congregate [96,97]. As a result, *N. capensis (L. capensis)* is a common species in urban areas that beneficially aid in decreasing insect populations attracted by city lights. Conversely, *P. hesperidus* is not very abundant and sparsely populated within its distribution from sub-Saharan Africa (Ethiopia down) to South Africa [98].

Betacoronaviruses—Sarbecoviruses with African Rhinolophids

Bat coronavirus sequences sharing similarity to human sarbecoviruses (SARS-CoV and SARS-CoV 2) have been identified throughout the geographic distribution of rhinolophid bats in Asia, Europe, and Africa. The highest genetic similarities between human and bat sarbecoviruses (Rp3, HKU3, WIV1, WIV16, ZXC21, ZC45, RaTG13, RmYN02) originate in Asia [5,76,77,82,99]. Bat species from the *Rhinolophus* genus are considered the main hosts for the genetic diversity of bat sarbecoviruses [16,66,88]. Some species occurring in Europe have also been reported from Northern Africa, such as *Rh. ferrumequinum* and *Rh. euryale*; and are known hosts of sarbecoviruses [66], but very few sequences with similarity to members of the *Sarbecovirus* subgenus have been identified in Africa (Figure 6A). Reports include partial RdRp sequences from two species (*Rh. hildebrandtii* and *Rh. clivosus*) from Kenya, Rwanda, and Uganda (non-universal surveillance region) with similarity to SARS-CoV [19,35,36,100]. Further sequencing of the complete genome of BtCoVKY72 detected from a *Rhinolophus* sp. from Kenya identified the virus as a member of the *Severe acute respiratory syndrome-related coronavirus* species within the *Sarbecovirus* subgenus [100].


Collapsed clade of norbecoviruses in figure 6B
Collapsed clade of norbecoviruses (26)
KT346238/BtCoV/Rousettus/EGY/2013/NRC_HKU_092
KT346239/BtCoV/Rousettus/EGY/2013/NRC_HKU_108
HM211099/BtCoV/Rousettus/CHN/HKU9-5-2 (NOBECOVIRUS)
EF065515/BtCoV/Rousettus/CHN/HKU9-3 (NOBECOVIRUS)
HM211098/BtCoV/Rousettus/CHN/HKU9-5-1 (NOBECOVIRUS)
HM211100/BtCoV/Rousettus/CHN/HKU9-10-1 (NOBECOVIRUS)
KT346242/BtCoV/Rousettus/EGY/2013/NRC_HKU_308
KT346240/BtCoV/Rousettus/EGY/2013/NRC_HKU_302
KT346241/BtCoV/Rousettus/EGY/2013/NRC_HKU_306
KT346243/BtCoV/Rousettus/EGY/2013/NRC_HKU_310
NC_030886/BtCoVRousettus/CHN/2014/GCCDC1 356 (NOBECOVIRUS)
Collapsed clade - Pteropus/MDG/2011
KX285253/BtCoV/TZA/2013/PREDICT_CoV_62_AATKH
MT586852/BtCoV/Hipposideros/GIN/2016/CCGU269
MG000872/BtCoV/Hipposideros/ZWE/2016/Zim035Mag
MG963188/BtCoV/Hipposideros/GAB/2013/13GB0273
MG963187/BtCoV/Hipposideros/GAB/2013/13GB0215
MG963186/BtCoV/Hipposideros/GAB/2013/13GB0214
MH170126/BtCoV/Hipposideros/KEN/2015/2368_Meru
KX284981/BtCoV/Hipposideros/CMR/2013/PREDICT_CoV_32 ECO70036
MT586840/BtCoV/Hipposideros/GIN/2016/CCGU86
FJ710054/BtCoV/Hipposideros/GHA/2008/Kwam_22
FJ710047/BtCoV/Hipposideros/GHA/2008/Kwam_20
FJ710043/BtCoV/Hipposideros/GHA/2008/Boo_348
JX174638/BtCoV/Hipposideros/GAB/2009/292
NC_025217/BatCoV/Hipposideros/CHN/2013/Zhejiang2013 (HIBECOVIRUS)
KC633207/BtCoV/Rhinolophus/FRA/FR0711B3
KC633206/BtCoV/Rhinolophus/ITA/It15
KC633198/BtCoV/Rhinolophus/ITA/It1
KC633199/BtCoV/Rhinolophus/ITA/It17
KC633200/BtCoV/Rhinolophus/ITA/2009/It2
KC633204/BtCoV/Rhinolophus/BGR/2008/BB98_92
KC633203/BtCoV/Rhinolophus/BGR/BB98-98
KC633201/BtCoV/Rhinolophus/BGR/BB98-22
KC633202/BtCoV/Rhinolophus/BGR/BB99-04
GU190215/BtCoV/Rhinolophus/BGR/2008/BM48-31
KY352407/BtCoV/Rhinolophus/KEN/2007/BtKY72 (SARBECOVIRUS)
JQ649535/BtCoV/Rhinolophus/RWA/2008/UP441
JQ649536/BtCoV/Rhinolophus/RWA/2008/UP445
KF367457/BtCoV/Rhinolophus/CHN/2012/WIV1 (SARBECOVIRUS)
NC_004718/HumanCoV/CAN/SARS_CoV/Tor2 (SARBECOVIRUS)
MY304486/CivetCoV/Paguma/CHN/SARS_CoV/SZ3 (SARBECOVIRUS)
DQ071615/BtCoV/Rhinolophus/CHN/2004/Rp3(SARBECOVIRUS)
MK211376/BtCoV/Rhinolophus/CHN/2016/BtRs_YN2018B
DQ412043/BtCoV/Rhinolophus/CHN/2004/Rm1 (SARBECOVIRUS)
MT040336/ManisCoV/CHN/2017/PangolinCoVGX_P5E (SARBECOVIRUS)
MT084071/ManisCoV/CHN/2019/PangolinCoVMP789 (SARBECOVIRUS)
MN988669/HumanCoV/CHN/2020/SARS_CoV_2/WHU02 (SARBECOVIRUS)
MT135044/HumanCoV/CHN/2020/SARS_CoV_2/235 (SARBECOVIRUS)
MT163721/HumanCoV/CHN/2020/SARS_CoV_2/WA9_UW6 (SARBECOVIRUS)
LC529905/HumanCoV/JPN/2020/SARS_CoV_2/TKYE6182 (SARBECOVIRUS)
MN996532/BtCoV/Rhinolophus/CHN/2013/RaTG13 (SARBECOVIRUS)
KY370052/RodentCoV/Meriones/CHN/2014/RtMmCoV1/IM2014
KY370076/RodentCoV/Rattus/CHN/2016/RfCoVGX2016
KY370072/RodentCoV/Apodemus/CHN/2014/SX2014
KM349744/RodentCoV/Rattus/CHN/2012/HKU24_R05010I
KU745562/HumanCoV/OC43/CHN/2015/4467A (EMBECOVIRUS)
KX344031/HumanCoV/MEX/2011/OC43_LRTI_238 (EMBECOVIRUS)
MN026164/HumanCoV/OC43/KEN/2018/KLF_01 (EMBECOVIRUS)
MN514976/CamelidCoV/MAR/2013/HKU23_CAC2753
MN514966/CamelidCoV/NGA/2013/NV1097
DQ811784/BovineCoV/BCoVDB2 (EMBECOVIRUS)
NC_003045/BovineCoV/BCoV_ENT (EMBECOVIRUS)
EF446615/EquineCoV/1999/NC99
AC_000192/RodentCoV/MHV_JHM (EMBECOVIRUS)
NC_006577/HumanCoV/HKU1/CHN/2005 (EMBECOVIRUS)
KP112152/HumanCoV/HKU1/KEN/2014/008 (EMBECOVIRUS)
KJ866065/HumanCoV/HKU1/THA/2013/CU_A396 (EMBECOVIRUS)
KX285250/RodentCoV/Mastomys/TZA/2012/PREDICT_CoV_64/AARZQ
JX899382/BtCoV/Nycteris/GHA/2011/KW2E_F82
JX899384/BtCoV/Nycteris/GHA/2011/KW2E_F53
JX899383/BtCoV/Nycteris/GHA/2011/KW2E_F93
MN183194/BtCoV/Nycteris/MOZ/2015/18970
MN183193/BtCoV/Nycteris/MOZ/2015/18971
MN183195/BtCoV/Nycteris/MOZ/2015/18974
MN183196/BtCoV/Nycteris/MOZ/2015/18972
MT586851/BtCoV/Nycteris/GIN/2016/CCGU235
KC869678/BtCoV/Neromicia/RSA/2011/PML_PHE1 (MERBECOVIRUS)
MF593268/BtCoV/Neromicia/RSA/2015/UP5038 (MERBECOVIRUS)
KF958702/HumanCoV/SAU/2013/MERS_CoV_Jeddah_1 (MERBECOVIRUS)
KU308549/HumanCoV/KOR/2015/Seoul_SNU1_035 (MERBECOVIRUS)
KJ713299/CamelidCoV/SAU/2013/CAMEL_376 (MERBECOVIRUS)
KJ713297/CamelidCoV/SAU/2013/CAMEL_503 (MERBECOVIRUS)
KM015348/HumanCoV/GBR/2013/England_2 (MERBECOVIRUS)
KF917527/CamelidCoV/SAU/2013/MERS_CoV_Jeddah_1 (MERBECOVIRUS)
KX574227/BtCoV/Pipistrellus/UGA/2013/PREDICT/PDF_2180 (MERBECOVIRUS)
NC_009020/BtCoV/Pipistrellus/CHN/HKU5_1_LMH03f (MERBECOVIRUS)
NC_009019/BtCoV/Tylonycteris/CHN/HKU4_1_B04f (MERBECOVIRUS)
AF124992/PorcineCoV/PTGV
NC_002306/FelineCoV/FIPV
NC_003436/Porcine epidemic diarrheavirus/CV777
Nobecovirus
Hibecovirus
Sarbecovirus
Embecovirus
Merbecovirus
Alphacoronavirus
0.2

(**A**)

(**B**)

Figure 6. (**A**,**B**): Bayesian *Betacoronavirus* phylogeny of a 294-nucleotide sequence region of the RdRp gene. Shorter sequences were omitted. Clades collapsed in A are shown in B (and vice versa), and the collapsed clade of *Eidolon* nobecoviruses may be viewed in Figure S2). Sequences in italics indicate formally recognized species (subgenera are indicated in capital letters at the end of sequence names); sequences in bold originate in Africa; red highlights human viruses; green indicate non-bat animal hosts; blue/italics indicate formally recognized bat species; orange indicate viral detections from hosts not typically associated with a particular group of coronaviruses. All sequence names were edited to conform to the correct convention, with the modification of the unique sequence identifier listed last due to convenience. Only posterior probabilities of greater than 0.5 are indicated. No unpublished sequences were included.

This limited detection of sequences similar to sarbecoviruses may be due to lacking surveillance of individuals within the *Rhinolophus* host genus. There are 38 extant *Rhinolophus* species in Africa, with approximately 728 individuals from 14 species included in published surveillance efforts from 11 countries (Table S4). However, very small sample sizes averaging between 1–62 individuals have been tested per species. To our knowledge, no bat coronaviruses sharing high similarity to the SARS-CoV 2 clade sarbecoviruses have been identified from African bats. In addition to betacoronaviruses, unclassified alphacoronaviruses have also been identified from four *Rhinolophus* species, suggesting large diversities of coronaviruses to be present in these bats [19,29,33].

Rhinolophids are taxonomically challenging to identify with frequent revisions to species due to highly convergent morphology [67]. Certain species are widespread and have distributions spanning into other continents, such as *Rh. clivosus* from Africa and into South West Asia [101]. These bats generally roost in caves, unused mines, and buildings [67] and are threatened by disturbances to roosts such as mining and the use of pesticides and insecticides [102], though provide valuable ecosystem services by decreasing the populations of crop-damaging insects [102].

Sequences with similarity to sarbecoviruses have also been reported from non-rhinolophid genera, including *Chaerephon* spp. in Kenya and hipposiderids in Rwanda, Cameroon, and the Republic of the Congo [26,30,36]. The latter hosts' detections were few and may represent transient spillover between hosts (*Rhinolophus* and *Hipposideros*), possibly co-roosting. In addition, some other studies have reported the detection of viruses with homology to SARS-CoV in hipposiderid bats, though these viruses were part of a more distant sister clade than rhinolophid SARS-related viruses. Moreover, this sister-clade was later formally classified as the *Hibecovirus* subgenus. Due to the thorough surveillance of hipposiderid bats, these viruses have been reported from various countries, including Ghana, Gabon, Nigeria, Kenya, Rwanda, Zimbabwe, Guinea, and Rwanda (Tables S4 and S5).

Betacoronaviruses—Nobecoviruses and Fruit Bats

Members of the *Nobecovirus* subgenus are not currently associated with any known zoonotic diseases, though much like the aforementioned molossid alphacoronaviruses warrant further investigation due to their widespread occurrence in several abundant fruit bat species [79]. Nearly two-thirds of all the unclassified sequences in Figure S2 likely represent members of this subgenus. Described species in this genus include two Asian bat viruses, *Rousettus bat coronavirus HKU9* and *Rousettus bat coronavirus GCCDC1* detected in species such as *Rousettus leschenaultia* [80,103], as well as *Eidolon bat coronavirus C704* in Cameroon [34]. The African detections sharing similarities to members of the *Nobecovirus* subgenus are indicated in Table S5. These detections have been widespread and predominantly reported from fruit bat genera such as *Rousettus, Eidolon*, *Micropteropus, Epomophorus, Pteropus, Epomops, Myonycteris* (formerly *Lissonycteris*), and *Megaloglossus* [19,26–30,34,36,39,47]. Additionally, similar sequences have been reported from several insectivorous bat species, though whether these represent active maintenance of the virus in these hosts or transient spillover is unclear. Recombination events have been detected between species of the *Nobecovirus* subgenus identified in *R. leschenaulti* in Asia and rotaviruses (*Reoviridae;* double-stranded RNA viruses) co-infecting the same species, leading to the acquisition of the P10 orthoreovirus fusogenic gene [86].

E. helvum migrates over large distances throughout much of sub-Saharan Africa (Senegal to Ethiopia and down to southern Africa) and are tree-roosting fruit bats that form aggregates of thousands to millions of individuals. Large urban colonies have been recorded in trees of various cities (e.g., Accra in Ghana) [67]. Excreta from these urban colonies would provide ample opportunities for human contact with contaminated fecal and urine. *E. helvum* is also heavily harvested for bushmeat, with estimates of 128,000 bats being sold per year in markets in Ghana alone [67,104]. *R. aegyptiacus* also has a broad

distribution throughout sub-Saharan and parts of Northern Africa, as well as South East Asia and the Western Palaearctic region [67]. This species is a cave-dwelling fruit bat that forms large colonies in the thousands (e.g., 5000 to 50,000), and may co-roosts with multiple insectivorous bat species. Opportunities for contact and possible viral sharing may thus arise between different bat genera, though possible exposure events to humans are more infrequent and generally arise due to human activities. These bats are often threatened by farmers who view fruit-eating bats as destructive to their crops as well as due to mining and other cave disturbances [67,105].

3.1.6. Investigating Factors Affecting the Maintenance of Bat Coronaviruses

Understanding how bat coronaviruses are maintained in their host populations allows determination of infection duration and times that may be at 'higher risk' for coronavirus spillover opportunities. 'High risk' periods coincide with increased excretion of viruses from bats in a colony and may be associated with reproductive or seasonal factors affecting the viral infection dynamics of the colony. For example, an increase of mating activity and accompanying hormonal changes may affect the susceptibility of hosts to infection, or the increase in immunologically naive juveniles at the start of a birthing pulse creates a large population of bats susceptible to infection [106–108]. Understanding these dynamics allows the formulation of management plans to mitigate risks and facilitate engagement with communities at risk of frequent contact with particular bat populations. Behavioral changes may assist in reducing the associated risks of exposure and possible spillover interactions [109].

Limited African studies (only 5) expanded data analyses to include correlations between bat biology, ecology, and viral status of hosts [30,36,38,40,44]. Those investigating increased infection among age classes agree that subadults are more likely to host coronaviruses than adults [30,36,44], consistent with reports from other continents [109]. A higher frequency of infection was also identified among lactating females [44], though also males [30]. Most disagreements center around seasonality, with either no correlation identified [36] or a higher chance of detecting coronaviruses in the dry seasons [30]. Longitudinal surveillance projects would be able to assist with such interpretations in the future.

Bats occupy a wide range of niches, including diverse roost preference (e.g., cave-dwelling or tree-roosting), eating habits (frugivores, nectivores, insectivores, etc.), population sizes (less than 10 to thousands), and level of social interaction between the same and different species (gregarious or non-gregarious). It may also be possible that factors affecting the maintenance of coronavirus infection among bat species may not be universal to all bat species. Thus, combining coronavirus data from different species may result in biased conclusions. For example, it has been suggested that bat coronaviruses may amplify within maternity colonies [108], though the reproductive seasons of diverse bat species do not all overlap, and certain species are capable of reproducing more than once a year, depending on the geographic regions. For example, *Rousettus aegyptiacus* displays two birthing pulses among populations along the North of Africa [110], while populations in Southern Africa have only one [111]. Thus, if coronavirus maintenance is linked to its host species' reproductive biology, viral shedding may be predictable for certain species in particular climate zones.

A recent study predicted high-risk periods for different host species utilizing available surveillance data from three countries (Rwanda, Uganda, and Tanzania) and Bayesian modeling [30,60]. Though several assumptions were made regarding the duration of lactation and weaning, they determined that juveniles recently weaned were 3.34 times more likely to shedding coronavirus RNA than juveniles that were not recently weaned. Even adults were nearly four times more likely to be shedding coronaviruses when juveniles were being weaned [60], possibly due to increased coronavirus excretion levels within the colony. As described in Wacharapluesadee et al., [109], increased coronavirus shedding

among juvenile bats may be due to vertical transmission from mother to pup, which coincides with studies describing viral shedding from lactating females with increased frequency compared to non-lactating females [112]. The higher frequencies observed in recently weaned juveniles may be due to the loss of maternally received antibody protection following weaning [60,108]. These conclusions require confirmation with longitudinal surveillance among investigated bat species as well as serological studies determining changing antibody levels between lactating mothers, weaning and non-weaning juveniles, as well as other adults in the colony.

3.2. Surveillance in Other Wildlife and Domestic Animals (Livestock)

Coronavirus nucleic acid surveillance among non-bat wildlife, livestock, or other domestic animals in Africa is very limited, both in the frequency of research, sample sizes of animals tested, locations targeted, and are frequently investigated for only specific coronaviruses. Nucleic acid testing in animal populations where the prevalence of infection may be very low would yield limited data, provided that sampling was performed at a time when animals are infected or actively excreting viruses [113]. We only identified four reports in which other animals were tested for coronavirus nucleic acids, including anthroponoses of HCoVOC43 between humans and chimpanzees in Côte d'Ivoire [48], MERS-CoV specific surveillance among 4248 livestock animals from Ghana (cattle, sheep, donkeys, goats, and pigs) [50], general surveillance among 731 wildlife animals (rodents, non-human primates, and ad hoc samples of other wildlife) in Gabon [40], as well as just over 27,000 animals (birds, domestic animals, carnivores, pangolins, swine, rodents, and non-human primates) as part of the PREDICT surveillance initiative (accessed via Healthmap.org) (Table 4). Though this seems like a significant number of individuals tested, the total species diversity among all 16 countries sampled is much larger than the fraction represented by this surveillance. Moreover, not all hosts listed were surveyed in all countries (Table 4), with mostly opportunistic sampling from accessible individuals. However, even though the total positives detected in relation to the total number sampled is <1%, it still shows the presence of coronaviral RNA from among non-human primates (14 chimpanzees), ungulates (1 bush duiker), carnivores (1 African palm civet) and rodent species (13 individuals) from opportunistic surveillance [48,56].

Two of these sequences, publicly available and corresponding to the universal surveillance region (excluding the anthroponoses of HCoVOC43 from the chimpanzees), were included in the phylogenies in Figures 5A and 6A (KX285508 and KX285250). Most of the detected African rodent coronavirus partial sequences are phylogenetically placed in the *Embecovirus* subgenus, with human coronaviruses OC43 and HKU1 and other rodent coronaviruses from Asia [18,30]. Divergent rodent alphacoronavirus virus RNA was also identified (KX285508), as well as highly divergent shrew coronaviruses [30]. The sequence information confirms surveillance data from Asia and Europe, namely that rodents and shrews likely harbour additional undiscovered diversity of coronaviruses. Improved systematic and longitudinal surveillance of wildlife and domestic populations will provide more data on the presence of coronaviruses among these animal groups. The research is too limited to make any conclusions regarding the absence of viral sharing between animal groups. Additionally, serological surveillance would complement nucleic acid surveillance by providing data on hosts not actively infected with coronaviruses.

Not included in Table 4 is the expansive surveillance of dromedary camel populations for MERS-CoV. MERS-CoV is not only endemic to the dromedary camel populations of the Middle East but also populations in Northern Africa (Burkina Faso, Ethiopia, Kenya, Mali, Morocco, Nigeria, Somalia, Sudan, Tunisia) [93,114]. Seroprevalence of adult dromedaries is high (80–100%) and may result in respiratory disease with viral shedding via nasal discharge [93,115]. Despite this widespread occurrence, MERS infections among people from camels have only been reported from the Arabian Peninsula [114,115]. Viruses from African dromedaries form a separate basal lineage to the two clades of MERS-CoV identified from infected people and camels on the Arabian Peninsula [114,116],

though still share antigenic similarities through cross-neutralization [114]. Furthermore, within this African clade, genomes from the West and North African dromedary populations (Nigeria, Burkina Faso, Morocco) display deletions in specific accessory genes [114,117,118]. It has been suggested that these accessory genes are not required for the adaptation of the virus to dromedary camels and may have been necessary for a more historical host [114]. Whether bat-borne MERS-related viruses established in dromedary camel populations can only be addressed with better surveillance of African bat and dromedary populations, especially where bat and camelid distributions overlap [32].

Table 4. Summary of animals (non-bat) tested for coronavirus nucleic acids.

Animals Groups	Birds [1] and Poultry/ Other Fowl	Carivores [2]	Cattle/ Buffalo [3]	Dogs [4]	Goats/ Sheep [4]	Non-Human Primates	Pango-lins [5]	Rodents/ Shrews	Swine [4]	Ungu-lates [7]	Other [6]	Grand Total
Cameroon	-	**67**	-	-	-	3475	79	**4653**	-	144	16	8434
DR Congo	7	6	10	-	16	1574	3	1848	1	15	2	3482
Ethiopia	-	-	-	-	-	454	-	-	-	-	-	454
Gabon	1	11	-	-	-	82	18	1141	-	548	37	1838
Ghana	-	-	1230	-	2194	496	-	532	716	108	-	5276
Guinea	-	-	-	6	321	-	-	904	8	-	-	1239
Ivory Coast	12	-	-	-	-	**59**	-	293	-	-	-	364
Kenya	-	-	-	-	-	334	-	369	-	514	-	1217
Liberia	-	-	-	-	-	-	-	205	-	-	-	205
Republic of Congo	-	2	-	-	-	352	-	**461**	-	14	-	829
Rwanda	-	-	-	-	-	762	-	**708**	-	-	-	1470
Senegal	-	-	-	-	-	253	-	**263**	-	-	-	516
Sierra Leone	-	5	-	318	938	15	-	369	1012	-	-	2657
South Sudan	-	-	-	-	-	-	-	46	-	-	-	46
Tanzania	-	8	53	120	105	444	-	**1513**	95	**39**	1	2378
Uganda	-	-	-	-	13	1238	-	**762**	1	83	-	2097
Grand Total	20	99	1293	444	3587	9538	100	14,067	1833	1465	56	32,502
Coronavirus nucleic acid	-	1	-	-		14	-	13	-	1	-	29

[1] Unspecified; [2] carnivores (genets, mongoose, and civets; domestic cats); [3] domestic and African buffalo; [4] domestic; [5] tree and long-tailed pangolins; [6] ungulates (including camels, duikers, and antelope among others); [7] 'other' (reptiles, snakes, tortoise, hyraxes, and elephants). For species information review [56]. Numbers shaded in bold indicate positive detections from an animal group and country. No recorded surveillance is indicated with a '-'.

4. Coronavirus Serosurveillance

Coronavirus serology is complex and faces several challenges—even among human coronaviruses [113]. Serological targets include the immunogenic nucleoprotein that is abundant during infections and the spike protein that allows for the detection of more specific antibody responses and neutralizing antibodies [119]. Targeting a more conserved protein (such as the nucleoprotein) may yield high seropositivity levels due to potential cross-reactivity of conserved epitopes among related coronaviruses, without being able to discern between different viral species (or genera). Depending on the assay target, cross-reactivity could complicate human coronavirus assays due to conserved motifs between seasonal human coronaviruses, SARS-CoV, and MERS-CoV [113], as well as between SARS-CoV and SARS-CoV 2 [120]. Serosurveillance among animal populations is similarly hampered with cross-reactivity as they may be exposed to unidentified coronaviruses. Due to the challenges of cultivating certain animal coronaviruses, virus neutralization tests to exclude cross-reactions are not readily feasible. A lack of specific animal coronavirus assays often leads to the use of human coronavirus assays (generally based on the spike protein). However, interpreting the results should be made with caution as cross-reactivity to unknown epitopes and modifications to validated assays may allow for false assumptions [113]. There is a great need to develop suitable assays for serological surveillance of diverse coronaviruses in wildlife and domestic animals. The lack of well-

characterized reference sera to determine cut-off thresholds and limited species-specific biologics also challenges new assay development.

Bat coronavirus serology is demanding for all the aforementioned reasons and is further complicated by the large diversity of bat coronaviruses. Of note is that not all bat coronaviruses utilize the same receptor molecules. Angiotensin-converting enzyme two or ACE2 is the known receptor for SARS-CoV, SARS-CoV 2, and only the most closely related bat sarbecoviruses. The receptor-binding regions and important motifs even differ greatly between SARS-CoV and SARS-CoV 2 (see Andersen et al. [13]). The spike receptors for the larger majority of bat sarbecoviruses lack the required binding sites and are largely incompatible with human ACE2. The spike proteins of BtCoVKY72 only share 68–72% amino acid similarity to the spike proteins of SARS-CoV and SARS-CoV 2 and their most closely related bat viruses (unpublished data). Though, protein similarity alone cannot be used to determine if cross-reaction will occur due to the glycosylation and conformational folding of spike proteins [113].

In comparison to the number of studies investigating bat coronavirus nucleic acid surveillance, minimal serosurveillance studies have been performed on the continent. These include mainly Muller et al. [121], wherein a SARS-CoV ELISA kit with minor modifications was used to tests bat sera from the Democratic Republic of the Congo (DRC) and South Africa, as well as a MERS-CoV pseudo-particle neutralization assay to test *Rousettus* sera in Egypt and Lebanon by Shehata et al. [27]. Though no MERS-antibodies were detected in *Rousettus aegyptiacus*, antibodies reactive to SARS-CoV antigens were identified in 6.7% of bats tested (7 of 26 species) from the DRC and South Africa. These species include pteropid bats (*Rousettus*, *Myonycteris,* and *Hypsignathus*) as well as other insectivorous bat genera like *Mops, Miniopterus,* and *Rhinolophus*; many of these genera have since been identified to host either alpha- or betacoronaviruses. The results were confirmed with western blots, though no neutralizing antibodies were identified [121], cross-reactivity between potentially related bat coronaviruses. Increased bat coronavirus serological surveillance would provide better overall estimates of population exposure levels [119,122] and reduce false-negative assumptions from non-actively shedding hosts.

Wildlife, livestock or domestic animal serological surveillance in Africa is more frequent than serological surveillance among bats. A broad search of the literature found mainly studies focused on MERS-CoV serology and dromedary camel populations among various countries (reviewed in Dighe et al., [93]). Among domestic animals, several studies investigated livestock in Ghana [49–51]. Bovine coronavirus was determined to possibly be widespread among ruminants such as cattle and capable of spilling over into sheep and goats [51]. Cattle, sheep, goats, donkeys, and swineherds have been found lacking any serological response toward merbecoviruses like MERS-CoV or the similar *Nycteris* bat betacoronaviruses [50], or indeed HCoVNL63 and related bat viruses [49]. The authors highlight the need for such surveillance to be conducted in countries such as Kenya, where similar viruses to HCoV229E or HCoVNL63 were identified in bats.

Limited serosurveillance has been performed in wildlife. Though no feline coronavirus serological responses were identified among 13 lions from Botswana sampled between 2012 and 2014 [123], feline coronaviruses (particularly the highly pathogenic feline infectious peritonitis virus) have historically been shown to be actively circulating among captive cheetahs in the USA and free-living cheetah populations from Eastern and Southern Africa [124,125]. This lack of thorough surveillance in animals that may act as intermediate hosts and detecting spillover infections creates a gap in data not only for Africa but globally. Moreover, to our knowledge, no studies have investigated human populations in Africa for serological responses to bat coronavirus spillover [126].

5. Factors Associated with the Potential Emergence of Coronaviruses

Opportunities for potential pathogen exposure between humans and animals, including wildlife, are increasing. In Africa, the main factors include deforestation, agricultural intensification, and the collection, hunting, and butchering of bushmeat [22,127,128].

Interactions that are more specific to bats include ecotourism, mining, guano collection for fertilizer [128], or bat species that roost in man-made structures, such as houses, warehouses, schools, etc. Coronavirus nucleic acids are still detectable in guano fertilizer several days after collection, even if kept at room temperature (though viral isolation was not attempted) [129]. Although some factors may create opportunities for spillover, the exact routes of transmission are not yet clear. [130]. Research investigating potential interfaces in Africa is limited.

The bushmeat trade represents one of the most prominent points of contact between humans and bats on various continents [131], though it may practically represent a low risk of transmission for coronaviruses. Bushmeat serves as an important source of protein and household income in many African, Asian, and South American countries [132,133]. Large bats from the *Eidolon* or *Hypsignathus* genera are predominantly hunted, though smaller bats (*Hipposideros, Rhinolophus,* and *Myotis,* among others) are not excluded [133]. For sub-Saharan Africa alone, 52 African bat species (Table S4) are reportedly hunted in countries across their distribution [133]. *Alpha-* and *Betacoronavirus* sequences have been reported from at least 12 and 14 of these bat species, respectively (Table S4). Notably, viral sequences putatively grouping within the *Duvinacovirus*, *Sarbecovirus,* and *Hibecovirus* subgenera have been detected in one of the hunted bat species, namely *Hipposideros ruber* [30,36,37]. A large number of species deemed as bushmeat have, however, not been included in any coronavirus surveillance studies, and thus, their propensity as viral hosts and associated risk to humans remains to be determined.

Live animal markets have been labelled as an ideal interface for human exposure and disease emergence and have been scrutinized due to the ongoing global COVID19 pandemic [134]. As in specific regions of Asia where such markets are commonplace, live or cooked bats are sold in selected African countries [104,135,136]. These bats may also be used in traditional medicine. Additionally, festivals in Africa focused on bats, such as those in Buoyem (Ghana) and Idanre (Nigeria), may provide opportunities for viral spillover [137,138]. The emergence of SARS-CoV and the SARS-CoV 2 pandemic has led to the banning of wet markets from selling live animals in China [132]. Both bans were eventually lifted and remains a point of debate [139,140].

Human-bat interactions are motivated by social, economic and cultural drivers, which form an integral part of infectious disease research. Different cultures have multifaceted perspectives concerning bats, which may be shaped by the local beliefs, use in traditional medicine, knowledge of bat biology, disease risk, or change during periods of food shortages [141–144]. Though limited information is available in Africa, several recent studies have considered the risk perceptions of human populations to bats and their associations with zoonotic diseases [142–147]. Overall, the results suggest that communities have limited knowledge of bats and do not generally perceive bats as a threat [142,143,145]. These perceptions may likely have changed following the COVID-19 pandemic.

With the known diversity of coronaviruses in bat species from Africa and the association of a number of these bats in human activities, exposure to these viruses is inevitable. There have to date not been any reports of novel coronavirus-associated diseases speculated to be of bat origin on the African continent, contrary to the link between bats and sarbecoviruses from Asia [5,16]. There is a clear overlap between practices in Asian and African countries with regards to animal trade. An intricate relationship between the factors associated with disease spillover from bats to humans is likely involved. Identifying the synergistic effects of these factors is simply the first step in understanding their roles in disease emergence.

6. The Future of Coronavirus Surveillance

The majority of African coronavirus surveillance has been focused on nucleic acid detection, estimating the genetic diversity of coronaviruses from bats and largely excluding other wildlife. Very limited epidemiological information is available to understand

and support current assumptions regarding coronavirus maintenance among bat populations (effects of reproductive biology and ecologic impacts). Surveillance among other wildlife species and domesticated animals is so limited that no further conclusions can be reached on their risks. It is clear that bats host the genetic diversity of coronaviruses [17,18,78], but surveillance should be expanded to other species that share the same ecosystem as potential reservoir species and spillover hosts.

Longitudinal surveillance is essential towards understanding how bat coronaviruses are maintained within a species, as well as the occurrence and duration of shedding [109]. Identification of high-risk shedding periods can direct additional surveillance in other species and allow the formulation of preventative mitigation measures by decreasing possible interactions between human, livestock, and bat population. Determining possible increased shedding times can also allow better planning for surveillance studies to avoid sample collection of cross-sectional studies during the lowest shedding periods. This can be readily accomplished with non-destructive sample collection (colony-level fecal, swabs, or fecal collection). In addition, more basic research is also required for neglected species (Table S4), different animal groups (particularly rodents and livestock) [18] to expand surveillance regions and increase sample sizes.

The reliability of nucleic acid surveillance approaches would be much improved with standardized usage of updated, validated assays such as the recently published assay by Holbrook et al. [148], which updated the widely used Watanabe assay. There has also been an increasing shift away from only publishing short sequences to additional characterization of longer extended sequences or genes. This is both beneficial to the quality of research as well as disadvantageous to having basic surveillance data available. Better characterization of African bat coronaviruses will enable classification of more bat coronaviruses and identifying detectable recombination events. However, this requirement also hampers the frequency of newly published surveillance studies due to escalating costs and sequencing challenges leading to gaps of understanding and unreported diversity among different animal populations. A lack of such standardized approaches also results in technically challenging troubleshooting to be performed in resource-limited laboratories.

Moreover, the cost of fieldwork and sample collection in often remote regions of African countries, as well as the follow-up sample analyses, can be very high, with very little remaining for additional sequencing. Researchers should also be encouraged to publish data on the absence of coronavirus detections to assess species or regions of lower risk. Though not ideal, unpublished nucleic acid surveillance data can also be submitted to NCBI with all relevant collection data. As of August 2020, the user-friendly Database of Bat-associated Viruses (DBatVir) repository contained over 4600 bat coronavirus entries globally [149]. This repository is updated bimonthly, and accessing such a centralized source for bat coronavirus surveillance data (both published and unpublished) will allow for a more comprehensive comparison of detected viruses, assessment of surveillance coverage, and highlight areas where research is required.

We propose that surveillance studies publishing short sequences be bolstered by shifting from detecting viral presence alone to investigating questions concerning the epidemiology and maintenance of coronaviruses in selected populations of different species (Table 5). Bat surveillance in a specific region can be initiated, though it is important that surveillance of other species sharing the same ecological niche be done either concurrently or followed as soon as possible, including potential spillover hosts. Sampling of other animal groups and assessing anthropological and human behavioral risks should be included in the planning and implementation phase. Communities must be at the center of studies to understand societal and cultural issues. Initial surveillance at preselected sites may only provide an overall estimation of animal host species present (bat and non-bat), host movement patterns, and viral excretion, allowing informative planning decisions to be made for proper longitudinal surveillance appropriate sites. Surveillance using short

nucleic acid sequences from an updated assay is thus used to identify diversity and monitor changing excretion fluctuations of viruses in populations over time—either seasonally or based on a predetermined time frame (e.g., monthly). This would allow surveillance of both the presence and diversity of coronaviruses among bats and other sampled wildlife/domestic animals and investigate factors involved in viral maintenance with the collection of ecological data. Additionally, such data can be used for a basic assessment of risk regarding potential opportunities for spillover.

Further research is required to characterize detected coronaviruses, including recovery of complete genomes, incorporation of serological studies among bat populations and spillover hosts, or determination of host ranges and zoonotic potential with pathogenesis studies. Issues of cost or technical challenge may be overcome by collaborating with international institutions. In-country expertise and capacity building are essential to build sustainable surveillance programs and require an interdisciplinary approach.

Table 5. Framework for activity planning when implementing coronavirus surveillance in bat populations, other wildlife species, domesticated animals, and impacted human settlements.

Planning phase	Basic surveillance	Longitudinal surveillance	Extended surveillance

Consideration	Activity
Formulate a strong research question around the aim of the research to be conducted.	Scope of the surveillance—only coronaviruses or broader surveillance. What will the primary focus of the project be? Assessment of risk for settlements near known colonies? Review the literature and determine important species to target.
Assemble an interdisciplinary team	Collaborate with experts in virology, taxonomists, field biologists, veterinarians, ecologists, specific community leaders, social sciences, and policy-makers. A large interdisciplinary team is essential for accurate long-term surveillance.
Identify high-risk species or animal populations based on a predetermined research question	As a starting point, collaborations can assist in identifying accessible locations of interests, such as specific roosts (day or maternity roosts, etc.) for bat host species considered higher risk (from literature). The roosts can be assessed for population presence over time to enable longitudinal surveillance planning. The region must be assessed for nearby human settlements and the occurrence of animals (farmed, free-roaming, or other wildlife).
Perform initial surveillance targeting either large roosts or multiple smaller roosts	Assess viral presence and diversity with once-off or seasonal surveillance (statistically significant). Population-level sampling of excreted samples such as fecal collection (beneath roosting bats) is simple and non-invasive. Proper species identification should be conducted with both barcodes and morphological identification.
Nucleic acid testing with a suitable assay	Review the literature and use a recently updated assay to ensure detection of all available diversity. Test the assay sensitivity for comparisons. Based on the scope of the project and resource

	conservation—consider a specific or randomly primed approach.
Plan longitudinal surveillance (duration, types of samples collected, measurement, and ecological data collection). Plan to survey animal species in the region preferably concurrently or sequentially following bat surveillance.	Based on initial findings, plan for longitudinal surveillance according to specified intervals (based on bat presence at roosts or species movements): seasonal or periodic (monthly). Sampling must occur across different reproductive stages. Surveillance can be done at the population-level (overall) and individual-level (to determine demographics of infection prevalence).
Serological surveillance	Review options for serological assays (commercial or developed assays). Collaboration with experts may be critical. Serological testing (bats, non-bat animals, and humans) is important to understand coronavirus antibody responses, duration of protection, and exposure—optimize suitable assays.
Viral characterization	Recover complete genomes of selected viruses for classification and functional studies. Assessing possible zoonotic potential with pathogenesis studies and protein modeling. Collaborate with specialists that can assist and help develop local capacity.
Investigate human-animal interactions	Perform observational and behavioural studies to assess human-wildlife-livestock interactions.

7. Conclusions

Surveillance of coronaviruses in wildlife and potential spillover hosts is complicated with logistical, technical, and practical challenges. Proper biosurveillance requires detailed planning ahead of time with well-formulated research questions [150] and essential resources such as highly skilled staff, funding, and operating within ethical and regulatory requirements. Availability of research tools such as appropriate diagnostic assays, standardized protocols, and correct species (specifically related to wildlife) identification is paramount. Studies based on nucleic acid detection have been more commonly used, given the lack of suitable or validated serological assays. The development of such assays is further complicated with issues concerning coronavirus culture in vitro and stringent biosafety Level III conditions. The latter limits research to only a few groups when additional characterization, pathogenicity investigations, and determination of the zoonotic potential of newly discovered bat, rodent, and wildlife coronaviruses is needed. The development of recombinant proteins for serological assays and reverse-genetics systems for coronavirus rescue, though technically complex, are some of the only available options at present.

Much of the coronavirus biosurveillance studies reported, particularly in wildlife, has been reactive to outbreaks/newly emerging viruses and very opportunistic. The current coronavirus research identified many coronavirus host species among bats and rodents and provided novel insights into the possible evolutionary origins of some human coronaviruses [25,35,54]. Moreover, specific groups of coronaviruses have been identified for further research due to lack of characterization and high coronavirus diversity among abundant host populations with opportunities for human contact. The studies mainly provided "snap-shots" of diverse coronaviruses among different species, time points, and geographical locations. Such approaches do not allow long-term monitoring of these viruses in host species toward understanding the factors involved in viral maintenance, nor

does it provide cues for interpreting increased risk of spillover. Systematic longitudinal investigations of both natural and potential spillover hosts are needed. Additional layers of investigation must include studying human behavior and anthropological influences and the roles of virus/host interactions, pathogenicity, and the natural ecology of the virus. Investigations of coronavirus diversity among other wildlife (particularly rodents) and livestock are at infancy, with much still unknown. As a result, the future of coronavirus research in African has many topics to cover and will expand continent-wide, requiring an interdisciplinary collaborative approach and significant resource investment.

Supplementary Materials: The following are available online at www.mdpi.com/1999-4915/13/5/936/s1, Figure S1: complete Bayesian BEAST phylogeny of African alphacoronaviruses, Figure S2: complete Bayesian BEAST phylogeny of African betacoronaviruses, Table S1: overview of bat surveillance nucleic acid detection studies, Table S2: molecular methodologies employed by the bat surveillance nucleic acid detection studies, Table S3: summary of bat host species tested for coronavirus RNA and positive species reported, Table S4: coronavirus surveillance performed per bat host species, Table S5: bat species from which coronaviruses have been reported (positive species).

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Appendix A

Details of Phylogenetics

Short sequence lengths may hamper the resolution of a phylogeny, resulting in poor support for certain clades. The phylogenies in Figures S1 and S2 (and Figures 5 and 6) were constructed to include as many African bat coronavirus sequences as possible while still allowing for sequence lengths that would yield well-supported clades. Therefore, sequences that would have resulted in alignments of less than 200 nucleotides were omitted with final lengths between 260 and 294 nucleotides, respectively. For simplicity, all sequence names were converted to the standardized convention with the modification of listing unique sequence identifiers last. Sequences were obtained from Genbank (NCBI) by searching the accession numbers listed in the publications identified as described in Table 1, or manually searching for the publication title. The accession numbers of all sequences included are provided in the phylogenetic trees. Sequence alignments and editing were performed with ClustalW in Bioedit [151]. Maximum clade credibility trees were constructed using suggested models selected from jModelTest2.org [152]. Phylogenetic analyses were performed with Bayesian phylogenetics using BEAST v. 1.10 using the general time-reversible model (GTR) plus invariant sites and gamma distribution substitution

model [153]. The CIPRES Science Gateway was used to run computationally expensive analyses such as alignments, jmodeltest, and BEAST [154]. The Bayesian MCMC chains of the alphacoronavirus phylogeny was set to 20,000,000 states, sampling every 2000 steps, and the betacoronavirus phylogeny was set at 25,000,000 states (sampling every 2500 steps). Final trees were calculated from the 9000 generated trees after discarding the first 10% as burn-in. Trees were viewed and edited in Figtree v1.4.2.

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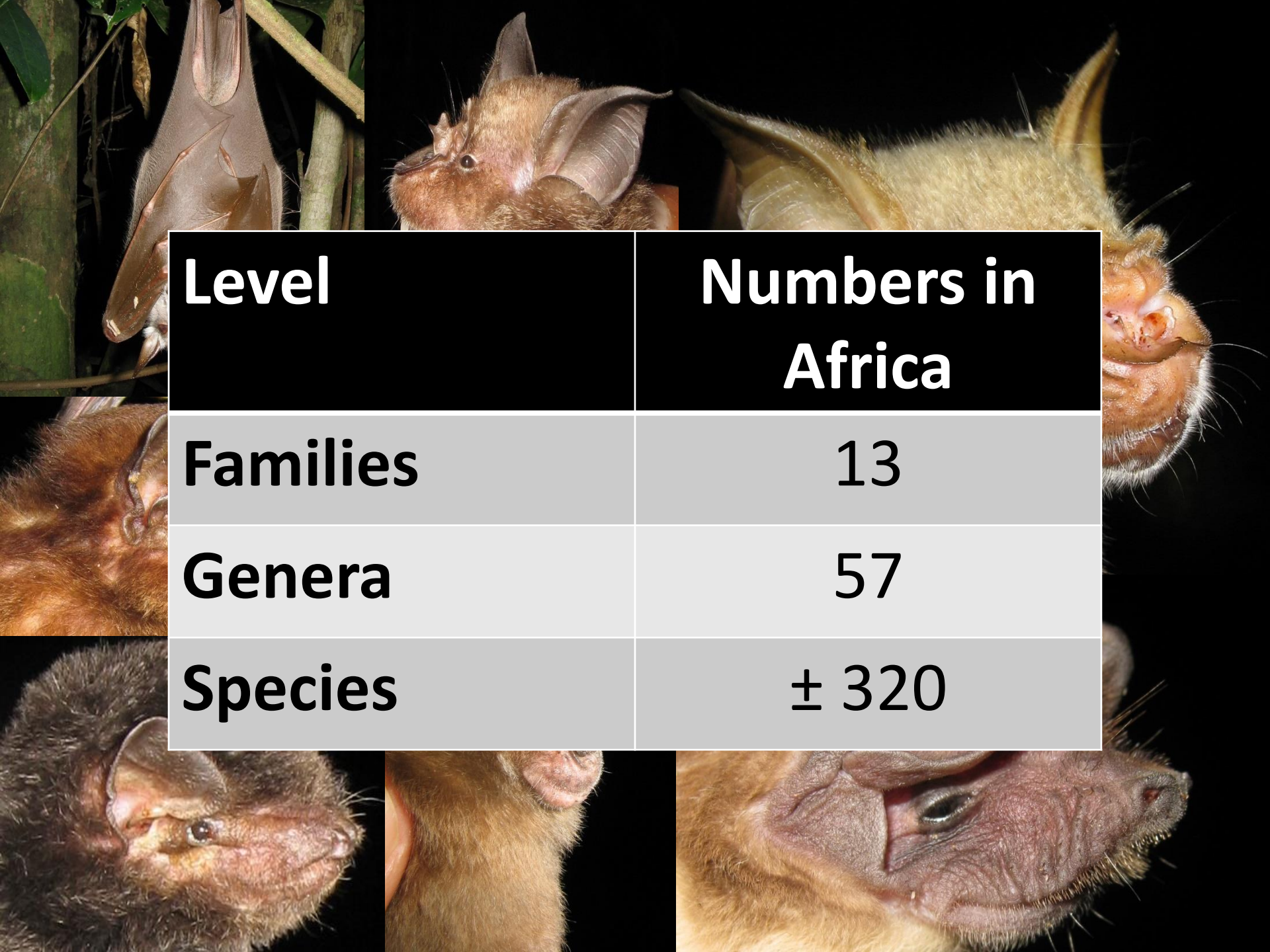
Bat identification workshop

Ara Monadjem

UNIVERSITY OF ESWATINI



Level	Numbers in Africa
Families	13
Genera	57
Species	± 320

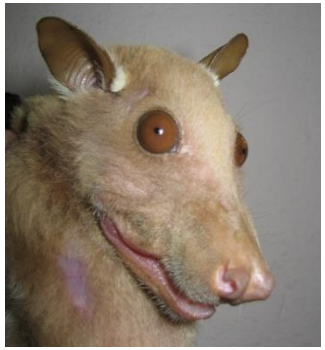
Can anyone identify this bat? Not easy!

How to proceed with identification?

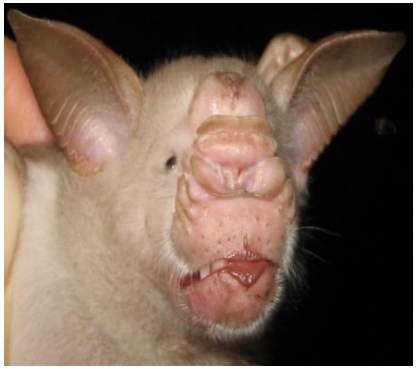
- So, what can we do?
- A useful approach is to identify the bat to a higher taxonomic level
- Class: Mammalia, Order: Chiroptera
- Family, Genus, Species

Southern African families

Pteropodidae



Hipposideridae

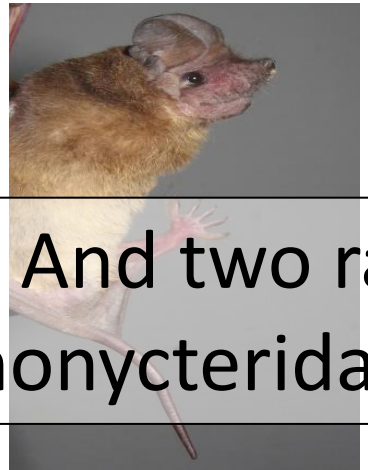


Rhinolophidae

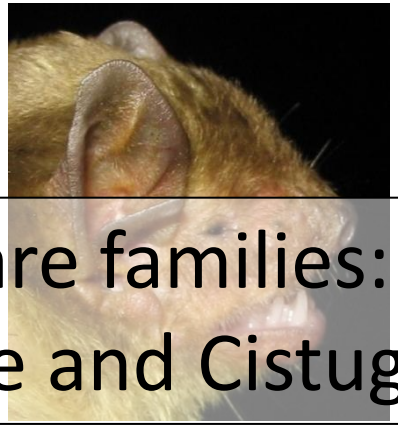
Emballonuridae

Nycteridae

Molossidae



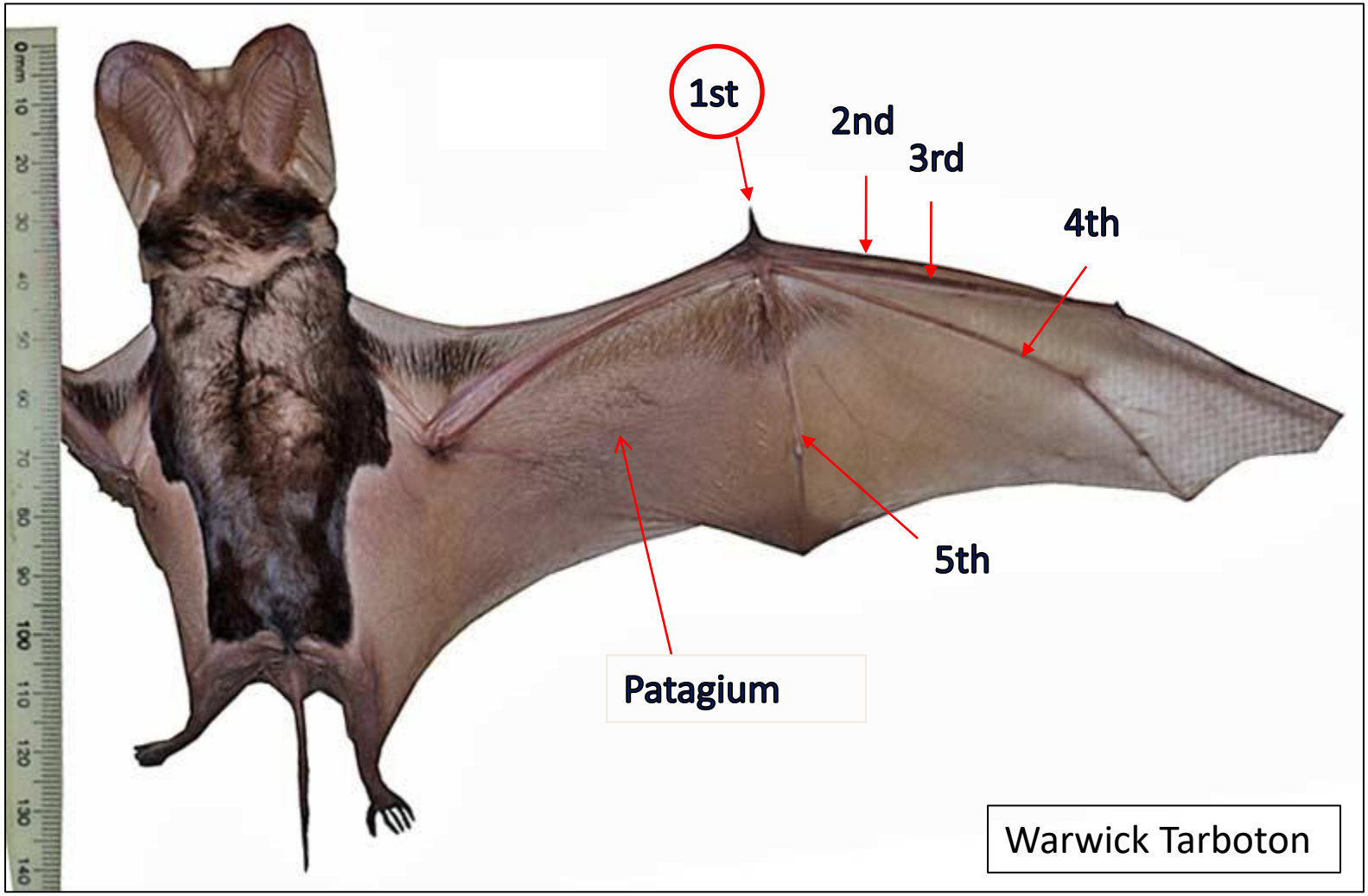
Vespertilionidae



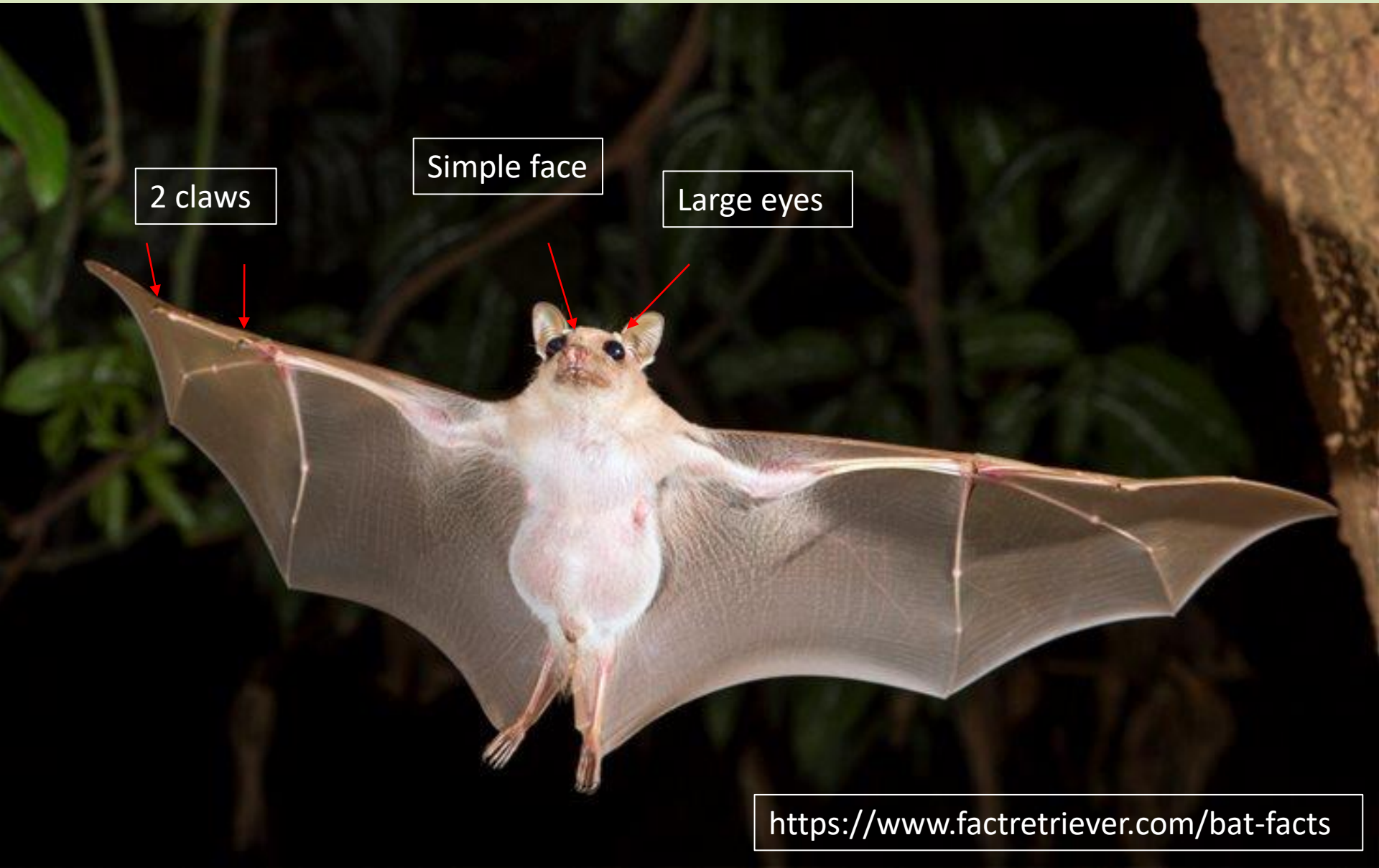
Miniopteridae

And two rare families:
Rhinonycteridae and Cistugidae

Note the claw on 1st digit



Pteropodidae (fruit bats)

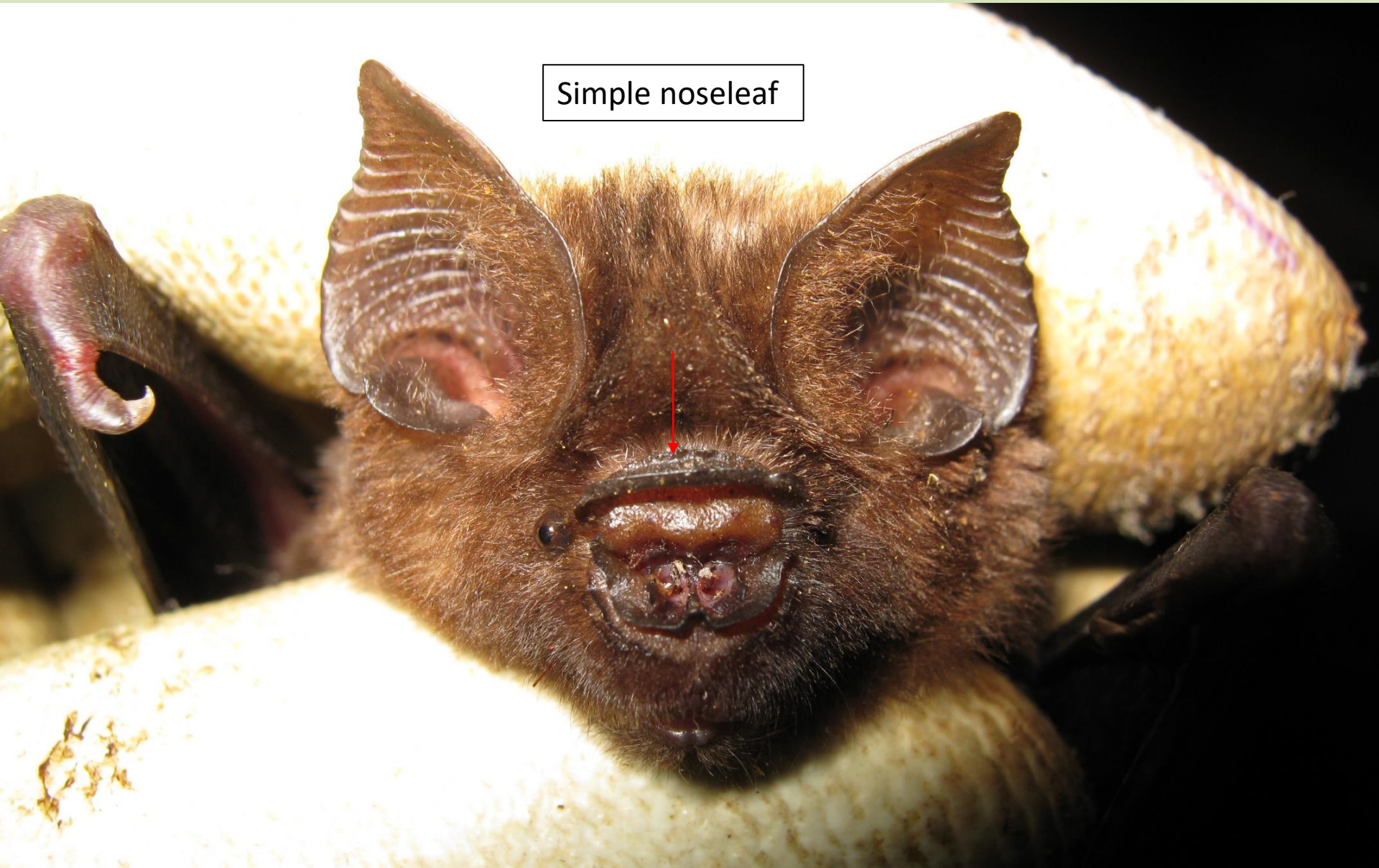


Typical genera/species

Epomophorus wahlbergi

Rousettus aegyptiaca

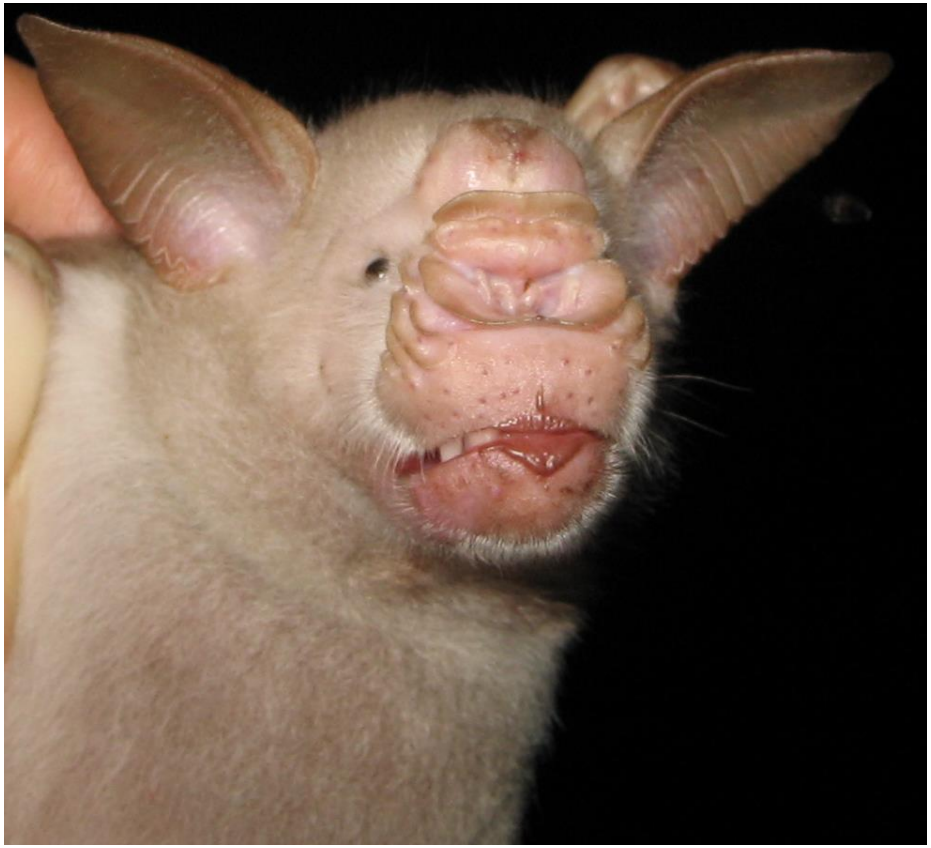
Hipposideridae (leafnose bats)



Typical genera/species

Hipposideros cafer

Macronycteris vittatus



Rhinolophidae (horseshoe bats)

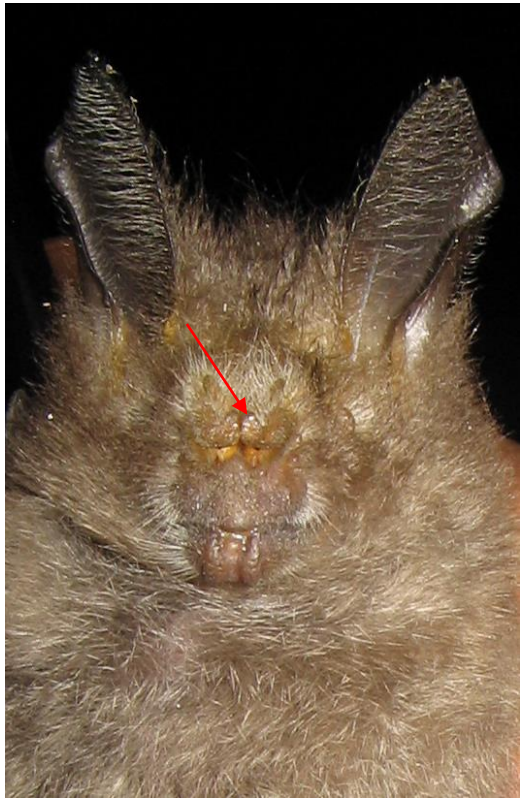
Complex noseleaf

Just one genus: *Rhinolophus* but many species!

Nycteridae (slit-faced bats)

T-shaped tail

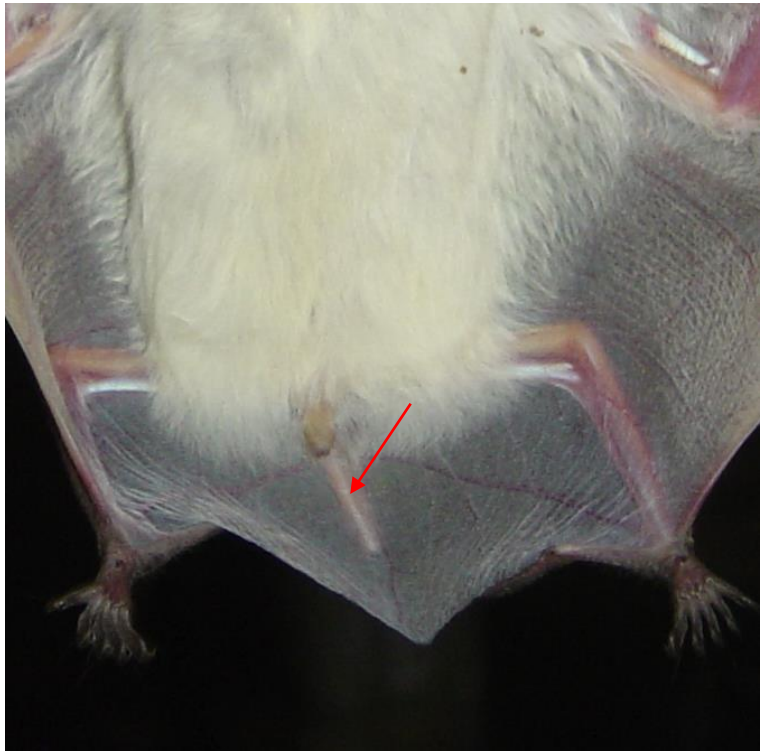
Noseleaf covered



Just one genus: *Nycteris*

Emballonuridae (tomb bats)

Tail protrudes above tail membrane



Large eyes

No noseleaf

Just one genus: *Taphozous*

Molossidae (free-tailed bats)

Tail protrudes beyond membrane

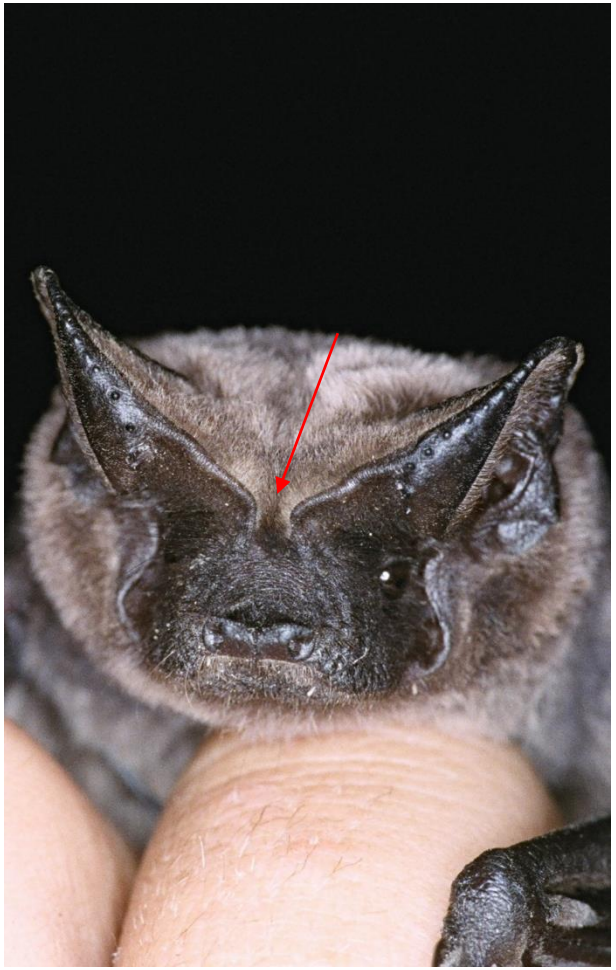
Bulldog-like face

Typical species (taxonomy not resolved)

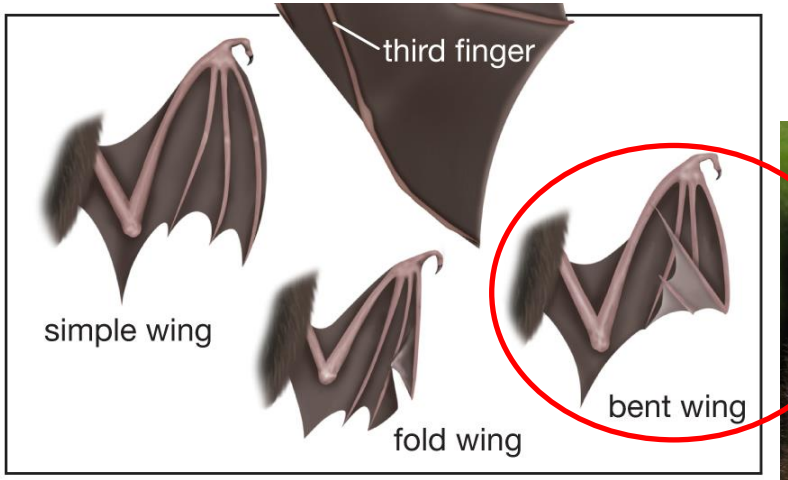
Chaerephon pumilus

Mops condylurus

Tadarida aegyptiaca



Miniopteridae (bent-winged bats)



'Bent' wings

No noseleaf

Domed head

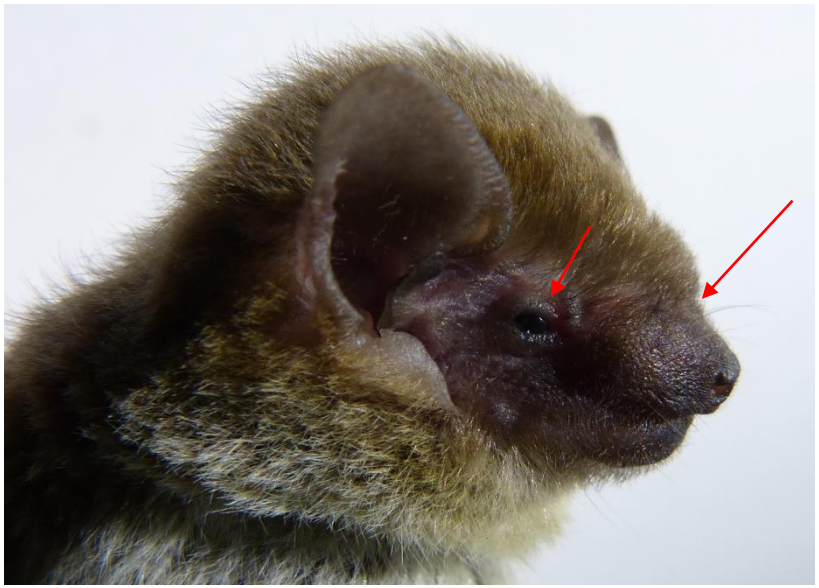
Just one genus: *Miniopterus*

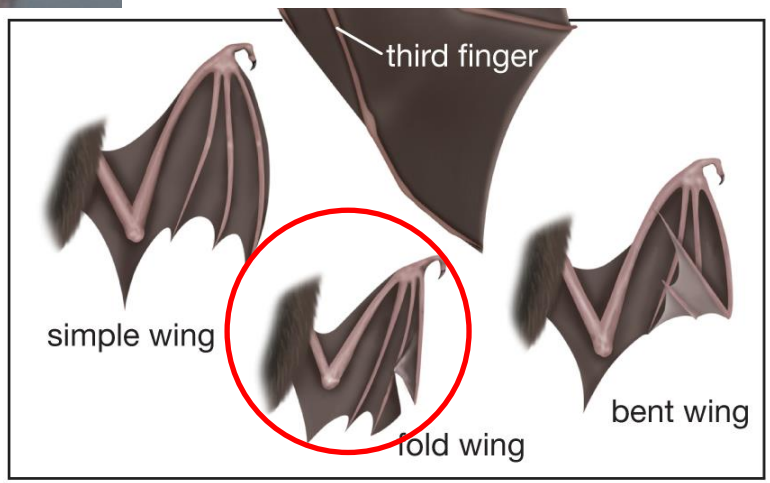
Vespertilionidae (vesper bats)

Folded wings

Small eyes

Simple face

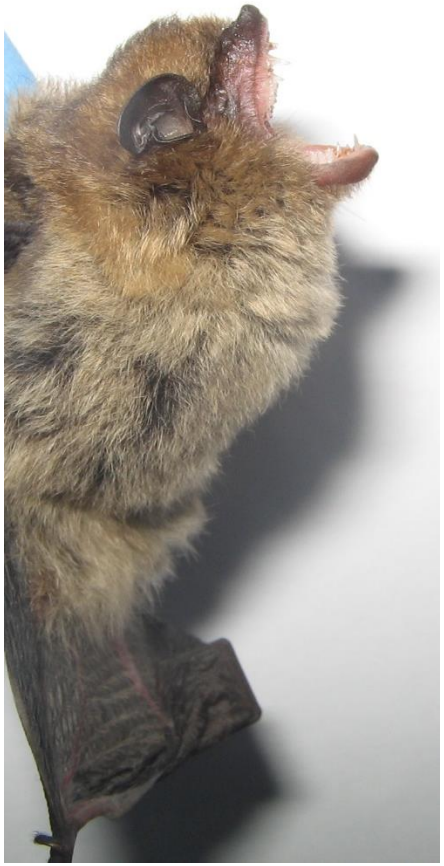




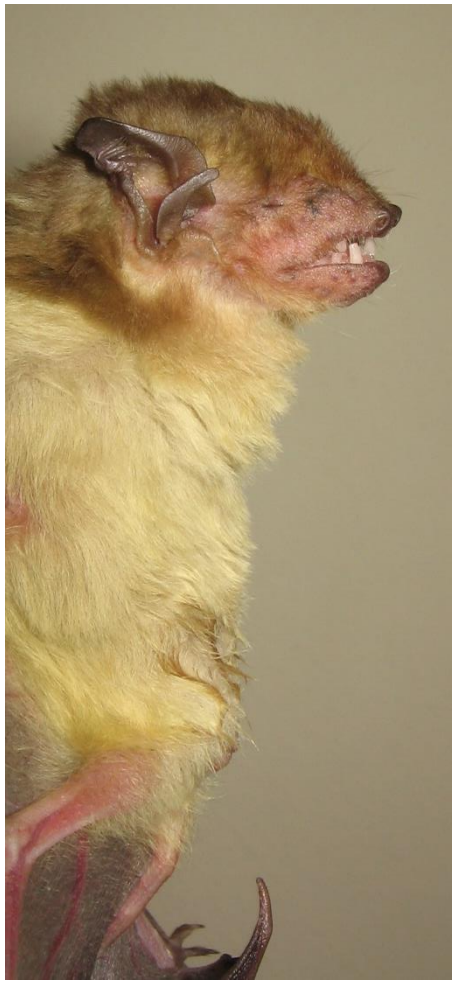
Many genera, and complicated!

Laephotis capensis

Afronycteris nana

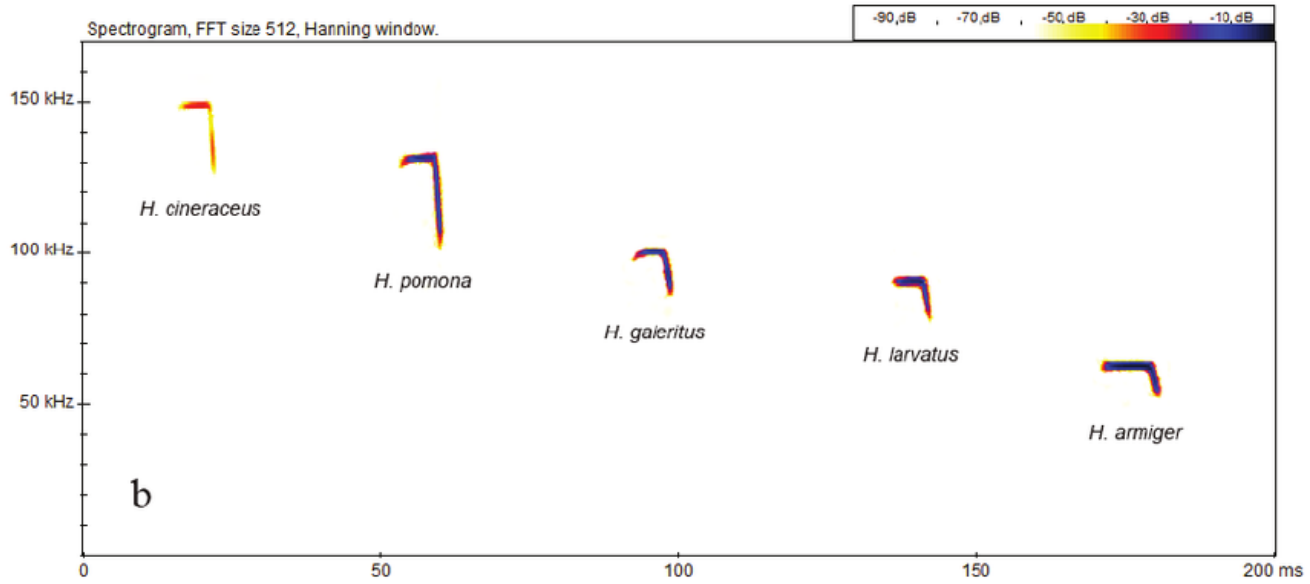


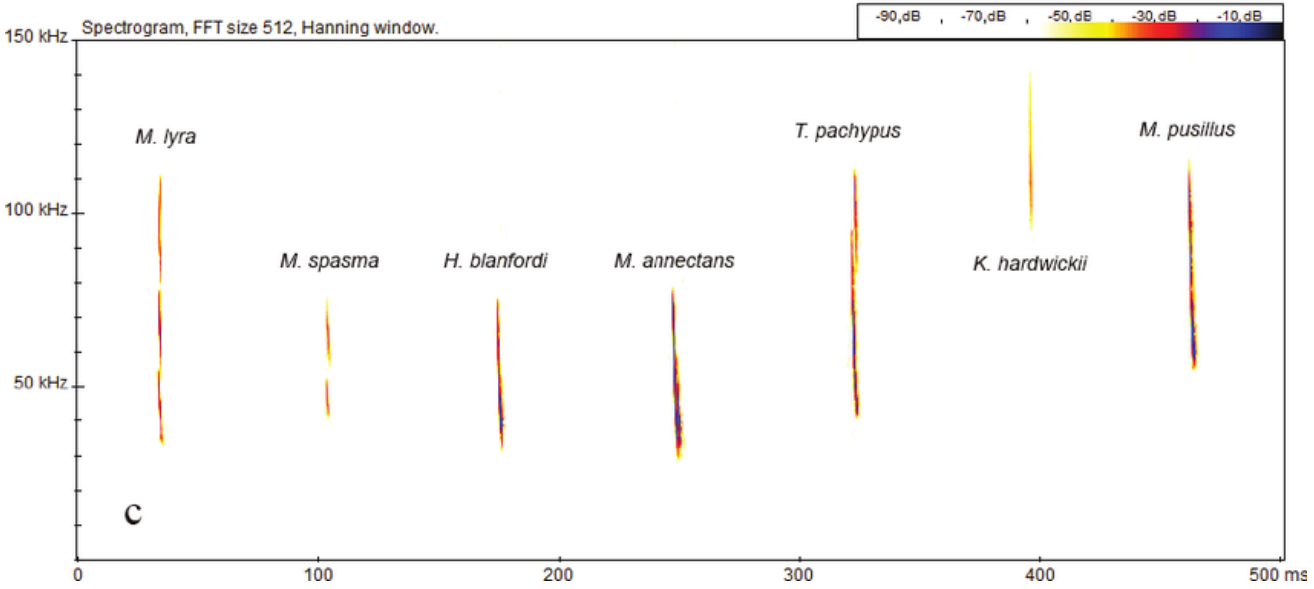
Scotophilus dinganii



Acoustic identification

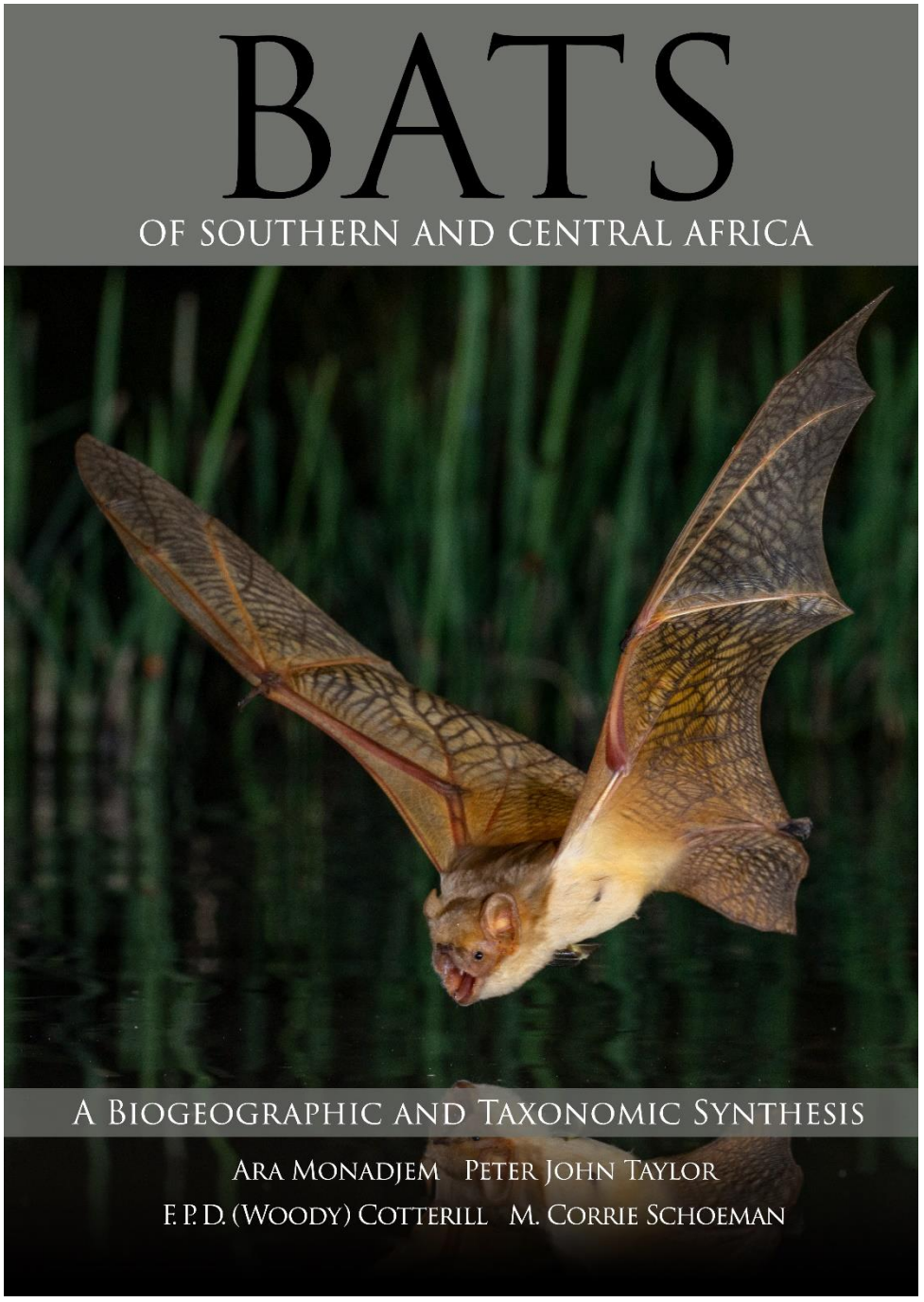
Different species have different calls





Phauk et al. 2013

Fully revised 2nd edition now available



From:	Wanda Markotter on behalf of Wanda Markotter <wanda.markotter@up.ac.za>
To:	Abednego Baker; Alexis Robinson; Alicia Cloete; Lopez, Angelica Patricia (Angie) CTR DTRA COOP THRT REDUCT (USA); antoinetteg@nicd.ac.za; Ara Monadjem; Barbra Bhebhe; Thompson, Chelsea L CIV DTRA CT (USA); Christelle Botma; Christopher Broder; Columbus Chaitezvi; colyngrobler12@gmail.com; Dineo Molewa; directoredc@gmail.com; drjubenkandamark@gmail.com; Jon Epstein <epstein@ecohealthalliance.org>; Eric Laing; Erika Strydom; Marike Geldenhuys; Grietjie De Klerk; Emily Hagan; inocencio s. chongo; ivanahory; Jacob Mabena; Jacqueline Weyer; Janusz Paweska; Jessica Coertse; Jessica Coertse; Teresa Kearney; Keneilwe Raseleka; Low de Vries <lowdevries@gmail.com>; lpmapaco@gmail.com; Madise, Dikeledi D; (b) (6) ; Marietta Bronkhorst; mariette.e.pretorius@gmail.com; marinda.moller@gmail.com; Marinda Mortlock; Mark Keith; Michelle Adams; more.spargo@gmail.com; motahanen@state.gov; Sarah Munro; Naazneen Moolla; PhD Kevin Olival; Jones, Omari A CTR (USA); Kendra Phelps <kendra.phelps@ttu.edu>; priscillat24@gmail.com; Riette Theron; Rochelle Rademan; ross@ecohealthalliance.org; Sara Achá; Bontle Sibiya; Spencer Sterling; tauya stansilous Muvengi; Tedson Nkoana; Thrishantha Munsamy; Mariette Pretorius; Matthew Wood; Wayne Ramkrishna; william.b.wheeler48.civ@mail.mil; Robert Young
Subject:	SABRENet Bat identification workshop
Date:	Tuesday, May 18, 2021 11:07:56 AM
Attachments:	viruses-13-00936.pdf Bat identification workshop_Monadjem.pdf

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Dear all

Thank you for attending today's workshop. If you could not attend, the recording is on the drive at the following link;

[redacted]

I am also attaching a copy of Ara's slides

Attached is also our first official publication link to the DTRA project that was published today. It provides a good summary of coronavirus surveillance in Africa and suggestions for future work.

Kind regards

Wanda

Prof. Wanda Markotter

Director: Centre for Viral Zoonoses

NRF-DSI South African Research Chair in Infectious Diseases of Animals (Zoonoses)

Research Chair at Future Africa: People Health and Place (One Health)

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www.futureafrica.science

Room 2-66, Pathology Building, 5 Bophelo Road, Prinshof Campus, University of Pretoria, Corner of Steve Biko and Dr Savage St, Pretoria 0001.

Specific Aims

About 60% of the known pathogens are zoonotic, and 75% are responsible for emerging outbreaks[1]. Spillover of zoonotic pathogens[2], i.e. naturally transmitted between animals, including humans[3], exists not only if pathogen and human populations inhabit the same region, but also if particular conditions enable transmission[2]. Changes in both land use global climate are widely recognized to influence the risk and emergence of zoonotic pathogens[1], driving shifts in the geographic distribution of habitat types and their biological communities[4,5]. The combination of these two forces creates new zones of contact between humans and wild and domesticated species, increasing the spillover risk of zoonotic pathogens to humans[3]. A result of this is the worldwide increase of spillover of zoonotic pathogens such as zika, hantavirus lung syndrome, Nipah, Ebola, and coronavirus (CoV), including SARS-CoV-2[6]. When the first COVID-19 outbreak occurred in December 2019, public health officials asked: What is the disease agent? How does it spread? Has it been seen before? Natural history collections may hold the answers to these questions. Collections across the globe contain more than three billion specimens that represent largely overlooked sources of data for pathogen surveillance[7-13]. Besides being spatiotemporal distribution maps of biodiversity, natural history collections can also be a toolkit for characterizing, mitigating, and predicting zoonotic infectious diseases, offering the short-cuts that public health responders are looking for in understanding the origins and distribution of emerging pathogens[7-11].

Bats are natural reservoirs for a variety of CoVs that cause serious disease in humans and animals[14]. Florida hosts 13 bat species, including *Eptesicus fuscus*, the big brown bat, a known reservoir of CoVs[15]. CoVs have been found previously in Florida[16]. Therefore, Florida faces the potential risk for spillback of SARS-CoV-2 from infected humans to wild animals, as occurred for both wild[17] and farmed[18-20] mink, and for the establishment of a new reservoir for SARS-CoV-2[21]. There is a need to determine what is the risk for Florida bat populations to be a suitable reservoir for SARS-CoV-2, as well as other pathogens. Animal-associated microbiomes are complex communities that play crucial functions for their hosts, including susceptibility to infections[22], but they can also reflect animal population dynamics, allowing us to understand responses to pathogens or global ecological changes[23]. Moreover, bat species foraging in anthropogenic landscapes hold clues regarding increased contact with the human population[24] and the potential zoonotic spillover risk. We ***hypothesize*** that shifts in bat gut microbiota can be used as a predictor for a bat population to be a suitable reservoir for a zoonotic pathogen, and this, together with the ecological traits of the bat species – morphology, diet, and roosting habits – can determine the risk for zoonotic spillover to humans. Our ***goal*** is to identify biomarkers – gut microbiome and diet species – that can be used for prediction of host suitability to be a reservoir of zoonotic pathogen and spillover/spillback risk. Two specific aims have been designed to accomplish our goal:

Aim 1. To identify gut microbiome biomarkers of bat populations from museum collection specimens. An interpretable machine learning framework will be developed for the analysis of the gut composition of 183 bat specimens from Florida collected between 1920-2020 using publicly available bat gut metagenomic data as a training set to identify bacterial biomarkers and predict presence/absence of CoVs.

Aim 2. To predict the spillover/spillback risk of zoonotic pathogens, including SARS-CoV-2, from bats to humans. We will develop a metric for spillover/spillback risk based on the ecological traits of bats, including morphology, roosting and diet habits, with the latter obtained by inverse metabarcoding.

The proposed framework is feasible, as gut microbiota composition has been successfully determined from museum specimens before[25], and microbiome as biomarkers was already proposed for cancer clinical investigations[26]. Moreover, this proposal is *significant* for several key reasons: 1) the findings will have immediate implications for public health during the ongoing pandemic as it will allow us to understand the potential of the bat population in the state to become a reservoir for SARS-CoV-2 or to be one for other CoVs; 2) the analyses can be partitioned according to roosting which is relevant because some species are more likely to be in close proximity to people, while others are colonial animals and transmission of viruses among the bats themselves may be higher; and lastly, 3) as the Florida Museum collection extends over many decades, it will give us unprecedent information on the spatiotemporal distribution ecological changes in the Florida bat population and extrapolate evolutionary shifts that might be associated with climate change in the last century.

Other aspects of this proposal are also *innovative*: 1) this will be the first time that gut bat microbiome data will be used as a biomarker for identifying reservoirs of zoonotic pathogens; 2) the new framework will allow us to detect bacteria species linked to pathogen presence that can be used as biomarkers to develop field diagnostics and predict the risk for bat populations to become suitable reservoirs of human-emerging pathogens not only in Florida and the U.S., but also in other countries; and finally, 3) this framework has the potential to be applied to forensics and ecological microbiome investigations on a worldwide basis adjusting the information to the area or pathogen of interest.

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From: Broder, Christopher on behalf of Broder, Christopher <christopher.broder@usuhs.edu>
To: epstein@ecohealthalliance.org
Subject: Fwd: Bat EID spillover risk research Zoom meeting
Date: Monday, May 3, 2021 12:52:34 PM
Attachments: Specific Aims Mavian bats museum.docx

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hi Jon,

here is there grant SAs she sent before,
in case its lost in your inbox

new zoom meet tomorrow AM 10:00

chris

---------- Forwarded message ---------
From: **Mavian,Carla Nartuhi** <cmavian@ufl.edu>
Date: Tue, Apr 13, 2021 at 1:02 PM
Subject: Re: Bat EID spillover risk research Zoom meeting
To: Broder, Christopher <christopher.broder@usuhs.edu>
Cc: cc: Parker, Tina (NIH/NIAID) [E] <parkerti@niaid.nih.gov>, Marini,Simone <simone.marini@ufl.edu>, epstein@ecohealthalliance.org <epstein@ecohealthalliance.org>

Dear Chris

Sure, I'm attaching here a draft of the specific aims that Simone and I drafted a while ago. As the project is evolving, they will change, but the main idea is there.

And thank you so much for connecting us to Dr. Epstein.

Dr. Epstein,

It's a pleasure to e-meet you. I'm looking forward to your meeting next week!

Best,

Carla

Carla Mavian, Ph.D. (She/Her/Hers)
Research Assistant Scientist
Emerging Pathogens Institute
Department of Pathology
College of Medicine
University of Florida

Southeast Asia is one of the world's highest-risk EID hotspots, and the origin of the SARS pandemic, repeated outbreaks of novel influenza strains and the spillover of dangerous viral pathogens such as Nipah virus. It is a wildlife 'megadiversity' region, where a rapidly expanding human population is increasing contact with wildlife, and increasing the risk of zoonotic disease outbreaks. The overarching goal of this proposal is to launch the **E**merging **I**nfectious **D**iseases - **S**outh **E**ast **A**sia **R**esearch **C**ollaboration **H**ub **(EID-SEARCH)** to analyze the diversity of key viral pathogens in wildlife, the frequency and causes of their spillover, and to identify viral etiologies of undiagnosed 'cryptic' outbreaks in people. EID-SEARCH includes leaders in the field of emerging viral pathogens at key US institutions, and in Thailand, Singapore, and the 3 major Malaysian administrative regions, whose collaborative networks span >50 clinics, laboratories, and research institutes across almost all SE Asian countries. This hub, and the network, will act as an early warning system for outbreaks - a way to exchange information, reagents, samples and technology, and a collaborative power-house for translational research. The long-term collaboration among the key personnel, and multidisciplinary skillsets from epidemiology, clinical management, lab analysis, through wildlife biology and data analysis **will act as significant assets when deployed to help counter outbreaks in the region.** The research goals of this EIDRC follow three specific aims:

Specific Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife. We will: 1) analyze some of the tens of thousands of archived wildlife samples at our disposal, conduct geographically- and taxonomically-targeted field surveillance in wild mammals (bats, rodents, primates), and use serological & PCR assays to identify known high-profile zoonotic pathogens, or close relatives with potential to infect people; 2) biologically characterize novel viruses that our analyses suggest have high spillover and pandemic potential; and 3) conduct *in vitro* receptor binding assays and cell culture experiments, and *in vivo* animal model infections using humanized mice and the collaborative cross mouse to assess their potential to infect people and cause disease.

Specific Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays. Assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities and approaches that can deal with the low statistical probability of identifying rare events. To achieve this, we will 1) conduct targeted cross-sectional serological surveys of human communities with extremely high geographic and cultural, occupational and behavioral exposure to wildlife-origin viruses; 2) design and deploy novel serological assays to identify baseline spillover of known or novel CoVs, PMVs and FVs in these populations; and 3) analyze and test hypotheses on the occupational, cultural and other risk factors for spillover (e.g. hunting wildlife).

Specific Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts. Our prior work provides substantial evidence of spillover leading to undiagnosed illness in people in the region. To test if these represent 'cryptic' outbreaks of novel viruses, we will conduct syndromic surveillance at regional clinics for the communities sampled in SA2. We will: 1) enroll and collect biological samples, and detailed survey data on risk factors, from patients presenting with influenza-like illness, severe respiratory illness, encephalitis, and other specific symptoms; 2) conduct molecular and follow-up serological diagnostic assays to test causal links between their syndromes and known and novel viral agents identified in SA1. Where viruses are identified, we will attempt to isolate and characterize them, then use the survey data, ecological and phylogenetic analyses to identify likely reservoir hosts/spillover pathways and inform intervention programs.

This research will advance our understanding of the risk of novel viral emergence in a uniquely important region. **It will strengthen in-country research capacity** by linking local infectious disease scientists **with an international collaborative network that has proven capacity to conduct this work and produce significant findings.** These include: testing of tens of thousands of samples from wildlife, humans and livestock in the region; discovery of hundreds of novel viruses from zoonotic viral families in wildlife; outbreak investigations in rural communities across SE Asia; discovery of the bat-origin of SARSr-CoVs; discovery of a novel bat-origin SADS-CoV killing >25,000 pigs in S. China; and development of novel serological and molecular assays for high-impact viruses, and state-of-the-art *in vitro* and *in vivo* assays to characterize viral pathogenic potential. This body of collaborative research provides proof-of-concept that EID-SEARCH has the background, collaborative network, experience, and skillset to act as a **unique early warning system for novel EIDs of any etiology threatening to emerge in this hottest of the EID hotspots.**

II. Research Strategy:

1. Significance: Southeast Asia is a critical hotspot for emerging diseases due to its high biodiversity of wildlife and their viruses, and the presence of key ecological and socioeconomic drivers of emergence **(Fig. 1)** (*1*). These include dense human populations living in networks of rural and urban communities, with strong cultural and occupational connection to wildlife and livestock farming and trading, and intimate connection to global travel networks (*2*). The region is undergoing rapid environmental and demographic change, both of which increase the risk of disease emergence and spread. Pathogens that emerge in this region often spread and sometimes enter the USA (e.g. prior influenza pandemics, SARS) and threaten global health security.

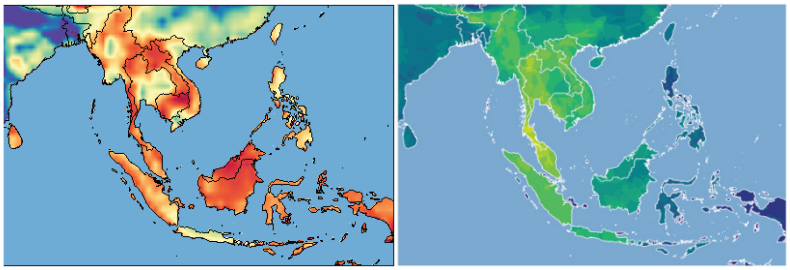


Fig. 1: Southeast Asia, and in particular, parts of Thailand and Malaysia are hotspots of risk for disease emergence **(left)**, and predicted high diversity of 'missing' or as-yet undiscovered viruses, yellow = highest diversity **(left)**. From (*1-3*).

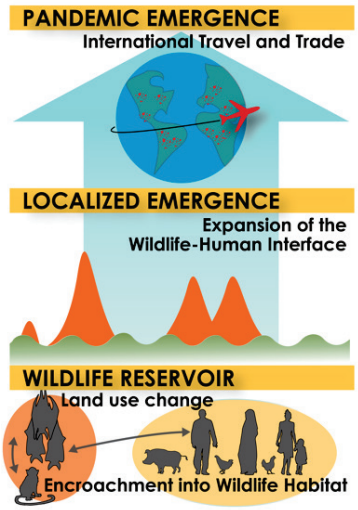
Not surprisingly novel viruses, including near-neighbors of known agents, have emerged in the region leading to often unusual clinical presentations **(Table 1)**.This adds to a significant background burden of repeated Dengue virus outbreaks in the region (*20*), and over 30 known *Flavivirus* species circulating throughout S. and SE Asia (*21*). The events in **Table 1** are dominated by three viral families: coronaviruses (CoVs), paramyxoviruses (PMVs - particularly henipaviruses) and filoviruses (FVs), which are initial examples of our team's research focus and capabilities. These viral groups have led to globally important emerging zoonoses (*4, 5, 22-31*), causing tens of thousands of deaths, and costing billions of dollars (*32-34*). Furthermore, near-neighbors of known pathogens in these families have been reported throughout the region, including by members of our consortium: henipaviruses in bats in Thailand, Cambodia, Philippines, Laos, Indonesia, China, Malaysia, Bangladesh and India (*35-41*); a novel henipavirus, Mòjiāng virus, in wild rats in Yunnan (*14*); serological evidence of FVs in bats in Bangladesh (*42*) and Singapore (*43*); Nipah- and Ebola-like viruses in *Hipposideros*, *Cynopterus* and *Rhinolophus* species in Malaysia (Hughes *et al.*, in prep.); evidence of novel FVs in bats in China (*44-46*), including Měnglà virus that appears capable of infecting human cells (*47*); novel PMVs in bats and rodents consumed as bushmeat in Vietnam (*48*); a lineage C β-CoV in bats in China using the same cell receptor as MERS-CoV to infect human cells *in vitro* (*49*); MERSr-CoVs in bat guano harvested as fertilizer in Thailand (*50*), and directly in bats (Wacharapluesadee *et al.*, in prep.); 172 related viruses.

Viral agent	Site, date	Impact	Novelty of event	Ref.
Nipah virus	Malaysia, Singapore 1998-9	~246 human cases, ~40% fatal	2nd emergence of a zoonotic henipavirus, 1st large outbreak	(*4-6*)
Melaka & Kampar virus	Malaysia 2006	SARI in family group, individual	1st disease due to bat-origin reoviruses, also Singapore, Vietnam etc.	(*7-10*)
Reston filovirus	Philippines 2008	Seropositive people killed pigs,	No prior FVs in pigs	(*11*)
Thrombocytopenia Syndrome virus	E. Asia 2009	100s of deaths in people	Novel tick-borne zoonosis with large caseload	(*12*)
Reston filovirus	Shanghai 2011	PCR positive pigs	Further evidence of pig RESTV infection	(*13*)
Mòjiāng virus	Yunnan 2012	Death of 3 mineworkers	1st evidence of rodent origin henipavirus in people	(*14*)
Nipah-like virus	Philippines 2015	Killed horses	No prior horse infection for NiV, known for HeV	(*15*)
SARSr-CoV & HKU10-CoV	S. China 2015	Seropositive people	1st evidence human infection HKU10 & SARSr-CoV	(*16*)
SADS-CoV	China 2017	>25,000 pig deaths seronegative people	Novel emergence of bat-origin CoV	(*17*)
Nipah virus	Kerala 2018, 2019	Killed 17/19 people 2018, 1 infected 2019	1st outbreak of NiV outside Bangladesh, W. Bengal focus	(*18, 19*)

Table 1: Recent emergence events in SE Asia indicating potential for novel pathways of emergence, or unusual presentations for known or

novel β-CoVs (52 novel SARSr-CoVs) and a new β-CoV clade ("lineage E") in bats in S. China that occur throughout the region (*24, 49, 51, 52*); 9 novel wildlife-origin CoVs and 27 PMVs in Thailand, and 9 novel CoVs in Malaysia reported by us from USAID-PREDICT funding (*51, 53*). These discoveries underscore the clear and present danger of zoonotic events in the region. The wide diversity of potentially pathogenic viral strains also has significant potential use in broadly active vaccine, immunotherapeutic and drug development (*49*).

Most high-impact zoonotic viruses originate in wildlife reservoirs, sometimes spilling over first into livestock 'amplifier' hosts, or directly into localized human populations with high levels of animal contact **(Fig. 2)**. Efforts to prevent emerging zoonoses have targeted these high-risk populations in regions prone to disease emergence (*31, 54*). However, these regions tend to be in the developing world, where nations often lack the resources to investigate novel zoonotic events, or wildlife reservoirs of zoonoses, and prioritize more immediate public health concerns such as Dengue virus or NTDs (*20*). Zoonotic disease surveillance and control is also hampered by inadequate information on basic disease ecology (e.g. range of wildlife reservoirs, risk factors for spillover) and pathogen biology (viral diversity, pathogenesis), and by logistical challenges sharing novel diagnostics, animal models, samples and reagents at sites where outbreaks begin (*55*). Countermeasure and vaccine development is challenged by the small number of isolates available, and the presence of closely-related strains, some with evidence of transmission to people but with unknown pathogenicity. For example, using NIH and other funding, we found serological evidence of exposure to African henipaviruses in 3-4% of a sample of people in Cameroon, with a significant risk factor being butchering of bats; and serological responses to bat SARSr-CoVs in 6/209 (2.87%) and bat HKU10-CoV in 2/412 (0.5%) people living close to a bat cave in Yunnan, all novel viruses with unknown clinical impact (*16, 56*). We discovered two novel bat-borne reoviruses, Melaka and Pulau viruses, and showed that 14/109 (13%) of people living in close proximity to bat roosts on Tioman Island, Malaysia were exposed to these viruses (*7*) as well as 12/856 (1.4%) of a random sample screened in Singapore (*8*). Previous studies in the Philippines found famers and butchers who had contact with sick pigs tested positive for Reston ebolavirus antibodies but had not experienced illness (*11*), and it was our team that discovered Philippine bats as natural reservoir hosts for Reston virus (*57*). In preliminary screening in Malaysia with funding from DTRA we found serological evidence of exposure to henipaviruses (Hendra, Nipah and Cedar) in 14 bats and 6 human samples, and Ebola (Zaire and Sudan)-related viruses in 11 bats, 2 non-human primates (NHP) and 3 human samples (Hughes, in prep.).



Even when spillover leads to outbreaks of illness and mortality, they are often unreported, undiagnosed or misdiagnosed. We initiated targeted syndromic surveillance of encephalitis patients in Bangladesh clinics and showed that Nipah virus (NiV) causes repeated annual outbreaks with an overall mortality rate of ~70% (*58*). NiV has been reported in people in West Bengal, India, which borders Bangladesh (*28*), in bats in Central North India (*59*), and now in people in Kerala, South India in 2018 and 2019, raising the specter of future spillover at other sites in the region (*18, 60*).

Fig. 2: Our Early Warning Approach: Most zoonotic viruses emerge from wildlife hosts, sometimes via livestock **(lower panel)**, to cause small scale outbreaks (green) or larger chains (red) of human-to-human transmission **(middle)**. In some cases, these spread more widely via air travel **(upper)**. Our EIDRC proposal targets each stage: **SA1** examines the diversity and spillover potential of viral threats in targeted SE Asian wildlife; **SA2** seeks evidence of their spillover into focused high-risk human populations; **SA3** identifies their capacity to cause illness and to spread more widely. Figure from (*54*).

Under a current NIAID R01, we have shown that we can use analytical approaches to target surveillance of wildlife and people and identify likely sites of spillover. We used phylogenetic analysis of bat hosts and their CoVs to identify a key cave complex in SW Yunnan Province that harbors all the genetic elements of SARS-CoV (*61*), as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics (*24, 49, 51, 52, 62-64*). We conducted risk factor surveys and biological sampling of human populations living close to this cave and found serological evidence of spillover in people highly exposed to wildlife (*16*). **This work provides proof-of-**

concept for an early warning approach that we will expand in this EIDRC to target other viral groups in one of the world's most high-risk EID hotspots.

The overall premise of this EIDRC proposal is that there is substantial evidence that: 1) diverse CoVs, henipaviruses and FVs related to known human pathogens are circulating in wildlife reservoirs in Southeast Asia; **2)** these viruses likely spillover regularly to people, are often unreported or misdiagnosed, and their clinical manifestations and potential to cause pandemics are unknown and underestimated; and **3)** our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence. To scale-up this approach, we propose to launch **EID-SEARCH** (the **E**merging **I**nfectious **D**iseases - **S**outh **E**ast **A**sia **R**esearch **C**ollaboration **H**ub), to better understand, and respond to, the risk of zoonotic viral emergence in Southeast Asia. We will initially focus on the diversity of known and novel CoVs, henipaviruses, and FVs in wildlife reservoirs. We will use this work to build our center's interactive network throughout the region, bridge barriers, provide early focal point for basic and translational studies, develop sample handling and storage routines, communication networks, cohort management, infrastructure development, and cross-training in surveillance and novel diagnostic platforms. We will develop and transfer technology to test these viruses' capacity to infect human cells and mouse models, develop new specific and sensitive serological and molecular diagnostic tools, conduct surveillance of human communities with high risk of exposure to wildlife, and clinical cohorts to identify evidence of spillover of viruses causing previously 'cryptic' clinical syndromes in people. We will test the capacity of novel viruses to infect human cells and/or mouse models, and thus asses potential for spillover and spread which will feed into our risk models. Our group also has extensive experience in other viruses, including vector-borne flaviviruses, and alphaviruses, and will use this experience to provide robust long-term surveillance, diagnostics, and basic science opportunities. While the current proposal focuses its basic and applied research agendas on this group of viral EID, we note that the EID-SEARCH is poised to rapidly respond to any outbreak. **Thus, FVs, henipaviruses and CoVs are examples of sentinel surveillance systems in place that illustrate EID-SEARCH's capacity to identify and initiate a rapid response targeting any novel microbial pathogen that emerges in this region.**

2. Innovation: Previous work by our consortium has developed strategies to target surveillance in wildlife and people to better anticipate and identify early spillover events and pre-empt outbreaks of emerging viruses. In the EID-SEARCH we scale up this approach in a central research "hub" made up of leading laboratories, clinics and surveillance sites in three critically high-risk EID hotspot countries in SE Asia. EID-SEARCH's reach is expanded through its regional network of collaborators that covers nearly all of the greater SE Asia region. The innovation of the EID-SEARCH is in: **1)** Its multidisciplinary approach that combines modeling to target geographic and taxonomic sampling targets with on-the-ground zoonotic disease surveillance in human and wildlife populations; **2)** the biological characterization approach that identifies how likely viruses are to be able to infect people, and enables evaluation of existing countermeasure technologies; an approach that is grounded in our successful work on SARSr-CoVs; and **3)** the strategic sampling of both high-risk communities with extensive animal contact at the front-lines of viral spillover *and* syndromic clinical cohorts that present with signs of novel viral emergence, to identify risk of viral spillover in an early warning system approach **(Fig. 2)**. **In Aim 1, we will target viruses in archived and newly collected wildlife samples, and characterize their risk of spillover to people**. We will use spatial and phylogeographic analyses to identify the geography and species of wildlife to test samples from, and to further characterize viruses we have recently discovered. We will use *in silico* methods (novel ecological-phylogenetic risk ranking and mapping of receptor binding domains), cell culture, and animal models to assess their potential for spillover into high-risk human populations. **In Aim 2, we will conduct targeted cross-sectional serological surveys of human communities with high levels of animal contact to find evidence of viral spillover, and identify occupational and other risk factors for zoonotic virus transmission.** We will use serological tests targeting viruses identified in Aim 1 to identify the baseline spillover of known or novel CoVs, henipaviruses and FVs in these populations. Serological findings will be analyzed together with subject metadata (including a simple animal-contact survey, location data, viral sequence/isolates etc.) to identify zoonotic risk factors and routes of exposure from specific wildlife or vector species. **In Aim 3, we will use syndromic surveillance of clinical human cohorts to identify evidence of viral etiology for otherwise 'cryptic' outbreaks.** We will enroll and

collect biological samples from patients in local clinics and hospitals who present with syndromes previously linked to known viral agents and live within communities at high risk for disease emergence. We will conduct molecular and follow-up serological diagnostic assays to identify likely viral etiology of syndromes that may represent otherwise cryptic events. Where viruses are identified, we will attempt to isolate or molecularly re-derive them, and use survey data, ecological and phylogenetic analysis to identify likely reservoir hosts and inform intervention programs. Our US partner BSL-4 laboratory, NEIDL, will attempt isolation and characterization of viruses requiring BSL-4 of containment (e.g. novel filoviruses, close relatives of Hev/NiV).

3. Approach: 3.1. Research team: The EID-SEARCH builds on long-term collaboration among world leaders in emerging virus research with proven experience collaborating internationally on field, lab and human surveillance research **(Fig. 3)**. Over the past two decades, our consortium partners have conducted high profile research on EIDs within the region and globally, including identifying the wildlife reservoirs for NiV and Hendra virus (HeV), MERS- and SARS-CoVs (*24, 27, 59, 61, 65-67*), discovering SADS-CoV (*17*), and developing an array of serological, molecular, *in vitro* and *in vivo* approaches to characterizing high-risk CoVs, henipaviruses, and FVs (*62-64, 68-81*). Our team has substantial experience conducting human surveillance in the community and during outbreaks **(Sections 2.2.a., 2.2.b., 2.2.c, 3.2.a, 3.2.b)**, including: USAID-PREDICT, and DTRA-funded viral surveillance of indigenous communities in Peninsular Malaysia and syndromic surveillance of hospital patients in Sabah (Co-I Hughes); collection of >2,000 serum samples from healthy blood donors and sanitation workers, febrile and influenza like illness (ILI), and diarrheal stool samples from children under 5 years of age across Borneo Malaysia (Co-I Kamruddin); collaboration on the MONKEYBAR project with the London School of Hygiene and Tropical Medicine to analyze risk and prevalence of the zoonotic malaria *Plasmodium knowlesi* through case control and cross-sectional studies in Sabah villagers (n=~800, 10,000 resp.) and clinical observational studies to determine the etiology of central nervous system infections and acute undifferentiated febrile illness in Sabah. (Co-Is William, Rajahram, Yeo); and community-based surveillance of hundreds of bat-exposed villagers in Thailand (Co-Is Wacharapluesedee, Hemachudha). As a proof-of-concept of how our team can rapidly scale up resources for diagnostics and surveillance in the face of a new EID, in 2016, we discovered a novel bat-origin HKU-2 CoV (*82, 83*) killing >20,000 pigs in S. China, designed PCR and serological assays, surveyed pig farmers for evidence of exposure, and conducted experimental infections, pathogenesis and molecular studies, **all in the span of 3 months** (*17, 84*).

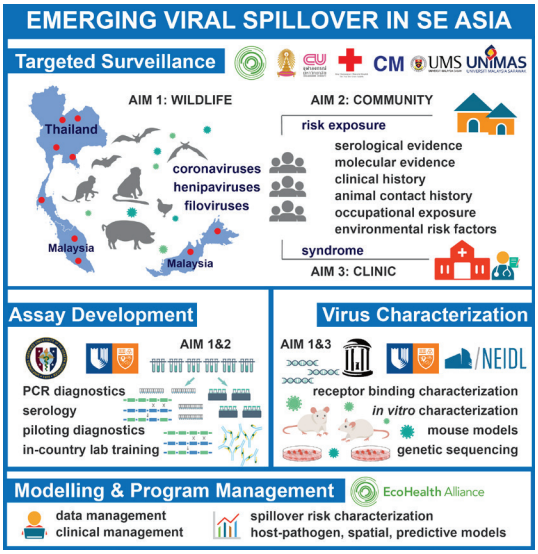


Fig. 3: EID-SEARCH scope, core institutions, and roles.

The administration of this center **(Section 4.1.)** will be centralized at EcoHealth Alliance (EHA) led by PI Daszak, who has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5-20 yrs on NIAID- and USAID-funded research. He will oversee all activities in this EIDRC, supported by Deputy lead, Dr. Olival – who has managed human and wildlife disease surveillance projects in SE Asia for >10 years, and a **Core Executive Committee (Section 4.1.a)**. Co-Is Olival and Zambrana are leaders infectious disease analytics, and head the modeling and analytics work for USAID-PREDICT. Co-Is Wang, Baric, Broder, Anderson, and Laing have developed a unique array of *in vitro* cell culture, serological and molecular reagents to identify and characterize emerging viruses in people and animals (henipaviruses and other PMVs, CoVs, FVs and many others). Co-Is Wacharapluesedee, Hemachudha, and Hughes and collaborators have coordinated clinical and community surveillance of people, and of wildlife and livestock within Thailand and Malaysia for the past 15 years, in collaboration with Ministries of Health, Agriculture and Environment. Finally, this consortium has extensive external funding from NIH, DoD, USAID, DHS and other USG agencies supporting laboratory, analytical and field studies directly related to the current proposal, and will bring substantial leverage to our EIDRC.

3.2. Geographical focus: The three core (hub) countries for this proposed EIDRC are Thailand, Malaysia and Singapore, three contiguous EID hotspot countries that stretch through one of the most significant foci of EID origins globally. Our proposed work includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sarawak and Sabah, and thus contains overlapping wildlife biogeography with India, Bangladesh, Myanmar, SW China, Laos, Cambodia, Vietnam, Philippines, and Indonesia. It encompasses diverse cultural and socioeconomic backgrounds which have correspondingly diverse and intensive interactions with wildlife reservoirs and the global travel network. We have already begun sampling of wildlife hunters, livestock workers, wildlife farmers and market workers, rural villagers living close to high risk wildlife species, people otherwise highly exposed to animals in these countries, and clinical cohorts, as part of current/prior NIH, DTRA, and USAID funding. Our core member's extensive network of collaborators in clinics, research institutes and public health laboratories in almost every other Southeast Asian country positions the EID-SEARCH within a much larger catchment area for emerging zoonoses and will allow us to rapidly scale up to include additional sites in response to an outbreak or shift in research priorities. We will conduct regular meetings with key research, public health and community leaders, including >50 leading laboratories/institutions in 10 countries **(Section 4.2)** to maintain these collaborative relationships with the core members of our consortium (**Fig. 4**). We will use these, and our annual meetings, to share information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks across the region.

Fig.4 : Map of Southeast Asia indicating three hub countries for this proposed EIDRC (**Red**: Thailand, Singapore, Malaysia) and countries in which Key Personnel have existing collaborating partners via other funded work (**Green**), indicating that EID-SEARCH provides access to key collaborators and sites throughout the region.

3.3. Capacity for linkage with other NIAID studies, and scaling up to respond to outbreaks: EID-SEARCH US-based partners include senior scientists with significant experience on NIAID-funded projects, extensive collaborations with other NIAID-funded scientists, and previous experience working with NIAID leadership through workshops, review of programs (e.g. PI Daszak's role in NIAID CEIRS external review), and work on study sections. These contacts ensure that the EID-SEARCH senior leadership will be ready and able to rapidly set up data, sample and reagent/assay sharing protocols with NIAID researchers, the other EIDRCs, the EIDRC CC and other NIAID research centers. EID-SEARCH in-country partners include senior medical doctors and infectious disease specialists at national and regional hospitals in each country and administrative state in this proposal. On news of potential outbreaks, **EID-SEARCH will rapidly mobilize its core staff and their extensive collaborative network across almost all SE Asian countries (Fig. 4) (Sections 4.1.a, 4.2. 4.3).** The core analytical approach can be used to rapidly identify sampling sites and targets to harvest vectors, vector borne arboviruses, birds and mammalian species that harbor most other viral threats. Through existing projects such as the USAID PREDICT Project and DTRA-funded biosurveillance projects, EHA is currently partnered with hospitals and researchers across South and Western Asia, West and Central Africa, and Latin America. Our partners already have capacity to conduct human and wildlife sampling and testing for novel viral agents, so expanding in geography or scale is readily achievable given sufficient resources.

Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife.

1.1 Rationale/Innovation: The vast majority of emerging zoonotic pathogens are linked directly or indirectly to wildlife host, particularly from important mammalian groups like bats, rodents and NHPs (*3*). Our previous work on wildlife origin viruses demonstrates that zoonotic viral emergence tends to occur in specific regions with high diversity of wildlife that harbor viruses of zoonotic potential, and human populations that have close contact to these wildlife **(Fig. 1)** (*2, 3*). In Aim 1 **(see Fig. 9 for overview)**, we will strategically conduct EID surveillance by applying risk analytics to prioritize the regions, wildlife species, and human populations that

have a particularly high risk of viral spillover. We will target high-risk zoonotic host reservoirs (bats, rodents and NHPs) in these regions and apply novel predictive models to estimate viral diversity and host range to identify wildlife species that have been ignored or under-sampled by previous zoonotic disease surveillance efforts. We will strategically collect specimens from these species and screen them, together with some of the tens of thousands of recently collected samples we have archived, for known and novel agents. We will begin screening using conserved PCR assays to identify known and novel viruses in high risk RNA viral families, *Coronaviridae*, *Paramyxoviridae*, and *Filoviridae*. We will expand to other groups of pathogens, including arboviruses, pending available resources and priorities established with the EIDRC coordinating center (EIDRC-CC) and other regional EIDRCs. Using risk ranking algorithms and a series of ecological and virological risk factors, we will triage the wildlife viruses we discover for further genomic characterization and viral isolation. On this subset of viruses, we will then use *in vitro* and *in vivo* approaches (viral rescue, cell lines, gene edited humanized transgenic mice and the collaborative cross genetic reference mouse population) previously developed and widely used by our team to predict capacity of novel viruses to infect people and spillover (*63, 85-88*). These high-risk viruses and their close relatives will be targets for human community serosurveys and clinical sampling in Aims 2 and 3, respectively.

1.2 Preliminary data: 1.2.a. Geographic targeting: EHA has led the field in analyzing where the highest risk of zoonotic spillover is across the globe, identifying SE Asia as a major EID hotspot (*1, 2*). These hotspots are sites where high wildlife biodiversity, human population density and rapid ecologic change collide **(Fig 1)**. Using host and ICTV viral data for all known mammalian viruses, and generalized additive models to correct for sampling bias, we are able to predict the relative number of yet-to-be-described viruses that a species harbors (*3*). This demonstrates substantial undiscovered viral diversity in mammals across Southeast Asia, and particularly in regions of Thailand, Singapore and Malaysia **(Fig. 1)**. Separately, we found that factors driving spillover (host diversity, climate) differ from those driving spread in human populations (population density, bushmeat hunting, livestock production) (*89*). We therefore analyze capacity for viral spread as a separate risk factor, using our unique demographic and air travel and flight data modeling software (*90, 91*). In the current proposed work, we will use all the above approaches to target wildlife sampling (archival and new field collections) in Aim 1, to inform sites for human sampling in Aim 2, and to assess risk of spread in Aim 3.

1.2.b. Reservoir host species targeting: Our analysis of all known mammalian host-viral associations demonstrates that bats, rodents and NHPs represent the three most important reservoirs for zoonoses, responsible for the highest proportion of known and predicted viral diversity of zoonotic potential (*3*). Thus, the bulk of our proposed wildlife sampling and testing will consist of these three taxa with a focus on bats, because this group is the known or hypothesized reservoir for the majority of high-impact zoonotic CoVs, PMVs and FVs (*50, 92-94*). Bats have the highest proportion of predicted zoonotic viruses in any mammalian group (*2*), and are exceedingly diverse in Southeast Asia (~500 species). To further prioritize taxonomic sampling targets within these diverse geographic hotspots, we used a novel Bayesian phylogeographic algorithm to identify host genera and species that are the most important centers of evolutionary diversity for a given viral group, e.g. bat genera for β-CoVs **(Fig. 5)** (*51*). This approach will be extended to other mammal taxa and viral families of interest, allowing us to identify the reservoir hosts of specific zoonotic viral groups, where viral diversity is likely highest.

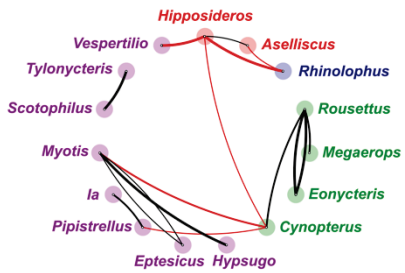


Fig 5: Preliminary analysis of sequence data collected under prior NIH funding, identifying SE Asian bat genera with the highest β-CoV evolutionary diversity. We will apply this Bayesian discrete phylogeographic model to identify taxonomic and geographic centers of viral diversification and sharing for relevant PMV, CoV, FV and other virus clades. Line thickness proportional to probability of virus sharing between two genera. Inter-family switches in red.

Finally, we have designed a strategy to estimate sampling gaps, using a viral 'mark-recapture' approach we previously published (*95, 96*) **(Fig. 6)**. Using prior data for bat, rodent and NHP viral discovery vs. sampling rates, we can identify the likely total viral diversity for specific host groups in a region, and use this to estimate sample sizes to discovering the majority of unknown viruses within a specific family or genus. **In the current**

proposal, we will use these analyses to estimate geographic and species-specific sampling targets **to more effectively identify new strains to support experimental infection studies and risk assessment.**

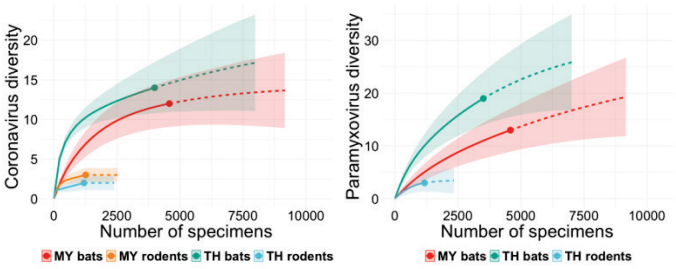


Fig. 6: Estimated CoV **(left)** and PMV **(right)** diversity in bats and rodents from Thailand and Malaysia, using data from PCR screening and RdRp sequences from >10,000 specimens in bats and 4,500 in rodents. Bats have 4X more viral species than rodents, controlling for sampling effort. We estimate that additional collection of 5k-9k bat specimens and testing of our archived bat and rodent specimens alone will identify >80% of remaining CoV and PMV viral species in these key reservoirs, yielding >800 unique viral strains.

1.2.c. Sample testing to identify known and novel viruses: We have conducted extensive wildlife surveillance for novel viruses across Southeast Asia in prior NIAID funded work and as part of the USAID PREDICT project. Under the 10-year PREDICT program globally, we identified 912 novel and 210 known viruses, more than the total number of viruses previously recognized in mammals by the ICTV (*97*). This work included collecting nearly 300,000 individual mammal samples from 14 EHA-led PREDICT countries, and PCR-screening of more than 60,000 individual specimens (*53*). In southern China alone, we identified 178 β-CoVs, of which 172 were novel, discovered a new β-CoV clade, "lineage E" (41), diverse HKU3r-CoVs (179 sequences) within a 'sister' clade to the SARS-CoV lineage, and a new bat-origin SADS-CoV, responsible for killing >25,000 pigs in Guangdong Province (*17*). We have collected 28,957 samples from bats, rodents and NHPs in Thailand and 47,178 in Malaysia, **but have only tested a minority of these using PCR.** We have identified 100 novel viruses in Thailand and 77 in Malaysia. **Furthermore, we have only sequenced a small segment of one gene for all novel viruses found. We have archived duplicate samples which are now available for use in this project**, including fecal, oral, urogenital, serum samples, and biopsies from bats, rodents, and NHPs. In the current proposed work, we will attempt to sequence, isolate and characterize those viruses that our analyses below suggest are most likely to be able to infect humans.

1.2.d. In vitro & in vivo *characterization of viral potential for human infection:* Using Coronaviridae as an example, we have conducted *in vitro* and *in vivo* experiments to characterize the pathogenic potential of novel SARSr-CoVs from bats. We isolated three SARSr-CoVs from bat feces: WIV1, WIV16 and Rs4874, with Spike (S) protein sequences that diverged from SARS-CoV by 3-7% (*24, 61, 98*). We conducted full-length genome sequencing of 12 other novel SARSr-CoVs, some highly similar to outbreak SARS-CoV in the most variable genes (*61*). Using our reverse genetics system we constructed chimeric viruses and rederived full length recombinant SARSr-CoV from in silco sequence. All 3 SARSr-CoV full length isolates and the two chimeric viruses replicated efficiently in Vero E6 cells and in HeLa cells expressing hACE2, civet and bat ACE2, but not in those without ACE2 (*24, 61, 98*). We used the SARS-CoV reverse genetics system (*72*) to generate a chimeric virus with a mouse-adapted SARS-CoV backbone expressing SHC014 S protein with 10% sequence divergence from SARS-CoV S. This and the other full length and chimera viruses replicated in primary human airway epithelium, using the human ACE2 receptor to enter into cells (*62*). Thus, **SARSr-CoVs with diverse variants of SL-CoV S protein without deletions in their RBD can use human ACE2 as receptor for cell entry.** The Baric lab has a well-established hACE2 and DPP4 transgenic mouse model that **we used to assess the capacity of novel SARSr-CoVs and MERS-CoV to infect humans and cause disease** (*85, 99*). Infection of bat SARSr-SHC014 or WIV1 caused SARS-like clinical signs in the transgenic hACE2 mouse model **that weren't reduced by immune-therapeutic monoclonals that attenuate SARS-CoV pathogenicity, nor after challenge following vaccination against SARS-CoV** (*62*) **(Fig. 7)**. We repeated this virus characterization approach with chimeras using HKU3r-CoV S proteins that are ~25% divergent from SARS-CoV S, **and found that they are unable to use the ACE2 receptor**. Additionally, we were unable to culture HKU3r-CoVs in Vero E6, or human cell lines.

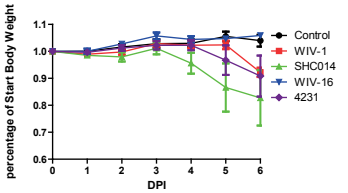


Fig. 7: Body weight change in hACE2 transgenic mice after SARSr-CoV infection, demonstrating that bat-origin SARSr-CoVs cause similar clinical signs to outbreak SARS-CoV strain (4231) (*62, 63*).

A similar approach to the above pipeline for CoVs will be applied to novel henipaviruses we discover during our research. The broad mammalian tropism of HeV and NiV (*68*) is likely mediated by their usage of highly conserved ephrin ligands for cell entry (*19, 100*). A third isolated henipavirus, Cedar virus does not cause pathogenesis in animal models. **Co-Is Broder and Laing** have developed a reverse genetics system and rescued a recombinant CedV to test ephrin receptor usage/tropism and pathogenicity during henipavirus infections (*77*). CedV is unable to use the Ephrin-B3 receptor (*77, 101*) which is found in spinal cord and may underlie NiV encephalitis (*102*) and that pathogenesis also involves virulence factors V and W proteins. The putative henipaviruses, Ghana virus and Mòjiāng virus, predict V and W protein expression, with GhV able to bind to ephrin-B2, but not -B2 (*103*), and the receptor for MoJV remains unknown (*104*) but is likely ephrin-B2 (*105*). Our group has also used similar methods to test the hypothesis that novel Filoviruses discovered in wildlife are able to infect people. NiV, EBOV and MERS-CoV all infect primary microvascular endothelial cells, which are available at Co-I Baric's lab for EID-SEARCH collaborators (*71, 106*). Primary human lung endothelial cells are highly susceptible to Ebolavirus infection (*107*), and Huh7 cells can be used to isolate virus from clinical samples (*108*) offering advantages for reverse genetic recovery of novel FVs (*109*). The three filovirus genera, *Ebolavirus*, *Marburgvirus* and *Cuevavirus*, use Niemann–Pick type C1 (NPC1) protein as cell entry receptor (*110*). For novel filoviruses, cell culture would be carried out under BSL-4, however they can readily be characterized following identification of their encoded GP sequences which can be pseudotyped into recombinant GP-bearing vesicular stomatitis virus (VSV) pseudovirions (*111*). These pseudovirions can be safely used to characterize the tropism and entry filoviruses mediated by GP without a need for BSL4 containment. **Co-Is Wang and Anderson** used cells originating from various mammalian species to determine spillover and/or zoonotic potential of MLAV, a newly discovered Asian filovirus (*47*). Despite the low amino acid sequence identity (22–39%) of the glycoprotein with other filoviruses, MLAV is capable of using NPC1 as entry receptor. Cells from humans, monkeys, dogs, hamsters and bats were able to be infected with a MLAV pseudovirus, implying a broad species cell tropism with a high risk of interspecies spillover transmission.

Mouse models. For novel viruses across all families, we will use the UNC collaborative cross mouse that captures 90% of the diversity within all mouse groups to assess the most suitable model for further experiments (*112*). We have used this model for CoV, FV (Ebola), Flaviviruses, and alphaviruses infections. Clinical signs such as weight loss, hemorrhage, encephalitis, and, acute or chronic arthritis were recapitulated in these mice following SARS, EBOV, West Nile virus and Chikungunya infections, respectively (*86-88, 113-116*). Note that CC OR3015 shows hemorrhagic disease phenotypes after EBOV infection **(Fig. 8)**. *Bat Models.* Duke-NUS has developed two models for bat *in vivo* studies: a colony of *Eonycteris spelaea* (cave dwelling small fruit bat) and a bat mouse model, which contains the bat immune system in an immunodeficient mouse (*117*). In a proof of concept study, **Co-Is Wang and Anderson** infected *E. spelaea* bats with MERS-CoV to demonstrate their susceptibility and suitability for infection with a bat borne virus under BSL3 containment. These bats and the bat mouse model will serve to complement and expand studies from the UNC collaborative cross mouse, pending further funding from EIDRC CC. Recombinant viruses, transgenic mouse models and experimental recombinant protein constructs described above will be made available to the EID-SEARCH consortium and other EIDRCs following standard procedures **(see Resource Sharing Plan)**.

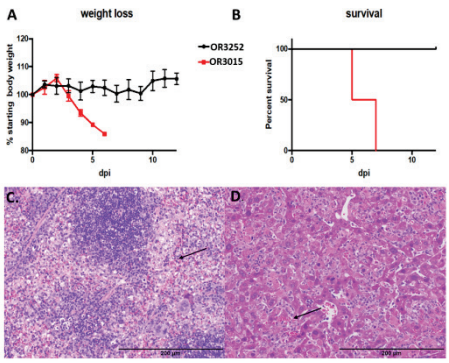


Fig. 8: EBOV Infection in Collaborative Cross Mouse. **Panel A/B:** Wt EBOV in two different CC lines demonstrate different disease patterns. **Panel C/D:** Hemorrhagic phenotypes on d. 6 (arrow) in spleen and liver, resp. From (*86*).

1.3. General Approach: We will conduct analyses to geographically- and taxonomically-target testing of samples from wild mammals that are most likely to harbor known high-profile zoonotic pathogens, or close relatives with potential to infect people. This includes selecting from tens of thousands of samples collected on recent projects and stored in freezers in our laboratories, and new sampling of wildlife in high risk locales. We will use viral family level PCR assays to identify and partially characterize viruses, then follow up sequencing of relevant receptor binding proteins to assess homology and predict binding affinity, then attempt to isolate and

biologically characterize viruses that phylogenetic and other data suggest have high spillover and pandemic potential. We will follow this with *in vitro* binding assays and cell culture experiments, and *in vivo* animal model infections to assess their potential to infect people and cause disease **(Fig. 9)**. EHA will lead the study design, targeted sampling, and data analysis; Co-Is Hughes and Wacharapluesadee will lead field sampling and in-country testing; Duke-NUS, UNC, USUS will lead the serological and PCR diagnostic development and the *in vitro* and *in vivo* analyses, as well as cross-training and tech transfer to the hub labs in Thailand and Malaysia. This will include via support of one Malaysian Ph.D student, Mei-Ho Lee (CM Ltd.) who has worked with the EID-SEARCH collaborators for 9 years. The focus of her study will be infection potential of novel CoVs and PMVs already found in bats in Malaysia under preliminary data for this proposal.

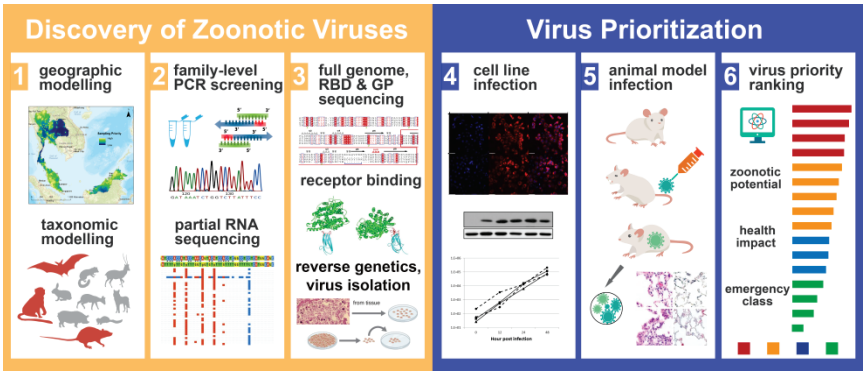


Fig. 9: Aim 1 will use analytical tools to target selection of archived and newly collected samples, conduct PCR and sequencing of novel viruses, recover by reverse genetics, and assess zoonotic potential using *in vitro* and *in vitro* models and analyses.

1.4 Wildlife samples: 1.4.a. Geographic and taxonomic targeting for newly collected wildlife samples: We will prioritize sites within each country that rank highest for both the risk of zoonotic disease emergence (*2*) and the predicted number of 'missing' zoonotic viruses (*3*). Our preliminary analysis **(Fig. 1)** suggests priority areas include: the Kra Isthmus (S. Thailand) and adjacent forests in N. Peninsular Malaysia, NW Thailand (near the Myanmar-Laos border), and lowland rainforests of Sarawak and Sabah. In each of these 'hottest of the hotspots' regions, we will identify 2-4 distinct sampling sites based on the geographic distribution of key hosts we prioritize for additional wildlife sampling. We will priority rank bat, rodent, and NHP species using host trait-based models (*3*). We will also identify species predicted to be centers of evolutionary diversity for CoVs, PMVs, and FVs using ancestral host reconstruction of available sequence data and Bayesian discrete phylogeographic models (*118-120*). We will leverage our strong collaborations with wildlife experts in Malaysia and Thailand, including many of whom are based at our core partner institutions, to ground-truth geographic and taxonomic model outputs with local expert knowledge to ensure the sampling plan is realistic based on current species distributions, population size estimates, seasonality, and accessibility to sites. Most zoonotic viruses are naturally carried by multiple wildlife host species, and there are strong, predictable relations between host phylogeny and viral taxonomy (*3*). We will use a combined network analysis and phylogeographic model to rapidly predict and prioritize new (unsampled) hosts for important, novel viruses we discover during our research. We have shown this relatively simple model (using just wildlife species range overlap and phylogenetic similarity between host species) is both generalizable to estimate host range for all mammalian and avian viruses *and* robust – accurately predicting hosts 98% of the time when cross-validated using data from known viruses (*121*).

1.4.b. Sample size justifications for testing new and archived wildlife specimens: We calculate initial sample sizes targets using our preliminary data on average PCR prevalence from screening tens of thousands of bat, rodent and NHP specimens for CoV and PMVs based on previous studies in Malaysia and Thailand. We will refine these sample size targets, in real-time, over the course of our project using viral discovery curve analyses **(Fig. 6)** to either scale-up or scale-back new sampling and testing of archived specimens for each species depending on viral capture rates. We will only collect additional specimens projected from each prioritized taxa to achieve statistically robust sample sizes and to complement our archived specimens. For example, *Rousettus* spp. bats, known FV reservoirs, were not adequately sampled under PREDICT research in Thailand. Preliminary analysis indicates that, for all viral target families, we will require ~14,000 new samples to identify 80% of remaining viral species diversity in our study region, ~5,000 samples from Thailand and ~9,000 samples from Malaysia **(Fig. 10).** Viral strain diversity from a sampling effort this size is expected to number in the hundreds of strains. For example, given ~6% prevalence of CoVs in bat species previously sampled under the PREDICT project, a target of 14,000 individuals would yield ~800 PCR positive individuals,

representing, an estimated ~600 novel sequences/strains. Similarly, for PMVs, which have an average PCR detection prevalence of ~1.5%, sampling of 14,000 individuals would yield ~200 positives and an estimated 150 unique strains. Filovirus estimates are not feasible yet due to the small number of positive samples in prior studies, but will be estimated as the project begins. From each mammal, we will collect serum, whole blood, throat swabs, urine and fecal samples or rectal swab using our previously-validated nondestructive methods. We will sample sites at different time-points throughout each year, and sample an equal number of males and females to account for seasonal or sex-specific differences viral shedding **(See Vertebrate Animals)** (*122, 123*). Dead or animals euthanized due to injury will be necropsied.

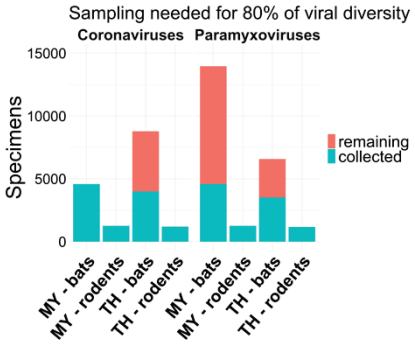


Fig. 10: Stacked bar plot showing number of currently-archived specimens **(blue)** and new specimens required **(orange)** to discover 80% of predicted number of CoVs and PMV species from high-risk bat and rodent taxa in Malaysia and Thailand.

1.4.c. Sample collection, testing, viral isolation: Wildlife will be captured and sampled using previously published techniques, by personnel wearing appropriate personal protective equipment (Tyvek suits or dedicated long external clothing, double nitrile gloves, leather gloves, an N95 or p100 respirator, and safety glasses) (*53, 93, 95, 96, 122, 124*). All samples will be placed in a vapor phase liquid nitrogen dry shipper immediately upon collection, and then transferred to a -80C freezer once back in the lab, until testing. Viral RNA will be extracted from bat fecal pellets/anal swabs. RNA will be aliquoted, and stored at -80C. PCR screening will be performed with pan-coronavirus (*125, 126*), filovirus (*127*) and paramyxovirus primers (*128*), in addition to virus specific primers where additional sensitivity is warranted in key viral reservoirs, i.e. Nipah virus or MERS virus specific primers (*129, 130*). PCR products will be gel purified and sequenced. We will attempt isolation on samples that contain viruses of interest (determined in **Aim 1.5 below**), using Vero cells and primary cell culture from the wildlife taxonomic order (bat, rodent and NHP cell lines). Over 70 bat cell lines are maintained at Duke-NUS from five different bat species. These include primary lung, brain, bone marrow, heart, kidney, intestine, liver, lymph node, muscle, spleen, PBMC and ovary/testes from *Eonycteris spelaea, Cynopterus bracyhotis, Rhinolophus Lepidus, Myotis muricola* and *Pteropus alecto* bats. In addition we have 8 immortalized cell lines, including those derived from lungs, therefore suitable for the isolation of respiratory viruses.

1.4.d. Moving beyond RNA viruses: To detect pathogens from other families, such as non-RNA viruses, we will combine conserved PCR assays with NGS. We will perform either unbiased NGS, or use a pan-viral enrichment library to sequence full-length genomes (*131*). We will conduct this work via EID-CC research projects or as part of outbreak detection/investigation in the region from DNA viruses or other agents.

1.4.e. Sequencing Spike Glycoproteins: Based on earlier studies, our working hypothesis is that zoonotic CoVs, FVs and henipaviruses encode receptor binding glycoproteins that elicit efficient infection of primary or continuous human cell lines (e.g., lung, liver, etc.) (*16, 61, 62*). Characterization these for SARSr-CoVs suggest that viruses up to 10%, but not 25%, different in the spike glycoprotein use human and bat ACE2 receptors for docking and entry. Uneven sampling gaps (e.g., no strains were found with 10-25% spike variation) prevent a thorough understanding of the transition point where the most divergent strains lose human ACE2 receptor usage. Our viral discovery curves **(Fig. 6)** suggest further sampling will reveal a rich diversity of as-yet-undiscovered SARSr-CoVs and strains of other viruses that fill in the phylogenetic tree between currently-described viruses. In this aim we will use phylogenetic and viral discovery analysis to specifically target bat species and regions that are under-sampled to allow sufficient power to identify and characterize missing strain diversity for potential zoonoses. We will sequence the S proteins of novel SARSr-CoVs to prioritize viruses for experimental work in Aim 3 to test this hypothesis. Likewise, we will test the hypothesis that phylogenetic related zoonotic receptor binding protein genes of MERS-related, Ebola-related and NiV/HeV-related viruses will also program efficient entry via human and ortholog receptors. For all novel virus strains, we will sequence the complete entry spike genes by amplifying overlapping fragments using degenerate primers as shown previously (*24, 61*). ***Reverse Genetics:*** Full-length genomes of selected CoVs, FVs or henipaviruses (representative across subclades) will be sequenced using NEBNext Ultra II DNA Library

Prep Kit for Illumina and sequenced on a MiSeq sequencer. PCR or MinIon sequencing will be performed to fill gaps in the genome. The full length spike gene sequences, including the amount of variation in the receptor binding residues that bind the appropriate human receptor will be used to select strains for Aim 3 experiments. We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-CoV cross neutralization, synthetically reconstruct a small subset (1-2) and evaluate growth in Vero, BHK and Huh7 cells (*63, 132*). From full length sequences, synthetic clones will be ordered to recover recombinant viruses using reverse genetics as described by our groups (*62, 63, 77, 109, 133, 134*). Recombinant virus growth will be accessed in Vero, BHK, Huh7 and select bat cells and recovered viruses used for downstream studies. Virus strain prioritization is described below.

1.5. Assessing risk for spillover. 1.5.a. Capacity to infect human cells: We will use a series of cell cultures to assess the capacity of target viruses to enter human cells. For CoVs, FVs and henipaviruses, we will use primary human ciliated airway epithelial cells (HAE) and lung endothelial cell cultures derived from the lungs of de-identified transplant recipients. HAE are highly differentiated and grown on an air-liquid interface for several weeks prior to use (*62, 63, 132*). We will prepare HAE and lung endothelial cultures from three different patient codes in triplicate in collaboration with the tissue procurement facility at the Cystic Fibrosis Center at UNC. Cultures will be inoculated with chimeric bat SARSr-CoVs or novel FVs or henipaviruses to assess efficient replication. At 72 hpi, cultures will be fixed for immunofluorescent staining using antisera to the SARS-CoV conserved nucleocapsid protein (N) or to structural genes of the other viruses (*64, 71*). SARSr-CoVs that differ significantly in S protein sequence (11-24%) from epidemic SARS-CoV yet replicate *in vitro*, will also be evaluated for sensitivity to neutralization in Vero cells by PRNT50 assays using broadly SARS-CoV cross reactive human mAbs S227.14, S230.15, S215.17, and S109.8 (*63, 135*) or monoclonal antibodies targeting the Ebola or Marburg E glycoprotein (Available through BEI), or NiV/HeV glycoproteins (*136*). As controls or if antisera is not available, the S genes of novel SARSr-CoV or spike entry genes of other novel viruses under investigation will be inserted into VEE 3526 replicon vectors (VRP3526-S), packaged and used to vaccinate mice (*137*). Polyclonal sera against test bat-SARS virus or other spike genes will be harvested and tested for ability to cross neutralize SARS-CoV, GD03, WIV-1, SHC014, WIV-16, other novel SARSr-CoV and HKU3-SRBD by PRNT50 assay (*63, 138, 139*). Using PRNT50 titers with sera (n=4 each) among these viruses, antigenic cartography (*140*) will allow comparison of antigenic vs. phylogenetic distance, identifying the transition at which SARSr-CoV strains escape SARS-CoV based vaccines, informing immunotherapeutic and vaccine design strategies (*141-143*). Similar approaches will be applied to novel MERS-related viruses, other CoV, FVs or henipaviruses. While most wildlife-origin viruses with unknown transmission potential or pathogenicity in people uncategorized, we use BSL-3 for isolation as standard, and cell culture for potentially BSL-4 pathogens will be undertaken at collaborating institute NEIDL. To facilitate this, duplicate samples in culture medium from animals PCR +ve for FVs and very close relatives of NiV or HeV will be shipped to NEIDL **(See letter of support)** for culture, characterization and sharing with other approved agencies including NIH Rocky Mountain Laboratories where PI Daszak and Co-Is Baric have ongoing collaborations. Similarly, some duplicate samples of animals PCR +ve for CoVs will be shipped to UNC for further characterization by Co-I Baric. Where feasible and appropriate, training opportunities at NEIDL and UNC for in-country staff will be given, thereby enriching hands-on collaboration across sites.

1.5.b. Host ACE2, DPPR, Niemann-Pick C1 (NPC1) and EphrinB2/EphrinB3 receptors: We will sequence relevant host receptors for select mammalian wildlife species we find positive for strains of high-priority CoV clades, and all new henipaviruses and FVs. Of particular interest is homology across 18 bat and human orthologue ACE2 contact interface residues that engage the SARS RBD as a potential indicator of SARSr-CoV cross species transmission potential and growth in human cells (*70*). Likewise, variation in the NPC1 receptor has been shown to alter EBOV ability to infect in humans and bats, so this can be structurally mapped for prediction and downselection of strains (*144*). Signatures of positive selection in bat NPC1 were concentrated at the virus-receptor interface, with the strongest signal at the same residue that controls EBOV infection in *Eidolon helvum* cells. Thus NPC1 is a genetic determinant of FV susceptibility in bats, and some NPC1 variations likely reflect host adaptations to reduce FV replication and virulence (*145*). NiV and HeV use ephrin B2 or ephrin B3 as a receptor, and variation affects its ability to use human vs bat receptor molecules (*103, 146*).

1.5.c. Host-virus evolution and predicting receptor binding: We will use Bayesian and Maximum Likelihood phylogenetic analyses of RNA-dependent RNA polymerase (RdRp) sequences from PCR screening, receptor binding glycoproteins, and/or full genome sequence data (when available) to reconstruct the evolutionary history of the novel bat SARSr-CoVs, bat MERSr-CoV, FVs and henipaviruses that we identify. We will run co-phylogenetic analyses to map out instances of host-switching (*147, 148*) and ancestral state reconstruction analyses **(Fig. 4)** to identify the most important species for shaping evolutionary diversity for these viruses (*51, 149*), allowing for more targeted future surveillance and spillover mitigation interventions. For receptor binding glycoprotein sequence data from characterized viruses, we will apply codon usage analyses and codon adaptation indices, as used recently on henipaviruses (*150*), to assess the relative contributions of natural selection and mutation pressure in shaping host adaptation and host range.

1.5.d. Viral strain prioritization: Of the expected 100-200 novel SARSr-CoV strains, and approximately 600 total CoV strains, we will down-select to prioritize for further characterization based on S genes that are: i) different from SHC014, WIV1, SARS-CoV with diversity ranges of 10-25%; ii) have virus S RBD that could use human/bat receptors; iii) have recombinant chimeric spikes indicative of gene flow between clade I and II strains; iv) have bat ACE2 or DPP4 receptors that might select for spike RBDs that can use human receptors for entry (15/18 conserved residues in human/bat ACE2 molecules that bind SARSs-CoV S RBD domains are likely more efficient receptors than 3/18 conserved sites). Using structural models based on the SARS S glycoprotein, the extent and location of antigenic variation will be annotated onto the structure, further resolving the locations of highly evolving and conserved structural motifs/epitopes that function in protective immunity (*135, 151-153*). For novel henipaviruses, we will examine the receptor-binding proteins (e.g. envelope attachment (G) proteins) for sequence and structural homology with particular attention to conversation of amino acids that mediate ephrin receptor binding. We will prioritize strains that are predicted to interact with ephrin-B2 and expression V/W proteins to determine whether these viruses represent zoonotic threats. Using the recombinant Cedar virus (rCedV) platform, we will be able to rescue rCedV that express heterotypic G proteins to explore receptor utilization and tissue dissemination in animal models. We will assess ability to infect human cells using a continuous lung epithelial cell line, Calu3, which our group has previously used to generate data on NiV and HeV growth curves and a variety of host expression changes after infection (*154*). We will also use primary human airway cultures and microvascular endothelial cells to assess human infection for FVs, henipaviruses and MERSr- and SARSr-CoVs (*155*) (*156*) . There is some evidence for NiV and HeV replication in mouse lung, low level titers in young animals, 10 fold higher titers in aged mice (*157, 158*). Thus it is likely these models could be improved in the collaborative cross mouse resource at UNC. We have identified CC lines that are highly vulnerable to wildtype SARS and others that develop hemorrhagic disease following mouse adapted Ebola infections (*86-88*).

1.5.e. Animal models: We will use transgenic mouse models and the UNC Collaborative Cross model to assess spillover potential of viruses. For work on CoVs, we will follow the protocols in the transgenic mouse model used by the Baric lab previously. Briefly, in the BSL3 lab, 10- to 20-week old hACE2 or hDPP4 transgenic mice will be intranasally inoculated with 5 x 10^4 PFU of full length wildtype rbat CoV, or chimeric SARSr-CoV or MERSr-CoV encoding different bat CoV spike proteins respectively, then monitored daily for weight loss, morbidity, and clinical signs of disease. Mice will be euthanized at 2, 4, and 6 dpi (endpoint of 14 dpi), organs harvested and virus quantified by plaque assay or genome (mRNA1) RT-PCR. After 7 days in formalin, tissues for histopathology will be removed from the BSL3 and stained with H&E, and for immunohistochemistry using polyclonal antibodies against the N protein. We will conduct limited evaluation of existing countermeasures using therapeutic monoclonal antibodies *in vitro*. Existing SARSr-CoV or MERS-CoV mAbs (*159, 160*) will be diluted in DMEM starting at 1:20, and serial dilutions mixed with an equal volume of 50 PFU of chimeric bat SARSr-CoVs encoding different spike proteins and RFP indicator genes, then incubated on Vero E6 cells at 37°C for 1 h. Antibody neutralization titers will be evaluated by plaque quantification at 4-5dpi. In select instances, hACE2 transgenic mice will be injected with SARS-CoV mAbs, and infected with chimeric bat SARSr-CoVs. Clinical signs and morbidity will be assessed and tissue pathology examined and compared with mice without treatment of mAbs to determine the therapeutic effect on SARSr-CoV infection, and protection of SARSr-CoV by wildtype SARS-S based vaccines assessed as described (*132, 161*). We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-

CoV cross neutralization, synthetically reconstruct a small subset (1-2), recover full length viruses and evaluate the ability of nucleoside analogues to inhibit growth in HAE cultures and/or *in vivo* (*63, 132*). For the Collaborative Cross model, we will infect six to eight mice from 6-8 Collaborative Cross mouse lines (10 weeks of age) with six test viruses/yr, intransasally or by subcutaneous infection with 1 x 10e4 virus. Animals will be followed for weight loss, morbidity, mortality and other clinical disease symptoms (e.g., hunching, hindleg paralysis). One half the animals will be sacrificed at day 3 and day 7 pi. to evaluate virus growth in various organs, and organ pathology. In highly vulnerable lines, additional experiments will be performed using more animals, evaluating virus growth, clinical disease symptoms, respiratory pathology, tropism and organ pathology through day 28 pi. We anticipate that some CC lines will prove highly vulnerable to select wildtype viruses, because of host susceptibility combinations that promote severe disease outcomes.

1.6. Potential problems/alternative approaches: Challenges with logistics or obtaining permission for wildlife sampling in sites we select. We have a >20-year track record of successful field work in Malaysia, and >10 years in Thailand, and have strong established partnerships with local wildlife authorities and communities to ensure access. We have existing permissions in place which will be renewed at the start of this project. Due to the abundance of wildlife habitats in the region, if access to a site is withdrawn, we will rapidly identify a suitable, complementary site with similar bat species composition and abundance. Finally, we have already collected tens of thousands of wildlife samples from the region and will focus on these while waiting for permission. **We may not identify novel viruses in our sample wildlife species due to seasonality of viral shedding or other factors.** We have calculated sample sizes based on realistic detection rates from our extensive preliminary data, and are confident that we will detect and identify large numbers of potentially zoonotic pathogens, particularly for CoVs and PMVs. We acknowledge that FV strain diversity may be harder to capture, given relatively low seroprevalence in bat populations – suggesting lower levels of viral circulation (*42, 43*). However, our team was the first and only group to sequence FV RNA from Asian wildlife species (*46, 47, 57*), and we are confident that with our targeted sampling and testing strategy offers the best chance globally to identify additional strains of Ebola and related viruses. The sampling regions we have selected are subtropical, and our previous data, and even published studies in temperate regions (*162*), do not suggest a strong pattern of seasonality in CoV shedding, and where seasonal patterns do occur, i.e. for henipaviruses in Thailand (*163*), or can be predicted from wildlife serology data, we will be sure to conduct sampling at different timepoints throughout the year to account for this.

Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays.

2.1 Rationale/Innovation: Rapid response requires early detection. Assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities that interface frequently with wildlife that harbor high risk emerging viral strains. To enhance the low statistical probability of identifying these rare events, In Aim 2 we will target rural human communities inhabiting regions identified in Aim 1 as having high viral diversity in wildlife, that also engage in practices that increase the risk of spillover (e.g. hunting and butchering wildlife). We will initially identify 4 subsites in each of Thailand, Peninsular Malaysia, Sarawak, and Sabah for sampling. We will design and deploy human risk factor questionnaires, and specific and sensitive serological assays to identify the baseline frequency, and causes of, spillover of known and related novel viral pathogens in these populations. **We will use the results to test a key hypothesis: that hunting wildlife increases the risk of exposure to novel viruses, compared to the rest of the community.** The alternative hypothesis is that seroprevalence in the general community is not statistically different to those who hunt and butcher wildlife, because exposure to wildlife pathogens is largely through inhabiting a biodiverse landscape where wildlife make indirect contact with people (e.g. via fomites). For participants who report symptoms related to 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever; or 6) unusual presentation of high fever with severe diarrhea; all within the last 10 days, an additional sample type will be collected, two nasal or oropharyngeal swabs. Samples will be marked for additional PCR-based assays to identify presence of known and novel CoVs, henipaviruses and FVs, and for isolation and biological characterization of potential pathogens, using the approach laid out in Aim 1.

2.2 Preliminary data on high-zoonotic risk human community surveillance: 2.2.a Biological sample collection in SE Asia: Our long-term collaboration in the region has included qualitative and quantitative survey data, and collecting large archives of banked human sera and other biological samples from high zoonotic-risk populations. For example, under the USAID-PREDICT project, EHA has collected 1,400 and 678 specimens in Malaysia and Thailand respectively from people living in zoonotic disease interfaces, all of which will be available for retrospective serological testing by EID-SEARCH which we have ensured is compliant with our existing IRB. Our core group has collected and tested many thousand additional specimens from other important community cohorts **(Table 2)**, and some of these will continue under EID-SEARCH **(Section 2.4)**.

Country, site	Lead	# enrolled, focus	Findings
Peninsular Malaysia	Hughes	9,800+ samples, Orang Asli indigenous pop., for PCR/serol.	25+ novel CoVs, influenza, Nipah ab+ve, FV ab+ve
Malaysia Sabah	Kamruddin	1,283 for serology	40% JE, 5% ZIKV ab+ve
Malaysia Sabah	William	10,800 for zoonotic malaria study	High burden of macaque-origin malaria
Malaysia Sabah	Hughes	150 bat cave workers	Ongoing
Malaysia Sarawak	Siang	500 Bidayuh and Iban people	8% PCR prevalence HPV in women
Malaysia PREDICT	Hughes	1,400 high zoonotic-risk communities for viral PCR, serology	Ongoing. Multiple known and novel CoVs, PMVs, others.
Thailand	Wacharaplu-esadee	100s of bat guano harvesters/villagers	Novel HKU1-CoV assoc. clinical findings
Thailand PREDICT	Wacharaplu-esadee	678 high zoonotic-risk communities for viral PCR, serology	Ongoing. Multiple known and novel CoVs, PMVs, others.
Singapore	Wang	856, for Melaka virus	7-11% MELV ab+ve

Table 2: Biological sample collection from healthy populations conducted by members of **EID-SEARCH** in our hub countries.

Of particular interest is the access to indigenous communities in Peninsular Malaysia (Orang Asli) and Sarawak (Bidayuh, Iban people), and the bat guano harvesters in Thailand. These communities hunt, butcher and eat wildlife daily, and work in supremely high-risk zoonotic interfaces (e.g. bat caves). One of our indigenous community sites is experiencing an ongoing outbreak of unknown etiology that we are investigating currently **(Section 3.2.a)**.

2.2.b Human Contact Risk Factors: EHA is the lead organization across all 27 USAID-PREDICT project countries for collecting data on, and analyzing the human aspects of, zoonotic spillover risk (*164, 165*). We conducted exploratory studies using standardized one-on-one semi-structured interviews and site observation data with people living in hotspot areas or engaged in high-zoonotic spillover risk activities, using these data to assess local social and cultural norms underlying wildlife contact (*165*). We then developed a risk factor survey to accompany human biological sampling to evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. We conducted cross-sectional studies in high risk populations in 27 countries, and study participants provided biological samples (oral/oropharyngeal swab, nasal/nasopharyngeal swab, urine sample/urogenital swab, fecal sample/rectal swab, whole blood, serum, plasma). For example, survey and biological samples were collected from: 1,585 participants from 7 sites in Yunnan, Guangxi, and Guangdong provinces, China; 1,400 participants from 4 sites in Malaysia; and 678 participants from 4 sites in Thailand. Samples were tested with novel serological assays and results analyzed against risk factors from survey data (*16*). In China, CoV-seropositive individuals were mostly farmers/peasants (8/9), living in Yunnan province (7/9), 41-60 yrs old (7/9), with domestic contact with rodents (6/9), that are male (6/9). Although these results are preliminary and don't provide detailed information on routes of exposure, they identify key subpopulations to target that would likely increase the opportunity for identifying evidence of spillover for other CoVs, PMVs and FVs. Data from PREDICT surveys have been used to help identify target populations in Aim 2, identify strategies to better target at-risk people, and conduct focused **human risk factor surveys and serosurveys to produce statistically significant findings for henipaviruses, CoVs and FVs.** In Aim 2, we will use combined biological sampling and risk factor surveillance in targeted populations within the community to 1) test the hypothesis that occupational (i.e. wildlife hunting) risk factors correlate with seropositivity to henipaviruses, FVs and CoVs; 2) assess possible health effects of infection in people; and 3) where recent

illness is reported, obtain biological samples for PCR testing and potential viral isolation and characterization. Obtaining this information could be a significant step in understanding the likelihood of recent 'hidden' spillover events and their public health impacts, as well as the risk of future emergence of viral disease.

2.2.c. Risk factors for illness: Our preliminary data in Thailand, Malaysia and other SE Asian countries revealed that frequent contact with wild (e.g. bats, rodents, NHPs) and domestic animals (e.g. poultry, swine) among local communities was associated with a high percentage of self-reported severe/acute respiratory illness (SARI/ARI) and Influenza-like illness (ILI) symptoms **(Section 3.2.b)**. In addition, people involved in crop production, hunting and butchering of wildlife reported statistically significantly higher SARI and ILI symptoms (Li *et al.*, in prep.). **These preliminary data suggest a target population and sites for current proposal with the correct targets for serological surveillance and risk factor surveys in Aim 2 to produce statistically significant findings**. Coupled with better serological tools from our team **(Section 2.2.d)**, we will be able to identify the likely routes of exposure to known or novel coronaviruses, henipaviruses, and FVs in the study region, as well as build data on the illnesses they cause in people.

2.2.d. Serological platform development: Most emerging viruses produced a short-lived viremia in people so that large samples sizes are required to provide enough PCR-positive individuals to analyze infection patterns. For Aim 2, we will focus on serological sampling in the community, because antibodies are typically long-lived and can be used to assess prior virus exposure or infection with much smaller samples sizes (*123*). Most serological assays target a single protein, and for emerging viruses, it's often unclear which viral antigen will be immunodominant during human infection, and therefore which to target in developing serological assays. Co-Is Wang, Anderson (Duke-NUS) and Broder, Laing (USUS) have developed a series of approaches to deal with this problem, and which will be used in testing human biological samples in Aims 2 and 3. Henipaviruses, FV and CoVs express envelope receptor-binding proteins that are the target of protective antibody responses. Co-I Broder has led efforts to express and purify henipavirus native-like virus surface proteins for immunoassay application (*166, 167*), developing monoclonal antibodies (*168, 169*) and as subunit vaccines (*170, 171*), has developed a novel, multiplex microsphere immunoassay (MMIA) allowing serum samples to be simultaneously tested for antibodies reactive with henipaviruses, FVs and CoVs. This MMIA uses a Luminex-based multiplex platform and custom designed virus receptor-binding proteins expressed in a mammalian cell-culture system and purified as native-like oligomeric proteins. These oligomeric antigens retain post-translational modifications and quaternary structures, allowing capture of conformational-dependent antibodies raised during a natural infection, which represent the majority of the protective humoral response. The known diversity of henipaviruses includes five presently described viruses, NiV, HeV, CedV, GhV and MojV and this assay has been expanded to include all described species **(Fig. 11)**. In the past, our collaborators have used NiV and HeV G protein antigens in a MMIA assay to identify a serological footprint of NiV-related African henipaviruses and exposure to virus(es) antigenically-related to NiV in livestock sampled in Bangladesh (*78, 79*). To strengthen serological data interpretation, the G protein from each virus is integrated into the MMIA so that virus-specific vs. henipavirus cross-reactive antibodies can be detected and address specificity of past virus exposure. Extensive cross-reactivity exists among ebolaviruses and between ebolaviruses and marburgviruses (*172-174*). We are validating the MMIA pan-FV assay for specificity, and as part of this, in collaboration with Duke-NUS, we have found serological evidence of past exposure to a filovirus(es) most antigenically-related to ebolaviruses (e.g. Ebola virus, Bundibugyo virus), but unreactive with Reston virus GP in three under-sampled fruit bat species, suggesting that novel FVs circulate within bat hosts in SE Asia (*43*). **This platform has now been set up for use in Malaysia and Thailand and will be available for use in this proposed work.** Co-Is Hughes, Broder, Laing have conducted initial screening of human, wildlife and livestock samples at high-risk interfaces in Malaysia, finding serological evidence of past exposure to viruses antigenically-related to NiV and EBOV in humans, bats and non-human primates (NHPs).

Fig. 11: Validation of multiplex microsphere immunoassay (MMIA) specificity and identification of immunologically cross-reactive viruses for A) NiV B) EBOV.

Co-Is Wang and Anderson (Duke-NUS) have developed a phage-display method to screen antibodies for all known and related bat-borne viruses, including henipaviruses, FVs and CoVs and have set up a similar Luminex-based assay. Additionally the Duke-NUS team has demonstrated capacity for rapid and cost-effective development of LIPS serological assays against novel viral agents **(Sections 2.6.a, 3.2.a)**. These will be used for verification and expanded surveillance. For molecular investigation (Aim 2 & 3), we will combine targeted PCR with a discovery-driven enrichment NGS strategy to not only survey currently known viruses, but also to discover potential novel viruses. Both the phage-display serological and enrichment platforms can be rapidly updated to include new viruses identified.

2.3 General Approach/Innovation: Our human sampling approach uses two approaches in Aims 1 & 2 (Fig. 12). In Aim 2 we will conduct community-based surveillance using a brief, focused questionnaire to identify the prevalence and frequency of risk-factors for viral spillover in tandem with collection of high quality biological samples to determine the seroprevalence of spillover viruses in at-risk human populations. We will use generalized linear models to analyze correlation between biological results and risk-factors for viral spillover. **In Aim 3**, we will conduct clinic-based syndromic surveillance and biological sampling at clinics/hospitals that are the catchment healthcare facilities for the communities sampled in Aim 2. Both community-based and clinic-based syndromic surveillance programs are cross sectional case-control studies designed with the sample sizes necessary to statistically quantify (with a power of 80%) risk factors and health impacts for viral spillover, linked to serological status and PCR prevalence in symptomatic patients. All human sampling conducted under Aims 2 and 3 will adhere to strict criteria for inclusion and recruitment and protection of human subjects from research risk **(see Human Subjects and Clinical Trials Information)**.

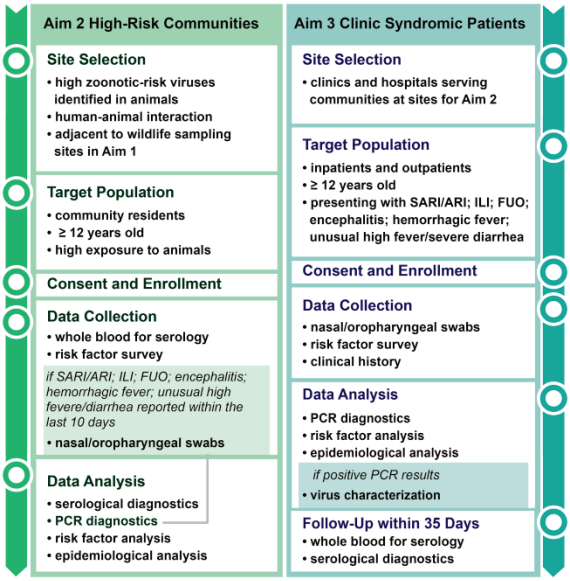


Fig. 12: Human sampling approach. Aim 2 **(left)** focuses on serology in high-risk communities to identify evidence of population exposure to novel zoonotic viruses. Aim 3 **(right)** sets up clinical cohorts at hospitals serving these high-risk communities to conduct syndromic surveillance and identify the etiology of new viral syndromes

2.4 Target population & sample sizes: We will target populations in the same geographic regions as those identified in Aim 1 that harbor wildlife with high potential zoonotic viral diversity, that are EID hotspots, and that are well-connected through regional travel and trade hubs to the global travel network **(Fig 13)**. Within each geographic region we will identify populations at four sub-sites, building on our preliminary data **(Table 2)**. We will target specific communities based on our analysis of previously collected behavioral questionnaire data, and other publicly available data (e.g. wildlife hunting licenses sold in a region, number of guano caves accessed for fertilizer etc.) to identify key at-risk populations and occupations with high exposure to wildlife. We will conduct concurrent sampling trips at visits to sub-sites during which members of the community will be enrolled and wildlife surveillance activities conducted. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock, or slaughtering wildlife will be considered for enrollment. Participants will complete a consent form and questionnaire in addition to having biological samples collected. During these community trips after data collection the field research staff will conduct community meetings to help inform the public health risk of zoonoses. **Target populations:** *Thailand (Co-I Wacharapluesadee):* 100 subjects of healthy high-risk community from 2 study sites, Chonburi (NiV, other PMVs and CoVs found by PCR in bats in our previous work), and Ratchaburi (bat guano harvester villages where we found a MERSr-CoV in bat guano (*50, 175*) and serological evidence of α-CoVs, HKU1-CoV in people (*175*). *Peninsular Malaysia (Co-I Hughes, CM Ltd.):* We will expand our sampling of the Orang Asli indigenous communities to include sub-sites in communities in Districts we have already sampled and

additional Districts in the States of Kelantan Perak, Pahang, and Kelantan all close to bat caves. Samples will be stored and tested at NPHL. *Sarawak (Co-I Tan, Fac. Med. Hlth. Sci. UNIMAS):* We will expand our sampling of the Bidayuh and Iban indigenous people to include the Dayak and Orang Ulu ("people of the interior") because this group has a more intimate connection to remote wildlife-rich areas than the other two, albeit all are hunters. Human sampling will be led by UniMAS in collaboration with CM Ltd / EHA. *Sabah:* We will conduct sampling at the Madai bat and bird cave that has 200 permanent residents who harvest bird's nests for soup, and >1,000 during the harvesting season and at Gomantong cave – one of the largest bat and cave swiftlet nesting sites in the world. *Sabah: (Co-I Hughes):* We will conduct sampling at the Madai bat and bird cave that has 200 permanent residents who harvest bird's nests for soup, and >1,000 during the harvesting season and at Gomantong cave – one of the largest bat and cave swiftlet nesting sites in the world. Protocols follow those for Peninsular Malaysia *Singapore: (Co-I Wang, Duke-NUS):* Active human surveillance will be not be conducted in Singapore, but archived human specimens e.g. from our Melaka virus serosurvey (*8*), will be made available to conduct retrospective serosurveys for specific pathogens that are identified as high interest to the EIDRC, NIH, and EID-CC.

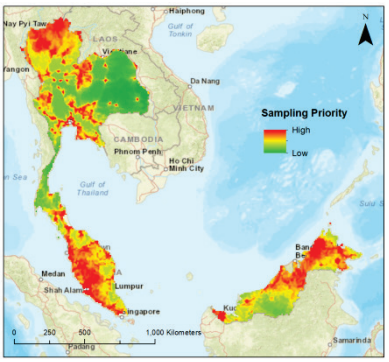


Fig. 13: Targeting high-risk sites for human zoonotic disease surveillance in Thailand and Malaysia. This map plots areas with high numbers of expected viruses in wildlife (*3*), greater spillover probability based on EID risk factors (*2*), and higher levels of connectivity (measured by road density data) that may facilitate person-person spread.

Sample sizes: From our previous work we conservatively anticipate that 10-30% of the community population will have had exposure to wildlife allowing us to capture highly exposed and non-exposure individuals at each site. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock farming, transportation, selling, or slaughtering wildlife in the surveyed areas will be targeted so that they make up ≥30% of the sampled population in each community. We will stratify sampling to ensure appropriate representation of sex, demographic, and socio-economic factors at each community site. In each of our regions, we will conduct sampling at four sub-sites in populations with high prevalence of high occupational and environmental risk of exposure to wildlife populations, sampling 175 persons at each sub-site. We will target populations with, on average, 30% or higher prevalence of occupational or environmental exposures to wildlife based on data from previous studies. This design will give us >80% power to detect 5 percentage point differences in seroprevalence against any of our viral groups between baseline and high-risk subgroups. We conservatively assume 5% base seroprevalence, as well as assuming high spatio-temporal variance amongst sub-sites, with baseline varying 0-25% amongst sub-sties and the fraction of the population at risk varying from 20-40%. (Power calculation conducted by 500 simulations of a generalized linear mixed model (GLMM)). For community-based surveillance, we will enroll 700 individuals per study region, allowing us to make county/province-level comparisons of differing effects; the total sample will be 3,500 participants over 5 years.

2.5 Data & sample collection: Following enrollment, biological specimens (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max 500 µL of whole blood and two 500 µL serum samples) will be collected by locally certified healthcare professional proficient in phlebotomy techniques and a short questionnaire will be administered by trained project staff that speak appropriate local language to all participants. Through the serological findings and the survey we will investigate the details of five risk factors related to high risk wildlife exposure, to maximize the power of the analyses, based on continuing analysis of our previous work, and will focus on: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. For participants who report the symptoms relating to 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever within the last 10 days, an additional sample type will be collected, nasal or oropharyngeal swab (2x). Blood of participants whose serum tests positive for emerging viral pathogens will be taken and Peripheral Blood Mononuclear Cells (PBMCs) frozen for future use.

These will be used for future harvesting of polyclonal and monoclonal antibodies as potential therapeutics/ diagnostics **(see Letter of Support NEIDL)**.

2.6: Laboratory analysis: 2.6.a Serological testing: We will screen all human specimens for henipaviruses, FVs and CoVs using our novel, multiplex microsphere immunoassay (MMIA) **(Section 2.2.d)**. We will use ELISA and serum neutralization tests (SNT) as confirmatory, where feasible and appropriate for the biocontainment level given sensitivity and specificity variation, and the need for live virus for SNTs **(See Select Agent Research)**. Serologic evidence of local spillover will be used to prioritize zoonotic strains for recombinant virus recovery from full length sequences. Preliminary data suggest ELISA will be effective for some viral groups. We previously developed a SARSr-CoV specific ELISA for serosurveillance using the purified NP of a bat SARSr-CoV (Rp3). The specificity of this assay was evaluated using polyclonal antibodies against HKU1, OC43, 229E, NL63, MERS-CoV and EBOV and no significant cross-activity detected (*16*). **This suggests that if we can expand our NP serology tests to cover other bat CoVs, and that doing so may identify many more seropositive individuals.** For CoVs, we will therefore use two serological testing approaches: 1) testing human sera collected from both community- and clinic- based sampling for a panel of bat CoVs that will include SARS-CoV, MERS-CoV, SADS-CoV and a range of bat SARSr-CoVs (*16*); 2) testing for antibodies to common human CoVs (HCoV NL63, OC43 – **Section 2.8**). We will test panels of NP and G recombinant proteins to assess if this approach will act as a comparison to MMIA for FVs and PMVs.

2.6.b RT-PCR testing. Specimens from individuals in the community who reported being symptomatic within the last 10 days **(Section 2.5)** will be screened using RT-PCR initially for CoVs, PMVs, and FVs following published protocols **(Section 1.4.c)**. Positive samples will be subjected to full genome sequencing and RT-PCR amplification of the glycoprotein genes. Samples from the clinic-based syndromic surveillance will also be tested using RT-PCR for Influenza A & B, HCoV NL63, OC43, HKU1, SARS-CoV & 229E, NiV, HeV, Ebola (WHO PCR protocol) based on the symptom as rule-outs.

2.6.c Biological characterization of viruses identified: Novel viruses identified in humans will be recovered by cultivation or viruses re-derived from full length genome sequences using reverse genetic approaches. Viruses will be characterized for growth in primary human cell lines and in mouse models, including the Collaborative Cross Mice or humanized mice expressing human receptors from related strains. The approaches are discussed in more detail under Aim 1.

2.7 Epidemiological analysis: We will conduct a cross sectional case-control study to identify risk factors for Henipavirus, FVs, and CoVs spillover. “Cases” are defined as participants whose samples tested positive for Henipavirus, FVs, or CoVs by serological tests. “Controls” will be selected from the pool of participants admitted to the studies and whose samples tested negative for Henipavirus, FVs, or CoVs. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models and least absolute shrinkage and selection operator (LASSO) regression analyses (*176*) to analyze correlation between biological results, serological/PCR status, and risk factors related to high risk wildlife exposure including: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) home or work proximity to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms lifetime and past 12mo. Additionally, we will use this procedure to determine how clinical presentation differs between Henipavirus, FVs, or CoVs in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms.

2.8 Potential problems/alternative approaches: Rarity of spillover events means it may be difficult to identify sufficient seropositives to statistically analyze human risk profiles. First, we will be targeting our community-based surveillance to subpopulations with high-levels of wildlife exposure, at sites selected for diverse and prevalent viruses to increase likelihood of finding positive individuals. Second, our serology testing will include a multi-plex panel of assays for a large diversity of viruses, increasing likelihood of positives. For example, in previous work our overall bat CoV PCR prevalence in the community was, 11.8%; β-CoV, 3.4%; α-CoV, 9.1%). Thus, using this broad serological panel to screen individuals prone to contact with species of higher zoonotic risk increases the potential for detecting spillover with enough power for statistical analyses,

and will shed light on behaviors that predispose to spillover. Third, we will include common human CoVs, PMVs, and influenza viruses in our panel, so that even if low prevalence of wildlife viruses is found, we will be able to conduct a valuable cross-sectional study of the seroprevalence of more common human viruses. Finally, we will be able to assess relative measures of human-wildlife contact from our survey work. We will analyze intensity of contact with high-risk taxa against other risk factors to provide useful proxy information for spillover risk. **Serological testing may be difficult to interpret in the presence of known and novel viruses.** We will use the three-tiered serological testing system outline in **2.6.a** to try to identify exposure to these 'novel' viruses. However, we know exposure to a range of related viruses generates antisera that are cross-reactive with known, related virus antigens - a serious challenge to serological surveillance and diagnostics. Our collaborations have already demonstrated that SE Asia bat sera samples screened with our pan-filovirus MMIA have been exposed to an Ebola-like virus, that is antigenically-distinct from known Asiatic filoviruses, Reston virus and Měnglà virus (*43*). Using these three serological platforms we will address inherent challenges of indirect virus surveillance through antibody capture by testing multiple binding assay platforms and antigens, using positive control samples to establish serological profiles against known viruses that will aid in our interpretations of cross-reactive antisera responses likely representative of 'novel' viruses.

Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts.

3.1 Rationale/Innovation: Our previous research **(Table 1)** and other data suggests that there are substantial numbers of outbreaks or clinical cases for which the etiologic agent is not diagnosed, e.g. (*177, 178*). To investigate this in SE. Asia, we have assembled a multi-disciplinary team that includes senior infectious disease doctors and clinicians and will work directly with local clinics and regional hospitals to identify the 'cryptic' outbreaks or cases that occur in the region. **In Aim 2, we will conduct serology in high zoonotic-risk communities to find evidence of prior exposure in people,** i.e. evidence of viral spillover into the community. In Aim 3 we identify members of these communities presenting at clinic with undiagnosed illness that may have high-impact zoonotic viral etiology. **Therefore, in Aim 3 we focus on PCR to identify the putative viruses that are replicating in these actively-infected patients (Fig. 12).** We will enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and who present at regional clinics or hospitals with symptoms typical of high impact zoonotic viruses. We will collect detailed survey data to assess their contact type and frequency with wildlife and livestock and conduct molecular and serological diagnostic assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. We will conduct molecular viral discovery assays on patients who present with symptoms indicative of a high-risk zoonotic virus. If novel pathogens are identified by PCR, we will attempt to isolate, obtain full genome sequence and/or biologically characterize the wildtype or molecularly re-derived pathogen, using the *in vitro* and *in vivo* strategy laid out in Aim 1.

3.2 Preliminary data clinical surveillance: 3.2.a. Clinical surveys and outbreak investigations: Our longterm collaboration in the region has included the following activities investigating clinical syndromes: *Peninsular Malaysia*: **At the time of writing, an outbreak of suspected undiagnosed viral illness in the indigenous Batek Orang Asli ("people of the forest") community living at Gua Musang ("civet cat cave") district, in Kelantan, Malaysia, close to Taman Negara – a National Park containing the oldest known rainforest**. This community is one where we have conducted prior routine surveillance and biological sampling, demonstrating proof-of-concept that our two-pronged high-risk community/clinic approach will work. Dozens of people are affected and the outbreak is currently being investigated by our group in collaboration with the Malaysian NPHL. The Orang Asli continue to practice traditional subsistence hunting of wild animals (bats, rodents, primates). These communities are remotely located, in heavily forested areas, with limited access to medical services. This outbreak, and others that will continue to emerge just like it, underscore the critical need for our EIDRC - to improve the technological tools, training, and human capacity for rapid EID surveillance, diagnosis, and response at the frontlines of disease emergence in SE Asia. **Investigating this outbreak is a key priority if EID-SEARCH is funded.** *Sabah*: Co-I Kamruddin (BMHRC) is leading an asymptomatic leptospirosis study on sanitation workers, an ILI study in a Kota Kinabalu clinic and diarrheal study in children under 5 yrs. Co-Is William, Tan and others have conducted clinical observational studies to determine the etiology of central nervous system infections and acute undifferentiated febrile illness in Sabah.

Co-Is William and consultant Yeo have collaborated with Co-I Wang to enroll 90 adult and over 100 pediatric participants with central nervous system infections, negative tests for malaria and dengue from 3 district hospitals in Sabah to determine the various etiologies. While a proportion tested positive for rickettsial agents, the majority had no clear diagnosis. Co-Is William, Rajahram, and Yeo have conducted clinical studies of over 200 inpatients with acute febrile illness with negative tests for malaria and dengue from 3 district hospitals in Sabah to determine the various etiologies. While a high proportion tested positive for rickettsial agents, the majority had no clear diagnosis. *Sarawak:* Co-I Siang (UNIMAS) is conducting a survey of high-risk female Kelabit indigenous people and has found 8% with HPV52 as the dominant strain. He is working on clinical cohorts of hand, foot and mouth disease and will recruit patients from that study in the current proposed work. Finally, Co-Is Siang, Kamruddin are conducting a long-term study of rotavirus and other viral etiologies of acute gastroenteritis in Kuching, Sarawak that will include swine herders and farmers. The Baric lab is a global leader in norovirus VLP diagnostic reagents to detect serologic evidence of norovirus gastroenteritis, as well as RT-PCR detection of novel noroviruses in humans, and has identified novel bat noroviruses, suggesting further opportunities for expansion of this work (*142, 179-181*). This work is particularly relevant in Malaysia because pig farms are shunned by the government and local villagers to they are placed far away from town centers, deep in the forest, and it is this scenario that led to the emergence of Nipah virus in 1998-9 (*6, 22*). *Thailand:* Co-Is Hemachudha, Wacharapluesedee (TRC-EID, Chulalongkorn Univ. Hosp.) work in coordination with the Thailand Ministry of Health to investigate outbreak etiology, including the first case of imported MERS-CoV in Thailand, **for which we produced sequence confirmation within 24 hours from acquiring the specimen**. Three imported MERS cases have been detected in Thailand since 2015, and all cases were confirmed at the TRC-EID. We worked during the Zika outbreak to identify > 500 PCR-positive Zika patients with sequence confirmation. We have also worked on returning and suspected Ebola travelers, syndromic surveillance for SARI, encephalitis, Hand Foot and Mouth disease in children and viral diarrhea. Consultant Hickey (US CDC, Thailand), screened specimens collected between 1998 and 2002 from a large cohort of children from Kamphaeng Phet Province to examine Dengue virus and JE epidemiology (*182, 183*). Among the 784/2,574 convalescent sera henipavirus-specific antibodies were detected in 13 serum samples (1.7% seroprevalence), suggesting prior exposure to endemic henipaviruses or a related PMV in rural Thailand. *Singapore:* Duke-NUS has worked with the Ministry of Health to investigate Zika cases (*184*), and other EIDs. With EHA and others, Duke-NUS has developed novel CoV assays for clinical surveillance, and for expedited analysis during outbreaks. Our pipeline of novel virus detection, reservoir host identification, serological assay development, and serological testing is as rapid as several months, with the development of novel serological assays from sequence data in 7 days (*17*). **For example, with the discovery of SADS-CoV, we identified and fully sequenced a new virus, screened archived wildlife specimens to identify the natural reservoir host species (*Rhinolophus* spp. bats), developed LIPS serological assays, and tested human and swine samples to assess seroprevelance in less than 3 months (*17*).**

3.2.b Analysis of self-reported illness: We have developed a standardized approach for analyzing data from study participants on self-reported symptoms related to target illnesses: fever with cough and shortness of breath or difficulty breathing (severe acute respiratory illness - SARI); fever with muscle aches (fevers of unknown origin (FUO); and, cough or sore throat (influenza-like illness - ILI). For all of our prior human clinical surveillance in 20+ countries, including in Malaysia and Thailand, we used a least absolute shrinkage and selection operator (LASSO) regression to identify associations between participants who reported ILI and/or SARI symptoms and demographics, occupation, and contact with animals in the last year. Results have clear biological relevance. For example, in Yunnan, China, factors strongly associated with prior disease involve animal hunting and consumption **(Fig. 14).** We will expand this approach for all clinical syndromes in Aim 3, and use targeted questions to assess patient's exposure to wildlife in terms that are relevant to each specific country.

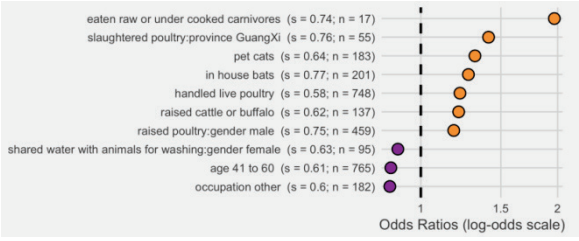


Fig. 14: Factors associated with self-reported ILI & SARI in prior 12 months (s = bootstrap support; n = #+ve out of 1,585 respondents). **Orange circles** = odds ratios > 1 (+ve association); **purple** = odds ratios <1 (-ve association).

3.3 General Approach: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent has not been identified. To capture these, we will expand on our current syndromic surveillance to enroll, and collect high quality biological samples from patients who present at regional clinics and live in rural communities linked to Aim 2. Case definitions and enrollment criteria include: 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever; or 6) high fever/diarrhea with unusual presentation. We will collect survey data to assess contact with wildlife, and occupational and environmental exposures, conduct molecular and serological assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. If novel pathogens are identified by PCR, we will attempt to isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work. We will then use behavioral survey data, ecological and phylogeographic analyses to assess the likely reservoir hosts to inform intervention programs.

3.4 Clinical cohorts. 3.4.a Cohort selection: We will initiate active clinic-based syndromic surveillance at 2 town-level level clinics and 2 provincial-level hospitals in each Thailand, Peninsular Malaysia, Sabah and Sarawak. These serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2 and are inclusive of wildlife sampling sites in Aim 1. Patients ≥ 12 years old presenting at the health facility who meet the syndromic and clinical case definitions above will be recruited into the study after completing a consent form. We will aim to enroll 300 participants per hospital, with that we will have a 95% probability of detecting live virus in patients in each hospital assuming a population prevalence of 1%. This sampling effort will total of 4,800 individuals for clinical syndromic surveillance. We assume up to 40% loss from follow-up, therefore yielding 2880 participants that will be available for follow-up blood sampling **(Section 3.4.b)**. While enrollment is limited by the number of patients that come to the healthcare facility presenting with relevant clinical symptoms, in our work in the PREDICT project we enrolled an average of 100 participants per year, so we are confident this is an achievable enrollment target. Study data will be pooled across country regions, as clinical patients are limited by the number of individuals presenting at hospitals. "Cases" will be determined as participants whose samples tested positive for either CoVs, henipavirus, or FVs, PCR tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. Sites: *Thailand:* We will work with two primary hospital, Phanat Nikhom Hosp., Chonburi district (Key Pers. Dr. P. Hemachudha) & Photharam Hosp., Ratchaburi Province, and a large tertiary hospital, King Chulalongkorn Memorial Hospital, Bangkok Thailand (Co-I Dr. T. Hemachudha). *Peninsular Malaysia:* **Focusing immediately on the current outbreak in Orang Asli, we will work closely with the Malaysian Ministry of Health to support their investigation, and with 6 Orang Asli clinics which focus solely on this indigenous community.** Co-I Sellaran (Lintang Clinic, Kuala Kangsar) refers patients to Sungai Siput Hospital and Gen. Hospital, Ipoh. Key Pers. l Lotfi (Pos Betau clinic, Kuala Lipis) will continue collaboration with Co-I Hughes in the communities at that site. *Sarawak:* Key Pers. Diyana (Director, Bario Clinic) refers patients to Miri Hospital (Co-I Utap) and serves people from the Kelabit, and nomadic tribes in the region, as well as populations within a number of large towns. *Sabah:* We will continue our collaboration with Queen Elizabeth Hospital, QEH (Key Pers. Lee, Rajahram) and Hospital University Malaysia Sabah, HUMS (Co-I Lasimbang, Director). Both clinics include our targeted sites for **Aim 2** in their catchment, and both have ongoing collaboration with the Borneo Medical Health Ctr (Co-I Kamruddin, Director). *Singapore:* Currently, there are no plans to conduct clinic surveillance based on budget constraints. However, in the event that that is an evolving need for clinic surveillance, enrollment, and sampling, we have close working relationships with all hospitals in the country, and the central infectious disease reference lab (key clinicians at which are aware of our proposal). This would allow us to rapidly on-board a hospital based site.

3.4.b Clinic enrollment and follow-up: We will recruit inpatients and outpatients to participate in the study after initial screening to determine if they meet our clinical case definitions. Once consented and enrolled, biological samples will be collected by trained and locally certified hospital staff and a questionnaire administered by trained hospital or research staff that speak appropriate local language. Every effort will be made to take samples concurrently when collecting samples for normative diagnostics. For both inpatients and outpatients, samples will be collected no more than 10 days of reported onset of illness to increase the chance

of PCR detection of viruses (*185*). Where possible, we will follow up 35 days after enrollment to collect an additional two 500 µL serum samples conduct a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. This gives adequate time for development of IgG, which occurs <28 days after onset of symptoms for SARS patients (*186-188*). Serum samples will be used to screen panels of high risk human and local zoonotic Filovirus, Henipavirus and Coronavirus strains as described in Aim 2. These samples may prove valuable to collaborators in that the PBMC could be used in production of therapeutic antibodies.

3.4.c Sampling and clinical interview: Biological specimens whole blood (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples) and two nasal or oropharyngeal swabs will be collected from all participants, and a questionnaire will be administered. We will investigate five risk factors, to maximize the power of the analyses, all related to high risk wildlife exposure, based on continuing analysis of our previous work, and will include: 11) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With additional consent considerations for participants enrolled from clinical settings, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology. Blood will be taken for future harvesting of Peripheral Blood Mononuclear Cells (PBMCs), as per **Section 2.5**.

3.5 Sample testing: The standard syndromic diagnostic PCR assays for common pathogens will be conducted and the results will be shared with the hospital with 48 hours. Specimens will be further tested for 3 viral families; CoV, PMV and filovirus by consensus PCR **(Section 1.4.c)**. Necropsy samples from deceased patients will be further characterized by NGS if previous PCRs are negative and unable to identify the cause of infection. Co-Is Wang, Anderson will conduct a VirScan serological assay using paired serum samples at Duke-NUS that identifies antibodies with a four-fold rise in titer in the convalescent sample compared with the acute sample, thereby indicating a possible etiological agent. A PCR test with the specific primer from each virus antibody positive case will be further tested from acute specimens to confirm infection.

3.6 Viral risk characterization and potential for pandemic spread: We will use statistical models built from collated biological, ecological, and genetic data to assess the likelihood of pathogenicity for the range of diverse viruses we will characterize. The aim is to quickly identify and assess which viruses pose the most significant risk to human health and thus prioritize additional resources to more fully characterize, better understand pathogenicity, and invest in our animal model pipeline and ultimately therapeutic development for those pathogens. We will combine two independent but related statistical models to predict pathogen spillover risk and pathogenicity. First, we will expand a simple generalized additive modeling approach (*3*) to assess spillover risk probability to a new virus that is currently not known to infect people, when we have only limited genetic data from initial PCR screening and sequencing. Additional data from receptor binding protein homology and cell line infection experiments will be incorporated into the model framework as available. Second, given high likelihood to spillover and infect humans, or direct evidence of human infection from our clinical surveillance, we will then assess the likelihood of pathogenicity based on traits for phylogenetically related nearest neighbor viruses as a first approximation and incorporating more detailed data from genomic characterization and animal model experiments (Aim 1). Epidemiological trait data for ~300 viral species known to infect people (e.g. case fatality rate, global estimates of number of human cases per year or decade, R_0, infectious period, and primary symptoms) have been collated by recent studies and will be used as predictor variables (*189*). Thirdly, we will apply tools already developed, and being refined by EHA under DTRA and DHS supported research, to predict pandemic spread for viruses using global flight models, as well as additional datasets on human movement and connectivity across Southeast Asia (*90, 91*) **(Fig. 15)**.

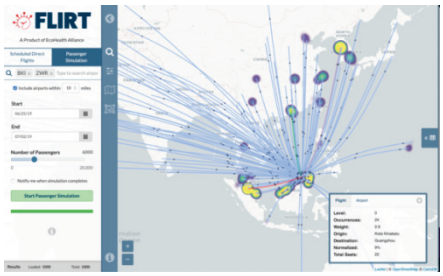


Fig. 15: Probability feed from EHA's Flight Risk Tracker tool (FLIRT). This image is of the likely pathways of spread and establishment for a Nipah-like virus emerging from Sabah, based on human movement and airline flight data (*90, 91*).

3.7 Potential problems/alternative approaches: Patients visiting clinics may have cleared virus, but not yet developed IgG antibodies, reducing seropositive cases. Our 35 day follow-up sampling should account for this because the maximum lag time between SARS infection and IgG development is ~28 days and Ebola virus infection and IgG approximately 20 days (*185-188, 190*). We also expect that patients in rural communities will only visit clinics when symptoms have progressed, likely coinciding with late illness and onset of IgG. We will also have data from our community study that can be analyzed in conjunction with, or in the absence of, clinical data from the hospital data to identify PCR- or seropositives. Finally, the risk of limited detection is outweighed by the potential public health importance of discovering active spillover of new henipaviruses, FVs or CoVs infection.

4. Administrative Plan

4.1. Project management: 4.1.a. Administrative core: The EID-SEARCH organizational partner roles are laid out by Specific Aim above **(see Fig. 3)**. EcoHealth Alliance (EHA) will lead the administrative core of the EID-SEARCH, including: coordination and management of all human and animal research; communication with consortium partners, NIH, other EIDRCs, and the EIDRC-CC; data management and quality assurance; modeling and analysis; and compliance with all US, international, and institutional rules and regulations. The administration will be led by PI Daszak, who has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5-20 yrs on NIAID- and USAID-funded research **(Fig. 16)**. He also has direct experience as one of two external reviewers of one of the CEIRS partners for NIAID (Program officer Diane Post). PI Daszak will be assisted by Deputy Lead, co-I Olival, who has overseen zoonotic disease research in Southeast Asia for >10 years and working directly with our partners in Thailand and Malaysia for 15 years. Senior program manager (Chmura) and full-time program assistant (Hamel) will provide administrative support. The EID-SEARCH data management, epidemiological analysis, and modeling activities will be led by co-Is Olival and Zambrana who have led all modeling and analytics on USAID USAID PREDICT for the last 10 years. They will overse a programmer/data scientist (Mendelsohn), evolutionary biologist/bioinformatician (Latinne), and human epidemiologists (Dawson, Li, Martinez). Our human epidemiology team will work directly with country teams in Malaysia and Thailand to manage human surveillance, IRB applications and approvals, and ensure compliance with all human subjects work. EHA's senior veterinarian (Field) and wildlife field biologist (Phelps) have extensive experience working that bats, rodents, and primates in SE Asia and will coordinate all training and field research in Malaysia and Thailand and ensure compliance with IACUCs.

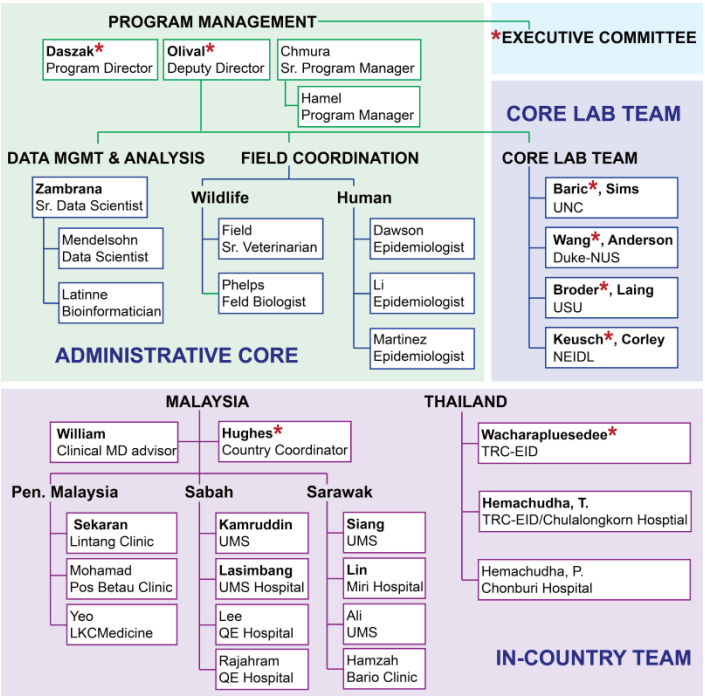


Fig. 16: Administration and program management for the **EID-SEARCH**

The EID-SEARCH core laboratory team includes global experts in virology, molecular pathogenesis, and the development of assays, reagents, and therapeutics for high consequence viral zoonoses (Co-Is Wang, Anderson at Duke-NUS; Baric, Sims at UNC; Broder, Laing at USU; Keusch, Corley at NEIDL). The core laboratory team will work with in-country diagnostic labs in Thailand (Wacharapluesedee, Hemachudha at the TRC-EID, Chulalongkorn University) and Malaysia (Hughes, Lee, CM Ltd.). Wacharapluesedee and Hughes will additionally serve as country coordinators for the EID-SEARCH, liaising directly with EHA on weekly conference calls, and working with in-country partners to ensure the smooth operation and coordination of clinical and community surveillance and wildlife research in Thailand and Malaysia, respectively. **A Core Executive Committee** comprising PI Daszak, Deputy Lead and Co-I Olival, and leads from each core partner and country: Co-Is Hughes, Wacharapleusadee, Baric, Wang, Broder, Keusch (or alternates), will conduct

regular conference calls and in-person meetings to facilitate rapid decision making within the EID-SEARCH. **This committee will also convene to manage EID-SEARCH response to outbreaks.**

4.1.b Project Management in Thailand and Malaysia: Wacharapluesedee and Hughes have collaborated directly with EHA for >15 years, including acting as country coordinators on the USAID PREDICT project for the last 10 years (project end date Sept. 2019). They maintain strong ties with Ministries of Health (MOH), Agriculture and Environment, multiple universities and research institutions, clinics, and hospitals, in their respective countries and across the region. The EID-SEARCH will use these connections to disseminate results, obtain permissions to conduct sampling, and also rapidly respond to and assist with outbreaks as they happen. Peninsular Malaysia, Sarawak, and Sabah are the three main Malaysian administrative regions, and effectively operate as three separate countries, with different regulations and government structures. We therefore provide specific details on the management of EID-SEARCH activities in each:

Coordination among Peninsular Malaysia, Sabah and Sarawak will be led by co-I Hughes (Conservation Medicine Ltd), and follow a successful model we implemented under USAID-PREDICT. **On Peninsular Malaysia** this project will be administered through the Zoonosis Technical Working Committee (ZTWC) established under the PREDICT project with a binding MOU among EHA, CM Ltd. and ZTWC, and including officers from MOH, Dept. of Veterinary Services, and PERHILITAN (the Govt. wildlife agency). EHA will communicate weekly with Co-I Hughes to coordinate and monitor implementation of research and reporting to ZTWC. Co-I Hughes will coordinate activities at all other Peninsular Malaysia institutions: NPHL, the National reference laboratory for diagnostic confirmation of pathogens, will manage molecular and serological screening (BioPlex) of Orang Asli samples, and serological screening of syndromic samples from Sabah and Sarawak; the PERHILITAN molecular zoonosis laboratory will store and conduct molecular and serological screening on wildlife samples; and Universiti Putra Malaysia (UPM) Faculty of Veterinary Medicine will conduct molecular and serological screening (BioPlex) of livestock samples, should these be required. **For Sabah & Sarawak**, work will be administered through the Sabah Zoonotic Diseases Committee (SZDC), a working technical committee comprising appointed and authorized officers from Sabah State Health Dept., Department of Veterinary Services, Sabah Wildlife Dept. (SWD), Universiti Malaysia Sabah (UMS) and EHA, all of which are also committed through a signed MOU. Co-I Hughes will oversee work at all other partners in Sabah, including: the Kota Kinabalu Public Health Lab (KKPHL) for molecular screening of syndromic samples from Sabah and Sarawak; the SWD Wildlife Health and Genetic and Forensics Lab for molecular screening of Sabah wildlife samples; The Borneo Medical Health Research Center (BMHRC) for screening some Sabah wildlife and livestock samples, if required, and human syndromic samples from Sabah and Sarawak. **In Thailand** all human community and wildlife research and testing will be coordinated by co-I Wacharapluesedee from the TRC-EID center. Clinical surveillance will be overseen by senior clinical physician and co-I T. Hemachudha.

4.1.c. Approval and release of results: In our experience, it is critical when working in resource-poor countries, on potentially important pathogens, to strictly adhere to protocols for release of results. EID-SEARCH will liaise with existing points of contact in the Ministries of Health, Environment, and Agriculture in each our administrative areas to approve and release project findings publicly. Results from human screening will be shared with participants when they become available, as per our IRB agreements ensuring no violations to anonymize data requirements (**see Protection of Human Subjects**).

4.2. Flexibility to extend the EID-SEARCH to new sites as needed: The EID-SEARCH consortium partners maintain extensive working relationships with leaders in EID outbreak control, clinical investigations and research at over 50 clinics, research institutes and public health laboratories across Southeast Asia. Due to space constraints, we haven't listed each of these, nor have we solicited >50 Letters of Support for this project. However, each core EID-SEARCH partner has contacted their networks and obtained permission for inclusion in the broader goals of the EIDRC. As examples of these contacts, our core partner, the Thai Red Cross Emerging Infectious Disease Health Science Centre (TRC-EID) at Chulalongkorn University, also serves as the WHO Collaborating Centre for Research and Training on Viral Zoonoses and has ongoing research collaborations across WHO SEARO countries including Bangladesh, Bhutan, Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, (Thailand), Timor-Leste; and has recently served as a training hub for scientists from Malaysia, Myanmar, Laos, the Philippines, and China to learn methods of wildlife sampling and diagnostic screening. Our Thai clinicians (Co-I T. Hemachudha and KP P.

Hemachudha) provide regular case consultations and clinical trainings for doctors across SEARO countries, including with Yangoon General Hospital and the National Health Lab in Myanmar, 2018. To maximize leverage of this broad network, EHA has budgeted for annual meetings in SE Asia, in addition to regular smaller network meetings, with our core team and key public health experts from network labs in each of the 10 SE Asian countries**.** Additionally, we will set up a listserv and an internal communication network to facilitate collaboration and information exchange, including on the first reports of new disease outbreaks. Our annual and smaller network meetings will critically allow face-to-face meetings of the EID-SEARCH that will foster greater sharing of information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks in the region, guided by the PI, Deputy and the Executive Committee.

4.3. Outbreak response: EHA collaboration with expert networks around the world allows us to mobilize and enhance effective One Health response to disease emergencies (*191*), ranging from real-time situation updates and risk analyses to on-the-ground investigations (*192-194*). We will adopt management tools from Emergency Operating Center (EOCs) (*195*) and Incident Management Systems (IMS) (*196*), to shift resources where necessary to help respond to novel zoonotic outbreak events and other public health emergencies. EHA has extensive experience working with governments in low and middle income countries (LMIC) applying these principles of epidemic preparedness during outbreak responses we've been involved with under the USAID-PREDICT project. For example, at the request of the government of Bangladesh, we provided technical field and laboratory support for Nipah virus and avian influenza outbreak investigations, assisting with wildlife sampling as part of the outbreak response alongside human and domestic animal sampling. In India, we provided technical assistance in response to the Nipah virus outbreak in Kerala in 2018. Last month in Indonesia were assisted the Ministry of Health's Center for Health Laboratory in Makassar to provide technical assistance in a mysterious outbreak in a small village in South Sulawesi that killed 4 villagers and infected 72. Our network partners include the key government and govt. approved laboratories that would be directly involved in public health emergency response in their respective countries. The serological and PCR platforms that EID-SEARCH develops will be made available to the main government outbreak investigation teams for clinical work and research during the outbreak. EID-SEARCH will also offer assistance training and conducting animal sampling during an outbreak, epidemiological analysis and modeling to help identify likely reservoirs or likely pathways to spread. Technical and material support for lab, field and analytical activities during an outbreak will be provided by EHA, UNC, USU, Duke-NUS, and NEIDL, as well as in-country partners. Any clinical samples, viral isolates and sequence data will be shared among partners to promote the rapid development of new diagnostic assays, reagents, and therapeutics that can be deployed to the region or other regions as part of the larger NIH EIDRC network.

Finally, while the initial pathogen focus of our group is on CoVs, PMVs and FVs, our broad collaborative group has multidisciplinary expertise on a number of virus-host systems. For example: PI Daszak was PI on a subaward from PI Laura Kramer's U01 on Poxviruses and Flaviviruses, managing a multidisciplinary research project on West Nile virus ecology. He was also co-I on a 5-year NSF-funded project to understand West Nile virus dynamics and risk in the USA (*197-201*); Co-I Baric is a global leader in Norovirus research leading to the development of vaccines and therapeutics (*202-205*); Co-I Wang has conducted significant work on bat immunology, therapeutic, and reagent development, as well as being involved in a range of outbreak investigations, viral discovery programs and other research on a wide diversity of viral groups (*206-215*). Additionally, the serological and PCR-based diagnostic platforms being developed by Co-Is Wang and Broder are adaptable to other viral targets. The modeling tools developed by Co-Is Olival and Zambrana-Torrelio can be used to predict the emergence and spread of diverse viral targets, including influenza, antimicrobial resistance, and vector-borne diseases (*216-221*). Our clinicians working in Thailand and Malaysia have a wide range of infectious disease investigations to adapt to any outbreak situation.

4.4. Communications: EHA will coordinate communication among all co-Is and key personnel, including:

- Multiple meetings per week with PI, Deputy Lead, Senior Program Manager (on project and task status)
- Weekly web/phone meetings between Program Manager and subawardee admin. staff
- Monthly web/phone conferences between EHA PIs and all subawardee PIs.

- Monthly web conferences between key personnel (research presentations/coordination)
- In-person Annual meetings with partner leads, key personnel at EHA and two in-person partner meetings annually between subawardees.
- Annual in-person meeting among all key personnel

4.5. Problem identification and resolution: Regular planning, monitoring, and evaluation meetings will be the primary mechanisms for problem identification. Minor problems (e.g. delays in sample availability or test results) will be dealt with internally through appropriate action and resolution monitored by PI Daszak and co-I Olival, and our Senior Program Manager. In the event of significant problems, such as prolonged poor productivity, inadequate scientific collaboration, or major disputes regarding research direction or resource allocation, EHA will assist with resolving the problem through negotiation with relevant co-Is and consultation with the Executive Committee. Should a resolution not be forthcoming, consultation with the EIDRC-CC, additional external technical advisors, and NIH staff may be warranted.

4.6. Adaptive management and risk mitigation: Maintaining a timeline and meeting milestones will require strict and continuous oversight of all project phases, frequent and regularly scheduled communication, and the ability to make decisions and implement strategies. To maintain our timeline on all projects, including the EID-SEARCH, we use an adaptive management approach to continually evaluate these trade-offs, to make decisions about when iteration is appropriate and when it is necessary to move forward with current information. Our ethos is that regular, scheduled communication among all staff, partners and collaborators will go a long way towards mitigating risks, especially if the process is collaborative and transparent.

5. Data Management Plan

EHA will house the Data Management and Analysis (DMA) team for EID-SEARCH, led by Co-PIs Olival and Zambrana-Torreilo and include Key Personnel Latinne and Mendelsohn. EHA has served as the data and analysis hub for numerous multi-institutional, multi-sectoral, international disease research groups, including acting as Modeling and Analytics lead for the PREDICT project (*122*), the Western Asia Bat Research Network (*222*) and EHA's Rift Valley Fever Consortium. We will leverage our experience and infrastructure from those projects to benefit the EID-SEARCH. **5.1. Project Database:** We will create a dedicated, centralized EID-SEARCH database to ingest, store, link, and provide for analysis all data associated with the proposed study and other expanded projects associated with the research network. The database will be SQL-based and use encrypted, secure cloud hosting services and enable export to archival and platform-independent formats. It will ensure data and metadata compatibility between project components, track data versioning and annotations. The system will be designed to work with both with both paper- and tablet-based field data entry and with the Lockbox laboratory information management systems **(Section 5.2)** in place in individual partner labs. The database will use existing metadata standards, including NCBI standards for genetic and molecular data and Ecological Metadata Language (EML) for field and wildlife data, as well as other standards and formats designated by the EIRDC CC. This will enable rapid publication and deposition of data. Granular security and privacy controls will be applied so that specific expansion projects undertaken in the network may be managed while maintaining data confidentiality as needed.

5.2. Biological Specimen Management: Project laboratories will use the Lockbox Laboratory Information Management System (LIMS), to manage the security, traceability, and quality of biological specimens. The LIMS will support sample barcoding at creation, tracking through transport, storage/inventory, and use via portable scanners. Lockbox supports CLIA and ISO 17025 as well as direct export to NCBI formats such as Sequence Read Archive. We will use the Lockbox LIMS application programming interface (API) to link to the central project database and associated samples with field and ecological data. We note that the project focuses on highly pathogenic viruses, including select agents; Lockbox LIMS supports sample tracking and movement compliant with US Select Agent Regulations and US Department of Commerce Pathogen Import and Export Control Regulations, and includes all necessary encryption, security, and backup protocols.

5.3. Training: Members of the DMA team will team will develop documentation and provide training for field and laboratory teams at all partner institutions in data management, metadata standards and data hygiene best practices. The DMA team will act as trainers and consultants for partner institutions in experimental

design, power analysis, data analysis, and computational and reproducibility issues, and visit each partner institution and/or field team base for training workshops and analysis consultations.

5.4. Data Identification and Privacy: For human clinical data and questionnaires, data will be identified by a unique identification code assigned to each individual and only this, de-identified code will be accessible in the project database, and destroyed at the end of the project - as per details provided in the Clinical Management Plan and Protection of Human Subjects forms.

5.5. Computing Resources: EHA operates a cluster of high-performance servers for data analysis activities, as well as infrastructure to launch cloud-based computing environments (**see EHA Facilities**). Our servers host all necessary software for statistical and bioninformatics work that is available to the DMA and partners anywhere in the world. We use a mixture of cloud services (AWS, Azure, Backblaze, GitHub) to provide redundancy, backup, version control, and rapid post-disaster recovery, and will be available to all project partners for analysis and training.

5.6. Data and Code Sharing: See details provided in the **Resource Sharing Plan.**

6. Clinical Management Plan

6.1. Clinical site selection: Our consortium partners have been conducting lab and human surveillance research, including during outbreaks, for >20 years and have developed strong relationships with local clinical facilities and processes in SE Asia and in LMIC globally. The plan for selection of clinical sites will begin in the same geographic regions as those identified in Aim 1 with high zoonotic viral diversity. Clinical sites will additionally serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2. We have already developed successful working relationships with the major healthcare facilities in Thailand and Malaysia and will use these established partners to rapidly gain appropriate permits and begin data collection quickly. Focusing on these EID hotspots in select biogeographic areas (see **Fig 13**) also reduces the number of additional sites needed to meet our goals within our geographic range. The capacity and capability requirements for clinical sites are fairly minimal, and include ability to enroll patients that meet the clinical case definitions of interest, collect and temporarily store biological samples, and follow standards for data management and subject protection with locked filing cabinets to store all paper records and an encrypted computer. We will be enrolling inpatient and outpatient participants within 10 days of the presentation of symptoms to increase the chance of PCR detection of viruses so we will not wait for advanced normative diagnostic tests, that are likely unavailable in the more remote sites, to be completed. We will work with clinical sites to determine the best process for project staff, in most cases supporting the time of currently hired staff at each site. We will recruit and train hospital staff in project-specific procedures including enrollment of patients, sample types to be collected, storage of samples, administration of the questionnaire, and data management plans. During the development and onboarding of new clinical sites we will assess the physical needs of the site and what supplies will be required for collection of data.

6.2. Standardized approach, oversight, and implementation: Management and oversite for all study sites will be undertaken by the local country coordinator with support from our Core Administrative team at EHA. Our research team has over 10 years of experience building capacity on human subjects' research and has developed training resources and materials for standardized implementation of community and clinical research and SOPs for screening, enrollment, and retention of participants. The country coordinator will conduct regular site visits to the clinical sites and annual visits to observe, monitor and evaluate the research process, and conduct follow-up training if required. Through our work with clinical sites under the USAID-PREDICT project we have developed culturally appropriate screening measures for clinal sites that do not disrupt the flow or quality of medical treatment received by the patient. This was done by working collectively with clinical staff to evaluate current procedures and patient flow at the site to determine the most efficient while minimal invasive inclusion of our study into the daily working of the clinal site. Most efficiently this was done by adding minimal basic screening questions to the current clinal intake forms, which allows the clinal research officer to quickly scan charts or logs for potential patients to enroll avoiding potential enrollees from being overlooked if staff are too busy or not on duty. Patients will be enrolled following established clinical criteria (**see Section 6.3**), samples collected and brief surveys conducted to assess the participants contact with wildlife through: 1) occupational exposures; 2) animal contact; and 3) the environment. With permission

from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology. Additionally, we will be following up with clinical participants to determine how clinical presentation differs between CoV, henipavirus, or FV in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. The country coordinator will be continually monitoring the project database to ensure we hit target sample sizes. While patient's enrollment is limited by the number of individuals presenting at hospitals, in previous research we enrolled an average of 105 patients per year, ranging from 77-244.

6.3. Clinical cohort setup, recruitment, enrollment: We will recruit inpatients and outpatients from clinical sites to participate in the study after initial screening to determine if they meet the clinical case definitions for 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever, of unknown etiology or severe diarrhea with unusual presentation for symptoms to increase the chance of PCR detection of viruses. Once enrolled, biological samples will be collected by trained hospital staff. When possible, samples will be taken concurrently with samples being collected for normative diagnostics. We will collect 5mL of blood for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples and two nasal or oropharyngeal swabs will be collected. Controls who test positive for CoVs, FVs, or Henipaviruses will be selected from the pool of participants admitted to the studies and whose samples tested negative for coronaviruses, henipavirus, or filoviruses. We will follow up 35 days after enrollment to collect an additional 5mL blood draw for collection of two 500 µL serum samples and a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. Following up 35 days post initial collection gives adequate time for 1) development of IgG and IgM, which occurs <28 days after onset of symptoms to evaluate the immunological progression of disease and 2) further the risk assessment of the participants to monitor contact with wildlife, people, and to assess the likely reservoir hosts to collect information to inform potential intervention programs.

6.4. Utilization of collected data: Sampling of wildlife and the enrollment of community surveillance participants and a clinical cohort of participants give us the ability to assess the viruses that are circulating in each of the three populations in a similar geographic region. We will use phylogenetic analysis to compare the relationship between wildlife viruses found and viruses fond in human participants. Additionally, the questionnaire data will allow us to assess relative measures of human-wildlife contact that we will analyze the intensity of contact with species known to be at a higher risk for spillover against other risk to provide useful proxy information for spillover risk. The clinical cohort will be split into cases and controls. "Cases" are defined as participants whose samples tested positive for either CoV, henipavirus, or FV via PCR tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. In clinical study, we will use this procedure to determine how clinical presentation differs between virus-exposed and -unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. We will model the outcomes to analyze correlation between biological results and risk factors related to high risk wildlife exposure. With this collective data set we aim to quantify and detect novel viruses likely to spillover regularly to people, are often unreported or misdiagnosed as their clinical manifestations, and are unknown to detect cryptic outbreaks causing previously 'hidden' clinical syndromes in people. Our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence.

6.5. Development of reagents of value to the community. Members of the EID-SEARCH consortium have substantial experience producing reagents, assays, and other products that are used widely by the clinical and research community, and some of which are on a pathway to commercialization. These include: PIs Daszak and Co-I Olival have produced software for analyzing the spread of novel viral agents through air travel networks; Co-I Baric has collaborated with a Norovirus surveillance collaboration with surveillance cohort at CDC and has developed therapeutics that have reached phase 2 and 3 clinical trials, He is currently working with Takeda Sanofi Pasteur on a Dengue therapeutic and with NIH on a tetravalent vaccine; Co-I Broder

developed a Hendra virus subunit vaccine that was commercially produced by Zoetis for horses and is labeled for human use under compassionate circumstances during outbreak situations.

6.6. Potential expansion: Expansion of the research project to other clinical sites or research areas could happen as new information is gathered, either through our research, the EID-SEARCH information network, or an outbreak being identified in the region by other organizations. If expansion is required we would rapidly shift research activities towards the clinical or community sites where the outbreak is active, using the same process we used to set up initial research locations. First, by working with local stakeholders to create a working relationship and on-boarding new clinical research staff through advanced trainings of project aims, SOPs on data collection and storage, and ethical guidelines. The network of infectious disease researchers in the community and hospital settings would allow for an accelerated response to any potential infectious disease outbreaks as staff are well trained on ethical guidelines for working with human subjects, data and specimen collection, storage, and transpiration, project staff would be on the front lines ready for the call to action. Ideally, our testing for viruses in priority viral families may lead to the discovery cryptic outbreaks before it spreads in the general public. Biological results coupled with survey data on key risk factors will provided necessary information for granular understand of disease spread.

7. Statistical Analysis Plan:

7.1. Framework: Statistical analyses across the project will be conducted under a common Bayesian framework. These models provide a unified, probabilistic approach best-suited for estimating effect sizes in heterogenous populations of human clinical and wildlife subjects in observational studies. Within this Bayesian framework, we will use generalized linear mixed models to estimate population prevalences and seroprevalences, and estimate the effects of demographic, occupational and environmental factors affecting these. We will use occupancy models (*223*) to estimate total viral species and strain diversity and completeness of sampling within the human and wildlife sub-populations, and discrete phylogeographic models to identify taxonomic and geographic centers of viral diversification. All statistical analyses will be performed reproducibly using scripted, programmatic workflows (e.g., the R and Stan languages) and maintained under source code version control (git). As with data management, the DMA team will act as trainers and consultants for exploratory data analysis, power analysis, and study design with project partners, and the EHA computing cluster will be available for partners undertaking additional or expansion studies. Power analyses, current and expansion, are performed via simulation approaches allowing planning for complex, hierarchical variation in study populations. Power analyses and specific analytical components of this study are detailed under each Specific Aim.

7.2. Data Quality Control and Data Harmonization: All data will be are examined at entry by field and lab teams upon data entry, followed by examination by DMA team members at upload and integration, for complete de-identification, completeness, accuracy, and logical consistency. The DMA will provide field and lab teams with reports, produced automatically, of data summaries, including aggregates, distribution, detected outliers and possible mis-entries. On a regular basis (quarterly or as-needed during data collection), DMA team members will review reports with field and lab teams to identify errors and update collection and entry procedures as necessary.

7.3. Statistical Considerations for Behavioral Questionnaires and Clinical Metadata: The data collected from the questionnaire will be analyzed to assess the reported measures of contact for each risk group under study, related to1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, rodents, and primates in/around house and other livestock animals (e.g. farming, butchering, slaughtering); 3) proximity of residence or workplace to environments of increased risks (e.g. nearby bat roosts); 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12 months and lifetime. Specific measures we are interested in are the proportion of respondents indicating they consume wildlife, where wildlife is obtained for consumption, have hunted wildlife, butchered or slaughtered live animals, seen wildlife in their homes, been bitten or scratched by animals, etc. Comparisons of measures of exposure contacts and types between men and women, children and adults, different study regions will be conducted in order to explore the occupational, environmental, and demographic factors (gender, age, socioeconomic status (SES)) that influence contact with animals and to determine who is

most at-risk. Statistical analysis will be employed to identify differences between groups with a 95% probably of detecting a difference. Measures of contact related to different activities within specific groups will be compared to determine the activities that put groups at most risk. As appropriate multivariate analysis (e.g. ordinary linear regression, logistic regression, non-normal distributions of outcome, least absolute shrinkage and selection operator (LASSO) regression, etc.) will be utilized to evaluate the relationship between the outcome variables, positive biological results (PCR or serology) key measures of contact and the factors that influence frequency and types of human-animal contact.

8. Project Milestones and Timelines

8.1 Milestones: <u>End of Year 1:</u> Aim 1: Sample targeting locations, species (for wildlife), sample size justifications completed for whole project and reported to in-country teams; Sample testing, viral isolation, NGS, glycoprotein sequencing begun for all archival and some newly-collected samples; *in vitro* work begun; host-pathogen dynamic analyses; animal model work begun. **Aim 2:** Target human community populations identified and sample sizes calculated for some sites in each country; Community data collection, serological testing and RT-PCR testing begun; first epidemiological analyses of data begin in last quarter. **Aim 3:** Clinical cohort selection underway; clinical enrollment, data collection and sample analysis begun. First Annual meeting in last quarter. First publications submitted by end of year, summary overview papers or reviews.

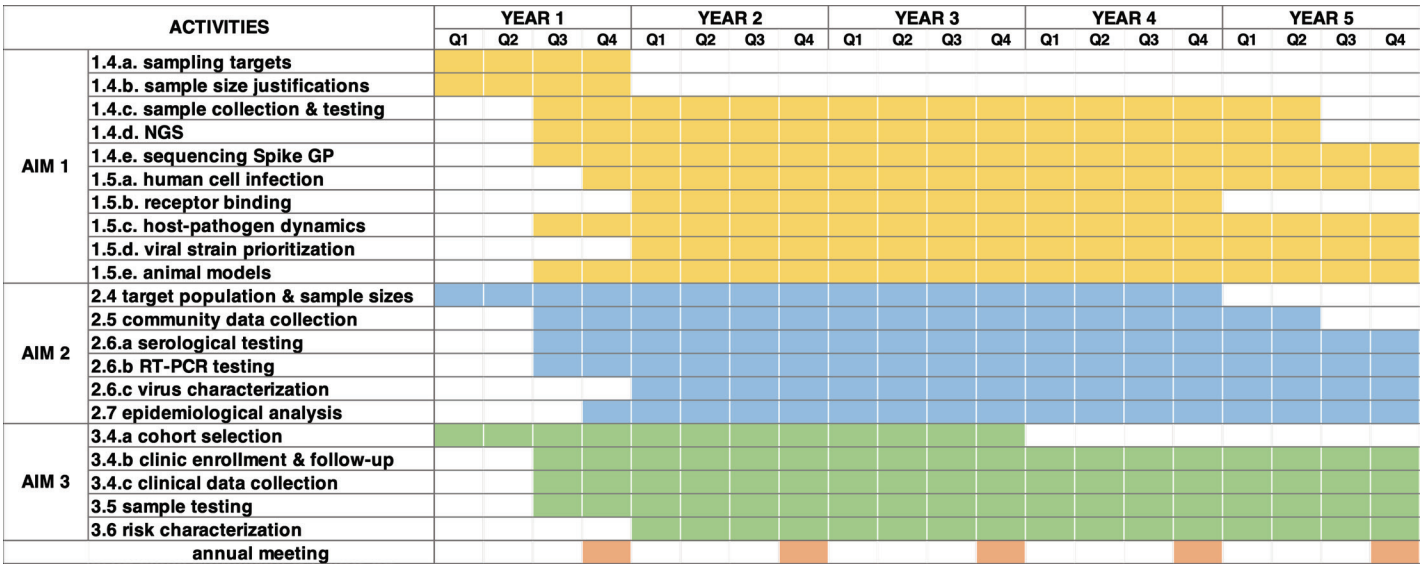
<u>End of Year 2:</u> Aim 1: No sample targeting or sample size justification analyses needed. All other aspects underway **Aim 2:** All aspects underway **Aim 3:** All sub-aims underway. Second Annual meeting in last quarter. Further 2 publications submitted by end of year, including first data papers.

<u>End of Year 3:</u> Aim 1: No sample targeting or sample size justification analyses needed. All other aspects underway **Aim 2:** All aspects underway **Aim 3:** All sub-aims underway. Third Annual meeting in last quarter. Further 3 publications submitted by end of year, largely data papers.

<u>End of Year 4:</u> Aim 1: No sample targeting or sample size justification analyses needed. All other aspects underway **Aim 2:** All aspects underway. Receptor binding work completed. **Aim 3:** No further cohort selection required; all other sub-aims underway. Fourth Annual meeting in last quarter. 3 further publications submitted, including first papers analyzing risk factors, pathogenic potential of novel viruses submitted.

<u>End of Year 5:</u> Aim 1: No sample targeting or sample size justification analyses needed. No receptor binding assays continuing. Serological and PCR testing completed end of 2nd quarter. Glycoprotein, *in vitro* and *in vivo* analyses, analysis of viral risk continue to end of project. **Aim 2:** No further community targeting or sample size work. Community data collection completed at end of 2nd quarter. All other aspects continue to end of project **Aim 3:** All sub-aims underway. Final Annual meeting in last quarter. Further 3 publications submitted.

8.2. Timeline:



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PROJECT SUMMARY/ABSTRACT

Southeast Asia is one of the world's highest-risk EID hotspots, the origin of the SARS pandemic, Nipah virus, and repeated outbreaks of influenza. This is driven by high diversity of wildlife and rapidly expanding demography that brings human and wildlife populations closer. This proposal will launch the **E**merging **I**nfectious **D**iseases - **S**outh **E**ast **A**sia **R**esearch **C**ollaboration **H**ub **(EID-SEARCH)**, a collaboration among leaders in emerging disease research in the USA, Thailand, Singapore and the 3 major Malaysian administrative regions. These researchers have networks that span >50 clinics, laboratories and research institutions across almost all SE Asian countries and will use the EID-SEARCH as an early warning system for outbreaks involving exchange of information, reagents, samples and technology, and a collaborative power-house for fundamental and translational research. The EID-SEARCH will also act as **a significant asset to scale-up and deploy resources in the case of an outbreak in the region.** This EIDRC will conduct research to: **1)** Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife, by analyzing previously-archived wildlife samples, conducting targeted wildlife surveillance, and using serology & PCR assays to identify novel viruses. These will be characterized to assess risk of spillover to people, and a series of *in vitro* (receptor binding, cell culture) and *in vivo* (humanized mouse and collaborative cross models) assays used to assess their potential to infect people and cause disease; **2)** Collect samples and questionnaire data from human communities that live in EID hotspots and have high cultural and behavioral risk of animal exposure (e.g. wildlife hunting, bat guano collection). These will be tested with serological assays to identify evidence of novel virus spillover, and analyzed against metadata to identify key risk pathways for transmission; **3)** Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts. We will conduct syndromic surveillance at clinics serving the populations in Aim 2, enroll patients with undiagnosed illness and symptoms consistent with emerging viral pathogens, and test samples with molecular and follow-up serological assays to identify causal links between these syndromes and novel viruses.

This research will advance our understanding of the risk of novel viral emergence in a uniquely important region. **It will also strengthen in-country research capacity** by linking local infectious disease scientists **with an international collaborative network that has proven capacity to conduct this work and produce significant findings.** The large body of high impact collaborative research from this EIDRC leadership team provides proof-of-concept that EID-SEARCH has the background, collaborative network, experience, and skillset to act as a **unique early warning system for novel EIDs of any etiology threatening to emerge in this hottest of the EID hotspots.**

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Subject:	Re: CREID TP
Date:	Monday, March 15, 2021 9:01:59 PM
Attachments:	EIDRC SE Asia Specific aims v6 FINAL_FINAL.docx EIDRC Southeast Asia v7 FINAL_FINAL.docx Project_Summary-Abstract_EIDRC_RFA-AI-19-028_(PI_Daszak)_v2 FINAL_FINAL.docx

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Hi Eric,

Please see the attached.

Best, Hongying

Hongying Li, MPH
Senior Program Coordinator & Research Scientist

EcoHealth Alliance
520 Eighth Avenue, Ste. 1200
New York, NY 10018

1.917.573.2178 (mobile)
www.ecohealthalliance.org

EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.

On Mon, Mar 15, 2021 at 8:54 PM Laing, Eric <eric.laing@usuhs.edu> wrote:

> Hi everyone,
>
> Could someone who has the technical proposal that was cleaned up and submitted easily on hand share it with me so I don't have to root through emails and drive folders?
>
> - Eric
>
> Eric D. Laing, Ph.D.
> Research Assistant Professor
> Department of Microbiology and Immunology
> Uniformed Services University
> 4301 Jones Bridge Road
> Bethesda, MD 20814
> cell: (301) 980-8192
> office: (301) 295-9884
> lab: (301) 295-9618
>
> eric.laing@usuhs.edu

CURRICULUM VITAE
Christopher C. Broder, Ph.D.

(b) (6)

BUSINESS ADDRESS: Department of Microbiology & Immunology
Uniformed Services University
4301 Jones Bridge Road
Bethesda, Maryland 20814-4799

Telephone: Office: 301-295-3401, Fax: 301-295-1545
Laboratories: 301-295-3313 / 9618 / 9616
Mobil: 301-535-2943
E-mail: christopher.broder@usuhs.edu / (b) (6)

EDUCATION:

1983 BS, Biological Sciences, with honors. Florida Institute of Technology, Melbourne, Florida.
1985 MS, Molecular Biology, Florida Institute of Technology, Melbourne, Florida.
1989 PhD, Microbiology and Immunology. College of Medicine, University of Florida, Gainesville, Florida.

TRAINING / POSITIONS:

1983 - 1985 Graduate student, Florida Institute of Technology, Melbourne. (Adv: Kenneth L. Kasweck, PhD)

1985 - 1989 Graduate student, Department of Immunology and Medical Microbiology, University of Florida. Gainesville. (Adv: Michael D.P. Boyle, PhD)

4/89 - 10/89 Postdoctoral Associate, Department of Medicine, University of Florida. (Adv: Richard Lottenberg, MD)

11/89 - 1/90 Microbiologist (GS-11), Laboratory of Viral Diseases (LVD), National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD. (Adv: Bernard Moss, MD)

1990 - 1992 National Research Council Research Associate, LVD, NIAID, NIH. (Adv: Bernard Moss, MD)

1993 - 1996 IRTA Fellow, LVD, NIAID, NIH. (Adv: Bernard Moss, M.D. and Edward A. Berger, PhD)

1996 - 2000 Assistant Professor, Department of Microbiology and Immunology; and Molecular and Cell Biology (secondary), USU, School of Medicine, Bethesda, MD.

2000 - 2005 Associate Professor (Tenured), Department of Microbiology and Immunology; and Emerging Infectious Diseases and Molecular and Cell Biology (secondary) USU, School of Medicine, Bethesda, MD.

2005-present Professor (Tenured), Department of Microbiology and Immunology, Joint appointment, Emerging Infectious Diseases Graduate Program, USU, Bethesda, Maryland.

2006-2018 Director, Emerging Infectious Diseases Graduate Program, USU, Bethesda, Maryland.

2018-present Chair, Department of Microbiology and Immunology, USU, Bethesda, Maryland

HONORS / AWARDS:

1987, 88 National Institutes of Health Training Grant Award.
1989 Medical Guild Graduate Research Award.
1990-92 National Research Council Research Associateship Award.
1993-96 National Institutes of Health Intramural Research Training Award Fellowship.
1996 The Fellows Award for Research Excellence, Office of Science Education, NIH.
1996 Breakthrough of the Year, Science; American Association for the Advancement of Science.
1997 Newcomb Cleveland Prize, American Association for the Advancement of Science.
2001 Outstanding Instructor in Virology, USU, School of Medicine Class of 2003.
2008 The Henry Wu Award for Excellence in Basic Science Research.
2013 The 3rd Sidney Pestka Lecture; 22nd Annual Philadelphia Infection & Immunity Forum.

2013 The 2013 Federal Laboratory Consortium (FLC) Award for Excellence in Technology Transfer.

2013 The CSIRO Chairman's Medal. The Commonwealth Scientific and Industrial Research Organisation (CSIRO); Australia's national science agency.

2014 The Cinda Helke Award for Excellence in Graduate Student Advocacy.

2016 The James J. Leonard Award for Excellence in Translational/Clinical Research.

2017 USU, Impact Award for outstanding contributions to the School of Medicine.

2018 USU, Impact Award for outstanding contributions to the School of Medicine.

2019 The 2019 Federal Laboratory Consortium (FLC) Award for Excellence in Technology Transfer.

2019 USU Outstanding Biomedical Graduate Educator Award.

2019 Selection to the University of Florida, College of Medicine, "Wall of Fame".

2019 Dean's Award for Leadership, University of Florida, College of Medicine.

2020 Inaugural, 2020 Federal Laboratory Consortium (FLC) Impact Award.

2020 Military Health System Research Symposium (MHSRS) 2020 Outstanding Individual Research Accomplishment by an Individual Senior Researcher

PATENTS, INVENTIONS, LICENSES:

- Bacterial Plasmin Receptors as Fibrinolytic Agents: **U.S. Patent No. 5,237,050.**
- Oligomeric HIV-1 Envelope Glycoproteins (gp140): **U.S. Patent Nos. 6,039,957 and 6,171,596.** Methods for Production, Purification, and Use as an Immunogen in Mammals.
- CC Chemokine Receptor 5 (CCR5) DNA, New Animal Models and Therapeutic Agents for HIV Infection. **U.S. Patent No. 7,151,087.**
- Cells Expressing Both Human CD4 and a Human Fusion Accessory Factor (CXCR4) Associated with HIV Infection: **U.S. Patent No. 6,197,578.**
- 4G10, a Monoclonal Antibody against the Chemokine Receptor CXCR4, Raised against the N-terminal Sequence of CXCR4. DHHS Reference No. E-340-2002/0. **Licensed to Santa Cruz Biotechnology 2002.**
- Compositions and Methods for the Inhibition of Membrane Fusion by Paramyxoviruses: **U.S. Patent Nos. 7,666,431 and 8,114,410.**
- Soluble Forms of Hendra and Nipah Virus G Glycoprotein. **Australian Patent No. 2005327194. U.S. Patent Nos. 8,865,171; 9,045,532; 9,056,902; 9,533,038, 10,053,495.**
- HIV-1 Envelope Glycoprotein Oligomer and Methods of Use. **U.S. Patent No. 8,597658.**
- Soluble Forms of Hendra and Nipah Virus F Glycoprotein and Uses Thereof: **Australian Patent No. 2013276968. U.S. Patent Nos. 10,040,825; 10,590,172.**
- Human Monoclonal Antibodies against Hendra and Nipah viruses. **U.S. Patent Nos. 7,988,971; 8,313,746; 8,858,938**
- Antibodies against F glycoprotein of Hendra and Nipah viruses. **U.S. Patent Nos. 9,982,038 and 10,738,104.**
- Hendra sG: **Licensed to Zoetis, Inc. (formerly Pfizer Animal Health)**. Equivac ® HeV; Nov, 2012, Australia.
- Human antibody m102.4 therapy against Hendra and Nipah virus infection; Phase I clinical trial completed in May 2016, Queensland Health, Queensland, Australia.
- Hendra sG: **Licensed to Auro Vaccines, Aurobindo Pharma USA**. Nipah/Hendra virus human vaccine.
- Cedar Virus and Methods of Use: **U.S. Patent No., 10,227,664**.

PROFESSIONAL SOCIETIES:

American Society for Virology (ASV)
Association of Medical School Microbiology and Immunology Chairs (AMSMIC)

DEPARTMENTAL RESPONSIBILITIES (Teaching), Graduate and Medical Education:

Annual: Lecturer; Medical School Integrated Curriculum; Viral Zoonoses; Antiviral Drugs; Gastrointestinal Viral Diseases; Viral Vaccines; Viral Infections of the central nervous system.

Biannual: Lecturer; virus entry, virus receptors, negative-stranded RNA viruses, emerging viruses.

Current and Former Postdoctoral Trainees:

Krishnamurthy Govindaraj, PhD, Institute of Medical Sciences, Lucknow, India. 1999-2004. (Research Associate, Henry M. Jackson Foundation for the Advancement of Military Medicine)

Hong Chen, MD, Hunan Med. Uni. Hunan, China. 1997-00. (Scientist, AscentGene, Inc., College Park, MD)

Sanjay Phogat, PhD, University of Delhi South Campus, New Delhi, India. 2000-2001. (Principal Scientist, Immunogen

Design, International AIDS Vaccine Initiative (IAVI), New York)

Tzanko S. Stantchev, MD, Varna Institute of Medicine, Rousse, Bulgaria. 1998-2008. (Research Scientist, Division of Monoclonal Antibodies, CDER, FDA, Silver Spring, MD)

Anil Choudhary, PhD, University, Rohtak, India. 2001-2006. (Scientist, Profectus, Inc. Baltimore, MD).

Antony S. Dimitrov, PhD, The University of Tokyo, Japan. 2004-2006. (Senior Staff Scientist, Profectus BioSciences, Inc. Baltimore, MD; Department of Microbiology and Immunology, Unformed Services University)

Matthew I. Bonaparte, PhD, SUNY Upstate Medical University, NY, 2005-2007. (Scientist, Global Clinical Immunology Sanofi Pasteur, Swiftwater, PA)

Dimple Khetawat (Harit), PhD, University of Calcutta, India. 2003-2011. (Research Associate, UNC Eshelman School of Pharmacy, Division of Molecular Pharmaceutics, University of North Carolina at Chapel Hill)

Yee-Peng Chan, PhD, The University of Malaya, Kuala Lumpur, Malaysia, 2005-2014

Vidita Choudhry, PhD, Jawaharlal Nehru University, New Delhi, India 2006-2014 (NMRC, Silver Spring, MD)

Bang Vu, PhD, Free University of Brussels, Belgium, 2010-2017

Moushimi Amaya, PhD, George Mason University, VA, 2016-

Current and Former Graduate Students:

Donald J. Chabot, PhD (Microbiology and Immunology-97'; 2000), (Microbiologist, Clinical Research Management, Inc./ Team Akimeka, USAMRIID, Bacteriology Division, Fort Detrick, MD)

Agnes Jones-Trower, PhD (Molecular and Cellular Biology-97', 2001), (Staff Fellow, Division of Viral Products, CBER, FDA, Bethesda, MD (Ret.)

Katharine N. Bossart, PhD (Microbiology and Immunology-98'; 2003), (President & Owner; Integrated Research Associates, LLC. San Rafael, CA)

Jared Patch, PhD (Emerging Infectious Diseases-01'; 2007), (Research Scientist, Food Animal Vaccine Development, Elanco, Inc., Greenfield, IN)

Julie A. Pavlin, MD, PhD, MPH, COL, USA, Ret. (Emerging Infectious Diseases-00'; 2007), (Director, Board on Global Health, Health and Medicine Division, The National Academies of Sciences, Engineering, and Medicine, Washington, DC)

Kimberly Bishop, PhD (Emerging Infectious Diseases-02': 2007), (Deputy Head, Genomics Dept., Biological Defense Research Directorate (BDRD) Naval Medical Research Center (NMRC), Fort Detrick, MD)

Andrew Hickey, PhD, MPH, LCDR, USPHS (Emerging Infectious Diseases-03': 2009) (LT, United States Public Health Service, Chief, HIV/STD Laboratory Research Section, CDC-Thailand).

Stephanie Petzing, PhD (Emerging Infectious Diseases-05': 2012), (AAAS Science & Technology Policy Fellow, U.S. Department of Defense, Threat Reduction Program Oversight Office)

Dawn L. Weir, PhD, LCDR, USN (Emerging Infectious Diseases-07': 2013), (LCDR, United States Navy, Medical Services Corps), Presently; Naval Research Laboratories, Washington, DC)

Deborah L. Steffen, PhD (Emerging Infectious Diseases-07': 2013) (Faculty, Stone Ridge HS, Bethesda, MD; Ret.)

Eric Laing, PhD (Emerging Infectious Diseases-10:' 2016) (Research Assistant Professor, Department of Microbiology and Immunology, USU, 2019)

Chelsi Beauregard, PhD (Emerging Infectious Diseases-13': 2020), (Assistant Professor of Biology, Southern New Hampshire University, Manchester, NH)

Sofia Da Silva, PhD (Emerging Infectious Diseases-13': 2020), (ORISE fellowship; Division of Research, Innovation, and Ventures (DRIVe). Biomedical Advanced Research and Development Authority (BARDA))

Graduate Thesis Committees (other):

Uniformed Services University:

Emerging Infectious Diseases Graduate Program (USU)

Sharon Wen, PhD; 06'

Gabriel DeFang, PhD, LCDR, MSC, USN; 07'

Trupti Brahmbhatt, PhD, CAPT, MSC, USN; 07'

Shana Miles, MD, PhD, LT, MC, USN; 10'

Claire Wernly, PhD; 10'

Aura Garrison, PhD; 12'

Michael Washington, PhD, LTC, MEDCOM, USA; 14'

Tonia Zangari, PhD; 14'

Kate Mastraccio, PhD; 18'

Trung Ho, MD-PhD-candidate
Adrian Paskey, PhD; 20'
William Valiant, PhD; 19'

Molecular and Cellular Biology Graduate Program (USU)
Randall Merling, PhD; 07'
Mark Serkovich, MS; 06'
Mark Smith, VMD, Diplomate ACVP, PhD, LTC, VC, USA; 14'

External:
Philippa J. Miller, PhD; 04' (The University of Melbourne, Victoria, Australia)
Yee-Peng Chan, PhD; 05' (The University of Malaya, Kuala Lumpur, Malaysia)
Tonya Colpitts, PhD; 07' (University of Texas Medical Branch, Galveston, Texas, USA)
Stephanie L. Foster, PhD-candidate (University of Texas Medical Branch, Galveston, Texas, USA)

UNIVERSITY SERVICE:

1998-2014	Uniformed Services University Merit Review Committee (USU study section)
1997-1998	Research Committee for the LCME report to the Board of Regents
1997-2000	Chair, Bio-Instrumentation Center Committee, Uniformed Services University
2000-2001	Faculty Senator, Basic Sciences
1997-2001	Comparability and Faculty Welfare Committee
2006-2018	Graduate Program Director (Ph.D.): Emerging Infectious Diseases
2006-	EID, Executive Committee
2006-2018	Graduate Education Committee
2007-2015	MD/PhD Admissions and Curriculum Committee
2008-2009	USU, School of Medicine 5-year Evaluation
2009-10	University Space Committee
2009-	Basic Science Chairs Committee (Chair, 2010-11, 2015-16)
2009-10	USU School of Medicine Strategic Planning Committee
2010-11	USU School of Medicine Curriculum Reform Clerkship Committee
2011-14	Dean's Advisory Group
2011	Neuroscience Graduate Program Director Search Committee (Chair)
2011-	Board of Academic Counselors
2012-2015	Committee on Appointments, Promotions and Tenure (CAPT committee)
2014-	School of Medicine Endowment Committee Meeting
2016-	Joint Patent and Technology Review Group (JPTRG)
2018-	USU Building F "Emerging Infectious Disease/Global Health" Scientific Neighborhood Team
2019-	USU, Names and Honors Committee.
2019-20-	Department Chair Search Committees; Department of Anatomy, Physiology and Genetics; Psychiatry.
2019	Vice President for Research (VPR), USU, Search Committee.

OUTSIDE ACTIVITIES AND SERVICE:

National and International Committees and Boards:

1997	Board Member: Source Evaluation Board for Biotechnology of the National Institute of Standards and Technology, United States Department of Commerce, Advanced Technology Program.
1999	*Ad hoc* Member: Special Emphasis Panel on *HIV Neuropathogenesis* for the National Institute of Neurological Disorders and Stroke, National Institutes of Health.
2000	*Ad hoc* Member: Scientific Board of the Dutch Aids Fund, Netherlands.
2001	Program Reviewer, The Pasteur Institute: for the Unit of Viral Immunology, France.
2000-03	Member: Study Section: *Molecular Biology and Pathogenesis of HIV*. The University-wide AIDS Research Program. Office of the President of the University of California.
2003	*Ad hoc* Member: Experimental Virology (EVR) Study Section, NIAID, NIH.

2003 *Ad hoc* Member: AIDS Molecular and Cellular Biology Study Section, NIAID, NIH.

2003-14 Management and Oversight Committee Member. Middle Atlantic Regional Center of Excellence in Biodefense and Emerging Infectious Diseases Research.

2004 *Ad hoc* Member: Source Evaluation Board for Biotechnology of the National Institute of Standards and Technology, United States Department of Commerce, Advanced Technology Program.

2005 Review Committee Member; The National Screening Laboratory for the Regional Centers of Excellence for Biodefense and Emerging Infectious Disease, Harvard Medical School, Boston, MA.

2005 Program Reviewer, new research unit: "Host-Virus Relationships", in The Pasteur Institute: France.

2009 Member, National Veterinary Stockpile Nipah virus Countermeasures Workshop; United States Department of Agriculture; (Geelong, Australia; March 17-19).

2009 The Health Research Council of New Zealand, program reviewer.

2007- Editorial board, *Journal of Virology*.

2010- Editorial board, *Virology*.

2011- Editor, *Viruses*.

2011- Editorial board, *Pathogens*.

2012- Editor, *Virologica Sinica*

2011 Member, Discontools Nipah Virus Infection Panel Expert Group. Gap analysis. International Federation for Animal Health Europe, Brussels, Belgium.

2011 Invited expert for the National Academies. Evaluation of the updated site-specific risk assessment for the National Bio- and Agro-Defense Facility (NBAF) in Manhattan, Kansas.

2016 Netherlands Organisation for Scientific Res., Dutch national science council, Gravitation Programme review.

2017 Member, BSL4ZNet Expert Panel Meeting for Henipaviruses and Ebolaviruses; Canadian National Centre for Foreign Animal Disease, Winnipeg (November).

2017- World Health Organization (WHO) taskforce; Nipah virus research and development (R&D) roadmap; with University of Minnesota (CIDRAP) and Wellcome Trust. March 1-2, July 9-10, 2018.

2018- Nipah Therapeutics Protocol Team; ICMR, NIAID, WHO.

2018- Nipah Task Force (CEPI).

***Ad hoc* Reviewer for the Following Journals:** PNAS; J.Virol.; J.Infec.Dis.; Virology; J.Virol.Meth; Nat.Struc.Bio., Nat.Micro.Rev., PlosPath.; PlosNegTrop.Dis.; Viruses, Viro. J.; Anti.Agents and Chemo.; AntiviralRes., Monoclonal Antibodies, Virologica Sinica, Science, Sci.Trans.Med., Pathogens, Frontiers.

RESEARCH EXPERIENCE AND INTERESTS:

M.S. (85'): "Analysis of Thymidine Kinase mRNA and Construction of a cDNA Library from Mouse L5178Y Cells".

Ph.D. (89'): "Isolation and Characterization of a Group A Streptococcal Receptor for Human Plasmin".

Current: Interactions between pathogenic human and zoonotic enveloped animal viruses and host cells: virus receptors; envelope glycoprotein structure/function; vaccines; antivirals; virus assembly, bio-surveillance.

ACTIVE SUPPORT: (10)

Grant Title: "Advancement of Vaccines and Therapies for Henipaviruses"
Grant Number: U19 AI142764-01. Center of Excellence for Translational Research (CETR)
Grant Period: 03/20/19-02/29/24
Total Direct: $24,587,556 Agency: NIH/NIAID, Role: Overall Center PI, Director of the Administrative Core, and PI.
Partners: Profectus Biosciences, Inc. (Auro Vaccines, LLC), Mapp Biopharmaceuticals, Inc., Vanderbilt University, and University of Texas Medical Branch.

Grant Title: Serological Biosurveillance for Spillover of Henipaviruses and Filoviruses at Agricultural and Hunting Human-Animal Interfaces in Peninsular Malaysia
Grant Number: HDTRA1-17-10037
Grant Period: 05/01/17-04/30/22
Total Direct: $910,000 Agency: DTRA, DoD: With: Ecohealth Alliance, New York, NY. Co-PI, with J Epstein.

Grant Title: A Subunit Vaccine (HeV-sG) to Protect against Nipah and Hendra Diseases
Grant Period: 07/01/18-06/30/2028

Total Direct: CRADA to USU $538,046. (Total award to Profectus Biosciences 23 million). Agency: CEPI (Coalition for Epidemic Preparedness Innovations (CEPI): Role: Co-PI, with J Eldridge.

Grant Title: Collaborative development and evaluation of an equine vaccine against Hendra virus
Principal Investigator: Christopher C. Broder, Ph.D.
Agency: Pfizer (Zoetis)/CRADA Period: July 1, 2012 to September 30, 2040
(CRADA): Development and evaluation of an equine vaccine against Hendra virus.

DTRA BTRP (Ecohealth Alliance, New York, NY. Co-PI, with J Epstein.)
Title: Malaysia Partners Luminex Training and Research Preparedness
1 Year Requested, POP: 01/01/2020-11/30/2020
Subcontract, Total Award: $90,777. Role: Co-Principal Investigator

DHA IDCRP (Burgess, T.)
Epidemiology, Immunology, and Clinical Characteristics of Emerging Infectious Diseases with Pandemic Potential (EpICC-EID)
2 Years Requested, POP: 05/01/2020-04/30/2022
Subaward, Total Award: $1,078,273. Role: Associate Investigator

DARPA PREEMPT (A. Peel)
Preempting Spillover of Novel Coronaviruses from Bats to Humans
1 Year Requested, POP: 06/01/2020 – 05/31/2021
Subaward, Total Award: $120,318. Role: Collaborator

DTRA BTRP HDTRA12010025 (with W. Markotter, Uni Pretoria, S. Africa)
Title: Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa
5 Years Requested, POP: 10/01/2020 – 09/30/2023
Subaward, Total Award: $1,116,971. Role: Co-Investigator

DTRA BTRP (Ecohealth Alliance, New York, NY. Co-PI, with J Epstein)
Title: Biosurveillance for Spillover of Henipaviruses and Filoviruses in Rural Communities in India
3 Years Requested, POP: 10/01/2020 – 09/30/2023
Subaward, Total Award: $888,721. Role: Co-Investigator

NIAID U01AI151797: Centers for Research in Emerging Infectious Diseases (CREID)
(Ecohealth Alliance, New York, NY. Co-PI, with P. Daszak.)
Emerging Infectious Diseases - South East Asia Research Collaboration Hub; 02/01/2020-03/31/2025
Subaward, $539,119. Role: Co-investigator

PENDING SUPPORT: (2)

(b) (5)
[redacted]

PREVIOUS SUPPORT: (22)

Grant Title: HIV-1 Fusion Cofactors
Grant Number: R29 AI41411
Grant Period: 04/01/97-04/30/99
Total Direct: $512,407 Agency: NIH/NIAID Role: Principal Investigator

Grant Title: Structural and Functional Analysis of HIV-1 Entry Cofactors
Grant Number: R0 73FG-01
Grant Period: 10/01/96-09/30/99
Total Direct: $81,000 Agency: USUHS/DOD, Role: Principal Investigator

Grant Title: HIV-1 Fusion Cofactors
Grant Number: R01 AI043885
Grant Period: 07/15/98-01/31/11
Total Direct: $2,167,550 Agency: NIH/NIAID, Role: Principal Investigator

Grant Title: Analysis of Oligomeric HIV-1 Envelope Glycoproteins
Grant Number: R21 AI42599-01

Grant Period: 11/01/97-10/31/00-expiring
Total Direct: $300,000 Agency: NIH/NIAID, Role: Principal Investigator

Grant Title: HIV-1 Envelope-CD4-Coreceptor Complexes as Vaccines
Grant Number: R21 AI47697-01
Grant Period: 7/01/00-6/30/02
Total Direct: $300,000 Agency: NIH/NIAID, Role: Principal Investigator

Grant Title: Program title: Broadly Effective Neutralization and CTL against HIV-1
Project 2 title: HIV-1 gp140 Oligomers as Vaccine immunogens
Grant Number: PO1 AI48380
Grant Period: 09/01/01-06/31/06
Total Direct: $1,261,561 Agency: NIH/NIAID, Role: Principal Investigator, Project 2

Grant Title: Nipah Virus and Hendra Virus Subunit Vaccines
Grant Number: R21 AI065597
Grant Period: 07/01/05-06/30/07
Total Direct: $275,000 Agency: NIH/NIAID, Role: Principal Investigator

Grant Title: Nipah Virus and Hendra Virus Peptide Therapeutics
Grant Number: U01 AI056423
Grant Period: 09/15/03 – 08/01/08
Total Direct: $2,025,326 Agency: NIH/NIAID, Role: Principal Investigator

Grant Title: Biodefense and Emerging Infectious Diseases Research: (MARCE)
Program II: Emerging Virus Entry into Host Cells: Strategies for Inhibition
Project II-3: Hemorrhagic Fever Viruses / Australian Bat Lyssavirus Tropism Entry and Host Factor Dependence
Grant Number: 2U54 AI057168
Grant Period: 08/01/03 – 02/28/14
Total Direct: $1,661,546 Agency: NIH/NIAID, Role: Principal Investigator, Project II-3

Grant Title: Characterization of the Envelope Glycoproteins of Beilong and J-virus
Grant Number: R073NN
Grant Period: 10/01/06-09/30/09
Total Direct: $60,000 Agency: USUHS/DOD, Role: Principal Investigator

Grant Title: Emerging Viruses and Host Cell Interactions in Bats
Grant Number: R073SA
Grant Period: 10/01/09-09/30/12
Total Direct: $60,000 Agency: USUHS/DOD, Role: Principal Investigator

Grant Title: High Potency HIV-1 Broadly Cross-Reactive Neutralization
Grant Number: U01AI078412
Grant Period: 04/01/2008 – 03/31/2013
Total Direct: $3,000,000 Agency: NIH/NIAID, Role: Co-Principal Investigator

Grant Title: Vaccines and Therapeutics for Nipah and Hendra virus
Grant Number: U01AI077995
Grant Period: 06/01/2008 – 8/31/2014
Total Direct: $5,617,562; Agency: NIH/NIAID, Role: Principal Investigator

Grant title: Development of sG as a human vaccine against Nipah/Hendra
Principal Investigator: T. Fouts, A. Dimitrov / Co-PI: Christopher C. Broder, Ph.D., Subaward.
Period: May 1, 2012 to April 30, 2017
Total Direct: $225,000; Agency: NIH/NIAID, Type: 1R01AI098760-02.

Grant title: Preclinical Development of m102.4, a Human Anti-Hendra and Nipah Antibody
Principal Investigator: T. Fouts, A. Dimitrov / Co-PI: Christopher C. Broder, Ph.D., Subaward.
Period: May 1, 2011 to April 30, 2016
Total Direct: $225,000; Agency: NIH/NIAID, Type: 1R01AI093346-03.

Grant Title: Nipah & Hendra virus Nonhuman Primate Model & Therapeutics Development
Grant Number: U01 AI182121
Grant Period: 03/15/2009 – 02/28/2015
Total Direct: $6,940,076. Agency: NIH/NIAID, Role: Co-Principal Investigator (with T. Geisbert, UTMB).

Grant Title: Analysis of the entry and egress of Cedar virus a new species of Henipavirus
Grant Number: R0732012
Grant Period: 10/01/12-09/30/15
Total Direct: $60,000 Agency: USUHS/DOD, Role: Principal Investigator

Grant Title: Identification, Countermeasures, and New Therapies Toward Biological Threat Agents;
Component Project: Soluble Trimeric Filovirus Envelope Glycoproteins
Grant Number: #HT9404-13-1-0021
Grant Period: 10/01/14-03/31/17

Total Direct: $78,085. Agency: BDRD/NMRC (DoD), Role: Component Project PI (with J. Czarnecki, NMRC)

Grant Title: Therapies for Neurotropic Viral Biothreat Pathogens
Grant Number: PPG. HT9404-13-1-0008
Grant Period: 10/01/13 – 9/30/17
Total Direct: $1,950,882.
Agency: USUHS, Role: Component Project PI (with B. Schaefer, USU)

Grant Title: Nipah Virus and Hendra Virus Entry and Virion Assembly
Grant Number: R01 AI054715
Grant Period: 04/01/06-09/30/18
Total Direct: $2,225,000 Agency: NIH/NIAID, Role: Principal Investigator

Grant Title: A Recombinant Cedar Virus-based Henipavirus Replication Platform for High-throughput Inhibitor Screening
Grant Number: R21 AI137813
Grant Period: 04/01/18-03/31/20
Total Direct: $275,000 Agency: NIH/NIAID, Role: PI

Grant Title: Chulalongkorn Luminex Training and Research Preparedness
Grant Number: DTRA STEP HDTRA1-17-C-0019
Grant Period: 01/05/19 – 06/01/19
Total Direct: $195,178: Agency: DTRA, DoD, Role: PI

INVITED LECTURES (>100):

1994. Department of Pathology and Lab Med., University of Pennsylvania, Jan 13. ***"HIV-1 Envelope Glycoprotein Mediated Cell Fusion: Structural Features of CD4 and Involvement of Accessory Components"***.

1994. GSF-Forschungszentrum für Umwelt und Gesundheit, GmbH, Neuherberg. Institut für MolekulareVirologie, Oberschleißheim, Germany. Current Advances In Molecular Biology Seminar Series. Aug 10. ***"Factors Associated with the Selective Fusogenic Activities of HIV-1 Envelope Glycoproteins for Specific CD4$^+$ Cell Types"***.

1995. Department of Microbiology, campus-wide series. University of Pennsylvania Feb 22. **"*Molecular Characterization of Viral Glycoprotein Mediated Membrane Fusion"***.

1996. 3rd International Workshop on HIV and Cells of Macrophage Lineage. Villa Monastero, Piazza Venini, Varenna, Italy. Oct17. ***"HIV Tropism: Distinct Accessory Fusion Factors for Different CD4$^+$ Cell Types"*.**

1998. Molecular Basis of Disease / Molecular and Cellular Biology Program, Medical College of Ohio, Health Education Building, Toledo, Ohio. February 17. ***"HIV: Envelope Glycoprotein and Membrane Receptors"***.

1999. Division of Retrovirology, WRAIR, Dec 10. ***"HIV Envelope and Virus Entry"***.

2000. Division of Viral Products, seminar series-CME credit approved, CBER, FDA, NIH, Bethesda, MD. February 24. ***"HIV-1 Envelope Glycoprotein: Receptor Interactions and Refined Subunit Immunogens"***.

2000. Center for Immunology & Microbial Disease, CME approved. Albany Medical College, Albany New York. March 27. ***"HIV-1 Envelope Glycoprotein-Receptor Interactions and new Subunit Immunogens"***.

2000. 2nd Frederick Workshop on the Cell Biology of Viral Entry. May 7-10, NCI-FCRDC, Frederick, MD, Invited Chair, Session I: ***"Virus-Receptor Interactions and Entry"***.

2001. Indiana University School of Medicine, Department of Microbiology, Indianapolis, IN, Mar 15. ***"Virus-Receptor Interactions: Tropism, Entry, and Refined Subunit Immunogens"***.

2001. Department of Microbiology, campus-wide seminar series-CME credit approved. University of Pennsylvania School of Medicine, Philadelphia, PA. October 3. ***"Functional and Structural Studies on Hendra and Nipah viruses - Newly Emerging and Highly Lethal Zoonotic Paramyxoviruses".***

2002. 2nd Collaborative Research Seminar on HIV and other Viral Entry Inhibitors. New York, NY. May 5. ***"Hendra and Nipah Viruses – Newly Emerging and Highly Lethal, Zoonotic Paramyxovirus Threats".***

2002. 3rd Frederick Workshop on the Cell Biology of Viral Entry. NCI-Frederick Cancer Research and Development Center, MD. May 7. ***"Hendra and Nipah Virus Envelope Glycoprotein-mediated Fusion.***

2002. Department of Microbiology and Immunology, Georgetown University, Washington, DC. Nov. 8. ***"Nipah and Hendra Viruses Emerging Zoonotic Paramyxovirus Threats".***

2002. Division of Viral Products, seminar series-CME approved, CBER, FDA, NIH, Bethesda, MD. Nov. 14. ***"Nipah and Hendra Viruses Emerging Zoonotic Paramyxovirus Threats".***

2003. 2003-Biodefense Vaccines, Therapeutics and Diagnostics: *Policy, Funding,* Development, Testing, Production, and Distribution. Biodefense Vaccines: The State of the Science. June 2-4, Washington, D.C. "***Hemorrhagic Fever and Emerging Viruses: Vaccines and Antiviral Agents".***

2003. NIH Research Festival. Mini-Symposia. Virus Entry – Virus Receptor Interactions. NIH, Bethesda, MD. Oct. 15. "***Nipah Virus and Hendra Virus Fusion, Entry, and its Inhibition".***

2003. Norman P. Salzman Fourth Annual Symposium in Virology: **Highly Pathogenic Viruses: Potential Agents of Bioterrorism.** FDA and the Foundation for the National Institutes of Health. ***"Nipah Virus and Hendra Virus: Emerging Zoonotic Paramyxovirus Threats".*** Nov 20, Cloisters Chapel, Building 60, NIH Campus, Bethesda, MD.

2003. 6th Asia Pacific Congress of Medical Virology. "***Nipah Virus and Hendra Virus Fusion, Entry, and its Inhibition".*** Dec. 6-10. Kuala Lumpur, Malaysia.

2004. USAMRIID, Fort Detrick, MD. Mar 9. **"*Nipah and Hendra: Emerging Viral Threats".***

2004. First Annual Regional Centers for Biodefense and Emerging Infectious Diseases Research meeting. Bethesda, MD. April 19-20. ***"Middle-Atlantic RCE Research Program 2: Emerging Viruses".***

2004. Department of Microbiology and Immunology, SUNY Upstate Medical University, Syracuse, New York. April 29. **"*Nipah and Hendra: Emerging Viral Threats".***

2004. 4th Frederick Workshop on the Cell Biology of Viral Entry. NCI-Frederick, MD. May 4. ***"A Soluble Hendra Virus Attachment Envelope Glycoprotein Blocks Fusion".***

2005. Second Annual Regional Centers for Biodefense and Emerging Infectious Diseases Research meeting. Galveston, TX. March 13-15. ***"Receptor Binding, Fusion Inhibition, and Induction of Cross-Reactive Neutralizing Antibodies by a Soluble G Glycoprotein of Hendra Virus".***

2005. 2005 ASM Biodefense Research: Symposium: Advances in Molecular Pathogenesis of Threat Agents Baltimore, MD. March 23. ***"Biology of Nipah and Hendra Viruses: Implications for Development of Vaccines and Therapeutics".***

2005. 2005-Biodefense Vaccines & Therapeutics Symposium: State of the Science. Arlington, VA. ***"Antibodies, Vaccines and Therapeutics for Emerging Virus Threats".***

2006. University of Virginia, Jan 10. Charlottesville**. *"Hendra and Nipah Viruses: Different and Dangerous"*.**

2006. Third Annual Regional Centers for Biodefense and Emerging Infectious Diseases Research meeting. New York City, NY. Mar 28. ***"A feline model of acute Nipah virus infection and protective vaccination with a soluble G glycoprotein".***

2006. University of Kentucky, Department of Molecular and Cellular Biochemistry *Lexington, Kentucky, Oct10.* ***"Hendra and Nipah viruses: From membrane fusion and receptors to potential therapeutic strategies"***

2006. Filoviruses: Recent Advances and Future Challenges: (ICID Global Symposia), Winnipeg, Canada. Sept 17-19. ***"Henipaviruses: From membrane fusion and receptors to Therapeutic Strategies".*** Plenary.

2006. 7th Asia Pacific Congress of Medical Virology. "***Nipah Virus and Hendra Virus Fusion, Entry, and its Inhibition".*** Nov 12-15. New Delhi, India. Plenary session.

2007. University of Texas Medical Branch. August 12. ***"The Envelope Glycoproteins of Hendra and Nipah viruses: Multifunctional molecules, vaccine immunogens and therapeutic targets***".

2007. University of Pittsburg, Center for Vaccine Research Seminar Series. September 26. "***The Envelope Glycoproteins of Hendra and Nipah Viruses: Multifunctional Molecules, Vaccine Immunogens and Therapeutic Targets".***

2007. University of Maryland, Department of Microbiology and Immunology. October 3. ***"The Envelope Glycoproteins of Hendra and Nipah viruses: Multifunctional molecules, vaccine immunogens and therapeutic targets".***

2008. The 3rd International Symposium of Emerging Viral Diseases. Oct 26-28. ***"Nipah and Hendra Virus Glycoproteins and Receptor Interactions."*** Plenary. Wuhan Institute of Virology, Chinese Academy of Sciences. Wuhan, China.

2008. American Society of Tropical Medicine and Hygiene (ASTMH) annual meeting. Session co-organizer and co-chair. Henipaviruses. ***"Nipah and Hendra Virus Receptor Binding and Entry."*** December 7-11. New Orleans, Louisiana.

2009. National Veterinary Stockpile Nipah virus Countermeasures Workshop; United States Department of Agriculture; (Australian Animal Health Laboratory, CSIRO, Geelong, Australia; March 17-19 2009). ***"Status of Vaccines and Therapeutic Countermeasures against Hendra and Nipah viruses."***

2009. Division of Viral Products Seminar Series-CME approved, March 26, CBER, FDA, NIH, Bethesda, MD. ***"Nipah and Hendra Virus Entry and New Animal Models of Infection and Pathogenesis."***

2009. NIH, Virology Interest Group seminar series. May 7th, NIH, Bethesda, MD. Nipah and Hendra Virus: ***"Receptor Binding and Entry, and New Animal Models of Infection."***

2009. WHO/FAO/OIE Workshop on Henipaviruses and Ebola-Reston Virus. Twin Waters, Queensland, Australia, Oct. 12-16, 2009 ***"Status of Vaccines and Therapeutic Countermeasures against Hendra and Nipah viruses."***

2009. Penn State, Bortree Lecture Series, October 7, ***"Nipah and Hendra viruses: From Receptor Binding and Entry to New Animal Models of Infection".***

2009. Juniata College, Huntingdon, PA. Oct 8, ***"Emerging Infectious Diseases: Graduate Education, USU".***

2009. New England Regional Center of Excellence in Biodefense and Emerging Infectious Diseases (NERCE-BEID) Workshop on Primate Infectious Diseases. Oct 28, ***"Hendra and Nipah virus –Therapeutics and new Primate Models".***

2009. Chemical, Biological, Radiological and Nuclear Countermeasures seminar series. BARDA, HHS, Nov. 17. ***"Vaccines and Therapeutic Countermeasures against Hendra and Nipah viruses".***

2009. IBC's 7th Annual International Conference: Antibody Therapeutics, San Diego, CA, Dec 8-10. ***"A Neutralizing Human Monoclonal Antibody Therapy for Nipah and Hendra Virus Infection".***

2010. Department of Microbiology and Immunology, Uni. of Illinois at Chicago, Mar 15. ***"Nipah and Hendra viruses: Receptor Binding and Virus Entry Studies Lead to New Therapeutics and Animal Models".***

2010. Dept of Pediatrics and Dept of Micro&Immun, Emory University School of Medicine, Atlanta, GA, Mar22. ***"Nipah and Hendra viruses: Studies on Receptor Binding and Entry Lead to New Therapeutics and Animal Models".***

2010. Dep. of Molecular and Microbiology, National Center for Biodefense & Infectious Diseases, George Mason University, VA. ***"New Animal Models and Countermeasures against Nipah and Hendra Virus".***

2010. 4th International Symposium of Emerging Viral Diseases. Oct 26-28. ***"Nipah and Hendra viruses: Studies on Receptor Binding and Entry Lead to New Therapeutics and Animal Models"*** Plenary. Wuhan Institute of Virology, Chinese Academy of Sciences. Wuhan, China.

2010. The 2nd International Conference on Infections of the Nervous System. Dec 2-6. ***"The New Non-Human Primate and Ferret Models for Nipah and Hendra Virus Pathogenesis and the Evaluation of Vaccine and Therapeutic Countermeasures"*** Plenary. St. Denis, Reunion Island.

2011. National Cancer Institute, Antibody Interest Group Seminar Series. NIH. Feb 25. ***"A Cross-Reactive Human Monoclonal Antibody Therapeutic for Hendra Virus and Nipah Virus".***

2011. 14th Annual Conference on Vaccine Research; the National Foundation for Infectious Diseases. May 16 ***"Bridging Animal and Human Health in the Search for Countermeasures for Henipaviruses"*** Plenary. Baltimore, MD.

2011. Colloquium Series on Infectious Disease; Institute for Infectious Diseases and Zoonoses, University of Munich Ludwig-Maximilians-Universität, June 1. ***"Bridging Animal & Human Health in the Development of Vaccines & Therapeutics against Hendra and Nipah Virus"***, Munich, Germany.

2011. Canadian Science Centre for Human and Animal Health. ***"Bridging Human and Animal Health in Developing Henipavirus Countermeasures"***. Oct 4. National Microbiology Lab, Public Health Agency of Canada, Winnipeg.

2012. Informa's Empowered Antibodies Congress 2012. June 13. ***"Successful Recombinant Human Monoclonal Antibody Therapy against Nipah and Hendra Virus Disease"***, Berlin, Germany.

2012. Vaccines and Diagnostics for Transboundary Animal Diseases," Sept 17-19, ***"Status of Passive and Active Vaccination Strategies against Hendra and Nipah viruses"***, Center for Food Security and Public Health, College of Veterinary Medicine, Iowa State University, Ames, Iowa.

2012. Infectious Disease & Immunity Colloquium, Sept 25 ***"Henipavirus Envelope Glycoproteins: Structural Studies and Ephrin Receptor Mediated Entry"***, Center for Biodefense and Emerging Infectious Diseases, UTMB, Galveston.

2012. Seminars at Huazhong Agricultural University, Oct 24, ***"The Present Status of Passive and Active Vaccination against Hendra and Nipah viruses"***, Wuhan, China.

2012. 5th International Symp.on Emerging Viral Diseases, Oct 25, ***"Henipavirus Envelope Glycoproteins: Structural Studies and Ephrin Mediated Entry"***. Plenary. Wuhan Institute of Virology, Chinese Academy of Sciences. Wuhan, China.

2012. 1st Ann. Host Pathogen Interactions in Biodefense and Emerging Infectious Disease, Nov 13, ***"Equine Hendra vaccine on the market-and a human monoclonal antibody therapy against Hendra and Nipah virus progresses further"***. George Mason Uni. VA.

2013. Annual meeting, Association of Medical School Microbiology and Immunology Chairs (AMSMIC), Jan 24, ***"Hendra and Nipah viruses: From Discovery to a Vaccine (From Bench to Bed- and Paddock-side)"***. Marco Island, FL.

2013. 11th ASM Biodefense and Emerging Diseases Research, ***"Tackling the Henipavirus Transboundary Threats by***

Passive and Active Immunization Approaches" Feb 25-27, Washington, DC.

2013. 3rd annual Sidney Pestka Lecturer, Annual Philadelphia Infection and Immunity Forum. ***"Immunization approaches succeed against the transboundary Hendra and Nipah virus threats"*** May 10.

2013. Program in Emerging Infectious Disease, Duke-NUS Medical School, Singapore. ***"Henipavirus Envelope Glycoproteins and Receptor Interactions: Structure, Function, and Therapeutic Targets"*** Jul5.

2013. Ruijin Hospital, School of Medicine Shanghai Jiao Tong University, Shanghai, China. ***"Passive and Active Immunization Approaches Succeed against the Nipah and Hendra virus Transboundary Threats"*** July 12.

2013. Infections of the Nervous System Pathogenesis and Worldwide Impact, Chinese University of Hong Kong. Gordon Research Conferences. *"**Combating the Hendra Virus and Nipah Virus encephalitic zoonoses by passive and active immunization"*** July 7-12.

2013. Basic Microbiology and Infectious Disease Training Program and the Department of Molecular Genetics & Microbiology Distinguished Lectures. University of Florida College of Medicine. Gainesville, FL. ***"Immunization strategies succeed against the transboundary Hendra and Nipah virus threats"*** Nov 25.

2014. The Hendra virus Team and Vaccine Retreat, Mornington Peninsula, Victoria, Australia. ***"From Virus Entry Studies to Hendra then Nipah then therapeutics-- What Next?"*** Feb 24.

2014. Division of Biotechnology Products CDER/FDA, Bethesda, MD. ***"Henipavirus Envelope Glycoproteins and Receptor Interactions: Structure, Function, and Countermeasure Targets"*** Apr 14.

2014. Informa; Antibodies Congress 2014: Recombinant and Bi-specific antibodies. Jun 18. ***"Combating the Hendra and Nipah virus emerging biothreats: A human monoclonal antibody therapy advances to clinical trial"***, Barcelona, Spain.

2014. 6th International Symposium on Emerging Viral Diseases, Oct 30, ***"Envelope glycoproteins of henipaviruses, Australian bat lyssavirus and rabies virus as targets of neutralizing human monoclonal antibodies"***. Wuhan Institute of Virology, Chinese Academy of Sciences. Wuhan, China.

2014. Norman P. Salzman 16th Annual Symposium in Virology. Foundation for the National Institutes of Health. *"**Next at Bat: A licensed vaccine and human monoclonal antibody therapy to combat the Hendra and Nipah virus Threats"*.** Nov 13, The Natcher Conference Center, Ruth Kirchstein Auditorium, NIH Campus, Bethesda, MD.

2014. ASTMH 63rd Annual Meeting Symposium: Bats and emerging viruses. ***"Development of the Hendra virus vaccine: A One-Health approach to Hendra virus control in Australia"*,** November 2-6, New Orleans.

2015. 18th Annual Conference on Vaccine Research; the National Foundation for Infectious Diseases. ***"Nipah virus and Hendra virus Animal Vaccines"*** April 13-15, Bethesda, MD.

2015. National Cancer Institute, National Institutes of Health, WebEx meeting, Apr 22. ***"A Human Monoclonal Antibody Therapy for People and a 'One Health' Vaccine for Horses as Countermeasures against the Hendra virus and Nipah Virus Threats"*** Bethesda, Maryland.

2016. Northwestern University Feinberg School of Medicine, Feb 8 ***"A 'One Health' Vaccine Approach and Human Antibody Therapy against Hendra and Nipah Viruses".*** Chicago.

2016. Division of Viral Products, CBER/FDA, June 23. ***"A human antibody therapy and a 'One Health' vaccine approach against Hendra virus and Nipah Virus"*** White Oak, MD.

2016. College of Pharmacy, Shandong University of Traditional Chinese Medicine. Oct 18. ***"Hendra Virus and Nipah Virus Active and Passive Vaccines"*,** Jinan, China.

2016. 7th International Symposium on Emerging Viral Diseases Oct 19-21, ***"Hendra and Nipah Virus: Passive and Active Vaccines"***, Wuhan Institute of Virology, Chinese Academy of Sciences. Wuhan, China.

2016. 4th International One Health Congress & 6th Biennial Congress of the International Association of Ecology and Health. Dec 3-7. Invited Keynote presentation: ***"A human antibody therapy and a 'One Health' vaccine approach against Hendra virus and Nipah Virus"***, Melbourne, Australia.

2017. Dept. of Tropical Medicine, Medical Microbiology and Pharmacology, University of Hawaii at Manoa; ***"A Vaccine and Therapy for Nipah virus and Hendra virus"***, October 10, 2017.

2017. NIAID, Division of Microbiology and Infectious Diseases, Rockville, MD; ***"A Nipah Virus and Hendra virus Vaccine and Therapy"***, October 19, 2017.

2018. Dept. Microbiology and Immunology, University of Illinois at Chicago; ***"Vaccines, Therapies and New Research Platforms for Nipah and Hendra Viruses"***, March 30, 2018.

2018. Aug 6-8, Indian Council for Medical Research / World Health Organization (ICMR-WHO): Workshop on Research

Roadmap for Nipah Virus Disease for India. ***"Monoclonal antibody therapeutic for Nipah and Hendra virus infection"***

2018. Aug 23, NIAID Technology Transfer Office, Rockville, MD. ***"Hendra-Nipah countermeasures"***.

2018. Oct 2-4, ***"Nipah Virus Returns: progress towards treatment and prevention"***. Infectious Disease Society of America (IDSA); IDWEEK-2018, San Francisco.

2018. Oct 18, ***"Nipah and Hendra Viruses: Countermeasures, New Tools and Surveillance"***. College of Pharmaceutical Science, Shandong University of Traditional Chinese Medicine, Jinan, China.

2018. Oct 21, ***"Nipah Virus Returns: progress towards treatment and prevention"***, 8th International Symposium on Emerging Viral Diseases, Wuhan, China.

2018. Oct 30, ***"Nipah and Hendra Viruses: Countermeasures, New Tools and Surveillance"***. Department of Microbiology, Perelman School of Medicine, University of Pennsylvania.

2019. Mar 4, *"**Vaccines and Therapeutics for Nipah Virus and Hendra Virus".*** Disease X: Advanced Diagnostics for Emerging Threats, Bangkok, Thailand.

2019. Mar 6, ***"An Antibody Therapeutic and a Nipah virus and Hendra Vaccine for Human Use"*** Thailand Ministry of Public Health (MOPH) – US, CDC, Bangkok.

2019. Apr 19, ***"An Antibody Therapeutic and a Vaccine for Nipah virus and Hendra virus"*** Cell Biology & Molecular Genetics Department, University of Maryland. College Park, MD.

2019. Apr 24, ***"Nipah and Hendra Viruses: Countermeasures, New Tools and Surveillance"*** COE seminar series FDA, White Oak Campus, MD.

2019. May 9, ***"Tackling Nipah and Hendra Virus: Countermeasures, New Tools, and Surveillance"*** WRAIR, Silver Spring, MD.

2019. Sept 25, ***"Bats, Pigs, Horses, and People...One Health Approaches against Nipah Virus and Hendra Virus"*** Department of Microbiology and Immunology, University of Maryland School of Medicine. Baltimore, MD.

2019. Oct 4, **"Bats, Pigs, Horses, Oh My! Battling emerging zoonotic viruses"** Notable Alumnus Lecture, University of Florida College of Medicine, Alumni Weekend. George T. Harrell, M.D., Medical Education Building. Gainesville, FL.

2019. Oct 31, ***"Bats, Horses, Pigs, People....One Health Countermeasures against Hendra and Nipah Viruses"*** World Vaccine Congress, Barcelona, Spain.

2019. Nov 18, ***"Bats, Horses, Pigs, and People....One Health Countermeasures against Nipah and Hendra Viruses"*** National Health Research Institutes (NHRI), Taiwan.

2019. Dec 10, ***"Monoclonal Antibody Countermeasures for Pathogenic Henipaviruses"*** Nipah virus international Conference; Nipah@20. Singapore.

2020. Oct 22, ***"A One-Health approach against Hendra Virus in Australia Leads to a Nipah virus vaccine for people"*** Department of Veterinary Medicine, VA-MD College of Veterinary Medicine, University of Maryland.

PUBLICATIONS: (total citations; >22,000; h-index, 68)

1. Lottenberg, R, **CC Broder**, and MDP Boyle. Identification of a Specific Receptor for Plasmin on a Group A Streptococcus. Infection and Immunity. 55(8):1914-1918, 1987.
2. **Broder, CC**, R Lottenberg, and MDP Boyle. Mapping of the Domain of Human Plasmin Recognized by its Unique Group A Streptococcal Receptor. Infection and Immunity. 57(9): 2597-2605, 1989.
3. Appelgate, ML, MM Moore, **CC Broder**, A Burrell, G Juhn, K.L. Kasweck, P-F Lin, A Wadhams, and JC Hoizer. Molecular Dissection of Mutations at the Heterozygous Thymidine Kinase Locus in Mouse Lymphoma Cells. Proc. Natl. Acad. Sci. USA. 87(1):51-55, 1990.
4. **Broder, CC**, R Lottenberg, GO vonMering, K Johnston and MDP Boyle. Isolation of a prokaryotic plasmin receptor: relationship to a plasminogen activator produced by the same microorganism. J. Biol. Chem. 266:4922-28, 1991.
5. McCoy, HE, **CC Broder**, and R Lottenberg. Strepokinases Produced by Pathogenic Group C Streptococci Demonstrate Species-Specific Plasminogen Activation. J. Infect. Dis. 164:515-521, 1991.
6. Lottenberg, R, **CC Broder**, MDP Boyle, SJ Kain, BL Schroeder, and R Curtiss III. Cloning, Sequence Analysis, and Expression in *Escherichia coli* of a Streptococcal Plasmin Receptor. J. Bacteriology. 174:5204-5210, 1992.
7. **Broder, CC**, DS Dimitrov, R Blumenthal, and EA Berger. The block to HIV-1 envelope glycoprotein-mediated membrane fusion in animal cells expressing human CD4 can be overcome by a human cell component(s). Virology. 193:483-491, 1993.
8. Dimitrov, DS, **CC Broder**, EA Berger, and R Blumenthal. Calcium Ions are required for Cell Fusion Mediated by the CD4-HIV-1 Envelope Interaction. J.Virol.67:1647-52, 1993.

9. **Broder, CC**, and EA Berger. CD4 Molecules with a Diversity of Mutations Encompassing the CDR3 Region Efficiently Support HIV-1 Env Glycoprotein-mediated Cell Fusion. J.Virol. 67:913-926, 1993.
10. Nussbaum, O, **CC Broder**, and EA Berger. HIV-1 Envelope Glycoprotein/CD4 Mediated Cell Fusion: A Novel Recombinant Vaccinia Virus-Based Assay Measuring Activation of a Reporter Gene by Bacterio-phage T7 RNA Polymerase Selectively In Fused Cells. J.Virol. 68:5411-5422, 1994.
11. **Broder, CC**, O Nussbaum, WG Gutheil, WW Bachovchin, and EA Berger. Evidence against CD26 Involvement in HIV-1 Envelope Glycoprotein/CD4-Mediated Cell Fusion. Science. 264:1156-1159, 1994.
12. **Broder, CC**, PL Earl, D Long, B Moss, and RW Doms. Antigenic Implications of HIV-1 Envelope Glycoprotein Quaternary Structure: Oligomer-Specific and -Sensitive mAbs. Proc Natl Acad Sci USA. 91:11699-11703, 1994.
13. **Broder, CC**, PE Kennedy, F Michaels, and EA Berger. Expression of Foreign Genes in Cultured Human Primary Macrophages Using Vaccinia Virus Vectors. Gene. 142:167-4, 1994.
14. Earl, PL, **CC Broder**, D Long, S Lee, J Peterson, S Chakrabarti, RW Doms and B Moss. Native oligomeric forms of HIV-1 envelope glycoprotein elicit a diverse array of mAb reactivities. J. Virol. 68: 3015-26, 1994.
15. Nussbaum, O, **CC Broder**, L Bar-Lev Stern, S Rozenblatt, B Moss, and EA Berger. Functional and Structural Interaction between Measles Virus Hemagglutinin and CD46. J. Virol. 69:3341-3349, 1995.
16. **Broder, CC** and EA Berger. Fusogenic Selectivity of the Envelope Glycoprotein is a Major Determinant of HIV-1 Tropism for CD4+ T-Cell Lines vs. Macrophages. Proc Natl Acad Sci USA. 92:9004-08, 1995.
17. Golding, H, DS Dimitrov, J Manischewitz, **CC Broder**, J Robinson, S Fabian, D Littman, and C Lapham. PMA-induced down modulation of tailless CD4 Receptors Requires Prior Binding of gp120 and Suggests a Role for Accessory Molecules. J.Virol. 69:6140-6148, 1995.
18. Richardson, TM, BL Stryjewski, **CC Broder**, JA Hoxie, JR Mascola, PL Earl, and RW Doms. The Humoral Response to Oligomeric HIV-1 Envelope Protein. J.Virol.70:753-62, 1996.
19. Feng, Y, **CC Broder**, PE Kennedy, and EA Berger. HIV-1 Entry Cofactor: Functional cDNA Cloning of a Seven-Transmembrane, G Protein-Coupled Receptor. Science. 272:872-877, 1996
20. Alkhatib*, G, C Combadiere*, **CC Broder***, Y Feng*, PE Kennedy*, PM Murphy, and EA Berger. CC CKR5: a RANTES, MIP-1α, MIP-1β Receptor as a Fusion Cofactor for Macrophage-Tropic HIV-1. Science. 272:1955-1958, 1996. (*equal contribution).
21. Alkhatib, G, **CC Broder**, and EA Berger. Cell Type-specific Accessory Factors Determine HIV-1 Tropism for T-cell Lines vs. Primary Macrophages. J. Virol. 70:6487-6494, 1996.
22. Rucker, J, M Samson, BJ Doranz, F Libert, JF Berson, Y Yi, RG Collman, **CC Broder**, G Vassart,, RW Doms, and M. Parmentier. Regions in β-chemokine Receptors CCR-5 and CCR-2b that Determine HIV-1 Cofactor Specificity. Cell. 87:1-10, 1996.
23. Edinger, AL, A Amedee, K Miller, BJ Doranz, M Endres, M Sharron, M Samson, Z-h Lu, JE Clements, M Murphey-Corb, SC Peiper, M Parmentier, **CC Broder**, and RW Doms. Differential utilization of CCR5 by macrophage and T cell tropic simian immunodeficiency virus strains. ProcNatlAcadSci. 94:4005-10, 1997.
24. Earl, PL, **CC Broder**, RW Doms, and B Moss. Epitope Map of HIV-1 gp41 derived from 47 Monoclonal Antibodies Produced by Immunization with Oligomeric Envelope Protein. J. Virol. 71:2674-2684, 1997.
25. Lee, S, Peden, K, Dimitrov, DS, **Broder, CC**, Manischewitz, J, Denisova, G, Gershoni, JM, and Golding, H. Enhancement of HIV-1 envelope-mediated fusion by a CD4-gp120 complex-specific monoclonal antibody. J.Virol. 71:6037-43 1997.
26. Bazan, HA, G Alkhatib, **CC Broder**, and EA Berger. Patterns of CCR5, CXCR4 and CCR3 usage by envelope glycoproteins from human immunodeficiency virus type-I primary isolates. J. Virol. 72:4485-91, 1998.
27. Wang JM, Ueda, H, Howard, OM, Grimm, MC, Chertov, O, Gong, X, Gong, W, Resau, JH, **Broder, CC**, Evans, G, Arthur, LO, Ruscetti, FW, Oppenheim, JJ. HIV-1 envelope gp120 inhibits the monocyte response to chemokines through CD4 signal-dependent chemokine receptor down-regulation. J. Immunol. 161:4309-4317, 1998.
28. Lee, B, J Rucker, RW Doms, M Tsang, X Hu, M Dietz, R Bailer, LJ Montaner, C Gerard, N Sullivan, J Sodroski, TS Stantchev, **CC Broder**. β-Chemokine MDC and HIV-1 Infection. Science. 281: 487a, 1998.
29. Golding, H, J Ouyang, M Zaitseva, **CC Broder**, DS Dimitrov, and C Lapham. Increased association of glycoprotein 120-CD4 with HIV type 1 coreceptors in the presence of complex-enhanced anti-CD4 monoclonal antibodies. AIDS Res. Hum. Retroviruses. 15:149-159. 1999.
30. Dimitrov, DS, D Norwood, TS Stantchev, Y Feng, X Xiao, and **CC Broder**.A Mechanism of Resistance to HIV-1 Entry:Inefficient Interactions of CXCR4 with CD4 and gp120 in Macrophages.Virology 259:1-6, 1999.
31. Sugiura, W, **CC Broder**, B Moss, and PL Earl. Characterization of conformation-dependent anti-gp120 murine monoclonal antibodies produced by immunization with monomeric and oligomeric HIV-1 envelope proteins. Virology. 254:257-267, 1999.
32. Xiao, X, L Wu, TS Stantchev, Y-R Feng, S Ugolini, H Chen, Z Shen, **CC Broder**, QJ Sattentau, and DS Dimitrov.

Constitutive cell surface association between CD4 and CCR5. PNAS. 96:7496-7501, 1999.

33. Chabot, DJ, P-F Zhang, GV Quinnan, and **CC Broder**. Mutagenesis of CXCR4 Identifies Important Domains for HIV-1 X4 Isolate Envelope-Mediated Membrane Fusion and Virus Entry and Reveals Cryptic Coreceptor Activity for R5 Isolates. J. Virol. 73:6598-6609, 1999.
34. Chabot, DJ, H Chen, DS Dimitrov, and **CC Broder**. N-linked Glycosylation in CXCR4 Masks Coreceptor Function for CCR5-Dependent HIV-1 Isolates. J. Virol. 74:4404-4413, 2000.
35. Lee, S, CK Lapham, H Chen, L King, J Manischewitz, T Romantseva, H Mostowski, TS Stantchev, **CC Broder**, and H Golding. Coreceptor competition for association with CD4 may change the susceptibility of human cells to infection with T- and M-tropic isolates of HIV-1. J. Virol. 74:5016-5023, 2000.
36. Xiao, X, D Norwood, Y-R Feng, M Moriuchi, H Moriuchi, A Jones-Trower, TS Stantchev, **CC Broder**, and DS Dimitrov. Inefficient Formation of a Complex between CXCR4, CD4 and gp120 in U937 Clones Resistant to X4 gp120-gp41-Mediated Fusion. Exp. Mol. Path. 68:139-146, 2000.
37. Xiao, X, A Kinter, **CC Broder**, and DS Dimitrov. Interactions of CCR5 and CXCR4 with CD4 and gp120 in Human Blood Monocyte-Derived Dendritic Cells. Exp. Mol. Path. 68:133-138, 2000.
38. Stantchev, TS and **CC Broder**. Consistent and Significant Beta-chemokine Inhibition of HIV-1 Envelope-mediated Membrane Fusion in Primary Macrophages. J Infect Dis. 182:68-78, 2000.
39. Chabot, DJ and **CC Broder**. Substitutions in a Homologous Region in the ECL-2 of CXCR4 and CCR5 Alter Coreceptor Activities for HIV-1 Fusion and Entry. J. Biol. Chem. 275:23774-82, 2000.
40. Earl, PL, W Sugiura, D Montefiori, **CC Broder**, S.A. Lee, C. Wild, J. Lifson, and B. Moss. Immunogenicity and protective efficacy of oligomeric HIV-1 gp140. J Virol. 75:645-53, 2001.
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ANNEXURE: 3-RV-1/2019 (SOP-IEC-KGMU)

ONE PAGE CV FOR NON-KGMU INVESTIGATORS

Broder	Christopher	C.
Date of Birth (b) (6)):		**Sex: Male**

Study Site Affiliation (Co-Investigator)

Professional Mailing Address **Department of Microbiology and Immunology** **Uniformed Services University, B4152** **4301 Jones Bridge Rd, Bethesda, MD 20814-4799, USA**	**Study Sited Address** **(Include institution name)**
Telephone (office): +1-301-295-3401	**Mobile Number: +1-301-535-2943**
Telephone (Residence): Mobile	**Email:** christopher.broder@usuhs.edu (b) (6)

Academic Qualifications (Most current qualification first)

Degree / Certificate	Year	Institution, Country
BS, Biological Sciences	1983	Florida Institute of Technology, Melbourne, Florida, USA
MS, Molecular Biology	1985	Florida Institute of Technology, Melbourne, Florida, USA
PhD, Microbiology and Immunology	1989	University of Florida, Gainesville, Florida, USA

Current and Previous Relevant Positions Including Academic Appointments (Most current position first)

Month and Year	Title	Institution / Company, Country
August, 2018	Chair	Uniformed Services University, USA
January, 2005	Professor	Uniformed Services University, USA

Brief Summary of Relevant Clinical Research Experience:

I have been a in the area of enveloped virus-host cell interactions for over the past 32 years. I developed the first oligomeric HIV-1 gp140 envelope glycoprotein, established a vaccinia virus-based reporter gene assay for measuring viral glycoprotein-mediated membrane fusion, and defined the membrane fusion tropism of HIV-1 followed by the discovery of the HIV-1 coreceptors (CXCR4 and CCR5). In 1999, I established an international group of experts in Hendra virus and Nipah virus research. My lab's work includes the discovery of the entry receptors for Hendra and Nipah (ephrin-B2/B3), developed the feline, ferret and African green monkey models of Hendra and Nipah pathogenesis, the structural solutions of the F glycoprotein and the G-ephrin receptor interactions; the discovery and development of antiviral human monoclonal antibodies including one having a Phase I clinical trial completed in May, 2016, known as m102.4 that has been used by compassionate emergency protocol in 15 people in Australia and one in the United States because of significant exposure risk to Hendra or Nipah infection. I also developed the Hendra/Nipah subunit vaccine based on soluble Hendra G (HeV-sG); called Equivac® HeV (Zoetis, Inc.) which is the first commercialized vaccine to a BSL-4 agent. We have developed reverse genetics platforms for Australian bat lyssavirus and Cedar henipavirus that can be utilized at BSL-2. My lab is now doing virus surveillance using multiplex assays with native-like viral membrane proteins that measure antiviral humoral responses in animals and humans

Signature: (b) (6) **(Signature Re**	**Date:** (b) (6)

ANNEXURE: 3-RV-1/2019 (SOP-IEC-KGMU)

ONE PAGE CV FOR NON-KGMU INVESTIGATORS

Broder	Christopher	C.
Date of Birth (b) (6)):		**Sex: Male**

Study Site Affiliation (Co-Investigator)

Professional Mailing Address **Department of Microbiology and Immunology** **Uniformed Services University, B4152** **4301 Jones Bridge Rd, Bethesda, MD 20814-4799, USA**	**Study Sited Address** **(Include institution name)**
Telephone (office): +1-301-295-3401	**Mobile Number: +1-301-535-2943**
Telephone (Residence): Mobile	**Email:** christopher.broder@usuhs.edu (b) (6)

Academic Qualifications (Most current qualification first)

Degree / Certificate	Year	Institution, Country
BS, Biological Sciences	1983	Florida Institute of Technology, Melbourne, Florida, USA
MS, Molecular Biology	1985	Florida Institute of Technology, Melbourne, Florida, USA
PhD, Microbiology and Immunology	1989	University of Florida, Gainesville, Florida, USA

Current and Previous Relevant Positions Including Academic Appointments (Most current position first)

Month and Year	Title	Institution / Company, Country
August, 2018	Chair	Uniformed Services University, USA
January, 2005	Professor	Uniformed Services University, USA

Brief Summary of Relevant Clinical Research Experience:

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Signature: **(Signature Required)**	**Date:**

From: Broder, Christopher on behalf of Broder, Christopher <christopher.broder@usuhs.edu>
To: Ava Sullivan
Subject: Re: DTRA India: CV and form needed for ethical approvals
Date: Wednesday, January 20, 2021 6:01:06 PM
Attachments: BroderCV-full-01202021-Signed.pdf
Annexure 3_KGMU_IEC-CBroder-signed.pdf
Annexure 3_KGMU_IEC-CBroder.docx

Ava

not sure about what was needed in the last yellow box

On Wed, Jan 20, 2021 at 5:37 PM Ava Sullivan <sullivan@ecohealthalliance.org> wrote:

> Hi Chris and Rajesh,
> A gentle reminder. I hope to move forward with the as soon as possible,
> Thanks,
> Ava
>
> _____
> Ava Sullivan
> *Project Manager and Research Scientist*
>
> EcoHealth Alliance
> 520 Eighth Avenue – Suite 1200
> New York, NY 10018
> 212-380-4498 (direct office)
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> www.ecohealthalliance.org
>
> ***EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation***
>
> > On Jan 14, 2021, at 3:24 PM, Ava Sullivan <sullivan@ecohealthalliance.org> wrote:
> >
> > Hi Chris, Jon and Rajesh,
> > Ethical Approval documents through King George Medical University require all listed Co-PI's of the DTRA-India project to:
> >
> > 1) Provide a signed copy of their CV
> > 2) Fill a one-page summary CV (attached here in Word)
> >
> > Could you please provide these two docs to me in the next week, so we can move forward with this important step?
> > Thanks, and please write me with questions or concerns,
> > Ava
> >
> > <Annexure 3_KGMU_IEC.docx>
> >
> > _____
> > Ava Sullivan

Project Manager and Research Scientist

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Complementary regulation of caspase-1 and IL-1β reveals additional mechanisms of dampened inflammation in bats

Geraldine Goh[a,1], Matae Ahn[a,1], Feng Zhu[a], Lim Beng Lee[a], Dahai Luo[b,c], Aaron T. Irving[a,d,2], and Lin-Fa Wang[a,e,2]

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Bats have emerged as unique mammalian vectors harboring a diverse range of highly lethal zoonotic viruses with minimal clinical disease. Despite having sustained complete genomic loss of AIM2, regulation of the downstream inflammasome response in bats is unknown. AIM2 sensing of cytoplasmic DNA triggers ASC aggregation and recruits caspase-1, the central inflammasome effector enzyme, triggering cleavage of cytokines such as IL-1β and inducing GSDMD-mediated pyroptotic cell death. Restoration of AIM2 in bat cells led to intact ASC speck formation, but intriguingly resulted in a lack of caspase-1 or consequent IL-1β activation. We further identified two residues undergoing positive selection pressures in *Pteropus alecto* caspase-1 that abrogate its enzymatic function and are crucial in human caspase-1 activity. Functional analysis of another bat lineage revealed a targeted mechanism for loss of *Myotis davidii* IL-1β cleavage and elucidated an inverse complementary relationship between caspase-1 and IL-1β, resulting in overall diminished signaling across bats of both suborders. Thus we report strategies that additionally undermine downstream inflammasome signaling in bats, limiting an overactive immune response against pathogens while potentially producing an antiinflammatory state resistant to diseases such as atherosclerosis, aging, and neurodegeneration.

bats | AIM2 | caspase-1 | inflammasome | IL-1β

Bats are placental mammals which uniquely utilize powered flight for locomotion, harbor a diverse viral repertoire, and possess longevity exceptional to their body size. In recent years, bats have been implicated in major outbreaks caused by fatal zoonotic viruses, such as SARS-CoV and MERS-CoV, henipaviruses, filoviruses including Ebola and Marburg virus, and a high likelihood of the currently circulating SARS-CoV-2 (1–4). Ongoing outbreaks with significant mortality and morbidity in human and livestock have driven a targeted search for the originating hosts of these spillover pathogens and pivotal studies have identified bats as significant reservoir and ancestral hosts to more zoonotic diseases per species, against all other mammalian orders (2, 5). Of key interest is the bat innate and adaptive immune system, due to evolutionarily driven or yet undiscovered, altered interactions between the host–pathogen interface, leading to their tolerance of viral diseases.

Genomic and transcriptomic studies have identified disparities between bats and other mammals. Positive selection has been shown in critical innate immune, tumor suppressive, and DNA damage checkpoint genes of bats (6), including NLRP3, TP53, and ATM. Altered natural killer (NK) cell repertoires were found among *Pteropus alecto*, *Myotis davidii*, and *Rousettus aegyptiacus* bats, along with differential contraction of IFN-α genes and expansion of IFN-ω genes in *Pteropus*, *Myotis*, and *Rousettus* bat species (7, 8). Despite mounting genomic evidence that bats have unique alterations in innate immune pathways, experimental confirmation is rare. We recently demonstrated that NLRP3 is dampened in bats as a result of loss-of-function bat-specific isoforms and impaired transcriptional priming (9). The stimulator of IFN genes (STING), a key adaptor to the DNA-sensing cGAS protein, is also exclusively mutated at S358 in bats, resulting in a reduced IFN response to HSV1 (10). We previously reported a complete absence of Absent in melanoma 2 (AIM2)-like receptor (ALR) genes across all available bat genomes from both Yinpterochiroptera and Yangochiroptera suborders (11). As these modifications in bats signify shifts in cell signaling and immune regulation, we thus investigated the loss of the PYHIN or ALR gene family for implications on the bat DNA-sensing inflammasome response.

The ALRs are an essential group of germline-encoded pattern recognition receptors (PRRs) comprising 5 members in humans and 14 members in mice, with the most well studied being AIM2 (12, 13). AIM2 is the prototypical member of the ALR family and was shown to mediate intracellular dsDNA-responsive inflammasome signaling, typically of invading pathogenic origin or aberrant host cytosolic DNA (14, 15). There is extensive

Significance

Bats have been shown to dampen several key upstream pathogen and danger-associated molecular patterns, yet much of the downstream signaling is yet unknown. Here, we identify residues in caspase-1 which are critical for enzymatic activity and have been targeted for inhibition in *Pteropus* bats. Further, we discover cleavage-site flanking residues which lead to loss of IL-1β cleavage in *Myotis* bats. Thus, we report an inverse relationship between caspase-1 function and IL-1β cleavage, resulting in a consistent reduction of downstream signaling by the inflammasome across bats within the two suborders. In sum, we confirm that bats have targeted the inflammasome pathway at multiple levels and via heterogeneous strategies to reduce proinflammatory responses, thus mitigating potential immune-mediated tissue damage and disease.

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diversity across mammalian ALR families and AIM2 is the only member with preserved orthology among species (12, 16). Upon recognizing exposed intracellular DNA, it binds to the major turn of the DNA helix and recruits its adaptor apoptosis-associated speck-like protein containing a CARD (ASC), which forms aggregates (ASC specks) to complex with downstream procaspase-1 (15, 17). The caspase-1 proenzyme undergoes homodimerization and autocleavage for activation, conferring it the ability to bind, cleave, and mature IL-1β, IL-18, and gasdermin D (GSDMD) and trigger pyroptotic cell death (18–22). The mammalian AIM2 is crucial in its role in sensing intracellular foreign DNA, accompanied by a potential for either a pathological or protective inflammatory response in the host (23–25). Yet the consequence of its absence in bats, a unique animal model shown to down-regulate components of its inflammasome pathway, is poorly understood.

Caspase-1, or cysteine aspartic protease 1, is the central inflammasome effector for pyroptosis and cytokine secretion, playing roles in diverse cellular processes, including apoptosis/necrosis, metabolism, mitophagy, and autophagy (26–29). The propeptide consists of a caspase-recruitment domain (CARD), a p20 and p10 polypeptide sequence, and undergoes sequential autoproteolysis at aspartic acid residues into p20/p10 subunits which dimerize to achieve the activated conformation for substrate binding and cleavage (18, 21, 30). It is converged upon by all canonical inflammasome receptors, including NLRP3, NAIP/NLRC4, NLRP1, AIM2, and pyrin, and many other members of the NLR family such as NLRP6, NLRP7, and NLRP12, mediating critical proinflammatory host responses against microbes or autoimmune and autoinflammatory sequelae (31). Further, it is involved in multiple age-related diseases, including amyloid β accumulation in Alzheimer's disease and cardiac injury during acute myocardial infarction (32, 33). While intensive study of human caspase-1 inhibitors are currently ongoing, given its therapeutic potential, still little is known about the downstream activation of caspase-1 in bats, especially given their altered immune landscape and dampened inflammasome function.

Here, we confirm that genomic loss of AIM2 in bats dismantles the inflammasome adaptor recruitment responsive to dsDNA. Additionally, reconstitution of the human gene in a bat in vitro environment is sufficient to partly restore this intracellular pathway up to ASC level. However, we discovered an absence of the downstream cytokine release or cell-death initiation despite robust ASC speck formation in bat primary macrophages. We identify key residues in the bat caspase-1 responsible for dampened IL-1β cleavage, or altered IL-1β cleavage sites which significantly reduce its processing and maturation in bats. Importantly, we have elucidated multiple levels of disengagement within the bat inflammasome pathway with key implications in their response toward cellular stress, inflammation, and pathogenic detection.

Results

Absence of ASC Speck Induced by DNA Stimulation in Bat Kidney and Immune Cells Is Restored by Human AIM2. Given the ALR family members, including AIM2, are the only DNA sensors mediating the intracellular sensing of pathogenic and aberrant host DNA to activate the inflammasome, we hypothesized that absence of all ALR genes in bats would result in the inability for bats to trigger inflammasome signaling. Indeed, with exogenous dsDNA ligand PolydA:dT stimulation of bat bone-marrow-derived macrophages (BMDMs), we observed a lack of recruitment of ASC into aggregates (ASC speck), which could be seen in dsDNA-treated murine BMDMs (Fig. 1*A*). Further, using high-throughput image-based flow cytometry (Imagestream), we observed that the cytosolic ASC remained diffusely distributed in bat cells, unlike their aggregation into a speck-like morphology in mouse macrophages (Fig. 1*B*).

To investigate whether lack of ASC speck formation was attributable to the absence of gene and protein expression from the ALR family, we generated a human AIM2-mCitrine fusion construct cloned into a mammalian expression vector and rescued the gene in *Pteropus alecto* kidney-derived (PaKiS) immortalized cells (34). We selected the human AIM2 protein, as AIM2 is the only ALR gene with conserved evolutionary and functional orthology across species (16) and the human ortholog is closest in homology to the only nonfunctional PYHIN peptide fragment identified in bats (*Pteronotus parnelli*) (11). Transient overexpression of human AIM2 alone was sufficient to restore ASC speck formation in PaKiS cells stably expressing bat ASC (Fig. 1*C* and *SI Appendix*, Fig. S1 *A* and *B*), resulting in organization into a perinuclear inflammasome complex with colocalization of AIM2 and downstream ASC (Fig. 1*D*). To evaluate the activation in response to intracellular DNA, lentiviral delivery was used to generate PaKiS cells stably expressing both human AIM2 and bat ASC at low copy number. ASC speck formation was induced with addition of PolydA:dT DNA ligand and increased in a dose-dependent manner in AIM2-positive cells only (12-fold increase at 2.0 μg/mL compared to 0 μg/mL) (Fig. 1*E*). This was accompanied by increasing detection of AIM2 oligomerization (15-fold), signaling intact sensing of dsDNA by AIM2 and consequent formation of the inflammasome recruitment platform (Fig. 1*F*). Importantly, interaction of the adaptor ASC with the AIM2 sensor demonstrates the highly conserved nature of bat ASC to retain ability for recruitment to oligomerized human AIM2. This supports our previous observation whereby bat ASC is also conserved with the human ASC in its function and speck properties, including size, density, and shape in response to NLRP3 (9).

Human AIM2 Restores ASC Speck Formation but Not Caspase-1 Activation or IL-1β Release in Bat Macrophages. As the *P. alecto* kidney in vitro immortalized cell system lacks classic inflammasome machinery, we examined primary in vitro differentiated bat BMDMs for activation of the inflammasome as a consequence of AIM2 restoration. Transduction of lentivirus carrying a control vector or human AIM2 was performed in *P. alecto* BMDMs (PaBMDMs), and ASC speck formation was similarly quantified by Imagestream. Only AIM2 reconstituted ($AIM2^+$) bat macrophages treated with PolydA:dT were able to induce endogenous bat ASC aggregation, whereas minimal induction was observed in the mock (vehicle)-treated control vector or $AIM2^+$, or PolydA:dT-treated control vector, conditions (Fig. 2*A*). Imagestream analysis supported this finding with visible ASC speck in PolydA:dT-treated $AIM2^+$ BMDMs compared to vehicle-only (mock) controls (Fig. 2*B*). This suggested AIM2-dependent restoration of ASC speck induction in response to the DNA ligands.

Thus, we next looked for downstream IL-1β cleavage or induction of pyroptosis. Unexpectedly, mature IL-1β was unable to be detected in the supernatant despite ASC speck induction in DNA-treated AIM2-reconstituted bat BMDMs, in contrast to the mouse BMDMs (Fig. 2*C*). This was accompanied by minimal lytic cell death and low lactate dehydrogenase (LDH) activity levels in the cell supernatant (Fig. 2*D*). To measure the caspase-1 activity specifically in bat BMDMs, we utilized the 660-YVAD-fmk fluorescent-labeled inhibitor of caspase activation (FLICA) assay with relative specificity for the caspase-1 active site. We observed lack of substrate binding even in DNA-treated and $AIM2^+$ bat BMDMs, compared to the robust activity in treated mouse BMDMs which possess endogenous AIM2 (Fig. 2*E*). As a previous study similarly identified minimal secretion of IL-1β upon NLRP3 inflammasome activation in bat primary immune cells, we decided to investigate the downstream convergence of both sensing platforms onto caspase-1.

Failure in IL-1β Production Is Due to Substitution of Two Residues in *P. alecto* Bat Caspase-1. Lack of downstream activation in the presence of ASC specks can be attributed to either caspase-1, or

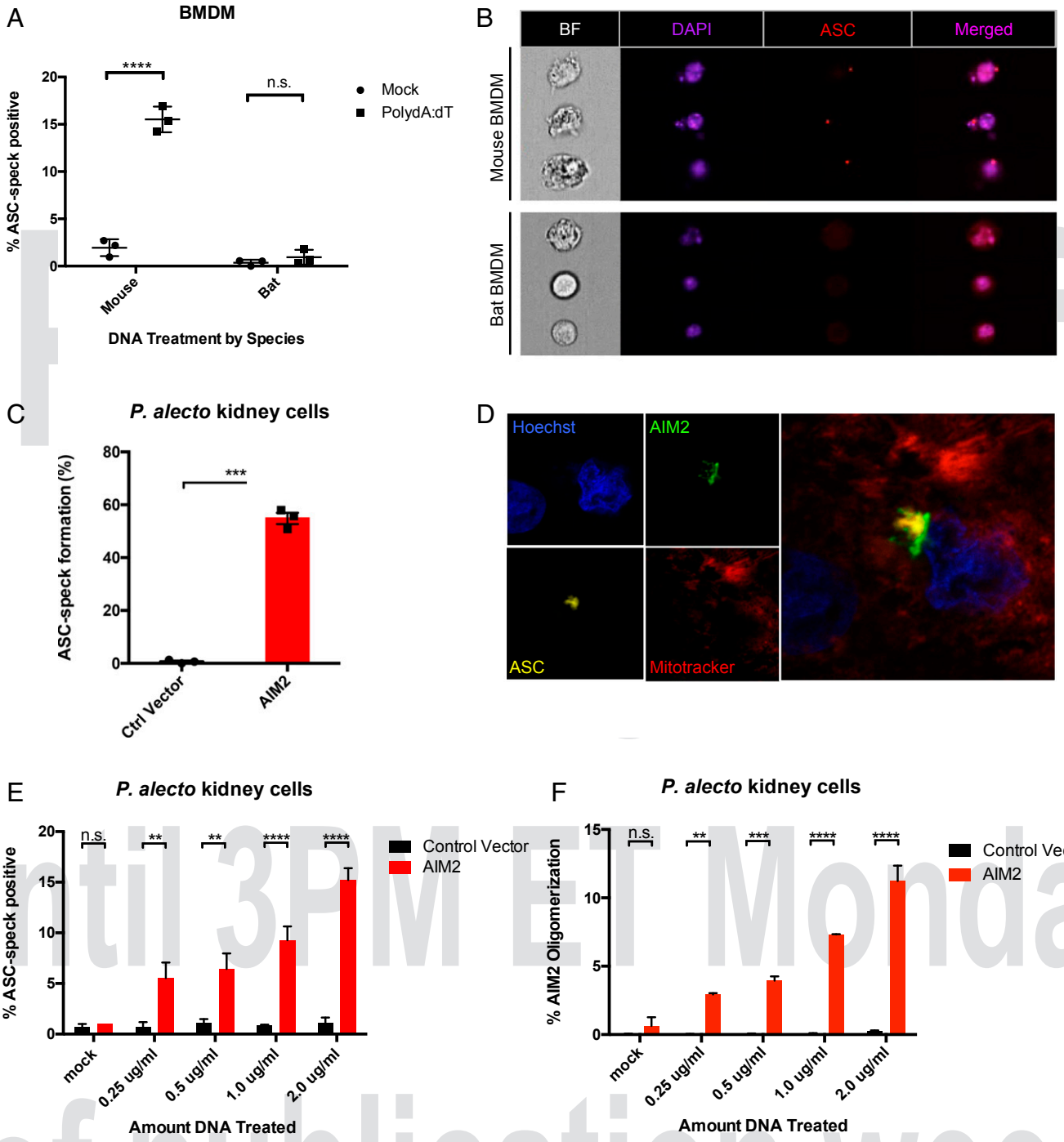

Fig. 1. Reconstitution of AIM2 restores DNA-triggered ASC speck in bat cells. (*A*) Measurement of ASC speck formation in mouse and bat BMDMs treated with either vehicle (mock) or transfected dsDNA (PolydA:dT, 1 μg/mL) for 4 h after 3 h LPS (mouse) or CL264 (bat) priming. (*B*) Single-cell imaging of mouse or bat BMDMs collected on Imagestream to visualize ASC speck aggregation or diffuse intracellular distribution, shown as bright field (BF), DAPI, and ASC signals. (*C*) ASC speck formation was quantified on Imagestream flow cytometry in *P. alecto* kidney cells (PaKiS) stably expressing bat ASC-mPlum and transiently expressing human AIM2. (*D*) Single-plane confocal imaging of transfected PaKiS cells showing ASC speck aggregation and association with cotransfected AIM2. Hoechst 33342 nuclear staining (blue), AIM2-mCitrine (green), ASC-mPlum (yellow), and Mitotracker (red). (*E*) Retroviral transduction of control vector or AIM2 was performed in ASC-mPlum stably expressing PaKiS cells, and DNA transfected in a dose-curve (mock, 0.25 to 2.0 μg/mL PolydA:dT). Imagestream flow cytometry was performed for triggered ASC speck. (*F*) AIM2 oligomerization induced in a dose-dependent manner quantified by Imagestream. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, n.s., nonsignificant; linear regression and two-tailed unpaired *t* test. Data are presented as mean ± SEM of three biological replicates (*A* and *B*) or three independent experiments (*C*–*F*).

IL-1β, or both. To dissect the mechanism, we reconstituted the entire AIM2 inflammasome axis in human embryonic kidney (HEK293T) cells, coexpressing AIM2, ASC, caspase-1, and IL-1β. Human genes were used for the upstream components (AIM2 and ASC) to standardize the system in a human cell line. Either control (empty vector), human, or *P. alecto* caspase-1 (HsCASP1, PaCASP1) proteins were expressed in increasing concentrations and the cell lysates were immunoblotted for

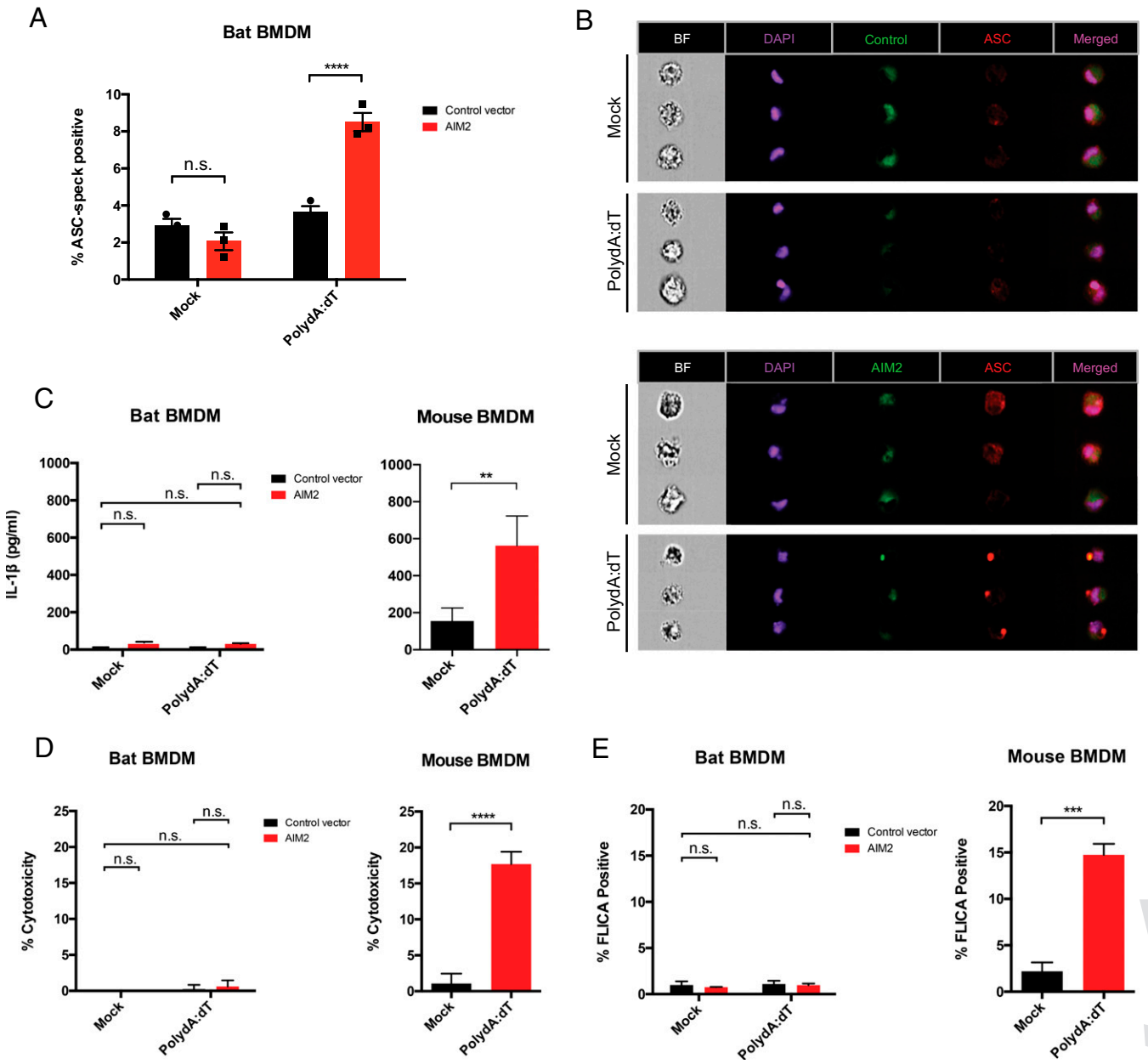

Fig. 2. Lack of caspase-1 or IL-1β signaling despite ASC speck restoration in AIM2-reconstituted bat macrophages. (*A*) Mouse or *P. alecto* BMDMs (PaBMDM) were differentiated in CSF-1 for 5 d and transduced with AIM2 or control vector lentivirus. At 48 h posttransduction, macrophages were primed with CL264 or LPS and treated with vehicle (mock) or DNA (PolydA:dT, 1 μg/mL) for 4 h and ASC speck was quantified via Imagestream. (*B*) Representative images for PaBMDMs stained for DAPI and anti-PaASC, shown is HsAIM2-mCitrine reconstitution and PaASC speck formation with either mock or PolydA:dT treatment. (*C*) LDH assay was performed on supernatant to measure cytolytic cell death in PolydA:dT-treated bat control or AIM2$^+$ BMDM (*Left*) or mouse BMDM (*Right*). (*D*) Similarly, IL-1β secretion was quantified in the supernatant of treated bat and mouse BMDMs using in-house bat ELISA protocol as previously published (14) and mouse IL-1β ELISA kit. (*E*) Fluorescent-labeled inhibitor of caspase activation (FLICA) assay was performed on bat and mouse BMDMs for caspase-1 activation upon DNA treatment, with staining for 1 h and flow cytometry analysis. Statistical analysis was performed using two-way ANOVA with Bonferroni's multiple comparisons test (*A* and *C*–*E*) and two-tailed unpaired *t* test (*C*–*E*). $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, n.s., nonsignificant. Data are representative of three biological replicates ($n = 3$) in *B* or mean ± SEM of three biological replicates ($n = 3$) in (*A*, *C*, and *E*).

cleaved IL-1β p17, representing the product of inflammasome activation. Notably, there was minimal detection of mature bat IL-1β p17 in the cell lysates despite increasing expression of PaCASP1 (Fig. 3*A*). In contrast, human caspase-1 showed robust cleavage of bat IL-1β, suggesting intact cleavage of bat IL-1β. We observed an overall decrease in pro-PaCASP1 expression in contrast to human protein expression, and an absence of PaCASP1 p32 or other intermediate self-cleavage products. Importantly, a clear reduction in PaCASP1 activity compared to HsCASP1 was observed, which suggested decreased functionality of bat caspase-1.

To confirm the loss of caspase-1 activity, we interrogated PaCASP1 for intact activation by FLICA assay with specificity to caspase-1 to detect for active site binding. Similarly, although HsCASP1 exhibited 10-fold times higher FLICA substrate retention and fluorescence (11.03% ± 0.201), PaCASP1 had minimal detection in activity (2.855% ± 0.991), with levels similar to the control vector (1.58% ± 0.218) (Fig. 3 *B* and *C*). To

further confirm that loss of inflammasome signaling was at the bat caspase-1 and not IL-1β level, we paired expression of either HsCASP1 or PaCASP1 with human or bat IL-1β. Assay of the lysates for either HsIL-1β or PaIL-1β indicated that HsCASP1, but not PaCASP1, was able to successfully cleave the IL-1β from both species (Fig. 3*D*). Altogether, this indicates that caspase-1, and not IL-1β, is dampened in the *Pteropus* bat inflammasome pathway, resulting in failure of IL-1β maturation and cleavage.

Structural studies in human caspase-1 have identified C285 and H237 as essential residues for catalytic activity, among others which form the substrate-binding active site (35, 36). Additional residues within the p10 and p20 fragments have been shown to mediate crucial interactions in dimerization, and mutations result in inability to bind and cleave cytokines for secretion (37). A recent study established that homodimerization of two p20/p10 subunits forms the active conformation of caspase-1, whereby autoprocessing of the caspase-1 p10 fragment is required for GSDMD cleavage and initiation of pyroptosis (22). Given the existing understanding of caspase-1 activation, we analyzed the caspase-1 gene sequence to determine the potential mechanism of its reduced activity in bats. Sequences of available bat caspase-1 genes across Yinpterochiroptera and Yangochiroptera suborders, and 10 nonbat mammalian species were aligned, and phylogenetic analysis by maximum likelihood (PAML) performed to identify lineages and sites acted on by selection pressures (38) (*SI Appendix*, Fig. S2*A*). We found one ancestral branch of the bats to have undergone positive selection pressures, and branch-site testing identified greater positive selection pressure exerted on two residue sites in its subsequent branch (*SI Appendix*, Fig. S2*B*). Compared to human, mouse, and other included species, the *P. alecto* and *Pteropus vampyrus* caspase-1 sequence showed alterations at residue 365 from Asp to Asn, and at 371 from Arg to Gln (red boxes). Both residues are localized within the p10 polypeptide and span five amino acids apart.

To understand the consequence of these substitutions, we performed site-directed reverse mutagenesis of the specific sites, replacing either one, or both residues in the bat with the equivalent human residues, resulting in N365D-only, Q371R-only, or double-mutant (DM) PaCASP1 (Fig. 3*E*). Likewise, the human HsCASP1 gene was mutated to individually or simultaneously replace both residues with those of *Pteropus* bat amino acid sites (D365N-only, R371Q-only, and HsCasp1 DM). Only the PaCASP1 DM, but not the single mutants, provoked reversal of the inactive PaCASP1 phenotype to rescue cleavage of IL-1β (Fig. 3*F*). Conversely for HsCASP1, single mutation of either site to the bat residues was sufficient to abrogate its ability to cleave IL-1β (Fig. 3*G*). Further, HEK293T cells expressing either HsCASP1 WT or mutant PaCASP1 DM showed increased cellular stress and death morphologically, while HsCASP1 DM and PaCASP1 WT exhibited increased viability regardless of ASC speck formation (*SI Appendix*, Fig. S3).

Bats also possess the GSDMD gene with conserved pore-forming N-terminal subunits and a caspase-1 recognition site $_{266}$FLSD$_{269}$; yet its function and cleavage potential by bat caspase-1 are still unknown. Thus, PaGSDMD was cloned and compared with HsGSDMD for cleavage by human and *P. alecto* wild-type (WT) or mutant caspase-1 variants. Similar to the pattern of IL-1β processing by PaCASP1, cleavage of PaGSDMD was undetected in WT PaCASP1, while PaCASP1-DM successfully restored cleavage as observed by the 31-kDa PaGSDMD-N fragment (Fig. 3*H*). Conversely, HsCASP1-D365N, R371Q, or the combined HsCASP1-DM led to abrogration of GSDMD cleavage (Fig. 3*I*). To provide the molecular basis of the loss-of-function mutations of D365N and R371Q, we examined the structure of human caspase-1 bound to GSDMD (Protein Data Bank [PDB] code: 6VIE) (39). In the substrate-bound caspase-1 state (p20/p10 dimer), R371 participates in electrostatic interactions with E367 of the opposite p10 subunit, therefore R371Q mutation results in unfavorable interaction across the interface (*SI Appendix*, Fig. S4). D365 forms part of the dimer interface and may stabilize the homodimeric interactions. As such, similar to R371Q, D365N mutation would weaken the caspase 1 dimer–dimer coordination, leading to inactivation of the enzyme (22, 39, 40). Taken together, our findings demonstrate that both sites in the p10 fragment are necessary for caspase-1 activation by the inflammasome complex, and both IL-1β and GSDMD substrate maturation is dampened in *P. alecto* bats in a caspase-1-dependent manner.

Complementation between Caspase-1 Activity and IL-1β Cleavage Results in Consistent Inflammasome Dampening across Bats. Bats, belonging to the order Chiroptera, are the second largest group of mammals with more than 1,000 species. Within the order Chiroptera, *Pteropus* bats are part of the Pteropodidae family in the suborder Yinpterochiroptera, which are distinct from the suborder Yangochiroptera, containing the rest of the microbat families (41, 42). To better understand if downstream inflammasome dampening is a consistent pattern across bats, we extended our study to include both the *Eonycteris spelaea* bat (cave nectar bat), also from Yinpterochiroptera, and the *M. davidii* (David's myotis) species from the Yangochiroptera suborder. Due to the ability of caspase-1 to signal via multiple upstream sensors, we reconstituted the NLRP3 inflammasome axis in HEK293T cells and expressed either human or relevant species of bat caspase-1 in a dose-dependent manner. We observed that *E. spelaea* caspase-1 (EsCASP1) retained the ability to cleave *P. alecto* IL-1β at reduced levels, and *M. davidii* caspase-1 (MdCASP1) demonstrated intact activity comparable to that of human at low-dose expression (Fig. 4*A*).

Next, we reconstituted the NLRP3 inflammasome axis and varied IL-1β of three different bat species in either a HsCASP1, EsCASP1, or MdCASP1 system (Fig. 4*B* and *SI Appendix*, Fig. S5*A*). Interestingly, we observed that *M. davidii* IL-1β possessed the least capacity for cleavage and *P. alecto* the highest, in contrast to their respective caspase-1 activity (highest in *M. davidii* and lowest in *P. alecto*). We hypothesized that efficient targeting of MdIL-1β for reduced cleavage may occur at or near its cleavage site. Therefore, we aligned the IL-1β amino acid sequence of *M. davidii* against eight other species of bats (*Myotis lucifugus*, *Eptesicus fuscus*, *Miniopterus natalensis*, *Desmodus rotundus*, *P. alecto*, and *P. vampyrus*, *R. aegyptiacus*, *Hipposideros armiger*) and six other model mammalian species (*Sus scrofa*, *Canis lupus*, *Pan troglodytes*, *Mus musculus*, and *Rattus norvegicus*) (*SI Appendix*, Fig. S5*B*). Among the 15 total species analyzed using branch site modeling in PAML, we discovered a Ser117 residue immediately adjacent to the cleavage site Asp^{115}-Ala^{116} which was under higher selection in the *Myotis* branch, which was a proline residue in all except three other species (*M. natalensis*, *S. scrofa*, and *C. lupus*).

We next hypothesized that residues conserved between *Homo sapiens*, *P. alecto*, and *E. spelaea* but differing in *M. davidii* may be responsible for the impaired cleavability of MdIL-1β. We thus additionally identified G110, S111, E113, and Q122 in MdIL-1β as distinct from equivalent IL-1β residues of the other three species. For a direct comparison of MdIL-1β with PaIL-1β (which is fully cleavable), we performed site-directed mutagenesis replacing each of the respective MdIL-1β residues into their *P. alecto* counterparts, along with a combined mutation containing all amino acid site substitutions (Fig. 4*C*). Notably, the MdIL-1β mutant S117P was able to strongly restore IL-1β cleavage, along with a partial restoration by a double mutant GS110/111DG; however, the combined mutant demonstrated strongest cleavage ability (Md > Pa 110 to 112) (Fig. 4*D*). Conversely, the PaIL-1β mutants DG110/111GS and P117S resulted in defective IL-1β cleavage (Fig. 4*E*). Further, both residues appear to have a partial effect either in restoration (MdIL-1β) or abrogation (PaIL-1β) of function, whereby the

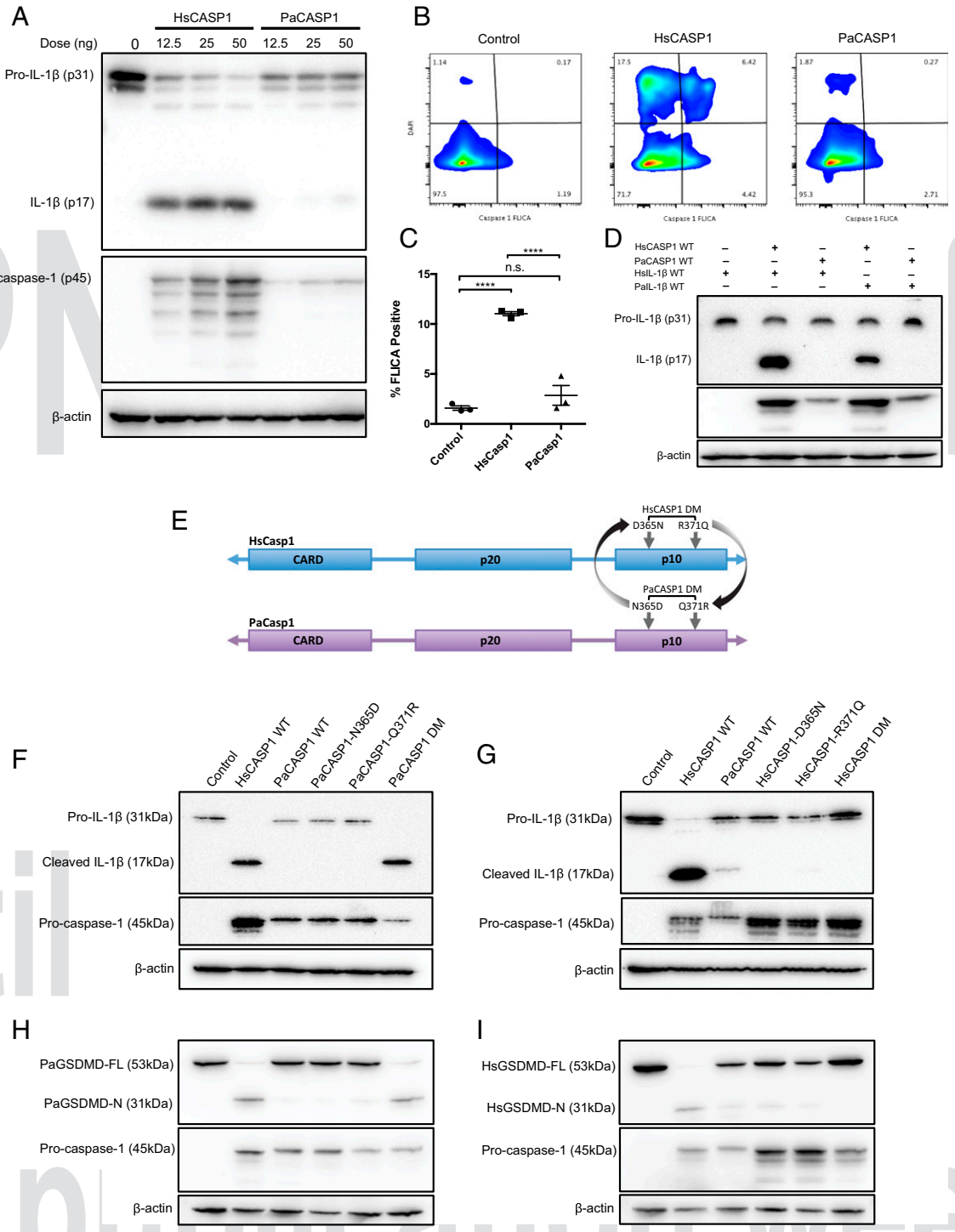

Fig. 3. Inactive PaCasp1 is rescued by substitution of N365D and Q371R in the p10 domain. (*A*) The AIM2 inflammasome axis was reconstituted in HEK293T cells using either HsCASP1 or PaCASP1 in a dose-curve. Cell lysates were stained for pro-IL-1β (p31) or mature IL-1β (p17). Procaspase-1 (p45) was assayed to compare expression, and lysates were normalized by β-actin. (*B*) Cells were reconstituted with the AIM2 inflammasome axis genes and incubated for 48 h, and stained with 660-YVAD-fmk caspase-1 FLICA substrate (Immunochemistry). Flow cytometry was performed to detect caspase-1 activation in control vector, HsCASP1 or PaCASP1 expressing cells, and data analyzed in FlowJo. (*C*) Quantification of FLICA-positive cells measured via flow cytometry. (*D*) Immunoblot of cell lysates crossing HsCASP1-3XFLAG with HsIL-1β-HA or PaIL-1β-HA, and PaCasp1-3XFLAG with HsIL-1β-HA or PaIL-1β-HA, coexpressed with human AIM2 and ASC. Cleaved human or bat IL-1β was measured with anti-HA antibody. (*E*) Schematic of site-derived reverse mutagenesis conducted in the p10 domain of PaCASP1 or HsCASP1. Targeted residues are shown, either single bat N365D or Q371R mutation, or containing both mutations (PaCASP1 DM); and D365N, R371Q, or HsCASP1 DM for the human gene. (*F*) AIM2 inflammasome axis was reconstituted in HEK283T cells, varying the PaCASP1 for WT, single-mutant N365D, Q371R, or double mutant DM. Cleaved IL-1β p17 was assayed via immunoblotting 48 h posttransfection. (*G*) Similarly, HsCASP1 WT, D365N, R371Q, or DM was reconstituted, and cleavage of IL-1β was analyzed by immunoblot. Shown are the pro-IL-1β (31 kDa), cleaved IL-1β (17 kda), and human or bat procaspase-1 (45 kDa). (*H* and *I*) Similarly, PaGSDMD was coexpressed with PaCASP1 (*H*) and HsGSDMD with cognate HsCASP1 (*I*) WT, single-mutants or double-mutant variants, in conjunction with upstream AIM2 inflammasome axis. Cleavage of GSDMD was detected by immunoblotting for the GSDMD-N-2xMyc domain after 48 h incubation in vitro as per *D*. Data are representative of three independent experiments in *A*–*G*. ****$P < 0.0001$, n.s., nonsignificant. Statistics were performed using unpaired Student's *t* test and presented as mean ± SEM of three independent replicates (*C*).

effect was strongest in the combined mutants. Thus, we observe that given a certain level of caspase-1 activity in any of the bat species (*P. alecto* low, *E. spelaea* medium, and *M. davidii* high), the cleavage potential of IL-1β occurs in opposite direction (*P. alecto* high, *E. spelaea* medium, and *M. davidii* low) (Fig. 4*F*). Taken together, this demonstrates a complementary mechanism whereby full caspase-1 activity is balanced by diminished cleavage potential of IL-1β, and vice versa, resulting in an overall, equivalent dampening of inflammasome signaling across multiple bat species from both suborders.

Discussion

We have confirmed that loss of AIM2/ALRs in bats results in inactive initiation of the inflammasome cascade in response to cytosolic DNA in vitro. Crucially, we reveal another layer of dampening through bat caspase-1, the principal cysteine protease responsible for cleaving inflammatory cytokines such as IL-1β and IL-18. We identified two inactivating alterations N365D and Q371R localized in the p10 sequence of wild-type *P. alecto* bat caspase-1 which when rescued by substitution of the human residues at equivalent sites, restored caspase-1 enzyme functionality. Simultaneously, when human caspase-1 was replaced with the equivalent bat amino acids, either residue change resulted in abrogation of IL-1β cleavage. Despite retention of caspase-1 activity in other bat species, corresponding reduction in IL-1β cleavage mitigated downstream inflammasome signaling. Thus, we have experimentally validated two additional residues of mammalian caspase-1, which are integral to its activity for substrate maturation, and demonstrated a proof of concept whereby downstream inflammasome activation in bats is dampened through a unique inverse relationship involving bat caspase-1 and IL-1β.

The discovery of dampened caspase-1 in bats has particular significance in their response to infection and immunity. As the classical inflammatory effector of the inflammasome complex, caspase-1 is converged upon by multiple upstream sensors, including NLRP3, NLRP1, AIM2, NLRC4, and others (43–47). These sensors are activated by a diverse array of cell- and pathogen-derived stimuli, including viral and bacterial nucleic acids, flagellin, ATP, and MSU crystals, and reactive oxidative species (ROS). Such signals trigger a systemic activation of alert and defense mechanisms, including pyroptosis, cytokine signaling, and the recruitment of neutrophils and macrophages into the affected tissue area (17, 48, 49). As caspase-1 cleaves cGMP-AMP (cGAMP) synthase (cGAS) to enhance host resistance to DNA viruses, and MAVS and TRIF to abolish IFN signaling (50), it is possible that bats may have evolved other compensatory mechanisms to resist viral pathogenesis by biasing cross-regulation of these pathways (7). Indeed, there is increasing evidence that AIM2 and other inflammasomes oppose type I IFN sensors, including cGAS, STING, and MyD88/IRF7 (51–54). As such, constitutive IFN expression in bats may, in part, be both an outcome and compensatory mechanism of inflammasome dampening, allowing them to mitigate viral pathogenesis. Further, as noncanonical caspase-1 substrates range from cytoskeletal components, enzymes in cell metabolism, and diverse other proteins involved in cellular stress responses and cell death pathway, it is possible that caspase-1 may retain residual baseline activity for regulation of these processes, thus conserving its expression in bats albeit at reduced function. Thus the effect of our findings on these nonimmune substrates would also warrant investigation.

It is notable that both identified caspase-1 residues acted on by high positive selection pressures possess noncharged (Asn, Gln) instead of charged (Asp, Arg) side chains, representing a substantial decrease in capacity for ionic bond formation. Both amino acids are situated within the p10-p10 interface of the caspase-1 $(p20p10)_2$ homodimer and are shown to participate in critical electrostatic interactions across the interface of the active conformation of caspase-1 (*SI Appendix*, Fig. S4). Loss of these charged residues likely results in weakened caspase-1 dimerization, preventing robust autoprocessing and substrate cleavage. To our knowledge, no prior study has identified either D365 and R371 in α6 as essential for caspase-1 activity in humans or other mammalian species, with early structural studies reporting the interface to only consist of residues 318 to 322 and 386 to 396 (35). Two noncompetitive inhibitors discovered decades earlier, gold thiomalate and auranofin, closely mimic this interface-disruptive mechanism but differ in residue specificity (35). While most widely used caspase-1 inhibitors to date utilize active site-specific mechanisms to impede function (18, 32, 35, 55–57), none of the marketable inhibitors yet allosterically perturb caspase-1 at the p10 dimer–dimer interface despite efforts (58, 59). Thus, our findings may prove to facilitate additional specificity in inhibitor design. Importantly, our findings provide evidence of evolutionary drivers of inhibitory mechanisms in nature informing insight into human caspase-1 and suggest another potential alternative strategy for caspase-1 targeting in human therapeutics.

Positive selection pressure occurred in both caspase-1 residues of the *Pteropus* bat genus but not in any other bat species. We observed retention of caspase-1 activity by *E. spelaea* and *M. davidii* bats from both bat suborders Yinpterochiroptera and Yangochiroptera, which may be explained by a lack of D365 and R371 substitutions in caspase-1 of both bats. Intriguingly, further downstream investigation for IL-1β maturation elucidated an inverse relationship between bat caspase-1 activity and IL-1β cleavage potential, whereby the cleavage of IL-1β within a bat species occurs in the opposite direction to its caspase-1 activity (e.g., in *M. davidii*, caspase-1 high and IL-1β low). The mechanism by which MdIL-1β cleavage is diminished was shown to occur through the S117 site immediately adjacent to the ^{115}Asp-Ala^{116} cleavage site, which when restored by mutation to S117P permitted cleavage of the MdIL-1β mutant into its 17-kDa fragment. This was followed to a smaller extent by the double mutant GS110-111DG of MdIL-1β, suggesting that all three residues might play a role in dampening maturation of the cytokine in *Myotis* bats. We demonstrate that by targeting either caspase-1 or IL-1β, bat species of both suborders possess dampening of important downstream inflammasome components.

Therefore, it is clear that differing strategies have been coopted by bats to dampen either caspase-1 activation, IL-1β cleavage, or both in a complementary manner, highlighting the importance of this phenotype across bats. Indeed, previous genomic or functional studies have found that different bat species exhibited varying or independent genomic strategies to dampen the AIM2 or NLRP3 inflammasome sensors, which culminated in an equivalent level of loss or reduction of activity (9, 11). However, cleavage of IL-1β alone is not sufficient to generate biological inflammasome functioning, and recent studies have demonstrated requirement of GSDMD for IL-1β and IL-18 secretion and activation of pyroptotic cell death (60–62). Thus, in bat species with intact caspase-1 activity, other significant functions may be retained such as GSDMD-mediated pyroptosis and secretion of IL-18, or activation of IL-37 and inactivation of IL-33, which may confer higher regulatory control of pro/antiinflammatory responses (63–65). As such, multiple other indicators of inflammasome functioning in bats remain unknown and require further investigation in the context of these findings.

In conclusion, we find strong experimental evidence pointing to diminished multisensor inflammasome signaling in bats, suggesting high selection pressures acting not only on single, but multiple levels in this pathway. Given the inflammasome functions at the forefront of innate immune signaling, such alteration of inflammasome signaling in bats has a critical role in viral disease tolerance and asymptomaticity (66). Inflammasome activation has been implicated in multiple coronavirus infections, including MERS-CoV, SARS-CoV, and SARS-CoV-2 (67), possibly

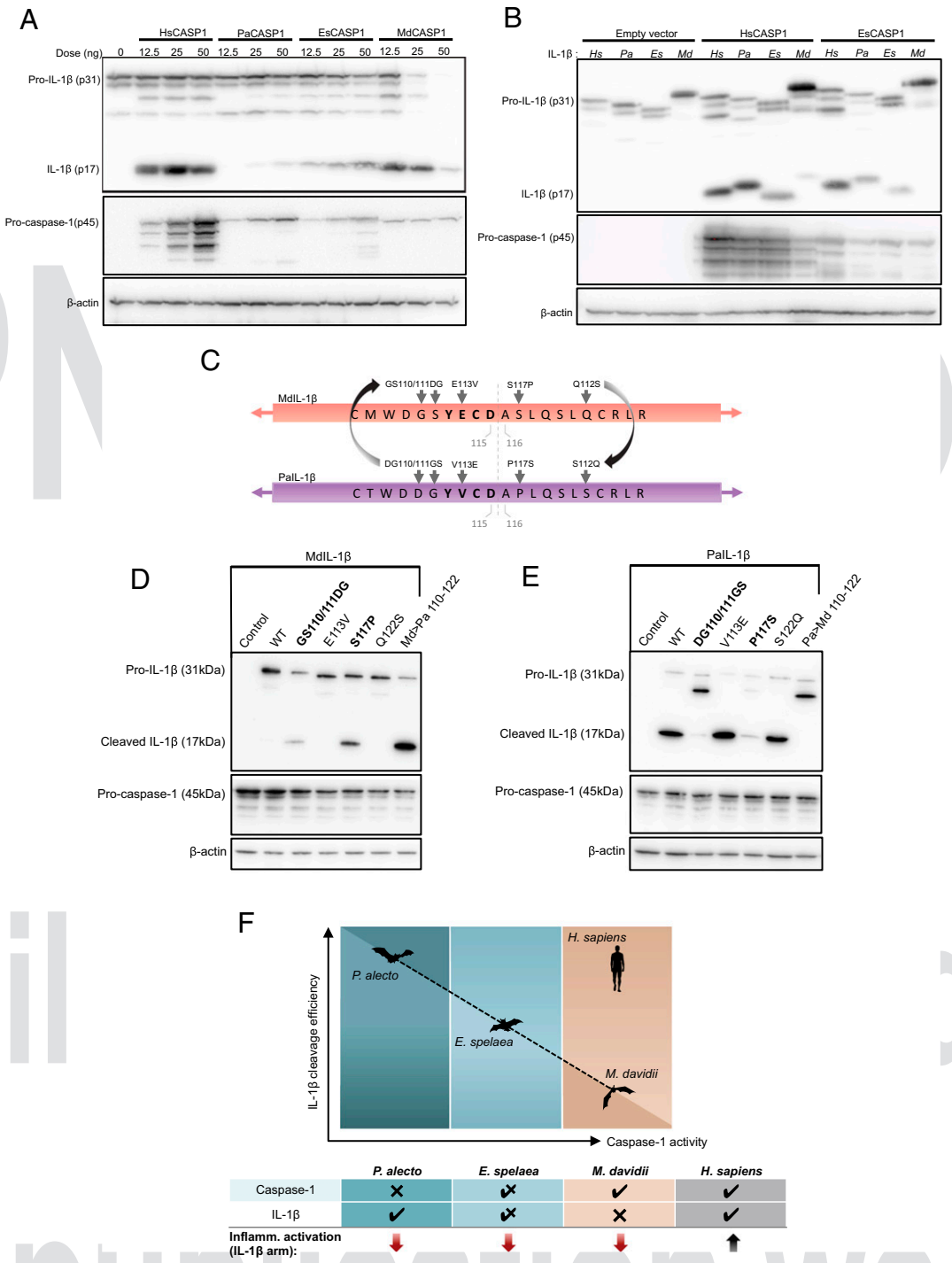

Fig. 4. Complementary relationship between *M. davidii*, *E. spelaea*, *and P. alecto* caspase-1 and IL-1β. (*A*) HEK293T cells were transfected with human NLRP3, ASC, pro-IL-1β for inflammasome axis reconstitution, and coexpressed with either *H. sapiens*, *P. alecto*, *E. spelaea*, or *M. davidii* caspase-1 in increasing concentrations (12.5 to 50 ng/well). Cells were incubated for 48 h and the cell lysate was harvested for Western blot. The amount of mature/cleaved IL-1β (p17) was compared between HsCASP1 and the three bat species and normalized by β-actin. (*B*) Coexpression of human, *P. alecto*, *E. spelaea*, or *M. davidii* IL-1β in HEK283T with reconstituted human NLRP3 and ASC. Cells were transfected with either empty vector, HsCASP1, or EsCASP1 (as indicated). Cell lysate was immunoblotted for pro-IL-1β (p31) and cleaved IL-1β (p17) with anti-HA, procaspase-1 (p45) with anti-FLAG, and normalized by β-actin. Figures are representative of three independent experiments (*A* and *B*). (*C*) Alignment of full-length IL-1β sequences was performed for *H. sapiens*, *P. alecto*, *E. spelaea*, and *M. davidii*, and site-directed mutagenesis was performed substituting identified MdIL-1β amino acid residues with equivalent sites from PaIL-1β near the Asp-Ala cleavage site (gray arrows). In bold, cleavage sites YECD and YVCD for MdIL-1β and PaIL-1β, respectively. (*D*) Full-length WT or mutant MdIL-1β was coexpressed with HsCASP1, AIM2, and ASC and incubated for 48 h; and lysates were assayed for successful cleavage of 17 kDa IL-1β via staining of C-terminal HA-tag. Md → Pa 110 to 122 denotes combined introduction of all identified *P. alecto* residues expressed by the MdIL-1β mutant protein. (*E*) Various mutant PaIL-1β was similarly expressed within the AIM2 inflammasome axis and incubated in vitro for 48 h, and various levels of cleaved PaIL-1β were detected by Western blot. (*F*) Diagram of inverse reciprocal relationship between *P. alecto*, *E. spelaea*, and *M. davidii* caspase-1 activity and IL-1β cleavage efficiency, displayed on the *x* axis and (*Left*) *y* axis, respectively. *Top* showing increasing pattern of caspase-1 activity (*P. alecto* < *E. spelaea* < *M. davidii* < *H. sapiens*) is countered by decreasing IL-1β cleavability (*M. davidii* < *E. spelaea* < *P. alecto* < *H. sapiens*). Table shows vertical summation of either caspase-1 or IL-1β cleavage and resultant function of the downstream inflammasome axis (IL-1β arm).

affecting the ability of bats to function as a reservoir host. With unique capacity for metabolically costly flight, bats could have adapted to elevated metabolic states by dispensing with this inflammatory arm (68–70). Further, inflammasome suppression improves longevity or prevents age-related decline in mice and promotes longevity in humans (71–75), which is in line with bats' long-lived mammalian phenotype. Taken together, our study contributes significant mechanistic understanding for strategies targeting inflammasome dampening in bats, offers potential insight in regulation of human inflammation, and further elucidates the ability of bats to harbor and transmit zoonotic pathogens without sustaining detrimental costs of immune activation.

Materials and Methods

Reagents. Reagents are as previously described (9). Ultrapure LPS-B5, CL264, and Hygromycin B Gold were obtained from InvivoGen. *P. alecto* ASC-specific monoclonal antibody (mouse IgG2b) was generated by GenScript's monoclonal antibody service. Rabbit polyclonal anti-ASC (AL177) (human/mouse) was purchased from Adipogen. Goat polyclonal anti-dog IL-1β (ab193852) (cross-reactive to *P. alecto*) and rabbit polyclonal anti-mouse IL-1β (ab9722) were from Abcam. mAb to β-actin (A2228) was from Sigma-Aldrich and mAb to GFP and variants (including mCitrine) were from Roche (11814460001). Anti-mouse/rabbit/goat horseradish peroxidase (HRP)-conjugated secondary antibodies were from Santa Cruz.

Plasmids. Generation of expression constructs for NLRP3, ASC-mPlum, IL-1β-HA, and empty vectors (control vectors) are as previously described (34, 76). Human AIM2 was cloned from human peripheral blood mononuclear cells (PBMC) cDNA using Q5 Polymerase (NEB) with *Age*I and *Not*I flanking primers (NUS-IRB reference code H-18–029). AIM2 was digested and ligated into pQCXIH (Clontech) vector containing C-terminal mCitrine or 3× FLAG. Procaspase-1 was cloned from the human pCI-caspase-1 construct (Addgene plasmid 41552) or Omniscript (Qiagen)-generated cDNA of *P. alecto* spleen. Caspase-1 was inserted into pQCXIH-mCitrine and pQCXIH-3× FLAG vectors. *P. alecto* or human caspase-1 mutants were generated by overlap extension PCR with primers containing the respective mutations. Similarly, *P. alecto*, *E. spelaea*, and *M. davidii* IL-1β were cloned by PCR of *bat* spleen cDNA into pQCXIH (Clontech) backbones containing C-terminal HA-tag. *P. alecto* and *M. davidii* IL-1β mutants were also generated by overlap extension PCR. Gasdermin D was cloned from human PBMC cDNA and *P. alecto* spleen cDNA with 2× MYC-tag on the N terminus into pQCXIH (Clontech) backbones containing C- terminal HA-tag. Primer sequences are listed in *SI Appendix*, Tables S1 and S2. All constructs were prepared with endotoxin-free plasmid maxi-prep kits (Omega Bio-tek).

Cells. All procedures utilizing animal samples in this study were performed in compliance with all relevant ethical regulations. Capturing and processing of bats (*P. alecto*) in Australia was approved by the Queensland Animal Science Precinct and University of Queensland Animal Ethics Committee (AEC#SVS/073/16/USGMS) and the Australian Animal Health Laboratory Animal Ethics Committee (AEC#1389 and AEC#1557). Where possible, wild bats with irreparable physical damage (torn wings) already scheduled to be killed were utilized. Processing of bats has been described previously (9, 69). Wild-type C57BL/6 mice were obtained with permission from the Singhealth institutional animal care and use committee. Harvesting and differentiation of bone marrow from *P. alecto* bats has been described previously and performed according to identical protocols (76, 77). Mouse bone marrow was harvested from C57BL/6 mice and frozen once in liquid nitrogen, thawed, and differentiated over 7 d in 10 ng/mLmacrophage colony-stimulating factor (M-CSF) as described previously (34). GP2-293 retroviral packaging cells were obtained from Clontech. GP2-293, HEK293T, and PaKiS cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco 11965092) medium supplemented with 10% fetal bovine serum (FBS). PaKiS (CVCL_YM14) is a spontaneous-immortalized kidney epithelial cell line with identical origin from the parental primary cells of PakiT03 (RRID: CVCL_DR89) (34). GP2–293 cell culture medium was supplemented with sodium pyruvate and nonessential amino acid (NEAA) cell culture supplement (Life Technologies) during retroviral packaging.

Reconstitution of AIM2 in Bat Macrophages. Retrovirus was generated by cotransfecting pVSV-G envelope protein with the plasmid containing the gene of interest (AIM2-mCitrine or mCitrine-only) at 1:1 ratio in GP293 cells grown at 70% confluency. Cells were incubated for 48 to 72 h in DMEM containing 10% FBS at 37 °C, and supernatant centrifuged and filtered through 0.45-μm PVDF sterile filters (Millipore). To further concentrate the retrovirus, either 100,000 MW Vivaspin columns (Sartorius) in a benchtop centrifuge or ultracentrifugation at 125,000 × *g* for 90 min (Optima X, Beckman Coulter) in a SW41-TI rotor was performed to 75 to 100× dilution. Retrovirus was titrated on HEK293T cells and added at multiplicity of infection 5 into PaBMDM media at day 5 of differentiation. Cells were incubated for 48 h, supernatant was removed, and cells were recovered in additional 24 h of PaCSF-1 RPMI with 10% FBS before treatment.

In Vitro dsDNA Stimulation. *P. alecto* immortalized kidney cells have been described previously (9). Cells stably expressing *P. alecto* ASC were transduced with mCitrine-only or AIM2-mCitrine retrovirus generated from GP2-293 cells. Cells were selected with puromycin for 5 d and recovered in 10% FBS DMEM media. AIM2-mCitrine/ASC double-positive cells were sorted by fluorescence activated cell sorting (FACS) using BD FACSAria for medium-to-low expression. Cells were grown to 70% confluency and transfected with increasing doses of PolydA:dT (Invivogen). For BMDM stimulation, differentiated cells were primed with 1 μg/mL CL264 (PaBMDM) or LPS B5 (MmBMDM) (Invivogen) for 3 h, washed with FBS-free RPMI (Gibco), and transfected with 1 mg/mL PolydA:dT using Lipofectamine 3000 (Thermo Fisher) in RPMI for 4 h. Supernatant was collected for LDH assay, IL-1β ELISA, and cells were stained for flow cytometry by Imagestream of FACS.

Imagestream Imaging Flow Cytometry. Cells were harvested for Imagestream imaging flow cytometry as previously described (9). Briefly, BMDM cells were harvested with 5 mM ethylenediaminetetraacetic acid (EDTA), washed once with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde, and permeabilized with 0.3% Triton-X + 2% FBS for 10 min at 4 °C. Cells were stained with primary ASC and prelabeled fluorescent anti-mouse antibodies for 1 h at room temperature, with DAPI for 15 min, and then washed twice with PBS and resuspended in FACS buffer. PaKiS were harvested by trypsinization and resuspended in FACS buffer directly. Events on the Imagestream X were acquired using using INSPIRE software on an Amnis ImageStreamX Mk II imaging flow cytometer using 40× magnification. At least 5,000 events were acquired per sample and analyzed with the inbuilt IDEAS software. Cells in focus were gated by brightfield r.m.s. values, single cells by aspect ratio by area values, and intact nuclei using DAPI staining. Double positive cells (AIM2-mCitrine, ASC-mPlum) were gated and analyzed for ASC speck formation plotted via mean fluorescence intensity by max-pixel intensity.

Confocal Microscopy. PaKiS cells were seeded into 24-well plates containing coverslips (#1.5 thickness). Cells stably expressing *P. alecto* ASC-mPlum were transiently transfected with human AIM2-mCitrine for 4 h, washed, and incubated for 48 h. Cells were stained for 30 min at 37 °C incubation with working concentration of Mitotracker according to manufacturer's instructions (Thermo Fisher). Mitotracker probe solution was removed and cells were washed with PBS 2× before fixing with 4% paraformaldehyde. Nuclear staining was performed with DAPI. Coverslips were mounted onto glass slides with Mowiol 4.88 and images acquired on a Leica TCS SP8 machine at 100× resolution. Images were processed using ImageJ 2.0.0 software.

IL-1β ELISA and LDH Release Assay. Supernatant collected from DNA-treated PaBMDMs was centrifuged to remove debris and frozen once at −80 °C. The supernatant was then measured by a sandwich ELISA protocol as previously described (9). Briefly, purified recombinant PaIL-1β protein was utilized for the standard curve, with goat anti-canine IL-1β primary antibody and rabbit anti-mouse IL-1β antibody used as capture and detection antibodies, respectively. IL-1β in mBMDM supernatants was detected using the BioLegend IL-1β Standard ELISA kit. LDH release assay was performed as previously described using a Cytotoxicity Detection Kit PLUS (LDH) from Roche (9). Calculations were performed as per manufacter's instructions, with low and high controls included for normalization of individual biological replicates.

Evolutionary Analysis of Mammalian Caspase-1 and IL-1β. Caspase-1 coding sequences (CDSs) were retrieved from National Center for Biotechnology Information (NCBI) for one armadillo (*Dasypus novemcinctus*) and many Boreoeutheria species, including Euarchontoglires and Laurasiatheria. Euarchontoglires species include two primates (human and *Pan troglodytes*), two rodents (rat and mouse), and one tree shrew (Chinese tree shrew, *Tupaia belangeri chinensis*) (*SI Appendix*, Table S3). Homologs of caspase-1 in the 15 bat genomes were identified by discontiguous MegaBLAST (BLAST + 2.7.1) with max e-value of 1e-5 and word size of 11. Similarly, IL-1β sequences for *M. davidii* and eight other species of bats (*M. lucifugus*, *E. fuscus*, *M. natalensis*,

D. rotundus, *P. alecto*, and *P. vampyrus*, *R. aegyptiacus*, and *H. armiger*) and six other model mammalian species (*S. scrofa*, *C. lupus*, *P. troglodytes*, *M. musculus*, *R. norvegicus*) were retrieved from NCBI or PCR-cloning and gene sequencing performed on bat cDNA (*SI Appendix*, Table S4). Alignment of the CDSs was generated by MAFFT (78) and used to plot the phylogeny tree by the maximum-likelihood method with the general-time-reversible (GTR) model and 1,000 bootstrap replicates in PHYML 3.0 software (38). The phylogeny tree and alignment file then served as input for performance of positive selection analysis on CodeML from the PAML package (version 4.9) (79), and branch-site models with relevant branches were marked on the tree. LRTs were performed in different substitution models, including 1) M0 (one-ratio), M1a (nearly neutral), M2a (positive selection), M3 (discrete), M7 (beta), M8 (beta and $\omega > 1$), and M8a (beta and $\omega = 1$) in site mod; 2) M0 (one-ratio) and two-ratio model assuming different ω for background and foreground branches in branch mode; and 3) positive selection along specified branches (model A) against a null model (model A null) that allows neutral evolution and negative selection for branch-site mode. Positive selection sites were scored by the Bayes empirical Bayes (BEB) method (80).

Caspase-1 Western Blot and FLICA Assay. The AIM2 inflammasome axis (HsAIM2, HsASC, human caspase-1, and IL-1β) was reconstituted into HEK293T cells at increasing doses using Fugene 6 (Promega) at 3:1 ratio with total DNA. Cells seeded into 96-well plates (Corning) were incubated for 48 h posttransfection and lysed in lysis buffer (79). cOmplete ULTRA protease inhibitor mixture and PhosSTOP phosphatase inhibitors (Roche) were added to lysis buffer before use. Proteins were separated on 12 to 15% SDS/PAGE gels and transferred onto 0.45-μm polyvinylidene difluoride (PVDF) membrane with a Trans-Blot Turbo transfer system (Biorad). Membranes were blocked in 5% bovine serum albumin (BSA) for 1 h and stained with primary antibody followed by HRP-conjugated secondary antibody. Membranes were developed with Amersham ECL Prime Western blotting detection reagent (GE Healthcare) on a myECL Imager (Thermo Scientific). For FLICA detection, cells were trypsinized, washed once in PBS, and stained with 660-Caspase-1 FLICA substrate (Immunochemistry) with occasional agitation for 1 h. Cells were washed three times with cellular wash buffer (provided), resuspended in PBS with 2% BSA, and analyzed via flow cytometry (LSRFortessa Cell Analyzer, BD Biosciences). Live/dead gating was performed using DAPI and subgated for AIM2-mCitrine and ASC-mPlum positivity, before gated for FLICA-660 positive staining (*SI Appendix*, Fig. S6). At least 10,000 cell events were collected per replicate and independently analyzed on FlowJo.

Data Availability. All study data are included in the article text and *SI Appendix*.

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To:	daszak@ecohealthalliance.org; christian.drosten@charite.de; christopher.broder@usuhs.edu; wil2001@columbia.edu
Subject:	re: bats, viruses - request for story comment
Date:	Thursday, October 22, 2020 5:34:12 PM
Attachments:	pnas.202003352.pdf

Hello, my name is Charles Choi, and I'm a science reporter in New York writing for science news service Inside Science, which is syndicated by MSNBC, Fox News and Discovery News, among others.

I'm interested in interviewing you about Linfa Wang et al.'s research on bats and viruses appearing in the next issue of the Proceedings of the National Academy of Sciences. I've spoken with several of you before regarding bats and viruses — good to contact you again.

My deadline is 2 p.m. ET on Sunday. Could you contact me via email to set up an appointment as soon as possible or call me at 1-917-328-7810?

I understand when the embargo on your research is -- my deadline allows my editor time to actually work on the story, as opposed to doing a rush job that can introduce inaccuracies into the piece. The story naturally will not get published until after the embargo lifts.

It would really help me out if you could answer a few questions:

x) What do you personally find most surprising or exciting about these results? What do you feel is the most important implication of these findings?

x) What specific potential clinical applications might this research hold? Perhaps by helping bodies not react in potentially harmful way to infections and other disorders?

x) Obviously COVID-19 is of concern right now. Might you talk about how we might think about these findings in relation to the current pandemic?

x) Are there any specific questions or criticisms you have about these findings?

x) What specific directions do you think research might or should go from here? What obstacles do you foresee in future research or development?

x) This shouldn't have to be asked, but can you for a lay audience talk about how the discovery that bats are reservoirs for so many diseases shouldn't mean that people should go out of their way to cull bats?

x) Are there any questions you would have liked to answer that I didn't ask you? Is there anything we didn't cover that you feel is important?

x) Since editorial style says I not refer to people by their institutional title (e.g. assistant professor of chemical engineering), what specialty might I refer to you by (e.g. virologist)?

x) Is there any research of yours that journalists have not reported on yet that might be interesting for a story?

Hope to hear from you soon! Thanks --

Charles Choi

Stories I've written:

Danger in the Forest:
http://www.sciam.com/article.cfm?id=drug-traffickers-endanger-preservation
At Trading Crossroads, Permafrost Yields Siberian Secrets:
http://www.nytimes.com/2004/01/06/science/06MUMM.html
The Worst Nuclear Plant Accident in History: Live from Chernobyl
http://blogs.scientificamerican.com/guest-blog/2011/03/15/the-worst-nuclear-plant-accident-in-history-live-from-chernobyl/

HDTRA1-14-24-FRCWMD-BAA,
CBEP-Thrust Area 6 – Cooperative Counter WMD Research with Global Partners
Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa

TECHNICAL PROPOSAL

I. ABSTRACT

Bat species, which are widespread in the Southern African region and beyond, have been identified as hosts of a number of viral agents, including filo- and viruses related to the Middle East respiratory syndrome (MERS) corona- and paramyxo- such as the henipaviruses. These viruses have proven to cause substantial human morbidity and mortality, threaten livestock and wildlife health and have pandemic potential. However, their distribution and potential to infect people and livestock in Southern Africa are unknown. This project will focus on building capacity and enhancing surveillance, including early detection programs for henipa-, filo- and zoonotic coronaviruses in Southern Africa. The project will specifically address the following: 1) regional training, including biosafety, bat capturing techniques, clinical sampling, viral detection and serological techniques, developing standardized protocols, ecological data collection and human behavioral studies; 2) establishment of serological and nucleic acid detection technologies in South Africa to develop sustainable regional diagnostic capacity that can be shared; 3) targeted serological and molecular surveillance in bats as well as serological surveillance in potential spillover hosts (livestock and humans); 4) assessing human behavioral risk for exposure to these agents; and 5) multi-stakeholder workshops to develop a sustainable regional biosurveillance strategy, analyze results and develop mitigation strategies and threat reduction policies for the region. Planning, training, execution and reporting will use an interdisciplinary One Health approach involving local government agencies of the respective countries including the Departments of Health, Agriculture, Environment, Veterinary and Wildlife Services to ensure long-term sustainability and stakeholder preparedness. The proposed project closely aligns with the aims of the Cooperative Biological Engagement Program with regards to support for biosurveillance, capacity building and threat reduction.

II. SCOPE

A. OBJECTIVE:

The overall objective is to detect filo-, henipa- and zoonotic coronaviruses in bat populations in Southern Africa and evidence of spillover in humans and livestock.
Specific objectives to achieve this are:
1) Regional training and establishment of networks in Southern Africa [Southern Africa Bat Research Network (SABRENET)] focused on biosafety, bat capturing techniques, bat taxonomy, virological sampling, viral detection techniques, developing standardized protocols, ecological and environmental data collection and observational and human behaviour studies.
2) Establishment of serological and nucleic acid detection platforms in key partner laboratories in human and animal health in South Africa that will sustainably build and enhance regional diagnostic capacity and will generate comparable data across the region.
3) Targeted serological and molecular surveillance and screening of samples in bats, as well as additional serological surveillance in potential spillover hosts (livestock and humans) at selected bat-human-animal interphases.
4) Assessing human behavioral risk for exposure to these agents at selected bat-human-animal interphases.
5) Multi-stakeholder workshops to develop a sustainable regional biosurveillance strategy, analyze results and develop mitigation strategies and threat reduction policies for the region.
We propose to test the following **hypothesis:** A diversity of potentially zoonotic henipa-, filo- and coronaviruses circulate in bats in South Africa and neighboring Mozambique and Zimbabwe, and may previously have infected livestock and people in areas where interactions between bats and such

hosts occur. Enhancing biosurveillance and building regional capacity will reduce biothreats by improving the ability to detect, diagnose and report emerging and remerging pathogens of biosecurity concern.

B. BACKGROUND:

Over the past four decades, approximately 75% of important emerging and re-emerging diseases were of zoonotic origin and most of these originating from Africa (Chan et al., 2010) and from a wildlife origin (Daszak et al., 2000; Olival et al., 2017). This includes paramyxoviruses in the genus *Henipavirus* such as Hendra (HeV) and Nipah virus (NiV), filoviruses that include Ebola (EBOV) and Marburg virus (MARV), as well as coronaviruses like severe acute respiratory syndrome coronavirus (SARS-CoV), all select agents, causing outbreaks with high mortality in humans. Fatal zoonoses have significant financial implications, with the World Bank estimating economic losses between 1997 to 2009 of US$80 billion, and that US$6.7 billion per year could be saved globally by preventing emerging disease outbreaks (Mazet et al., 2015). International agencies such as CDC, USDA, WHO and OIE have identified zoonotic diseases as a threat to global health security with pathogens not adhering to international borders and the ability to spread extensively and rapidly.

Filoviruses: The family *Filoviridae* are negative-sense, single-stranded RNA viruses represented by six genera of which two, *Ebolavirus* and *Marburgvirus* (Kuhn et al., 2019), cause fatal hemorrhagic fever outbreaks in humans. The diversity of filoviruses have also expanded in recent years with several novel viral detections in bats including Lloviu virus (LLOV) in Schreibers' long-fingered bats (*Miniopterus cf schreibersii*) in Spain and Hungary (Kemenesi et al., 2018), Mengla virus from China in rousette fruit bats (*Rousettus* spp.) and cave nectar bats (*Eonycteris* spp.) (Yang et al., 2019), as well as Bombali virus from Sierra Leone and Kenya associated with molossid bats [Angolan free-tailed bat (*Mops condylurus*) and the little free-tailed bat (*Chaerephon pumilus*)] (Goldstein et al., 2018; Forbes *et al.*, 2019). MARV was first identified in 1967 in green monkeys imported into Europe from Uganda after laboratory workers became infected (Olival and Hayman, 2014) and has since been detected and isolated in several African countries. Substantial evidence now exists that the Egyptian rousette fruit bat (*Rousettus aegyptiacus*) is a reservoir of MARV (Towner et al., 2009; Amman et al., 2012), and this species is widespread throughout Africa and into the Middle East. Historically, detections have been from central and eastern Africa, but recently MARV was also detected in *R. aegyptiacus* in the northern regions of South Africa (Paweska et al., 2018) and Sierra Leone. Detections have been opportunistic and sporadic, and not always corresponding to where human outbreaks have been identified or representative of the geographic range of the reservoir, which highlights a lack of surveillance. Longitudinal studies identified distinct viral pulses in juvenile bats that corresponded to the timing of human outbreaks, implicating birthing pulses as a driver of infection (Amman et al., 2012). Experimental infection studies of MARV virus in captive-bred Egyptian fruit bats reported viral RNA in oral and vaginal secretions, as well as excreta implicating these as potential routes of transmission (Swanepoel et al., 1996; Leroy et al., 2005; Amman et al., 2015). The first *Ebolavirus* spp. was discovered in 1976 and the genus now consists of six species: *Zaire ebolavirus*, Ebola virus (EBOV), *Bundibugyo ebolavirus*, Bundibugyo virus (BDBV), *Sudan ebolavirus*, Sudan virus (SUDV), *Tai Forest ebolavirus*, Taï Forest virus (TAFV), *Bombali ebolavirus*, Bombali virus (BOMV) and *Reston ebolavirus*, Reston virus (RESTV) (Kuhn et al., 2019) with potentially unique ecological niches. More than 25 human outbreaks have occurred with the most significant being the 2013-2016 outbreak in Guinea, Sierra Leone and Liberia where more than 11000 people died (Spengler et al., 2016). Index cases were reported to have had contact with dead wildlife including non-human primates and antelope, however, these species are not considered viral reservoirs since they also succumbed to the disease. Viral RNA for EBOV has only been detected in bat species with distributions restricted to West Africa and not occurring in Southern Africa, including the hammer-headed bat *(Hypsignathus monstrosus)*, Franquet's epauletted fruit bat (*Epomops franqueti)* and little collared fruit bat (*Myonycteris torquata)* (Leroy et al., 2005). However, a recent detection of EBOV has been reported in a Greater long-fingered bat (*Miniopterus*

cf inflatus) in Liberia (Epstein personal communication) expanding the potential geographic range for EBOV. Ebolavirus reactive antibodies are present in an even wider range of bat species, including *Epomophorus, Rousettus* and *Mops* spp. that do occur in Southern Africa (Olival and Hayman, 2014). RESTV had been detected in *Miniopterus cf schreibersii* in the Philippines (Jayme et al., 2015) again implicating the *Miniopterus* genus. Pigs are currently the only species of livestock known to be at risk of infection by an ebolavirus species as demonstrated by studies in the Philippines and China indicating that they are naturally infected with RESTV (Marsh et al., 2011). The reservoir and geographical distribution of EBOV and other Ebola virus species are still not clear with limited surveillance performed in the Southern African region in wildlife species due to the historical absence of human outbreaks.

***Paramyxoviruses*:** This is negative-sense single-stranded RNA viruses capable of infecting a diverse host range including mammals, birds, reptiles and fish (Virtue et al., 2009). HeV and NiV were first detected in the 1990s following outbreaks of fatal respiratory and encephalitic illness in Australia and Malaysia, respectively (Murray et al., 1995; Chua et al., 1999). During the initial emergence, both viruses spilled over into the human population from pteropid bats by means of intermediate hosts, i.e. horses and pigs respectively. Multiple NiV outbreaks have subsequently been reported from Bangladesh and India linked to repeated spillover events from flying foxes of the *Pteropus* genus directly to humans followed by a high rate of human-to-human transmission and high mortality (Gurley et al., 2007a; Chadha et al., 2006; Arunkumar et al., 2018). These viruses have been shown to be excreted in bat urine, saliva and feces (Chua et al., 2002; Smith et al., 2011). In Bangladesh, contamination of date palm sap by bats while feeding overnight, is the primary route of spillover, though other mechanisms of spillover may exist elsewhere (Gurley et al., 2007b). In Malaysia, pigs were infected by pteropid bats after eating dropped fruit contaminated with saliva, and subsequently infected farmers and abattoir workers (Chua et al., 1999; Paton et al., 1999). Initially, the distribution of henipaviruses was believed to be restricted to the geographical distribution of *Pteropus* spp. in Australia and South-East Asia, however, detections of henipavirus-related virus antibodies and nucleic acids in in African fruit bat species has expanded the geographical range (Hayman et al., 2008; Drexler et al., 2009). Serological evidence of these viruses in bat populations in several African counties including Zambia, Tanzania, Malawi, Madagascar, and Cameroon, as well as several islands in the Gulf of Guinea were reported (Peel et al., 2012; Peel et al., 2013; Brook et al., 2019). In addition, serological evidence in pigs (Hayman et al., 2011) and antibodies in Cameroonian locals were also identified (Pernet et al., 2014), highlighting the potential of spillover. A number of studies also reported the detection of henipa- and rubulavirus-related viral RNA in several bat species on the African continent (Drexler et al., 2012; Baker et al., 2012; Mortlock et al. 2015) including *Rousettus* and the Straw-coloured fruit bat (*Eidolon helvum)* (Peel et al., 2013). Sosuga virus (*Pararubulavirus* genus) has been the only paramyxovirus associated with human disease on the African continent, following the identification of the virus as the causative agent of a non-fatal febrile disease in a single human infection (Albarino et al., 2014), believed to have spilled over from *R. aegyptiacus* in Uganda (Amman et al., 2015). Various research studies, mostly targeted towards HeV and NiV, have reported strong seasonality associated with dry winters, correlations with bat densities, nutritional stress, and various events during the bat reproductive period (Plowright et al., 2008; Dietrich et al., 2015; Paez et al., 2017; Mortlock et al., 2019) as drivers of infection. The diversity and viral dynamics of henipa- and other potentially zoonotic paramyxoviruses in African bat species are poorly understood with only limited sporadic surveillance.

Coronaviruses: These are positive-sense RNA viruses divided into four genera, namely, *Alphacoronavirus, Betacoronavirus, Gammacoronavirus,* and *Deltacoronavirus*. A number of subgenera were recently assigned to each of these genera (Wong et al., 2019) due to an expansion of global diversity. These viruses can undergo recombination, creating new variants that may result in spillover infections and manifest as respiratory pathogens in humans that are transmitted through aerosols. Bat coronaviruses are predominantly excreted in faecal material, though oral swabs, and

urine have also tested positive implicating several routes of transmission. Severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) coronaviruses emerged in 2002 and 2012 respectively, and both pandemics resulted in significant morbidity and mortality (10% and 35%, respectively) in humans, which was exacerbated by global travel (Berry et al., 2015). Global coronavirus surveillance increased significantly after the detection of SARS. In the subsequent search for the reservoir, serological and partial nucleic acid detection in 2005 linked SARS-CoV to horseshoe bats (*Rhinolophus*) in China by identifying a closely related virus. Subsequent studies identified an even larger diversity of SARS-CoV-related viruses in horseshoe bats worldwide (Hu et al., 2017; Lau et al., 2010), however, the virus protein responsible for receptor-binding that mediates cellular entry (spike protein) (Li et al., 2005) was unable to interact with the SARS-CoV virus receptor. Thus, it is unlikely that any of these identified SARS-CoV related viruses were the source of the SARS-CoV human outbreak (Li et al., 2005). Recently a bat SARS-related CoV was found to possess a spike protein that facilitated binding to the same virus receptor as SARS-CoV (Ge et al. 2013; Yang et al., 2016), indicating the potential to cross species barriers and infect humans directly. In 2012, Middle East Respiratory syndrome virus (MERS) was identified in the Arabian Peninsula (Zaki et al., 2012), and a partial nucleic acid sequence was detected in an Egyptian tomb bat (*Taphozous perforates*) from Saudi Arabia (Memish et al., 2013). It has subsequently been shown that detected bat MERS-related CoVs are only distantly related to MERS-CoV and dromedary camels are considered the reservoir species transmitting the virus to people. Diverse coronavirus sequences were found in many different bat species worldwide, leading to the hypothesis that bats host the genetic diversity of the *Alphacoronavirus* and *Betacoronavirus* mammalian infecting genera (Woo et al., 2012), however, the risks and factors important to spillover is unknown (Hu et al., 2017; Anthony et al., 2017a). The diverse SARS-related coronaviruses circulating within the horseshoe bat (*Rhinolophus*) genus throughout their Asian, European or African distribution make this an important host genus to monitor for viral diversity and novel emergences.

Surveillance data for the Southern African region has been limited, with mostly opportunistic, once off sampling events focused on restricted geographical regions and selected bat species (Geldenhuys et al., 2013; Ithete et al., 2013; Cronje 2017; Bourgarel et al., 2018; Paweska et al., 2018; Mortlock et al., 2019). Southern Africa possesses a rich diversity of bat fauna including both frugivorous and insectivorous bat species (Monadjem et al., 2010) distributed across several biotic zones, but mostly concentrated in the eastern region of the continent, including areas in South Africa, Mozambique, Zimbabwe, Malawi and Zambia (Cooper-Bohannon et al., 2016). In addition to the taxonomic diversity, these bat species also exemplify a diversity of roosting behavior and site selection including trees, rock crevices and man-made structures. This include *Rousettus, Rhinolophus* and *Miniopterus* spp. as well as several free-tail bat species (Molossidae) implicated as potential hosts for high consequence pathogens in other studies. If pathogens are present, several factors play a role in spillover to other species and very few studies investigated this. This include host ecology, movement of bats (both foraging and long term movement), viral shedding and routes of transmission. These factors may be seasonal, emphasizing the need for longitudinal studies. Livestock such as cattle, goats, and pigs, are also present throughout Southern Africa and overlaps with bat diversity hotspots (Robinson et al., 2014; StatsSA2016). People with frequent close contact with bats due to religious, cultural or sociological reasons (Markotter, personal communication) also inhabit the same areas. This bat-human-animal interphase and risk for spillover has not been studied in the region. Habitat loss, primarily through deforestation has been increasing in the Southern African region (Brink and Eva, 2009), and there is evidence of increased bushmeat consumption (Regan et al., 2015; Lindsey et al., 2013). Understanding contact between bats, humans and other animals, including behavior analysis, are needed to understand risk in areas where these viral pathogens are present.

Several factors need to be considered when designing biosurveillance strategies in bats. Individuals do not generally exhibit signs of disease, populations can be large with low viral

prevalence and seasonality affect prevalence and transmission. Detection of viral nucleic acids can therefore be challenging and should be paired with **serological surveys**, which take advantage of long-term antibody persistence. Serology has some limitations including inherent antibody cross reactivity between target and related viruses, statistical interpretation of seropositive cut-offs, limited bat specific assays and control sera, and not being able to directly characterize the infectious agent involved. As a result, greater value can be achieved when combining serological surveys with molecular detection assays and subsequent viral characterization. When investigating potential spillover in non-bat hosts, such as domestic animals and humans, serology can be a more comprehensive strategy given the commercially available reagents that can be used to detect both past/convalescent (immunoglobulin G) and recent/acute (immunoglobulin M) infections. Serological assays for emerging zoonotic pathogens are limited and mostly based on in-house assays using different methodologies including virus neutralization, ELISA, western blots and indirect fluorescent assays (IFAs) and can only detect evidence of exposure to a single virus target. Multiplex serological assays using Lumenix xMAP-based technology offer the advantage to simultaneously detect virus specific and cross-reactive antibodies in a single test, saving time, resources and cost. Since the volume of bat sera collected in surveillance is limited, this is a major advantage. The Luminex system is based on the principles of flow cytometry, allows for multiplexing at least 100 analytes in a single microplate well and uses very small sample volumes (2 µl). This assay uses inactivated serum and recombinant reagents, which means the analysis can be performed in a Biosafety Level 2 laboratory. This technology has been successfully used in surveillance studies in bats and spillover hosts including detection of antibodies against paramyxo and filoviruses (Brook et al., 2019; Laing et al. 2018; Hayman et al., 2011; Peel et al., 2012; Chowdhury et al., 2014;) (See preliminary results for more detail). Broadly reactive **molecular RT-PCR assays** based on the conserved regions of viral genomes at family or genus level have been successfully used in surveillance studies for the viral families targeted in this proposal (See work plan for detail). These assays target short genomic regions that can be sufficient for determining phylogenetic relationships, but not formal genus or viral species demarcation that requires longer gene and/or genome sequences. For this, additional sequencing data is needed including targeting regions that may be important in spillover potential such as the receptor-binding and virulence factor encoding regions.

C. RELEVANCE; SCIENTIFIC AND THREAT REDUCTION IMPACT:

South Africa plays a prominent role in the Southern African region with laboratory capacity that can be expanded and shared to strengthen sustainable biosurveillance in the region. These pathogens can easily affect any country and therefore biosurveillance capacity must include regional networks and engagement with neighboring countries. Surveillance data will identify presence but also shedding of high consequence viruses in different seasons, bat species and biomes. Serological investigations will identify if spillover in livestock and humans occurred in the past and behavioral analysis will investigate risk factors for potential spillovers that may happen in the future. This project will strengthen South Africa's, and the region's, capacity to develop and implement surveillance strategies with appropriate biosafety and biosecurity principles, detect pathogens (both molecular and serological) in bats before outbreaks occur, recognize and diagnose outbreaks if they do occur and develop appropriate reporting strategies for the region. All this information can be used collectively to assess risk, predict potential outbreaks, and develop risk mitigation strategies that can be used throughout the region. It follows a One Health approach involving multi-sectoral stakeholders including government engagement and local communities to enhance communication and threat reduction activities. A regional network, Southern African Bat Research Network (SABRENET), will be established to improve communication and collaboration between countries and between disciplines. Sustainability and threat reduction will be specifically addressed by workshops involving all stakeholders from multiple Southern African countries and to align with the Global Health Security Agenda, and comply with the International Health Regulations (IHR) and other reporting guidelines. This project is closely aligned with the goals of the Cooperative Biological Engagement

Program (CBEP) and align with several of the Biological Threat Reduction Programme (BTRP) Lines of effort (LOE) including National Regulatory Frameworks (LOE3), Disease detection (LOE4), Laboratory diagnosis (LOE5), Epidemiological Analysis and investigation (LOE6) and Reporting and Communication (LOE7).

D. PRELIMINARY WORK AND ESTABLISHED COLLABORATIONS (See table 1 for explanation of abbreviations for institutions):

The two major partners, UP, South Africa (Markotter) and EHA, USA (Epstein) will combine their expertise in implementing surveillance programs and diagnostic sample testing. The major partners will also establish regional networks, and link with international networks including the Bat One Health Network (BOHRN), EcoHealthNet (an NSF-funded disease ecology research training program led by EHA), and the Western Asia Bat Research Network (WABNet). UP and EHA initiated discussions about possible collaborations five years ago and both UP and EHA currently collaborate with CEZPD-NICD. UP also collaborated with AfricanBats NPC (Seamark) and DNMNH (Kearney) since 2004. This includes training activities, joint fieldwork, co-supervision of students, as well as several joint publications. UP already has DAFF section 20 approval to research zoonotic pathogens in bats in Southern Africa and regularly communicate surveillance data to DAFF and NDoH. UP has been collaborating with USU (Broder) since 2017, and has initiated acquisition of Luminex reagents for filo- and henipavirus testing including a signed Material transfer agreement. EHA also have a successful collaboration with USU. EHA and CEZPD-NICD have a joint DTRA project on Rift Valley fever virus since 2014. UP has previously collaborated with the CVL laboratories in Mozambique and Zimbabwe, with joint publications on rabies as an output. Preliminary surveillance activities in small mammals were also conducted in Mozambique in 2017, and a preliminary visit to the CVL laboratory in Zimbabwe was conducted in 2018 to discuss future collaborations. Previous studies led by the South African team, including UP, CEZPD-NICD, DNMNH and AfricanBats have reported detections of several potential zoonotic pathogens in bats (Figure 1). This work was performed under the South African Research Chair of Prof. Markotter funded by the South African Department of Science and Innovation (ongoing) and the Global Disease Detection Programme of the Centers for Disease Control and Prevention (USA) 2016-2019. Biosurveillance on select agents in bats was only a small part of these projects, and therefore surveillance studies were sporadic and limited in scope to only specific bat species and limited

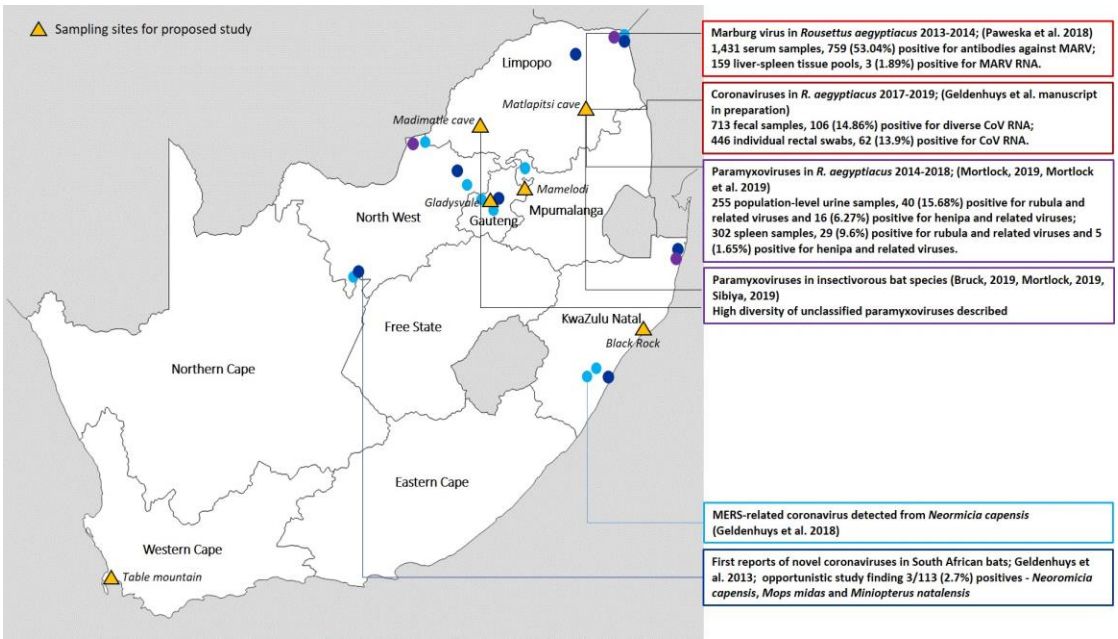

Fig 1: Summary of filo, corona and paramyxovirus nucleic acid detections in South Africa. Previous and proposed samples sites for this project are indicated (Unpublished).

geographical locations in South Africa. In addition, this surveillance only included molecular detection of pathogens and very limited serological investigations in bat species and none in potential spillover hosts. Preliminary studies indicate that seasonal reproduction of *R. aegyptiacus* drives viral infection dynamics, and that gradual loss of passive immunity among juveniles in this bat population increases the number of susceptible individuals, creating favorable conditions for viral spread (Figure 2). It also results in changes in virus shedding and seroconversion patterns, which are driven by sex, age and reproductive condition of female bats (Figure 2 and 3). Molecular assays to detect paramyxo-, corona- and filoviruses are established in the South African laboratories (UP and NICD) with some improvements needed (see detail under Work plan). Serology was not part of previous studies, with the exception of targeted Marburg virus serological studies. At the time, we had no feasible serological option available that could accommodate a multiplex platform suitable for low amounts of sera collected from bats and we initiated discussions to obtain luminex reagents from USU. Purified oligomeric, receptor-binding protein (G) of henipaviruses (e.g. HeV and NiV) have been used by the USU lab and collaborators for several years in antigen-based immunoassays to differentiate antibodies specific to HeV or NiV (Bossart et al., 2007), and in biosurveillance studies to examine exposure to these high priority viruses (Chowdhury et al., 2014; Peel et al., 2012) (Figure 4). With the isolation of new viruses including Cedar virus (CedV), Ghana virus (GhV) and Mojiang virus (MojV), the lab has continued to generate tetrameric henipavirus G for all presently described henipaviruses, and expand the HeV/NiV immunoassay into a pan- henipavirus multiplex assay capable of simultaneously

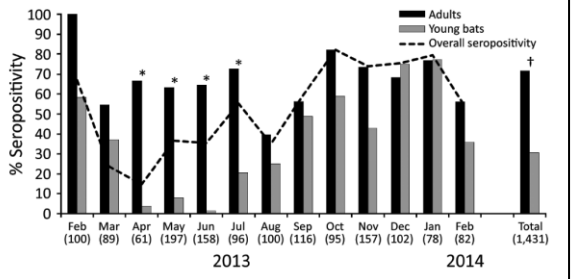

Fig 2: Seropositivity per month for Marburg virus as reported in Paweska et al., 2018

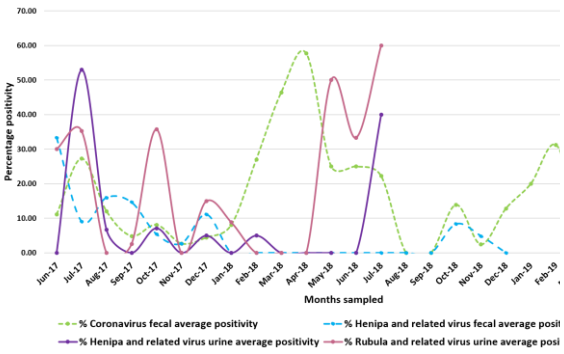

Fig 3: Detected viral RNA excretion dynamics in urine and fecal from the *R. aegyptiacus* bat at Matlapitsi cave

detecting and differentiating henipavirus-specific antibodies. Furthermore, USU constructed native-like glycoprotein antigens of all presently described filoviruses to generate a pan-filovirus antigen-based immunoassay that can be simultaneously used with the pan-henipavirus immunoassay to detect antibodies reactive with four of the WHO priority pathogens with pandemic potential. This pan-filovirus immunoassay was used by Laing et al. to detect serologic evidence of Asiatic filoviruses, antigenically-like ebolaviruses, in three fruit bat species collected in Singapore (Laing et al., 2018) (Figure 5).

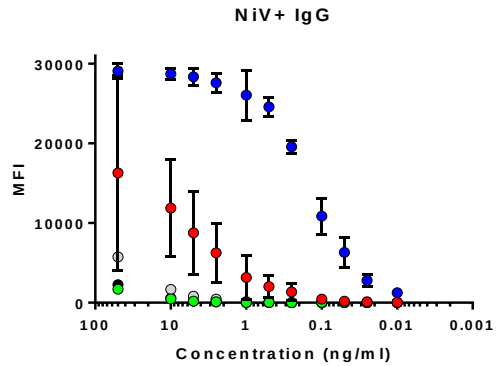

Figure 4: Pan-henipavirus immunoassay can specifically differentiate between antibodies reactive with heterologous viruses. Rabbits were immunized with virus specific G antigens. Antisera were screened with multiplex pan-henipavirus immunoassay in two independent experiments in technical triplicates; bars indicate 95% confidence intervals.

This assay was also used at the National Centre for Biological Sciences, Bangalore, India in a DTRA BTRP supported project to Duke-NUS researcher Dr. Ian Mendenhall, to detect exposure to Asiatic filoviruses in bats and humans hunting these bats for food, implicating likely cross-species transmission of novel filoviruses at this wildlife-human interface (Figure 6) (Dovih et al., 2019). This pan-filovirus/henipavirus immunoassay has been transferred to three laboratories in Peninsular Malaysia where it is being utilized in serology-based biosurveillance of wildlife (e.g. bats), livestock and indigenous human communities, funded by DTRA BTRP to Co-PI J. Epstein and contributed further evidence that a diversity of novel Asiatic

filoviruses, distantly related to Reston virus, but with unknown pathogenesis, circulate in certain species of bats.

In collaboration with DNMNH, a taxonomic database for bat species, including voucher specimens with morphological classification and genetic sequence confirmation, has been generated for most South African bat species, and for some species in Zimbabwe and Mozambique. UP, USU and EHA have developed several standard operation procedures and training material for past studies including

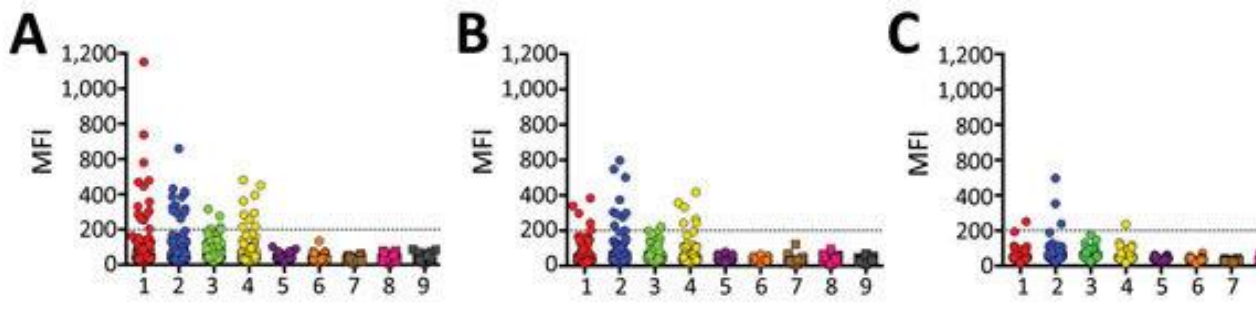

Figure 5. Serologic evidence of exposure to Asiatic filoviruses in 3 fruit bat species. (A) *Eonycteris spelaea* (B) *Cynopterus brachyotis* (C) *Penthetor lucasi*. Mean fluorescence intensity (MFI) values obtained from Bio-Plex assay (Bio-Rad, Hercules, CA, USA) screening of individual serum samples from bats of 3 species with soluble filovirus glycoproteins. Dashed line indicates the cutoff value at 200 MFI. 1, *Zaire ebolavirus*; 2, *Bundibugyo ebolavirus*; 3, *Taï Forest ebolavirus*; 4, *Sudan ebolavirus*; 5, *Reston ebolavirus*–monkey; 6, *Reston ebolavirus*–pig; 7, Marburg virus–Musoke; 8, Marburg virus–Angola; 9, Ravn virus.

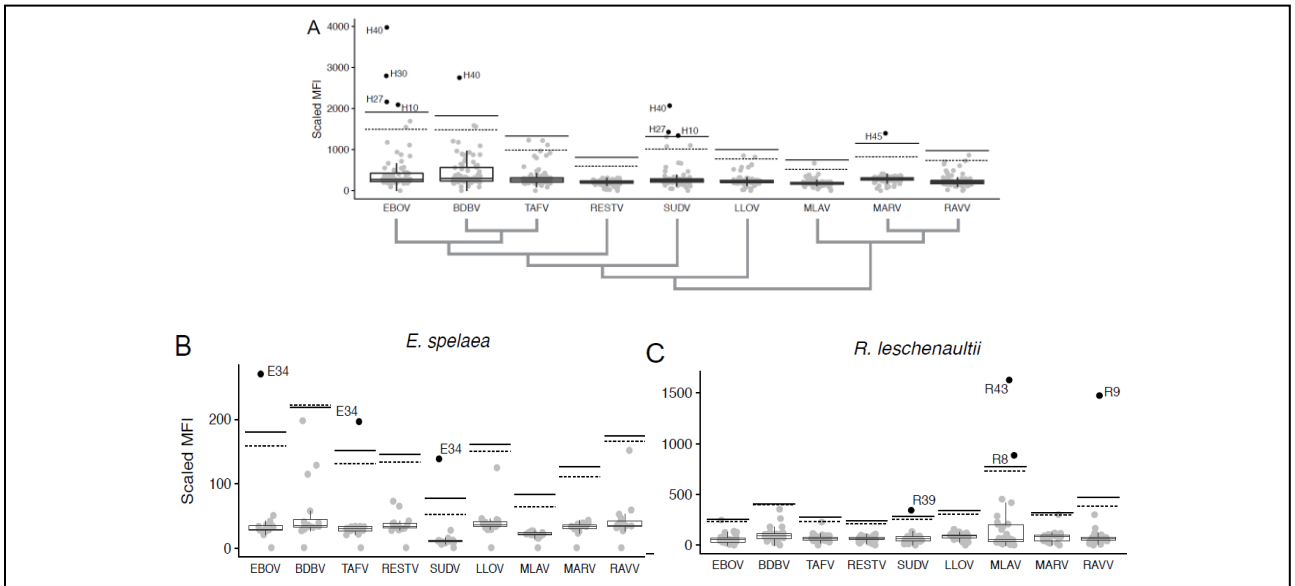

Figure 6. Serologic evidence of exposure to Asiatic filoviruses in humans and fruit bats, Nagaland, India. (A) Humans sampled; phylogenetic tree under X-axis is used to orient antigen-antigen relatedness (B-C) Fruit bat species indicated. (MFI) values obtained from MAGPIX assay (Bio-Rad, Hercules, CA, USA) screening of individual serum samples

biosafety, collection of bat samples, RT-PCR testing, DNA sequencing, bioinformatics analysis, luminex serological testing and behavioral risk assessment tools. This will be revised in Y1 and improved where necessary.

E. INSTITUTIONS AND ROLES (Summarized in table 1):

University of Pretoria (UP; prime (PI)), Pretoria RSA, Will finalize and implement the study design, coordinate overall project, coordinate and present training and workshops, develop and finalize SOPs, supervise Hons, MSc, PhD and post-doctoral students, obtain relevant regulatory and

ethical approvals in coordination with relevant government departments; coordinate and perform all field work, coordinate sample testing and testing of all animal samples, coordinate behavioural studies in coordination with relevant government agencies and partners; build diagnostic capacity in the UP laboratory (focused on animal sample testing) that can be shared including hosting and training of partners from neighbouring countries; data analyses, report and publish data; disease modelling and assessing risk mitigation strategies; and development of threat reduction policies. UP will be responsible for all contractual obligations with DTRA including reporting. **Staff**: Wanda Markotter (PI, Overall coordination – involved in all activities listed above), Jessica Coertse (Postdoctoral fellow, Bat filovirus surveillance), Marike Geldenhuys (Postdoctoral fellow, Bat coronavirus surveillance), Marinda Mortlock (Postdoctoral fellow, Bat paramyxovirus surveillance), Liz Basson (Grant administrator), Research assistants x 4 (fieldwork, processing and testing of samples (molecular and serology), database management) (To be hired), students to be identified (PhD, MSc and Honours),

EcoHealth Alliance (EHA; sub (Co-PI), NY USA, Will co-develop and implement the study design, coordinate and present training workshops, develop and finalize SOPs, co-supervise students, develop and implement human behavioural studies in coordination with relevant government agencies; fieldwork training and sampling (limited), data analyses, report and publish data; disease modelling and assess risk mitigation strategies; and development of threat reduction policies, develop awareness material. **Staff:** Jonathan Epstein (Co-PI – Involved in all activities listed above), Noam Ross (Disease modelling), Kendra Phelps (Fieldwork training and finalizing SOPs), Kevin Olival (Risk mapping, Developing SOPs & participating in training workshops, linkage with other bat networks e.g. WABNet), Emily Hagan (Develop and direct behavioural risk tools and analysis, assist in developing and implementing risk reduction strategies, community engagement and outreach material development), 1 postdoc and 1 PhD student who will work on disease modelling in bats and spatial analysis of movement data, respectively (To be identified).

Uniformed Services University (USU, sub CO-I), Maryland USA, Will develop and transfer Luminex reagents and SOPs to UP and NICD; train laboratory staff; co-supervise MSc and PhD students involved in serological projects; analysis and interpretation of filo-, henipa-, and coronavirus serological data; standardization of multiplex immunoassay and data interpretation. Assess risk mitigation strategies and development of threat reduction policies. **Staff:** Eric Laing (Co-investigator – Involved in all activities listed above), Christopher Broder (Head of the research group, scientific expertise and guidance), Laboratory technicians x 3 (Preparation of Luminex reagents at USU and assistance with training and data analysis).

National Institute for Communicable Diseases (NICD) Centre for Emerging Zoonotic and Parasitic Diseases (CEZPD) (CO-I), RSA, Develop SOPs specifically for laboratory diagnostics, fieldwork and reporting, co-supervise Hons, MSc, PhD and post-doctoral students, obtain relevant regulatory and ethical approvals pertaining to human studies in coordination with relevant government departments; coordinate and perform field work, involved in human behavioural and serological studies in coordination with relevant government agencies; build diagnostic capacity in the NICD laboratory that can be shared including hosting and training of partners, provide serological positive and negative serum panels, verify positive serology results obtained from the luminex, analyse results, report and publish data. Assess risk mitigation strategies; and development of threat reduction policies **Staff:** Jacqueline Weyer (Co-investigator – Involved in all of the above), Janusz Paweska (Head of the research group, scientific expertise and guidance), Naazneen Moolla (Medical scientist – Assistance and verification of luminex serological results), Postdoctoral fellow (Human and livestock serology) to be identified.

Table 1: Prime and partner institutions and summarized roles

	Study design and coordination	Contractual obligation with DTRA and in-country lead	Regulatory and ethical requirements	Coordinate and/or attend training	Development of SOPs	Collection of bat samples	Collection of host, environmental and ecological data	Collection of livestock samples	Collection of human samples	Molecular testing of samples	Transfer of Luminex technology and testing of samples	Human behavioral studies	Disease modelling	Co-analyze and reporting of data. Risk mitigation strategies and development of threat reduction policies
University of Pretoria (UP), Centre for Viral Zoonoses, South Africa (PI: Prof. Markotter)	■	■	■	■	■	■	■	■	■	■	■	■	■	■
EcoHealth Alliance (EHA), USA (Co-PI: Dr Epstein)	■			■	■	■	■					■	■	■
Uniformed Services University (USU), USA (CO-I: Dr Laing)				■	■						■			■
National Institute for Communicable Diseases (NICD), Centre for Emerging Zoonotic and Parasitic Diseases (CEZPD), South Africa (Co-I: Dr. Weyer)			■	■	■	■	■	■	■	■	■	■	■	■
Department of Agriculture, Land Reform and Rural development (DALRRD previously DAFF), South Africa together with KwaZulu-Natal, Limpopo and Western Cape Department of Agriculture and Rural Development				■	■	■		■						■
National Department of Health (NDoH), South Africa				■	■				■			■		■
National Department of Environment, Forestry and Fisheries (DEFF), South Africa				■	■									■
Central Veterinary laboratories (CVL), Agrarian Research Institute of Mozambique, Ministry of Agriculture and Food Security. Department of Veterinary Services and Wildlife services, National Ministries of Health, Mozambique and Zimbabwe				■	■	■	■					■		■
AfricanBats NPC, South Africa, Mr. Seamark				■	■	■	■							■
Ditsong National Museum of Natural History (DNMNH), South Africa: Dr Kearney				■	■	■	■							■

Department of Agriculture, Land Reform and Rural Development (DALRRD previously DAFF) together with KwaZulu, Limpopo and Western Cape Departments of Agriculture and Rural development, RSA, Identify representatives and attend training, meetings and workshops from the animal health sector, assistance with obtaining local regulatory documents and import permits, take part in and assist with field studies for collection of bat and livestock samples in South Africa, co-analyze data, report and publish, assess risk mitigation strategies and development of threat reduction policies. **Staff:** Grietjie de Klerk, DVM (Director - Involve in all of the above), Alicia Cloete (Epidemiologist, Involve in all of the above), State veterinarians for sampling areas; Limpopo, Western Cape and KwaZulu Natal RSA (assist and facilitate collection of bat and livestock samples and reporting).

National Department of Health (nDoH), RSA, Identify representatives and attend training, meetings and workshops from the human health sector, assist with collection of human samples and behavioral questionnaires in South Africa, co-analyze data, report and publish. Assess risk mitigation strategies and development of threat reduction policies. **Staff:** Wayne Ramkrishna (Epidemiologist - Involve in all of the above)

National Department of Environment, Forestry and Fishery (DEFF) RSA, Identify representatives and attend training, meetings and workshops from the environmental health sector, co-analyze data, report and publish, assess risk mitigation strategies and development of threat reduction policies. **Staff:** Abednego Baker - Involve in all of the above.

Central Veterinary laboratories (CVL), Agrarian Research Institute of Mozambique, Ministry of Agriculture and Food Security, Mozambique, Identify relevant trainees in animal health, attend training and workshops, coordinate field studies in Mozambique, collect and test samples collected in Mozambique in South Africa, assistance with obtaining local regulatory documents, co-analyze data, report and publish, assess risk mitigation strategies and development of threat reduction policies. **Staff:** Sara Achá (Veterinarian and researcher – Head of laboratory), Lourenço Mapaco (Veterinarian and researcher – Involved in training, fieldwork and testing of samples), Iolanda Vieira Anahory Monjane (Veterinarian, researcher and country coordinator for Mozambique study, involved in training, fieldwork and testing of samples).

Central Veterinary laboratories (CVL), Agrarian Research Institute of Zimbabwe, Ministry of Agriculture and Food Security Department of Veterinary Services and Wildlife services, Zimbabwe, Identify relevant trainees in animal health, attend training and workshops, coordinate field studies in Zimbabwe, collect and test samples collected in Zimbabwe in South Africa, assistance with obtaining local regulatory documents, co-analyze data, report and publish, assess risk mitigation strategies and development of threat reduction policies. **Staff:** Pious Makaya (Veterinarian – Laboratory Head), Babra Bhebhe (Veterinarian and coordinator for Zimbabwe study, involved in training, fieldwork and testing of samples)

National Ministries of Health, Mozambique, Identify relevant trainees in human health, attend training and workshops, coordinate human behavior studies in Mozambique, assistance with obtaining local regulatory documents, co-analyze data, report and publish, assess risk mitigation strategies and develop threat reduction policies. **Staff:** Inocêncio Salvador Chongo (focal point of One Health group at the Ministry of Health - Involve in all of the above)

National Ministries of Health, Zimbabwe, Identify relevant trainees in human health, attend training and workshops, coordinate human behavior studies in Zimbabwe, assistance with obtaining local regulatory documents, co-analyze data, report and publish, assess risk mitigation strategies and develop of threat reduction policies. **Staff:** To be identified

AfricanBats NPC, RSA, Coordinate and present training and workshops focused on bat biology and taxonomy, develop SOPs focused on these topics, coordinate all field studies focused on *Miniopterus* spp. and assistance with other fieldwork, assistance with RFID tracking and environmental monitoring at bat roosts, echolocation data analyses co-analyze data, report and publish data. Assess risk mitigation strategies and development of threat reduction policies. **Staff:** Ernest Seamark (Chief Executive Officer) and Dr. Mark Keith (Board of Directors) (Involvement of all of the above).

Ditsong National Museum of Natural History (DNMNH), RSA, Coordinate and present training and workshops focused on bat biology and taxonomy, developing SOPs focused on these topics, Involved in fieldwork, taxonomic identification of bats collected and administration of museum vouchers, comparison of morphological and genetic identification of bat species, co-analyze data, report and publish data. Co-supervision of students, assess risk mitigation strategies and development of threat reduction policies. **Staff:** Teresa Kearney (Research scientist/taxonomist – Involvement in all of the above).

F. CREDENTIALS (See detailed bio sketches of key staff for additional information):

University of Pretoria (UP) is a leading research-intensive university and is recognized nationally and internationally for the quality of its research and the extent of its research outputs. UP is also ideally situated in the executive capital of South Africa, Pretoria, in close proximity to the International airport (O.R Tambo) ensuring ease of travel to enhance collaborations and student exchange. One Health and zoonotic diseases is an important research theme and the Centre for Viral Zoonoses was established in 2016 to enhance multidisciplinary collaboration between the faculty of

Health Sciences, Natural and Agricultural Sciences and the only Veterinary Science faculty in South Africa. The UP has solid strength in the zoonosis research field and has in the last number of years made a series of significant financial and other contributions to support this research. Recently the Future Africa Institute was established to facilitate interdisciplinary research and this include state of the art training and meeting facilities. UP is also ideally situated close to several important collaborative networks including NICD, Onderstepoort Veterinary Institute (ARC-OVI), NDOH, DALRRD (DAFF) and DEFF. UP has a financial and grant management infrastructure and has successfully managed USA federal funding. Infrastructure include a biosafety level 3 laboratory, ultra-low temperature freezers in a biobank with back-up generator power, molecular and serological laboratories and Sanger DNA sequencing and next generation sequencing facilities on site. It also include information technology and bioinformatics infrastructure. Several established field sites exist as well as basic infrastructure and equipment to sample bats. **PI: Prof. Wanda Markotter** is the Director of the Centre for Viral Zoonoses and since January 2016 she is also occupying a South African Research Chair in "Infectious Diseases of Animals (Zoonoses)" funded by the South African government. She is a virologist who is involved in a multidisciplinary research programme on disease ecology in bat species in South Africa and other African countries leading to several high impact research outputs. Experience include molecular and serological diagnostics and fieldwork to collect samples. Her research group consist of support staff, postdoctoral fellows and several post graduate students that also have extensive experience in this field. She plays a key role in national governmental committees including the National Rabies Advisory Group and National One Health forum steering committee.

EcoHealth Alliance (EHA) is a scientific organization, working with local partners in over 30 countries at the nexus of health, biodiversity conservation and international development. EHA has a staff of 35 in New York, including scientists (e.g. social scientists, veterinarians, ecologists, analysts, IT experts, and economists), administration, and communications staff. EHA has an extensive record of publishing high quality, peer-reviewed papers, journals, briefing documents and reports, including seminal work on emerging infectious diseases and bat borne viral zoonoses with particular expertise in modelling of host-virus dynamics and human behavioural analyses. EHA's ability to produce highly utilized and understandable science-based outputs will contribute significantly to achieving project goals and provide objective methods for tracking project utilization of project findings. In 2014, EHA became the first foreign NGO to sign a Memorandum of Agreement (MOA) with the Government of Malaysia. The MOA is to study zoonotic disease in populations exposed to wildlife and includes three sectors of government: Ministry of Health, The Department of Wildlife and National Parks, and The Department of Veterinary Services. **Co-PI: Dr. Jonathan Epstein** is a veterinary epidemiologist and the Associate Vice President at EHA. He is a technical lead for surveillance and outbreak response under the USAID Emerging Pandemic Threats: PREDICT program, a $100 million effort focused on predicting and preventing pandemic diseases. Dr. Epstein is recognized internationally for his expertise on the ecology of emerging zoonotic viruses and currently directs research and surveillance programs in West Africa, South and Southeast Asia, and China. He has led investigations of NiV, EBOV, SARS CoV and MERS CoV in Asia, the Middle East, and Africa. He has also served as a consultant for WHO, FAO, OIE, and the Institute of Medicine.

Uniformed Services University (USU) is a US federal government health science university that trains military physicians through medical school, military masters of public health and military & civilian doctoral candidates in varying disciplines including emerging infectious diseases, and microbiology. USU hosts the Center for Global Health Engagement chapter, which has a mission to support the Department of Defense Global Health Engagement. USU has a world renowned health science faculty who have close ties with the proximally located Walter Reed Army Institute of Research and Naval Medical Research Centers. The Department of Microbiology and Immunology includes 12 full-time Faculty members, and has an overall focus on mechanisms of infectious diseases

and the host response/immunology. Faculty interests and active research programs at USU are diverse, with many nationally- and internationally-known investigators researching areas of viral immunology, vaccine and antiviral therapeutics, and animal model development. USU is also physically located directly across from the main NIH campus in Bethesda, Maryland. The overall broad scientific environment at both USU and the NIH is highly conducive to productive collaborations.

National Institute for Communicable Diseases (NICD), Centre for Emerging Zoonotic and Parasitic Diseases (CEZPD) The NICD is a national public health institute of South Africa, providing reference microbiology, virology, epidemiology, surveillance and public health research to support the government's response to communicable disease threats. The NICD serves as a resource of knowledge and expertise of communicable diseases to the South African Government, Southern African Development Community countries and the African continent. The institution assists in the planning of policies and programmes to support and respond to communicable diseases. CEZPD was established by the amalgamation of six former NICD sections: Special Bacterial Pathogens Reference Laboratory, Special Viral Pathogens Reference Laboratory, Arbovirus Reference Laboratory, Electron Microscopy, Parasitology Reference Laboratory and Vector Control Reference Laboratory. The CEZPD operates the only suit biosafety level 4 (BSL4) facility on the African continent, which places it both strategically and critically in a position to assist in the response of highly dangerous emerging and re-emerging zoonotic pathogens.

Department of Agriculture, Land Reform and Rural Development (DALRRD previously DAFF), RSA together with KwaZulu Natal, Limpopo and Western Cape Department of Agriculture and Rural Development and specifically the Animal Health section aim to manage risk associated with animal health by promoting, preventing and controlling animal diseases including zoonoses. This includes formulating policies and rendering epidemiological services for early warning and monitoring of animal diseases. Provincial departments and specifically the state veterinarians are involved in sampling animals.

National Department of Health (NDoH), RSA aim to improve the health status through the prevention of illnesses and the promotion of healthy lifestyles and to consistently improve the healthcare delivery system by focusing on access, equity, efficiency, quality and sustainability. The Directorate of Communicable Diseases include zoonoses and include multisector committees to report surveillance data and mobilize outbreak responses. This also include a National One Health forum including all stakeholders.

National Department of Environment, Forestry and Fisheries (DEFF), RSA aim to ensure an environment that is not harmful to people's health or wellbeing, and to have the environment protected for the benefit of present and future generations. To this end, the department provides leadership in environmental management, conservation and protection towards sustainability for the benefit of South Africans and the global community.

Central Veterinary laboratories (CVL), Mozambique, CVL is a technical department of the Directorate of Animal Science (DCA), of the Agrarian Research of Institute of Mozambique (IIAM) and is the national reference laboratory. The role is to perform animal diseases diagnostics and epidemiological studies; to identify and propose research area on animal diseases; to develop research methodologies and standardized techniques for animal diseases and zoonotic diseases; to guarantee the specialized training and follow-up of laboratory technicians and to provide technical assistance to the regional and provincial laboratories. There is a One Health Technical Working Group (TWG) established which includes different stakeholders: Ministries of Health, Agriculture and Food Security, Fisheries, Environmental and Wildlife.

Central Veterinary laboratories (CVL), Department of Veterinary Services and Wildlife services Zimbabwe, CVL is a branch under the Directorate of the Division of Veterinary Technical Services of the Department of Veterinary Services. Veterinary Services falls under the Ministry of Lands, Agriculture, Water, Climate, and Rural Resettlement. The role is to provide diagnostic support

for zoonotic diseases and diseases of economic interest in animals, epidemiological disease investigations and surveillance of diseases as well as Food safety monitoring and Port Health Services.

AfricanBats NPC, is registered within South Africa as a Not for Profit Company. The vision is to have a functioning, integrated program for education, capacity building, research and management for the conservation of bat populations in Africa. Activities include the publication of African Bat Conservation News and the African Chiroptera Report. AfricanBats NPC has been doing capacity building through workshops and training courses, attended by post-graduate students and emerging scientists undertaking research on bats. Research projects have focused on bat monitoring, bat cave conservation, bat systematics, taxonomy and life histories, bat in protected areas and anthropocene bats.

Ditsong National Museum of Natural History (DNMNH), formerly known as the Transvaal Museum was founded in 1892 and it has, since acted as custodian and documentation centre of South Africa's natural heritage. The Museum's collections and exhibits include fossils, skeletons, skins and mounted specimens of amphibians, fish, invertebrates, reptiles and mammals. It is one of the largest in South Africa and the largest collection of bat specimens in South Africa and extensive taxonomic expertise.

III. WORK TO BE PERFORMED

The project will focus on capacity building in the Southern African region (Table 2) and biosurveillance (Table 3) as summarized and 3 and explained below.

Capacity building will focus on South Africa and neighboring countries. Pathogens do not respect borders and a regional approach is needed for South Africa to effectively detect, predict and respond to infectious disease threats. The main activities will be: 1) training representatives from Southern African countries in development of effective surveillance strategies, standard operating protocols, biosafety, data analysis, disease modelling, field and laboratory investigations, effective reporting and communication following a One Health approach; 2) establish diagnostic technologies to detect filo-, henipa- and coronaviruses for the region at a central facilities in South Africa; and 3) build capacity between stakeholders including governmental representatives through joined meetings including development of mitigation and threat reduction strategies to inform policy. Although the biosurveillance part of the project will be focused on South Africa, capacity building will include training of colleagues from Mozambique and Zimbabwe. Trainees, representing animal, human, environmental health and academia, will be selected from each country in line with a One Health focus by the different stakeholders. Theoretical and practical training will take place in South Africa through workshops, at field sites and diagnostic laboratories. In addition to developing emerging researchers and postdoctoral fellows, projects will be developed for at least 4 PhD, 4 MSc and 4 honors students, including students from neighboring countries, with co-supervision between partners. Fellows and students will spend time with different team members to learn laboratory and analytical techniques. By including colleagues from neighboring countries, we will develop a workforce that can then initiate long-term surveillance programs in the respective countries. These projects will be supported in the long-term by the regional diagnostic capacity developed in South Africa in two institutions and access to these facilities for testing to the region. Both these institutions invest in staff and infrastructure independent of this project and funding. The South African Research Chair of Prof. Markotter is also a long-term commitment from the South African government [Department of Science and Innovation (DSI)] and the academic institution (UP). To also ensure sustainability there is close collaboration with all ministries and capacity building is focused on emerging researchers to ensure long-term continuity

Table 2: Workshops and training activities:

Activity	Location	Month and year	Duration in days	Chairs/ Trainers	Attendees	Proposed activities
Project Stakeholder meeting	UP, Pretoria, RSA UP, Pretoria, RSA Set up a video conference, Moz and Zim representatives will meet with South African colleagues in South Africa and USU and EHA will dial in from the USA New York, USA UP, Pretoria, RSA Set up a video conference, Moz and Zim representatives will meet with South African colleagues in South Africa and USU and EHA will dial in from the USA UP, Pretoria, RSA	June Y1 June Y2 June Y3 June Y4 June Y5	3 1 3 1 3	Markotter and Epstein	**~ 30 attendees** **At least one representative of each stakeholders mentioned below** South Africa: *UP, NICD, DALRRD, nDOH, DEFF, AfricanBats, DNMNH* Mozambique *CVL Moz, Ministry of Health), Academia, Ministry of Enviromental and Wildlife* Zimbabwe *CVL Zim, Ministry of Health* USA *EHA, USU, DTRA representatives*	**Y 1** Introduction to the project, roles and responsibilities, timelines Planning of surveillance strategies, standardized SOPs , standardize reporting policy Reporting of results and engagements with government (including between countries) Planning and design of human behavioral studies, communication strategy and awareness Biosafety training including packaging and shipping of samples Establishment of SABRENET **Y 2, 3 and 4** Share and discuss results and progress, reporting, publications Agree on activities for the next year Identify trainees and student involvement Develop mitigation and prevention policies, threat reductions based on preliminary results Draft a plan for sustainability of biosurveillance for the region and decide on implementation timelines **Y 5** Analyze and discuss all results, finalize mitigation strategies and policies for threat reduction Finalize the plan for sustainable biosurveillance in the region. Prepare final report and publications
Modelling and data analysis workshop	UP, Pretoria, RSA	June Y1	2	Olival, Ross, (EHA)	**~ 30 attendees** **At least one representative of each stakeholders mentioned above**	Epidemiology Risk analysis GIS Hotspot mapping Niche modelling Introduction to disease modelling
Introduction to bat biology and taxonomy training	UP, Pretoria, RSA	June Y1	4	Kearney (DNMNH), Seamark (African Bats), Phelps (EHA), Markotter (UP)	**~ 15 attendees** **At least one representative of each stakeholders mentioned above**	Introduction to bat biology, physiology, ecology, taxonomy, echolocation and environmental monitoring. The focus will be on Southern African bat species. Will include practical taxonomic exercises and sampling at both insectivorous and frugivorous bat roosts.
Bat field sampling training	Field sites in South Africa, Mozambique and Zimbabwe	Y1-Y4 various months	14	Markotter (UP), Postdocs (UP), Kearney (DNMNH) Seamark (African Bats), Phelps (EHA)	**At least one representative of each stakeholders mentioned above** A strong focus on training students and staff that will be involved in fieldwork	There will be continuous field training. This will consist of each trainee (Identified by the stakeholders) joining at least 2 field visits a year (part of the biosurveillance activity).
Laboratory diagnostic training	UP and NICD laboratories, RSA	Y1-Y5 various months	21	Markotter (UP), Weyer (NICD), Moolla (NICD) Laing (USU) Postdocs (UP and NICD)	**At least one representative of each stakeholders mentioned above** Focus will be on training students and staff involved in diagnostic testing	There will be continuous laboratory diagnostic training. This will consist of trainees visiting the diagnostic laboratories (NICD and UP), receive training and perform sample processing, testing and analyses of results.

Activity	Location	Month and year	Days	Chairs/ Trainers	Attendees	Proposed activities
Data interpretation workshop *	EHA, New York UP, RSA EHA, New York UP, RSA EHA, New York	Feb Y1 Feb Y2 Feb Y3 Feb Y4 Feb Y5	5	Markotter (UP), Epstein (EHA)	Markotter (UP), Epstein (EHA) E. Laing (USU) Postdocs UP, NICD, EHA	A smaller team that will do in depth data analysis that can then be discussed and analyzed by the broader group (Stakeholder meeting)

*Depending on data collected, additional people will attend the data analysis workshops.

Biosurveillance activities will be focused in South Africa with limited fieldwork collections in Mozambique and Zimbabwe (Y4). Activities will already start at the beginning of Y1 since ethical approvals and Department of Agriculture and Rural Development (Previously DAFF) Section 20 to perform biosurveillance in Southern Africa is already approved. Minor amendments will be applied for. Due to the proposed project start date (June 2020) no retrospective sample testing will be performed but previous results generated will be part of data analysis.

Sampling strategy: Targeted longitudinal surveillance in bats, as well as sampling in potential spillover hosts (cattle, goats, donkeys, and pigs), will be implemented in South Africa and once-off biosurveillance of bats in neighboring countries. The surveillance strategy has been designed based on species previously associated with high consequence pathogens in the literature as well as host and other ecological factors that may play a role in spillover (See background for detail). Cross sectional studies will focus on *Rousettus*, *Rhinolophu*s, *Miniopterus* and molossid bat species at several sites in South Africa (Figure 1) to determine the nucleic acid and seroprevalence of filo, henipa- and potentially zoonotic coronaviruses (Table 3). Prevalence depends on many factors, including sample type, seasonality, specific species targeted, target virus and sensitivity of the assay with studies reporting prevalence ranging from 0-100% and the prevalence of nucleic acids is usually much lower that the presence of antibodies. We assumed a nucleic acid and seroprevalence of 10% and sample sizes of individual bats were selected to detect positives with a 95% confidence interval based on estimated population sizes (Markotter, personal communication) and seasonality. Sampling in bats will be non-destructive where blood, swabs (oral and rectal) and urine samples will be collected and this sample strategy has proven to be successful in previous studies (Anthony et al., 2017a). Limited voucher specimens will be collected to confirm species identification where necessary. In addition, will we implement longitudinal surveillance in both *Rousettus* and *Miniopterus* spp. (implicated as filo- and henipavirus hosts), including during birthing periods to investigate seasonality of virus shedding. *Miniopterus* spp. move between Highveld roosts in urban areas during winter months and maternity summer roosts, and therefore several roosts are included to sample representative individuals over the study period. *Rousettus* roosts occur in different biomes in South Africa, and representative roosts are included specifically Black Rock and Table Mountain. Roost type differs and therefore caves, tree roosts, crevices and man-made structures are included in the selected sites and chosen because of a potential bat-animal human interphase. Matlapitsi is in a rural savanna area with free roaming livestock, vegetable and fruit farming. People visit the cave for cultural reasons, and there are fruit trees in-between human dwellings where fruit bats feed. Movement of *R. aegyptiacus* between Matlapitsi and Black Rock has been reported (>500 km) (Jacobsen and Du Plessis, 1976); Black Rock is in a tropical rainfall area, located inside a protected conservation area (Isimangaliso Wetland park), with human settlements and livestock within the foraging distance of the bats; The Table Mountain roost is located in the Table Mountain National Park, representing the fynbos biome and a winter rainfall with tourists visiting the area; Madimatle cave, Limpopo, South Africa is a maternity roost for *Miniopterus* spp., co-roosting with *Rhinolophus* spp. It is an important cultural site frequently visited by people travelling back and forth between this cave and major metropolitan cities in the most populated province in South Africa, Gauteng. It is also surrounded by wildlife farming areas. One-off sampling in Mozambique and Zimbabwe will focus on the cave dwelling *R. aegyptiacus* (implicated as a filo- and henipavirus host) and insectivorous bats co-roosting including know roosts such as Chinoyi caves.

Table 3: Summary of sampling sites, bat species targeted, sample numbers and sample frequencies.

	***Rousettus aegyptiacus*[#]**			***Miniopterus* spp[#]**			***Mollosid*s and *Rhinolophus* spp[#]**		
Site name (Fig 1)	**Year of sampling and frequency**	**Sample numbers**	**Estimated population sizes**	**Year of sampling and frequency**	**Sample numbers**	**Estimated population sizes**	**Year of sampling frequency**	**Sample numbers**	**Estimated population sizes**
Matlapitsi Limpopo, RSA*	Y1, Y2, Y3 Monthly	120/mo 1440/year Total=4320	Varies between 6000-20000 with lower numbers during the winter months	Y1, Y2, Y3 Seasonally (4 x/year)	120/visit 480/year Total=1440	Varies between 1000-2500 individuals	Y1, Y2, Y3 Seasonally (4 x/year)	120/visit 480/year Total=1440	Populations sizes are unknown but can be between 1000-2500 individuals
Black Rock KZN, RSA*	Y2 and Y3 Seasonally (4 x/year)	120/visit 480/year Total =960	Varies between 6000-10000 with lower numbers during the winter months	Y2 and Y3 Seasonally (4 x/year)	120/visit 480/year Total=960	Varies between 1000-2500 individuals	Y2 and Y3 Seasonally (4 x/year	120/visit 480/year Total=960	
Table Mountain Western Cape, RSA	Y2 and Y3 Seasonally (4x/year)	120/visit 480/year Total=960	A small population of about 1000-3000 individuals	Y2 and Y3 Seasonally (4 x/year)	120/visit 480/year Total=960	Varies between 1000-2500 individuals	Y2 and Y3 Seasonally (4 x/year)	120/visit 480/year Total=960	
Madimatle Limpopo RSA*	*R. aegyptiacus* not present	N/A	N/A	Y1, Y2, Y 3 Monthly for Oct – Feb	120/mo for 5 months 600/year Total=1800	Varies between 1000 in the winter months and 500 000 during birthing periods	Y1, Y2, Y3 Seasonally Only 5 month, 2 seasons	120/visit 240/year Total=720	
Other Miniopterus sites (Highveld) X 2 (Mamelodi and Gladysvale)	*R. aegyptiacus* not present	N/A	N/A	Y1, Y2, Y 3 Monthly for March – Sept	120/mo for 7 months 840/year Total=2520	Varies between 1000-2500 individuals	Y1, Y2 , Y3 Seasonally Only 7 month, 2 seasons	120/visit 240/year Total=720	
Mozambique*	Y4 Twice/year	200/visit 400/year Total=400	Unknown but *R. aegyptiacus* population estimated to be 2000-5000	Y4 Twice/year	200/trip 400/year Total=400	Unknown Varies between 1000-2500 individuals	Twice/year (Y4)	200/trip 400/year	
Zimbabwe*	Y4 Twice/year	200/visit 400/year Total=400	Unknown but *R. aegyptiacus* population estimated to be 2000-5000	Y4 Twice/year	200/trip 400/yea Total=400	Unknown Varies between 1000-2500 individuals	Twice/year (Y4)	200/trip 400/year Total=400	
Total bats sampled	**7040**			**8480**			**5600**		
	21 120								

Population densities of the bat species differ significantly and is different for the same species between sites and seasons. Estimations are indicated in the table.

*Sites that will be targeted for once of human behavior studies and collection of human and livestock serum for sero-surveillance. Only human questionnaire-based studies will be performed in Mozambique and Zimbabwe.

Human behavior (KAP analysis) and collection of human serum samples (n=150/site) will be implemented at three sites in South Africa in Y3 (Table 3) where potential human and livestock contact is a possibility. Sampling more than 100 per site will allow us to detect seropositivity with a 95% confidence at a seroprevalence of 3% assuming populations of 500. Livestock samples, including cattle, goats, pigs and donkeys (n=150/species/site), will also be collected in Y3 at South African sites. Only questionnaire-based human studies will be performed in Mozambique and Zimbabwe in Y4.

Collection of samples and testing*:* Sample collection will be performed in full personal protective equipment including disposable tyveks, double latex gloves, leather gloves, respiratory protection

(PAPRs) and gumboots. Oral and rectal swabs, urine and blood will be collected in duplicate. Onne sample set will be pooled for testing. All samples will be collected in inactivation buffer, DNA/RNA shield (ZymoResearch), at the site of collection, therefore avoiding any transport or storage of potential infectious samples (since viruses, bacteria, fungi and parasites are inactivated) and requirement of high biocontainment laboratories for processing of samples. This also protects the quality of nucleic acids by neutralizing nucleases and eliminates the need for cold-chain, if necessary. DNA/RNA shield abides by Center for Disease Control's (CDC) guidelines for pathogen inactivation and has been validated by various research groups to inactivate both enveloped and non-enveloped viruses such as parvoviruses, Chikungunya Virus, and West Nile Virus, Dengue Virus, Ebolavirus, Herpes Simplex Virus-1, Herpes Simplex Virus-2, Influenza A, Rhinoviruses, and MERS-coronavirus (Nowotny and Kolodziejek, 2014). Serum will be inactivated using 60°C for 15 min. This has been shown to be effective in inactivating viral pathogens (Van Vuren and Paweska, 2010).

Ecological and environmental data: Environmental (weather, rainfall and humidity) and ecological data of the bats (age, reproductive status, sex and measurements) will be collected, as well as population size estimates. We will employ marking and tracking technologies such as tattooing, pit tags (RFID), and telemetry systems to monitor virological status in individuals recaptured over time but also to determine foraging patterns (home ranges, distances traveled and habitat selection) and estimate population sizes. There is limited information about the habitat use and foraging patterns of *R. aegypticus*, and tracking this to estimate spillover risk will be a key component of this project. Movement data has been useful in understanding host and virus ranges and where bats may interact with people and livestock (Epstein et al., 2009; de Jong et al., 2013). EHA (Epstein) and the Movement Ecology laboratory (Hebrew University of Jerusalem) have extensive experience in using telemetry in fruit bats and will collaborate with UP on this specific part of the project.

Diagnostic testing of samples: Testing for viral antibodies and nucleic acids from all bats samples collected will be performed at UP and NICD, RSA. This will include follow up DNA Sanger sequencing of all positive amplicons. Molecular detection technologies are already established with some improvements needed (Table 4) and synthetic RNA controls will be used as positive controls and to determine sensitivity of the assays. In addition, these assays are conventional nested RT-PCRs, and the assay times are extensive. Given the scope of the proposed project and the high number of samples that will be tested, we aim to adapt these assays to use randomly primed cDNA, which can be used as template for all virus specific assays. This will save cost and processing time. Assays will be optimized on a SYBR Green real-time PCR platform, which will drastically improving throughput by reducing the processing times for sample testing, limits contamination risk and save on costs. Additional characterization of gene regions using Sanger (UP) and high throughput genome sequencing (UP) will be performed on all novel sequences identified. Luminex technology will be established in partnership with USU and will be transferred to both NICD and UP. This will include coupled antigens and controls for henipa- and filoviruses (Marburg and Ebola) and proteins are available for Hendra, Nipah, Cedar, Mojiang, Ghana henipavirus, Ebola virus, Sudan, Reston, Bundibugyo, Tai forest, Cueva, Lloviu, Marburg, Ravn, Mengla and Bombali virus. Currently, there are no antigens available for coronaviruses and cross reactivity complicates the value of such an assay and will therefore be excluded. Negative *R. aegyptiacus* sera available from previous experimental infections are available (NICD) to be used as additional controls to establish and verify cut-off values. NICD is the reference laboratory for human sample testing as well as for filovirus diagnostic testing and therefore the technologies will be established at both NICD and UP.

Data analyses, reporting and communication strategy: Data analyses will include statistics, bioinformatics, epidemiology and disease modelling. This will be done annually, including all stakeholders (Table 3). It will also include retrospective results generated before this project started. Standard operating procedures will be established for reporting and will include developing risk mitigation and prevention strategies. A communication strategy will be developed at the start of the project that will include education and awareness materials, also targeting communities.

Biobank and database: A secure and up to date database that will be shared by all partners will be established. UP has developed an in-house database that can function on a web-based system and can be adapted for this project. The PACS system is also available to manage sample information. For storage of samples the current biobank infrastructure at UP will be expanded.

Table 4: Detail of nucleic acid detection methods that will be used in this study

Paramyxoviruses						
Respiro-, *Morbilli-*, and *Henipavirus*	Hemi-nested RT-PCR (RMH assay)	Tong et al., 2008	10-100 copies of RNA (Tong et al., 2008); [Two-step protocol 10 copies of RNA (Bruck, 2019)]	Partial polymerase (L) gene	611 bp (first round); 494 bp (nested round)	The sensitivity and specificity of the assays were evaluated against a range of paramyxoviruses including several human viruses such as measles (*Morbillivirus*) and mumps virus (*Orthorubulavirus*), as well as the zoonotic bat-borne Hendra and Nipah viruses (*Henipavirus* genus) amongst others. These assays have been widely applied for biosurveillance in bats which led to the detection of viruses such as Ghanaian bat henipavirus (Drexler et al., 2012), bat mumps orthorubulavirus (Drexler et al., 2012) and viruses related to other human pathogens such as human parainfluenza virus 2 (Mortlock et al., 2019). Additionally, the family wide PAR assay has been successfully used in the USAID PREDICT program for paramyxovirus biosurveillance.
Avula- and *Rubulavirus*	Hemi-nested RT-PCR (AR assay)	Tong et al., 2008	10-100 copies of RNA (Tong et al., 2008); [Two-step protocol 300 copies of RNA (Mortlock et al., 2019)]	Partial polymerase (L) gene	272 bp (first round); 224 bp (nested round)	
Assay comment: Both the selected assays (RMH and AR) are very sensitive and capable of detecting a diversity of paramyxoviruses. The family wide assay (PAR) was is much less sensitive (500-1000 copies of RNA), hence the selection of the two more specific and sensitive assays for biosurveillance. All three assays were developed and published in 2008 and included the then known diversity of bat-borne paramyxoviruses (Hendra, Nipah, Menangle and Tioman viruses). However, these assays have not yet been updated or evaluated against more recently described bat- and wildlife-borne viruses such as Sosuga, Cedar, Mojiang and Ghanaian bat henipavirus. We aim to evaluate the sensitivity of these established assays against more recently described bat-borne viruses such as Sosuga, Cedar, Ghanaian bat henipavirus and the presumed rodent-borne Mojiang virus and update the primers if necessary.						
Filoviruses						
Diversity targeted	**Assay type**	**Reference**	**Sensitivity**	**Targeted gene region**	**Size of amplicon**	**Potential to detect zoonotic viruses**
Ebola- and Marburg virus and new diversity (Bombali and Mengla)	Qualitative real-time RT-PCR (probe based)	Panning et al., 2007; Rieger et al., 2016 (RealStar® Filovirus Screen RT-PCR Kit)	Panning et al., 2007: Virus dependent (500-500 RNA copies); RealStar kit: ~ 10 RNA copies	Partial polymerase (L) gene	293 bp	Capable of detection of all Ebola- and Marburvirus strains, limited for detection of other viruses/strains (Rieger et al., 2016; Emperador et al., 2019)
	Hemi-nested RT-PCR	Yang et al. 2019, 2017; He et al. 2015	Not determined	Partial polymerase (L) gene	214-478 bp	Capable of detection of Ebola virus strains and Mengla virus (not evaluated for detection of other filoviruses)
	Qualitative real-time RT-PCR (probe based)	Kemenesi et al. 2018	Not determined	Partial polymerase (L) gene	73 bp	Capable of detection of Lloviu virus (not evaluated for detection of other filoviruses)
	RT-PCR	Goldstein et al. 2018	Not determined	Partial polymerase (L) gene	680 bp	Capable of detection of Bombali virus (not evaluated for detection of other filoviruses)
	Quantitative real-time PCR (probe based)	Forbes et al. 2019; Jääskeläinem et al. 2015	6 RNA copies for *Zaire ebolavirus*	Partial polymerase (L) gene	108 bp	Capable of detection of *Zaire ebolavirus* and Bombali virus (not evaluated for detection of other filoviruses)
Assay comment: Most diagnostic assays target the *Ebola-* and *Marburgvirus* genera and to allow for detection and differentiation, most of these assays target the more variable genes i.e. glycoprotein (GP) and the RNA-dependent RNA polymerase (L) (reviewed in Emperador et al., 2019). Genomic sequences of filovirues of different genera differ from each other by more than 55% complicating assay selection for use in family-wide targeted surveillance and a combination of several assays (as listed) will be required to allow detection of the complete diversity. Although established assays have been reported to be successful for detection of novel filoviruses (for example Forbes et al. 2019), the majority has not been fully validated or evaluated for detection of all members of the *Filoviridae* family with important information regarding specificity and sensitivity lacking. Therefore, several assays will be required for surveillance; the Panning/kit assay will be used for detection of Ebola- and Marburgvirus strains in combination with the other assays listed to cover the entire diversity (i.e. Bombali-, Lloviu- and Menglavirus).						

Coronaviruses						
Diversity targeted	**Assay type**	**Reference**	**Sensitivity**	**Targeted gene region**	**Size of amplicon**	**Potential to detect zoonotic viruses**
Coronaviruses - focused on the mammalian infecting *Alpha-* and *Betacoronavirus* (includes SARS coronavirus) genera	Hemi-nested RT-PCR	Geldenhuys et al., 2018	The nested assay is capable of detecting 50 copies of synthetic control RNA (Nkambule, 2019)	Partial region of the RNA dependent RNA polymerase (RdRp) gene	443 bp (first round); 268 bp (nested round)	Capable of detecting the range of human coronaviruses (synthetic controls); detected a MERS-related coronavirus from an insectivorous bat species (Geldenhuys et al., 2018); SARS-related coronaviruses from Horseshoe bats in Rwanda (Markotter et al., 2019)
Assay comment: Members of the *Coronaviridae* family are highly diverse, which complicates sequence detection of all species with one nucleic acid detection assay. The RNA dependent RNA polymerase (RdRp) gene region (or nsp 12) is situated within ORF 1b of the genome, and is highly conserved and a number of nucleic acid detection assays (Stephensen et al., 1999; Poon et al., 2005; Woo et al., 2005; Watanabe et al., 2010; de Souza Luna et al., 2007; Tong et al., 2009; Gouilh et al., 2011; Geldenhuys et al., 2013; Geldenhuys et al., 2018) use a region located between approximately 14,800 and 15,600 nt depending on the genome (in reference to SARS coronavirus AY714217) for coronavirus RNA detection. Since the majority of assays use the same region, it allows overlapping sequence regions to be compared when investigating partial genes. Assays reported in Watanabe et al., 2010 and Quan et al., 2010 are used in the PREDICT project (Anthony et al., 2017a; Anthony et al., 2017b; Wacharapluesadee et al., 2015; Lacroix et al., 2017), though the sensitivity of these assays have not been reported in literature. The Geldenhuys et al., 2018 assay is a hemi-nested RT-PCR assay that is a multiplexed assay focused on diversity of alpha- and betacoronaviruses and correspond to the same regions targeted in these assays. The primers of the Geldenhuys et al., 2018 assay was updated in 2017, and included the latest diversity of coronaviruses (including human and major bat coronavirus lineages).						

IV. SUMMARY OF TASKS AND PERFORMANCE SCHEDULE

Table 5: Summary of the tasks that will be performed for the duration of the project. Detailed tasks are indicated in the attached Statement of work (SOW).

TASK	**Y1**	**Y2**	**Y3**	**OY4**	**OY5**	**TASK**	**Y1**	**Y2**	**Y3**	**OY4**	**OY5**
1. Build and enhance capacity in Southern Africa to conduct biosurveillance for filo-, henipa- and coronaviruses						**3. Conduct targeted observational and human behavior studies**					
1.1.Project stakeholder workshop						3.1.Design the study and questionnaires					
1.2.Establishment and sustaining a Southern African Bat Research network (SABRENET)						3.2.Apply for necessary ethical approvals and obtain community permissions					
1.3.Modelling and data analysis workshop						3.3.Collect and analyze data					
1.4.Introduction to bat biology and taxonomy						**4. Conduct targeted serological surveys in people and livestock**					
1.5.Bat field sampling training						4.1.Scoping visits to potential study sites					
1.6.Establishment of luminex serology technology at UP and NICD						4.2.Apply for necessary permits and ethical approvals					
1.7.Laboratory diagnostic training						4.3.Collection of samples					
1.8.Data interpretation workshop						4.4.Test and analyze data					
2. Implement biosurveillance in bats in Southern Africa and testing of samples						**5. Reporting and Communication**					
2.1.Cross sectional and longitudinal surveillance in bat species in South Africa						5.1.Discuss results, finalize and compile an annual report including all stakeholders					
2.2 Nucleic acid testing of samples and DNA sequencing of positives.						5.2. Develop a communication plan and implement it					
2.3. Serological testing of serum samples collected from bats and data analysis						5.3. Present at scientific conferences and prepare manuscripts for publication					
2.4.Characterization of additional genes or genome regions of positive samples, verify serological positive results						5.4. Annual report to all governmental partners and stakeholders					
2.5. Enter all data and results into a database and analyze						5.5. Annual report to DTRA					
2.6.Once off surveillance of bats in Mozambique and Zimbabwe						5.6. Attend DTRA technical review					

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Cc:	epstein@ecohealthalliance.org
Subject:	DTRA funding application
Date:	Wednesday, October 7, 2020 9:18:54 AM
Attachments:	DTRA Markotter Epstein Technical proposal.pdf

Dear colleagues

Hope you are well. As you may recall, we submitted a proposal to DTRA in 2019 for funding and you were listed as partners (See attached). The good news is that the funding was finally awarded. We would like to schedule an orientation project meeting on 19 October from 3-5 pm (Virtual). This will focus on an introduction to the project and activities proposed. We will have a discussion on the work plan proposed, responsibilities and any deviations that we need to make, keeping the current restrictions of the COVID -19 pandemic in mind.

I will also send a separate meeting invite. The meeting will also be recorded and if you cannot join, we will also make this recording available.

Almost a year has passed since the submission of this proposal, please let me know who needs to be included/excluded in this invite since there may have been staff changes since.

This is really an exciting project and we are looking forward to starting the conversations, activities and building capacity in Southern Africa.

Kind regards

Wanda and Jon

--
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Nipah virus dynamics in bats and implications for spillover to humans

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Nipah virus (NiV) is an emerging bat-borne zoonotic virus that causes near-annual outbreaks of fatal encephalitis in South Asia—one of the most populous regions on Earth. In Bangladesh, infection occurs when people drink date-palm sap contaminated with bat excreta. Outbreaks are sporadic, and the influence of viral dynamics in bats on their temporal and spatial distribution is poorly understood. We analyzed data on host ecology, molecular epidemiology, serological dynamics, and viral genetics to characterize spatiotemporal patterns of NiV dynamics in its wildlife reservoir, *Pteropus medius* bats, in Bangladesh. We found that NiV transmission occurred throughout the country and throughout the year. Model results indicated that local transmission dynamics were modulated by density-dependent transmission, acquired immunity that is lost over time, and recrudescence. Increased transmission followed multiyear periods of declining seroprevalence due to bat-population turnover and individual loss of humoral immunity. Individual bats had smaller host ranges than other *Pteropus* species (spp.), although movement data and the discovery of a Malaysia-clade NiV strain in eastern Bangladesh suggest connectivity with bats east of Bangladesh. These data suggest that discrete multiannual local epizootics in bat populations contribute to the sporadic nature of NiV outbreaks in South Asia. At the same time, the broad spatial and temporal extent of NiV transmission, including the recent outbreak in Kerala, India, highlights the continued risk of spillover to humans wherever they may interact with pteropid bats and the importance of limiting opportunities for spillover throughout *Pteropus*'s range.

bats | henipavirus | Nipah virus | *Pteropus* | disease modeling

Outbreaks of zoonotic diseases are often sporadic, rare events that are difficult to predict, but can have devastating consequences (1). Emerging viral zoonoses of wildlife that have become pandemic include HIV/AIDS, 1918 H1N1 influenza virus, severe acute respiratory syndrome (SARS) coronavirus, and the current COVID-19 pandemic caused by SARS-CoV-2 (2–5). Bats are important hosts for many zoonotic viruses, including Ebola virus, SARS-CoV, SARS-CoV-2, and Nipah virus (NiV); the ecological drivers and transmission dynamics of these viruses in their reservoir hosts are poorly understood (6–12). A better understanding of the transmission dynamics of zoonotic pathogens in their natural reservoirs may help anticipate and prevent outbreaks (10, 13).

NiV is an emerging zoonotic paramyxovirus (genus *Henipavirus*) that has repeatedly spilled over from bats to cause outbreaks in people and livestock with high case-fatality rates across a broad geographic range. To date, human NiV infections have been identified in India, Bangladesh, Malaysia, Singapore, and the Philippines (14–18). It has caused repeated outbreaks in Bangladesh and India, with a mean case-fatality rate greater than 70% (14, 19, 20). A single genus of frugivorous bats (*Pteropus*) appears to be the main reservoir for henipaviruses throughout Asia and Australia (21–25). This includes *Pteropus medius* [formerly *Pteropus giganteus* (26)], the only pteropid bat present in Bangladesh and India (16, 27–30). NiV has several characteristics that make it a significant threat to human and animal health: 1) Its bat reservoir hosts are widely distributed throughout Asia and occur within dense human and livestock populations, leading to widespread frequent spillover events and outbreaks; 2) it can be transmitted directly to humans by bats or via domestic animals; 3) it can be transmitted from person to person; 4) spillover has repeatedly occurred in highly populous and internationally

Significance

Nipah virus (NiV) is a zoonotic virus and WHO priority pathogen that causes near-annual outbreaks in Bangladesh and India with >75% mortality. This work advances our understanding of transmission of NiV in its natural bat reservoir by analyzing data from a 6-y multidisciplinary study of serology, viral phylogenetics, bat ecology, and immunology. We show that outbreaks in *Pteropus* bats are driven by increased population density, loss of immunity over time, and viral recrudescence, resulting in multiyear interepizootic periods. Incidence is low, but bats carry NiV across Bangladesh and can shed virus at any time of year, highlighting the importance of routes of transmission to the timing and location of human NiV outbreaks.

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connected regions; 5) it is associated with high mortality rates in people; and 6) there are currently no commercially available vaccines to prevent infection or drugs to mitigate disease (31–33). As a result, the World Health Organization has listed NiV in its R&D Blueprint as one of the 10 highest-priority pathogens for the development of medical countermeasures due to its potential to cause significant outbreaks (34). In May 2018, an outbreak of NiV encephalitis with a 91% mortality rate occurred in a new location—Kerala, India—more than 1,200 km southwest of previous Indian and Bangladeshi outbreaks (35). A single case was subsequently reported in Kerala in 2019, and while local *P. medius* populations have been implicated as the local source of infection, the route of spillover in both instances remains unknown (35, 36).

In Malaysia and Bangladesh, consumption of cultivated food resources contaminated with bat excreta, such as mangoes in Malaysia and date palm sap in Bangladesh and northeastern India, have been identified as the predominant cause of spillover to pigs and people, respectively (37). Human outbreaks occur almost annually in Bangladesh, and the seasonal timing (November to April) and spatial distribution of outbreaks coincide with patterns of raw date-palm-sap consumption in a region termed the "Nipah belt" (38). However, there is variability in the geographic locations and number of spillover events, as well as the number and magnitude of human outbreaks that occur each year (39, 40). Spillover has also occurred outside the predominant season and region of date-palm-sap consumption (41). Whereas no human outbreaks have been reported in eastern Bangladesh, despite date-palm-sap harvesting and consumption, human outbreaks have been reported in Kerala, India, where date-palm sap is not cultivated (38). These observations suggest an alternate route of spillover in certain locations and a critical need to understand the mechanisms of underlying viral infection dynamics in bats and the extent of genetic diversity within the virus—each of which may influence the timing, location, and epidemiology of human outbreaks (38).

Previous research on the transmission dynamics of NiV and Q:13 Hendra virus in *Pteropus* species (spp.) bats has produced mixed, and sometimes contradictory, findings. NiV, like Ebola, Marburg, Hendra, and some bat coronaviruses, is associated with seasonal spikes in infection that coincide with annual or semi-annual synchronous birth pulses (21, 42–48). Seasonal periods of Q:14 NiV shedding were observed in *Pteropus lylei* in Thailand, and seasonal spikes in NiV (or a related henipavirus) seroprevalence coinciding with pregnancy periods were observed in *Eidolon dupreanum* in Madagascar (49, 50), but not in *Pteropus vampyrus* or *Pteropus hypomelanus* in Peninsular Malaysia (25). Hendra virus prevalence in Australian pteropid bats has shown multiyear interepizootic periods, during which little virus can be detected, followed by periods of markedly increased viral shedding (51–53). It has been hypothesized that multiyear periodicity in the incidence of henipavirus infections could arise from a buildup and waning of herd immunity in the reservoir host, with reintroduction of virus via immigration, recrudescence, or viral persistence (11, 54–56).

There is a paucity of data related to henipavirus-associated immune dynamics in free-ranging pteropid bats, including the duration of immunity in adults and juveniles, which limits our understanding of population-level viral dynamics. Experimental infections of *Pteropus* bats with Hendra virus and NiV show that bats mount an antibody response following infection with Hendra virus and NiV (57–59). Waning of anti-NiV antibodies was observed in recaptured wild *Eidolon helvum*, a bat related to *Pteropus* spp., in Madagascar (60). Passive transfer and waning of maternal antibodies also occurred in captive *Pteropus* species, and, along with loss of immunity in adults, could contribute to the loss of herd immunity in wild populations (61). Some pteropid bat species are migratory, and interconnected colonies form a metapopulation, which could allow for viral reintroductions into susceptible colonies (10, 25, 62, 63). In addition, NiV recrudescence has been observed in wild-caught *P. vampyrus* and possibly also in *E. helvum* (64–66). Either of these phenomena could allow NiV to persist regionally during periods of high local immunity. However, no study has yet shown evidence in open, free-ranging bat populations that examines the influence of these factors on NiV transmission dynamics.

Here, we examine the distribution, dynamics, genetic diversity, and underlying drivers of NiV infection in *P. medius* in Bangladesh to improve our understanding of human outbreak patterns. Specifically, we analyze the spatial, temporal, and demographic variation in serological dynamics and viral shedding in bats over a 6-y period to determine the spatiotemporal drivers and dynamics of virus transmission. We also analyze the movement patterns of individual bats and analyze NiV phylogenetics to understand patterns of spatial mixing and virus strain diversity.

Results

Comparative Nipah Seroprevalence and Virus Infection Study in Bats Inside and Outside the Nipah Belt and Concurrent Longitudinal Bat Study Inside the Nipah Belt. In a cross-sectional spatial study conducted between January 2006 and July 2012, we caught and tested 883 *P. medius* (~100 per district) from eight colonies in different districts across Bangladesh. We detected anti-Nipah immunoglobulin G (IgG) antibodies in all colonies (Fig. 1). Q:15 Seroprevalence varied by location ($\chi^2 = 55.61$, $P < 0.001$). In most locations, adult seroprevalence exceeded juvenile seroprevalence; in Tangail and Rajbari, seroprevalence was similar across ages. Viral detection in individuals was rare; overall, we detected NiV RNA in 11 of 2,088 individuals and in three pooled oropharyngeal samples (representing five bats, but which could not be resolved to an individual) (Table 1). We detected viral RNA in individual bats in Faridpur and Rajbari and from pooled samples from Thakurgaon and roost urine samples from Cumilla. Of the 11 PCR-positive individuals, three had IgG antibodies (*SI Appendix*, Table S1). We also detected virus in pooled urine collected from tarps placed below bats at roosts associated with human outbreaks in Bhanga and Joypurhat. The viral prevalence in Rajbari in January 2006 was 3.8% (95% CI: 0 to 11%; $n = 78$). In Faridpur, which is adjacent to Rajbari and where we conducted an intensive longitudinal study (see below), viral prevalence estimates ranged from 0 to 3% (95% CI: 0 to 10%; $n = 100$ at each of 18 sampling times) (Table 1). NiV RNA was detected in bats from inside (Rajbari, Thakurgaon, and Faridpur) and outside (Cumilla) the Nipah Belt. There was no significant difference between NiV detection rates from individual bats by the two main sample types: urine/urogenital swabs, 0.37% ($n = 2{,}126$) and oropharyngeal swabs, 0.15% ($n = 1973$) ($\chi^2 = 1.92$ $P = 0.17$). The estimated detection rate from pooled urine samples, collected from tarps placed underneath roosts) across the entire study was 2.5% ($n = 829$), which was significantly higher than either sample type collected from individual bats ($\chi^2 = 55.6$, $P < 0.001$).

Factors Associated with NiV IgG Serostatus in *P. medius*. There was no statistical difference between seroprevalence in bats inside the Nipah Belt and outside (95% odds ratio [OR] 1.2, highest posterior density interval [HDPI] 0.47 to 3.1). Adults had higher seropositivity than juveniles (OR 2.4, 1.7 to 3.6 HDPI), and males greater than females (OR 1.6, 1.0 to 2.4 HDPI) (Fig. 2). There was weak evidence that seroprevalence was higher in pup-carrying (OR 4.0, HDPI 0.6 to 34) and pregnant (1.5 times, HDPI 0.85 to 2.8) individuals than other females. Neither mass, forearm length, nor the mass:forearm ratio (a proxy for age) Q:16 consistently correlated with seropositivity. However, bats with poor body condition (an assessment of pectoral muscle mass by palpation) were less likely to be seropositive (poor/fair body

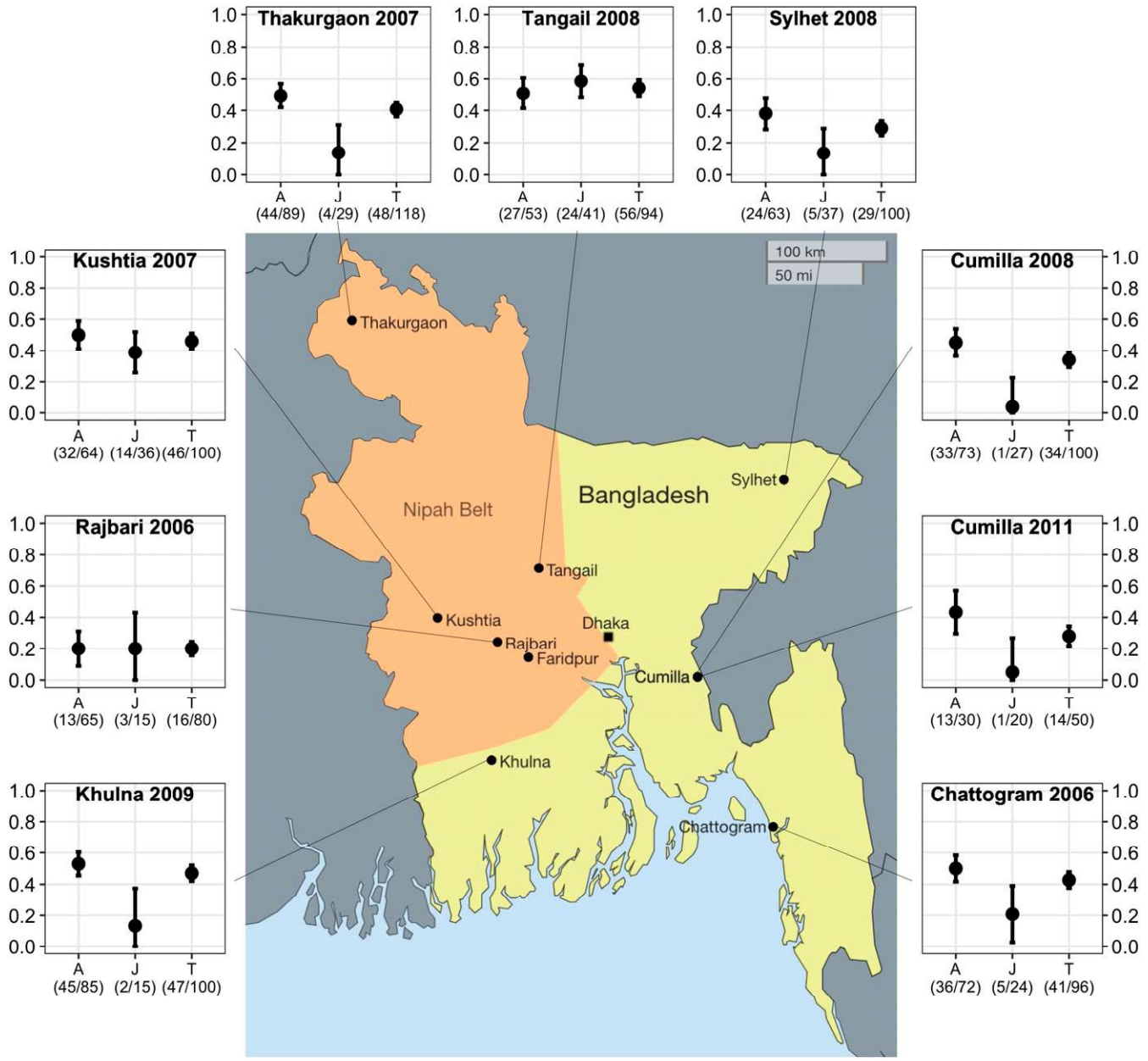


Fig. 1. Map showing age-stratified seroprevalence in *P. medius* colonies, Bangladesh. Bats from eight colonies were sampled and tested for anti-NiV IgG antibodies: four within the "Nipah belt" (orange shaded) and four outside. Seroprevalence of adults (A), juveniles (J), and total seroprevalence (T) are shown with 95% CI error bars. The shaded region represents the "Nipah belt," where previous human NiV outbreaks have been reported.

condition OR = 0.69, HDPI 0.49 to 0.96). Finally, serostatus was strongly correlated in mother–pup pairs; 39 of 41 pups with seropositive mothers were seropositive, and 32 of 39 pups with seronegative mothers were seronegative.

Longitudinal NiV Serodynamics in *P. medius*, Faridpur District (2006 to 2012). We sampled bats quarterly from a single population in the Faridpur district. We also microchipped a total of 2,345 bats between 2007 and 2012. We used generalized additive models (GAMs) to characterize changes in NiV seroprevalence over time. There were significant fluctuations in adult (>24 mo) and juvenile (6 to 24 mo) seroprevalence over the 6-y study period (Fig. 3*A*). Juvenile seroprevalence ranged from 0 to 44% (95% CI: 37 to 51%), and decreased over the first year of life for bats born in each year ("yearlings"), consistent with loss of maternal antibodies in juveniles. A more pronounced decrease occurred from mid-October to mid-December than other parts of the year. However, the GAM indicating this had only slightly better fit (ΔAIC [ΔAkaike information criterion] < 1) than one with a linear decrease over the whole year (Fig. 3*B*).

Adult seroprevalence ranged from 31% (95% CI: 20 to 46%) to 82% (95% CI: 77 to 87%) with three cycles of clear variability over the course of the study (Fig. 3*A*). We found no evidence of regular seasonal fluctuations; a GAM with annual cyclic terms fit worse than one without (ΔAIC > 10). Viral RNA was detected during periods of increasing, decreasing, and stable seroprevalence.

We fitted a series of age-stratified compartmental susceptible–infected–recovered models to examine different biological processes influencing serodynamics, including density-

Table 1. PCR detection of NiV RNA in *P. medius* 2006 to 2012

Location	Date	Bats tested	Throat swabs tested	Throat swabs positive	Urine tested	Urine positive	Rectal swabs tested	Rectal swabs positive	Bats positive	Prevalence	±95% CI
Spatial study											
Rajbari	Jan-06	99	79	3	78	0	79	1	3	0.04	0.11
Thakurgaon	Mar-07	118	115	3*	72	0	—	—	unk.	0.00	—
Kushtia	Aug-07	101	100	0	99	0	—	—	0	0.00	—
Tangail	Jun-08	100	61	0	77	0	—	—	0	0.00	—
Chattogram	Aug-06	115	19	0	—	—	—	—	0	—	—
Cumilla	May-08	100	0	0	50	0	—	—	0	—	—
Sylhet	Sep-08	100	100	0	49	0	—	—	0	0.00	—
Khulna	Jan-09	100	50	0	80	0	—	—	0	0.00	—
Cumilla	Mar-11	50	50	0	50	0	—	—	0	0.00	—
Longitudinal study											
Faridpur	Jul-07	102	64	0	50	0	—	—	0	0.00	
Faridpur	Dec-07	101	N/A	N/A	N/A	—	—	—	0		
Faridpur	Apr-08	100	64	0	88	0	—	—	0	0.00	
Faridpur	Jul-08	100	58	0	74	0	—	—	0	0.00	
Faridpur	Jul-08	100	98	0	99	0	—	—	0	0.00	
Faridpur	Feb-09	100	50	0	100	1	—	—	**1**	**0.01**	**0.10**
Faridpur	May-09	101	100	0	99	2	—	—	**2**	**0.02**	**0.10**
Faridpur	Aug-09	100	100	0	99	0	—	—	0	0.00	
Faridpur	Nov-09	100	100	0	82	1	—	—	**1**	**0.01**	**0.11**
Faridpur	Feb-10	100	100	0	100	0	—	—	0	0.00	
Faridpur	Jun-10	100	100	0	100	3	—	—	**3**	**0.03**	**0.10**
Faridpur	Sep-10	100	100	0	100	0	—	—	0	—	
Faridpur	Jan-11	100	100	0	100	0	—	—	0	0.00	
Faridpur	May-11	102	102	0	102	1	—	—	**1**	**0.01**	**0.10**
Faridpur	Aug-11	100	100	0	100	0	—	—	0	—	
Faridpur	Dec-11	100	100	0	100	0	—	—	0	—	
Faridpur	Apr-12	100	78	0	78	0	—	—	0	—	
Faridpur	Jul-12	100	100	0	100	0	—	—	0	—	
Faridpur	Nov-12	100	100	0	100	0	—	—	0		
Total		**2,789**	**2,088**	**6**	**2,126**	**8**	**79**	**1**	**11**	**0.005**	**0.02**
Outbreak investigation											
Pooled roost urine samples *n* = no. pos.											
Bangha	Feb-10		19	3							
Joypurhat	Jan-12		19	16[†]							
Rajbari	Dec-09		35	0							
West Algi	Jan-10		31	0							

Unk., unknown.

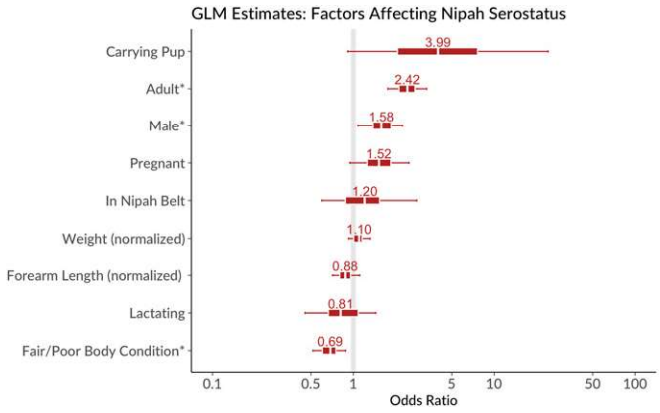
*NiV RNA was detected in three pooled oropharyngeal samples, confirmed by sequencing, although confirmation from individual samples could not be made. These data are not used in prevalence estimates.

[†]Detection by qPCR, Ct ranges 20 to 38.

vs. frequency-dependent transmission, recrudescence vs. immigration of infected individuals, and seroreversion (loss of antibodies) in both juveniles and adults (*Methods* and Fig. 4). Density-dependent models were a far better fit to the data than frequency-dependent models (difference in log-likelihood 10.0; ΔAIC = 20.0), suggesting that movements of bats and fluctuations in colony size alter spatiotemporal variation in the risk of NiV infection in bats. In Faridpur ("Domrakhandi/Khaderdi" in *SI Appendix*, Fig. S1) during the period of sampling, the roost population declined from ∼300 bats to 185, which decreased transmission potential in the fitted model: R_0 in adult bats was estimated to decrease from 3.5 to 2.1 as the number of bats in the colony decreased. As a result, over the 6-y study period, the fitted model predicted that the threshold for herd immunity (i.e., the seroprevalence below which the reproductive ratio $R_t > 1$) for adults fell from 72% (when bat counts were highest—in 2006) to 52% (when bat counts were lowest).

The fitted model suggests that serodynamics in juveniles were strongly driven by inheritance and loss of maternal antibodies. The estimated duration of maternal antibodies was 17.6 wk (95% CI: 13.7 to 25.0), which was much quicker than the loss of antibodies in adults (290.8 wk, 95% CI: 245.0 to 476.4) (*SI Appendix*, Table S2). Finally, models with recrudescence fit the data better than models without recrudescence (*SI Appendix*, Table S2; difference in log-likelihood 32.6; ΔAIC = 65.1), and models with recrudescence fit the data better than models with immigration (ΔAIC = 3.76).

Mark–Recapture and Seroconversion/Seroreversion. There were 56 recapture events over the study period (*SI Appendix*, Table S3). Thirty-one bats were recaptured at a nearby roost other than the original capture location. This network of roosts, or "roost complex," formed a polygon covering ∼80 km^2 and included 15 roosts sampled during the longitudinal study (*SI Appendix*, Fig. S2 *A* and *B*). Ten instances of seroconversion (change from IgG-negative to IgG-positive) and nine instances of seroreversion (positive to negative) were observed (*SI Appendix*, Table S3). The mean time between positive and negative tests in adults (excluding juveniles with maternal antibodies) was 588 d ($n = 6$) (range: 124 to 1,082 d).



Q:39 **Fig. 2.** Results of Bayesian generalized linear model of factors affecting Nipah serostatus in bats in cross-sectional study. Bars indicate ORs and 50% (inner) and 95% (outer) credible intervals for model parameters. Factors with asterisks (*) have 95% CIs that do not overlap one. Model intercept (predicted probability of seropositivity for a juvenile, female bat outside the Nipah belt of mean size and good body condition) was 0.26 (95% CI 0.12 to 0.56).

Home Range and Intercolony Connectivity Analysis. Home-range analysis of satellite telemetry data from 14 bats (mean duration of collar data transmission = 6.25 mo; range = 1 to 25 mo; *SI Appendix*, Table S4) showed that the majority of bats roosted within 10 km of where the bats were originally collared, in the Faridpur (Nipah belt) colony, and within 7 km from where the bats in the Cox's Bazaar colony were originally collared (315 km east of Faridpur). The average foraging radius was 18.7 km (SD 21.5 km) for the Faridpur bats and 10.8 km (SD 11.9 km) for the Cox's Bazaar bats (*SI Appendix*, Fig. S2). Home range analysis suggests that bats in Faridpur and Cox's Bazar (separated by approx. 310 km) would have a <5% probability of intermingling (Fig. 5). Home-range size was larger during the wet season than the dry season (2,746 km^2 vs. 618 km^2) (*SI Appendix*, Figs. S3 and S4).

NiV Phylogenetic Analysis. Phylogenetic analysis of NiV sequences from a 224-nt section of the N gene (nt position 12,90 to 1,509 [position ref gb|FJ513078.1| India]) suggests that strains from both India and Malaysia clades are present in bats in Bangladesh (Fig. 6). This finding was supported by an additional analysis of near-whole N gene sequences (~1,720 nt) from bats, pigs, and humans, including those from a subset of *P. medius* from this and a more recent study by our group (*SI Appendix*, Fig. S5) (67). Eleven 224-nt N gene sequences obtained from bats between 2006 and 2012 (all from the Faridpur population) were identical. Overall, the N gene sequences identified from the Faridpur, Rajbari and Bhanga colonies between 2006 and 2011 had 98.21 to 100% shared nucleotide identity. Sequences from Rajbari district obtained 5 y apart (January 2006 and January 2011) had only a single nucleotide difference, resulting in a synonymous substitution (G to A) at position 1,304, which was found in four other bat NiV sequences from this study, as well as in the NiV isolate from *P. vampyrus* in Malaysia. Five human NiV N gene sequences from various locations within the Nipah belt over the same time period as our bat study show more nucleotide diversity than those from the Faridpur *P. medius* population. Human sequences throughout Bangladesh and from Kerala, India, all nested within the diversity found in *P. medius* (Fig. 6). By contrast, the sequences found in *P. medius* from Cumilla, a location 150 km to the east of Faridpur, showed 80.8 to 82.59% shared nucleotide identity with sequences from *P. medius* in Faridpur and clustered within the Malaysia group of NiV sequences. The two Cumilla sequences were identical to each other and had up to 87.95% shared nucleotide identity to sequences from *P. lylei* in Thailand.

Discussion

Our findings suggest that NiV circulation occurs in bat populations throughout the country. We observed that virus can be shed by bats at any time of year and that viral dynamics are cyclical, but not annual or seasonal. Our models fit to serological data suggest that these cycles may be driven by demographic and immunological factors; the waning of herd immunity through turnover or individual waning in bat populations allows heightened viral transmission when seroprevalence passes below a critical threshold. Previous studies from Bangladesh suggested that human NiV outbreaks occur primarily within a defined region in western Bangladesh, termed the "Nipah belt," during a defined season (November through April) (41, 68). These observations raised the question of whether the timing and location of human infections are due solely to differences in the frequency and intensity of date-palm-sap consumption, or whether ecological factors such as the distribution and timing of bat viral infection also play a role (19, 38, 69). Our extensive survey of *P. medius*, which is common across Bangladesh and throughout the Indian subcontinent, demonstrates that viral circulation within their populations is not limited to the Nipah belt (16, 27, 30).

A number of mechanisms have been proposed for the maintenance of acute viral infections in bat populations, which are often formed of interconnected colonies, including synchronous birthing and subsequent loss of maternal antibodies (11, 43, 45); lowered immunity within pregnant females due to stress; nutritional stress and other factors (47); immigration of infected individuals from other colonies (62, 70, 71); and recrudescence within previously infected individuals (11, 64, 72). However, little is known about how henipaviruses are transmitted among wild bats. *Pteropus* species are typically gregarious and their roosts, often comprising multiple hardwood trees, and are highly socially structured, with individuals segregated by age, sex, and social dominance (69, 73, 74). Interactions among individuals are often dependent on their grouping, and the intensity of social interactions varies with specific behaviors such as mother–pup interactions, play (juveniles), territorial fighting (adult males), and mating (adults) (74). Our data and previous experiments show that henipaviruses can be shed orally, urogenitally, in feces, and in birthing fluids (59, 75). This suggests that multiple mechanisms for transmission are possible, including mutual grooming, fighting, mating, exposure to excreta or birthing fluids, and ingestion of food contaminated by saliva. Roost size also increases seasonally during mating and birthing periods, which the fitted models suggest would increase transmission, if seroprevalence is below the herd-immunity threshold (30, 73). While *P. medius* does not roost with other bat species, it does feed with other frugivorous bats, and it's possible that interspecies viral transmission occurs during feeding (76, 77). In Madagascar, henipavirus antibodies have been detected in multiple species of frugivorous bats, though it is unknown whether the same virus or antigenically related viruses was shared among them (60). While serological evidence suggests that it is possible henipaviruses circulate in other frugivorous bat species, our findings, as well as those of others (16, 59), suggest that in Bangladesh, *P. medius* is the main natural reservoir for NiV. Henipaviruses other than Nipah may be circulating in *P. medius* (28). We assumed that the anti-IgG antibodies detected by the serological assays used in this study were specific to NiV, but it is possible that the enzyme-linked immunosorbent assay (ELISA) used in the cross-sectional study may have detected antibodies against unknown henipaviruses, which could elevate NiV seroprevalence estimates. An advantage of the Luminex assay used in the longitudinal study is that we could compare median fluorescent intensity (MFI) values to multiple specific henipaviruses (Nipah, Hendra, and

Q:18 Q:19

POPULATION BIOLOGY

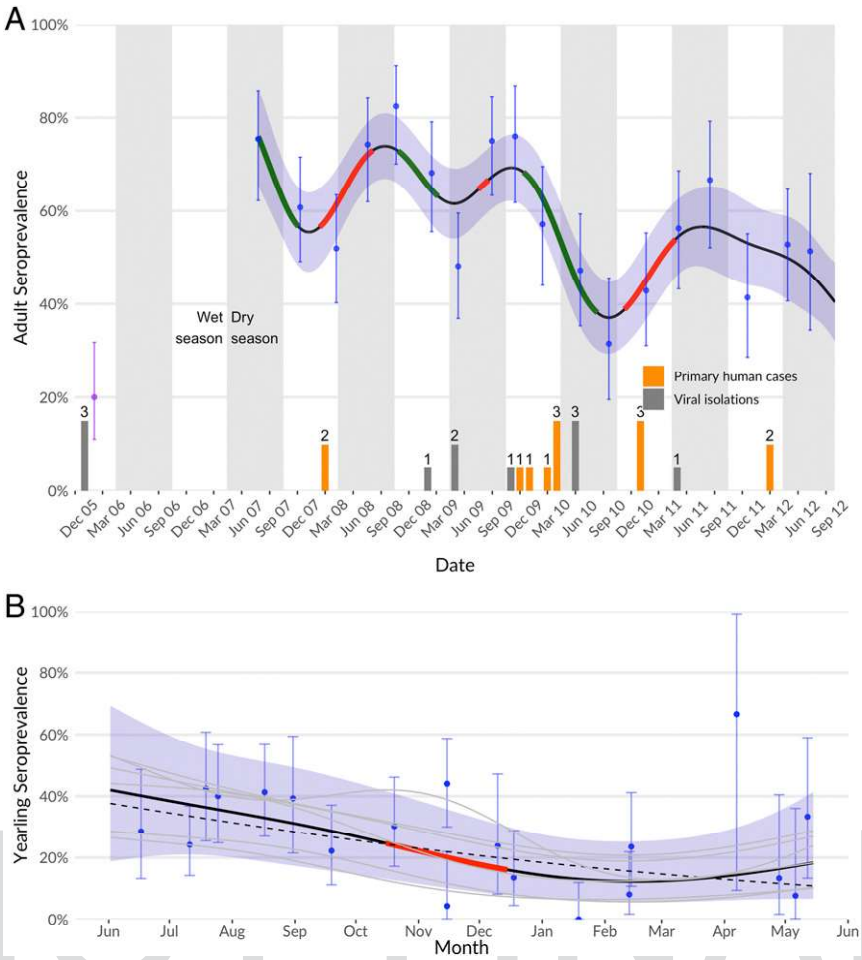


Fig. 3. Serodynamics of the Faridpur bat population, measured and fit to a GAM. (*A*) Adult seroprevalence over time, with measured values and 95% CI in blue and mean GAM prediction and 95% shown with line and surrounding shaded areas. Point from February 2006 (purple) is shown separately due to ELISA vs. Luminex measure. Periods of significant change (where GAM derivative 95% does not overlap zero) are shown in red (increasing) and green (decreasing). Periods of increase indicate viral-circulation events in the adult population; these do not occur with consistent periodicity or seasonality. Counts of primary human cases from local district (dark gray) and bat viral detections (orange; Table 1) are shown on bottom for comparison. (*B*) Juvenile seroprevalence during the first year of life ("yearlings"). All years' measurements are collapsed onto the scale of a single year overlain to show yearling dynamics. Measured values and 95% CIs are shown in blue, and mean and 95% CIs for the GAM model pooled across cohorts are shown with line and surrounded shaded areas. GAM realizations for individual years are in gray and overall effect in black. The period of significant decline in the GAM is shown in red. Juvenile seroprevalence decreases over the course of the year and is not distinguishable from a simple linear decrease (ΔAIC < 1, dotted line).

Cedar) and differentiate between specific reactions to NiV and reactions to the other viruses, which could indicate antibodies against an unknown henipavirus. Hendra and Cedar viruses are enzootic in Australian *Pteropus* spp. and are not known to occur in Bangladesh, so we considered reactions to these viruses NiV-negative results.

Our modeling indicates that NiV is primarily driven by immunity and density-dependent transmission dynamics among bats, with cycles of higher seroprevalence dampening intracolony transmission followed by waning of antibody titers within individuals and death of seropositive individuals. Waning humoral immunity against NiV has been consistently shown in henipavirus studies of African pteropodid bats (56, 60). Our recapture data provided reported evidence of the loss of detectable NiV IgG antibodies in individual free-ranging bats, which supports the fitted model suggesting limited duration individual immunity and the importance of population-level waning immunity. The consistently decreasing seroprevalence that we observed in juveniles suggests that they lose maternal antibodies over their first year (the fitted model estimates after 3 to 5 mo), consistent with other studies of maternal antibodies against henipaviruses in pteropodid bats (47, 56, 61, 65). Our analysis do not support the hypothesis (45) that seasonal pulses of these new seronegative individuals are sufficient to drive new outbreaks because high seroprevalence in adults limited transmission in several years (Fig. 4).

NiV reintroduction into a colony may occur from a persistently infected individual (e.g., via recrudescence) or immigration of an infected individual. Our analyses suggested that recrudescence was a more important driver of transmission dynamics than immigration. Recrudescence of henipavirus infection has been observed for NiV in captive *P. vampyrus* (64), for henipavirus in captive *E. helvum* (56, 66), and humans infected by NiV (78) and Hendra virus (79). It is difficult to know from serology alone whether wild-caught seronegative bats had been previously infected. Experimental infections comparing naïve to previously infected *P. medius* that have sero-reverted would provide a better understanding of how humoral immunity influences individual susceptibility to infection and inform dynamics models attempting to explain viral maintenance within bat populations (60).

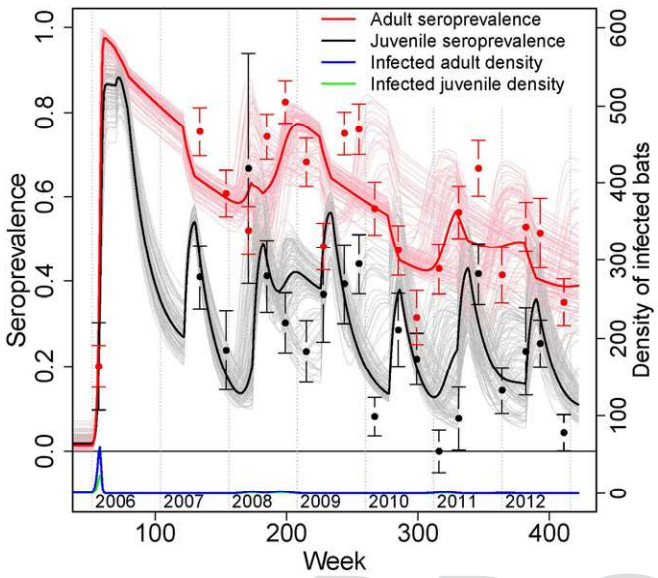


Fig. 4. Longitudinal data and fitted model for NiV serological dynamics in adult and juvenile bats. Red and black points show observed data (±1 SE), and solid lines show the fitted model (thick lines show the trajectory for the model with maximum-likelihood parameter estimates; thin lines show realizations for parameter estimates drawn from the estimated distributions) for the fraction of adults and juveniles seropositive for NiV (left axis), and the model-estimated number of infected adult and juvenile bats (bottom and right axes). See *Methods* for details of model structure.

Our longitudinal study was limited to a single population of interacting subcolonies and bat populations across Bangladesh likely represent a dynamic metapopulation. Our roost count data and recapture data from microchipped bats showed how roost sizes can fluctuate and that bats shift among local roosts. The fitted model strongly suggested that decreases in local roost counts substantially reduced local transmission potential of NiV. However, a larger study across multiple regional populations would be needed to understand how local shifts in bat colonies impact broader fluctuations in regional populations and spatial patterns of NiV transmission.

Understanding how bat populations connect across landscapes is important for understanding viral maintenance, and studying local and migratory bat movements can provide important ecological information related to viral transmission, including how bats move between different colonies (62, 80). Our satellite telemetry data suggest that *P. medius* exists as a metapopulation, like other pteropid species (11, 71). The numbers of individuals we collared represents a small sample size; however, they are comparable to other bat satellite telemetry studies of related species, and our data suggest that bat dispersal in Bangladesh may currently be more localized than other species elsewhere. *P. medius* appear to travel shorter distances and remain within a smaller home range (321.46 and 2,865.27 km^2 for two groups) than *P. vampyrus* in Malaysia (64,000 and 128,000 km^2) and *Acerodon jubatus* in the Philippines, both of which are similarly sized fruit bats (62, 81). Pteropodid bat migration is primarily driven by seasonal food-resource availability (63, 82–84). In Bangladesh, *P. medius* prefer to roost in human-dominated environments in highly fragmented forests, as opposed to less-populated, intact forested areas, such as in national parks (85). The conversion of land to villages and farmland over recent human history has likely led to increased food availability for *P. medius* and may have reduced the impetus for long-distance migration (37). This may reflect a similar adaptation to anthropogenic food resources, as observed over the last few decades in Australian *Pteropus* species (71). Home ranges were significantly smaller during the dry season, which corresponds to winter months and the time when most female bats are pregnant, likely resulting in them flying shorter distances to conserve energy. Genetic analysis of *P. medius* across Bangladesh has shown that, historically, there has been extensive gene flow and intermixing among populations, and we did observe a few instances of longer-distance flights; however, the movement data indicated that, overall, these bats had much smaller home ranges (80). Less connectivity among bat populations across Bangladesh may influence NiV transmission by creating longer interepizootic periods and larger amplitude fluctuations in population-level immunity in *P. medius* compared to more migratory species (71). Bat movement and population connectivity may also influence the genetic diversity of NiV found in different locations.

The potential existence of a more transmissible or pathogenic strain of NiV already circulating in bats further underscores the need to strengthen efforts to prevent spillover. While the overall strain diversity among NiV has not been well characterized due to a dearth of isolates, two distinct NiV clades have been described: a Bangladesh clade, that includes sequences identified in India and Bangladesh; and a Malaysian clade, that comprises sequences from Malaysia, Cambodia, The Philippines, and Thailand (18, 67, 86). Our findings of substantially different NiV sequences in Faridpur and Cumilla suggest that viruses from both clades are circulating in Bangladesh. Strains of NiV from these two clades are associated with differences in pathogenesis, epidemiological and clinical profiles in humans and animal

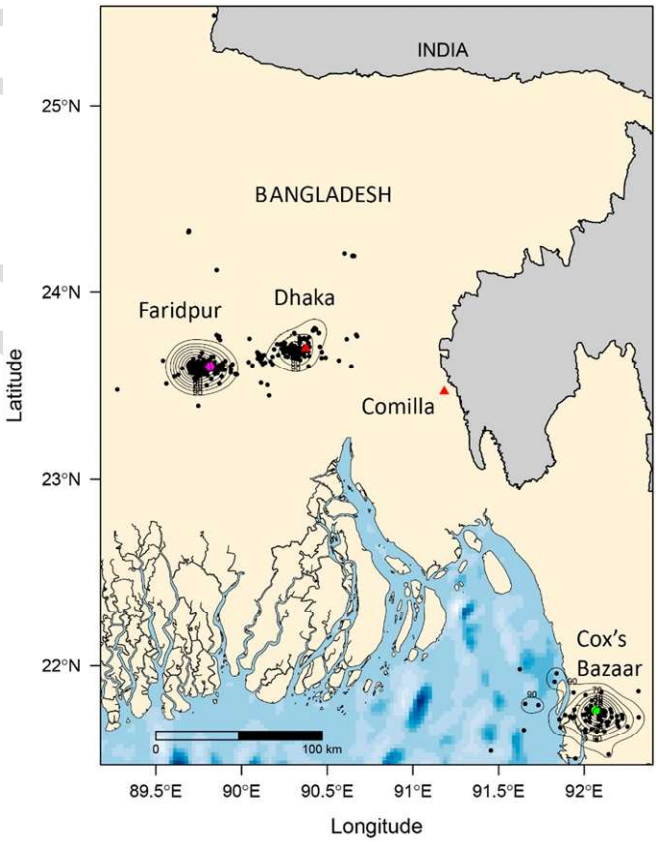


Fig. 5. Satellite telemetry and home-range analysis. Location data from satellite collars (n = 14) placed on 11 bats from Faridpur and 3 bats from Cox's Bazaar, Chattogram, collected between 2009 and 2011 were used to calculate local and long-range movement patterns and home range for these two groups.

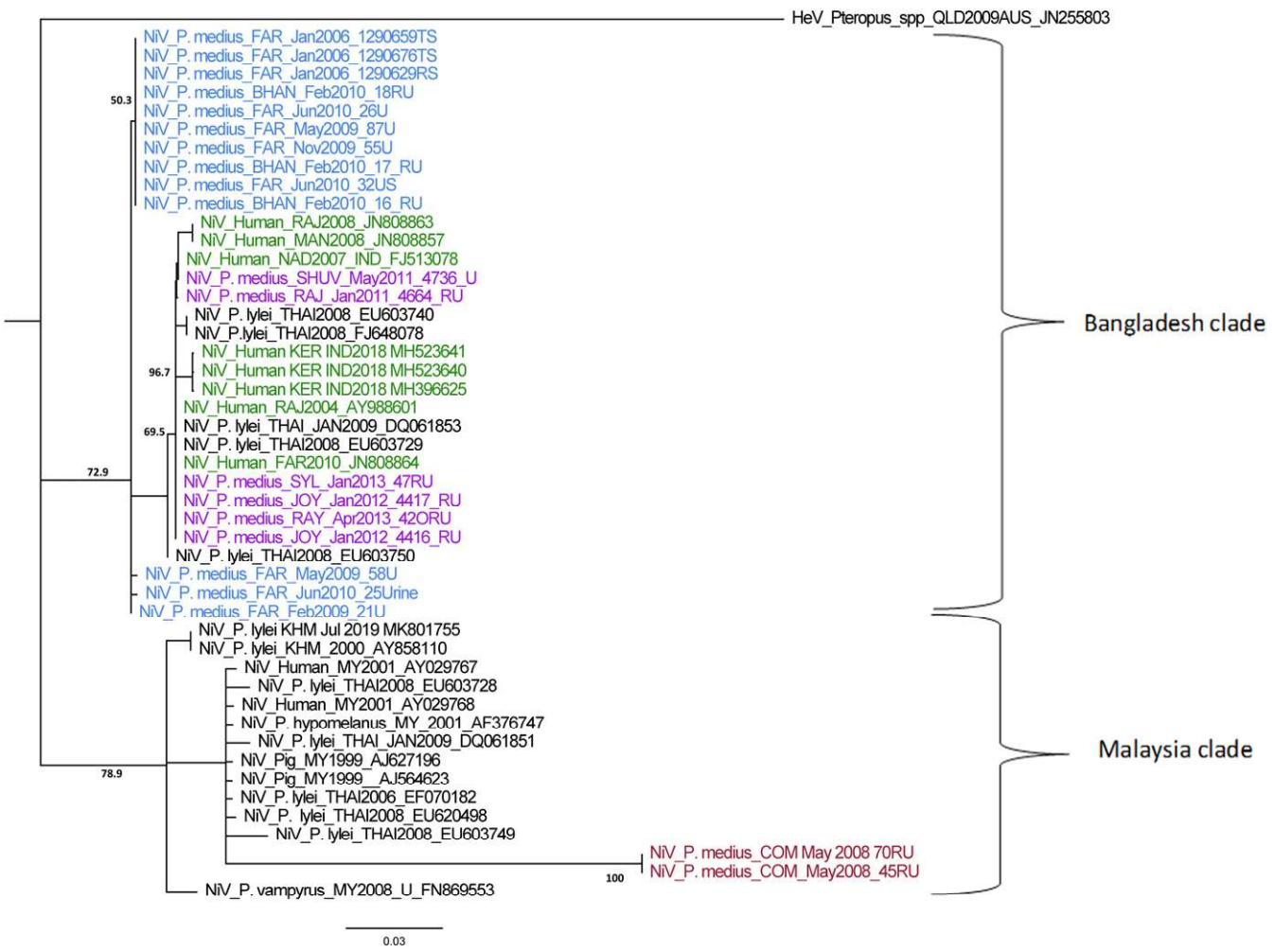


Fig. 6. NiV partial N-gene phylogeny (224 nt). Phylogenetic neighbor-joining tree created in Geneious Prime 2019 using a Tamura-Nei model with 1,000 bootstrap replicates and Hendra virus as an outgroup (117) is shown. Branch lengths are shown as the number of substitutions per site. Sample collection date, location, and GenBank accession numbers are included in the label for each sequence, except *P. medius* sequences we collected (GenBank accession nos. MK995284–MK995302). Blue labels indicate bat sequences from Faridpur and Bhanga (an outbreak response in Faridpur). Purple sequences are from *P. medius* from other roosts sampled during the longitudinal study. Red sequences are from *P. medius* in Cumilla. Green sequences are human NiV sequences from Bangladesh and India.

models, and observed shedding patterns in bats (49, 87–91). Phenotypic variation in NiV could influence human outbreak patterns by altering transmission to, or pathogenesis in, humans and the likelihood of smaller outbreaks being identified or reported (92). Human-to-human NiV transmission via contact with respiratory and other secretions has been regularly observed in Bangladesh and India, including the recent 2018 outbreak in Kerala (14, 68, 93), whereas transmission among people was not a common feature of the Malaysia outbreak, despite close contact between cases and health care providers (94, 95). NiV cases in Bangladesh have shown more strain diversity than in the Malaysia outbreak, which could be due to greater virus diversity in *P. medius* (96).

Until now, there have been very few Nipah sequences obtained from *P. medius.* We found that Nipah N-gene sequences from bats from the Faridpur population were nearly identical over time, compared to variation in N-gene sequences from bats and humans from other locations observed over the same time period (2006 to 2010). This suggests that there may be locally prevalent and stable NiV genotypes that persist within bat colonies. Human NiV genotype diversity is likely a reflection of the diversity of the NiV strains in the local bats that seed outbreaks (10). This is also supported by viral sequences obtained from humans and bats associated with the 2018 NiV outbreak in Kerala, India, where human NiV sequences were most closely related to local *P. medius* sequences (97).

Connectivity of pteropid bats in Bangladesh with those in Southeast Asia could explain the observed strain diversity in our study. Historical interbreeding of *P. medius* with pteropid species found in Myanmar, Thailand, and Malaysia and our telemetry findings showing bats are capable of flying hundreds of kilometers could explain the presence of a Malaysia clade NiV sequence in bats from Cumilla (80). NiV Bangladesh strains have also been found in *P. lylei* in Thailand (98). The N gene of the Cumilla NiV strain differs from those reported from bats in the Nipah belt by 20%, whereas NiV Malaysia and NiV Bangladesh differ by only 6 to 9% and are associated with different clinical profiles. Whole-genome sequence would have allowed for better characterization of the Cumilla strain; however, this was not obtained. Despite the short sequences used in our analysis, the N gene is generally conserved relative to other genes and is representative of the diversity across henipavirus genomes (86). We would expect the rest of the Cumilla viral genome to also be highly divergent, potentially even qualifying it as a different

henipavirus species. It is, therefore, plausible that the clinical profile of a 20% divergent Nipah-related virus differs significantly from known strains. Sequence information from an isolated human NiV case in Cumilla has not been reported, so comparison to the sequence we found in bats was not possible (41). Studies linking viral genotype to clinical outcomes in people would provide additional insight into the effect of strain diversity in bats on the potential for larger-scale human outbreaks.

Our study sheds light on the sporadic nature of human NiV outbreaks, with multiyear interepizootic periods in South Asia. PCR results show that overall NiV incidence in *P. medius* is low, consistent with previous studies of Hendra virus and NiV (25, 47, 52). The fitted model suggests that transmission increases when bat populations have become susceptible through waning immunity (11). In the current study, observed seroprevalence patterns and the fitted model suggest that three periods of transmission occurred over the 6 y of sampling, each of which followed periods of low adult and juvenile seroprevalence. Viral detection in bats has coincided with some human outbreaks, supporting the hypothesis that spillover is a sporadic event (67, 97). In our study periods, low seroprevalence in bats was not always followed by outbreaks in humans. We detected NiV RNA during periods of both increasing and high seroprevalence, consistent with recrudescence, which was strongly supported by the fitted model and has been demonstrated in captive animals (64–66). This likely contributes to the sporadic variation in human outbreaks (e.g., spillover events) from year to year in Bangladesh.

Overall, our results suggest that NiV outbreaks in humans stem from an interaction of four factors: 1) multiyear fluctuations in transmission intensity among bats driven by immunity and colony size/density-dependent transmission; 2) relatively localized bat movements creating spatially variable transmission dynamics; 3) occasional shedding by previously infected bats due to recrudescence; and 4) highly seasonal contact between bats and humans via consumption of raw date-palm sap. The timing of multiple factors involved in driving transmission dynamics needs to align for intracolony NiV transmission events and further align with human behavior and availability of a route of spillover for human outbreaks to occur, as previously hypothesized (99). We further conclude that NiV dynamics in bats combined with the seasonality and specific geography of date-palm-sap consumption in Bangladesh likely explains the sporadic nature of human outbreaks in the region (38).

These findings suggest that human NiV outbreaks in other regions of Bangladesh (and Asia) where *Pteropus* bats occur are also likely to be sporadic and rare, leading to underreporting or a lack of reporting. This is probably exacerbated by the fact that the clinical syndrome is similar to that of other common infections, such as Japanese encephalitis, malaria, and measles (100). Understanding whether some NiV strains are capable of causing mild or asymptomatic cases will provide important insights about why outbreaks have not been detected in areas such as eastern Bangladesh or other parts of Asia, where host, virus, and potential routes of spillover exist. One reason is that mild or asymptomatic cases would be unlikely to be detected by current surveillance systems. About half of all Nipah outbreaks in Bangladesh between 2007 and 2014 were unreported, suggesting that many cryptic spillover events have occurred (101). The 2018 and 2019 spillover events in Kerala, India, which were linked to local *P. medius* colonies and which occurred in an area that does not cultivate date-palm sap, further emphasize the point, but raise questions about the mechanism of spillover.

In the last two decades, the world has experienced large epidemics of bat-associated viruses, including Ebola in West Africa and Democratic Republic of the Congo, SARS coronavirus, and SARS-CoV-2. The World Health Organization has listed NiV and other henipaviruses as priority pathogens for vaccine and therapeutic research and development, along with Ebola viruses and coronaviruses. Surveillance for henipaviruses and antibodies in bats and people where they are in close contact will help determine spillover risk; characterize henipavirus genetic diversity; and understand the genetic determinants of NiV transmissibility and pathogenicity among humans. These measures may help target interventions that reduce spillover, substantially improving our ability to reduce the risk of a more transmissible strain of NiV emerging and causing a large-scale epidemic with significant human and animal mortality.

Methods

The study period was between January 2006 and November 2012. The study was conducted under Tufts University Institutional Animal Care and Use Committee protocol G929-07 and International Centre for Diarrheal Diseases Research, Bangladesh (ICDDR,B) Animal Experimentation Ethical Committee Protocol 2006-012 with permission from the Forest Department, Government of Bangladesh. Locations were selected based on whether the district had any previously recorded human NiV encephalitis clusters at the time of this study and was therefore inside the Nipah Belt (e.g., Rajbari, Tangail, Thakurgaon, and Kushtia) or whether they had not and were outside the Nipah Belt (e.g., Cumilla, Khulna, Sylhet, and Chattogram). The Thakurgaon study was conducted as part of an NiV outbreak investigation and coincided with ongoing human transmission (102). Between 2006 and 2012, three different studies of *P. medius* with similar bat-sampling protocols were performed: 1) a cross-sectional spatial study with a single sampling event in each of the eight locations listed above; 2) a longitudinal study of a Faripur bat colony with repeated sampling approximately every 3 mo from July 2007 to November 2012; and 3) a longitudinal study of the Rajbari colony with repeated sampling at a monthly interval between 12 mo period between April 2010 and May 2011. Opportunistic sampling of *P. medius* was also performed during this time period during NiV outbreak investigations (Bangha, Faridpur [February 2010], Joypurhat [January 2012], Rajbari [December 2009], and West Algi, Faridpur [January 2010]). Bats were captured using mist nets at locations within eight different districts across Bangladesh between January 2006 and December 2012 (Fig. 1).

Capture and Sample Collection. For the country-wide cross-sectional and Faridpur longitudinal study, ~100 bats were sampled at each sampling event, which lasted 7 to 10 d. This sample size allowed us to detect at least one exposed bat (IgG antibody-positive) given a seroprevalence of 10% with 95% confidence. Bats were captured using a custom-made mist net of ~10 m × 15 m suspended between bamboo poles, which were mounted atop trees close to the target bat roost. Catching occurred between 11 PM and 5 AM as bats returned from foraging. To minimize bat stress and chance of injury, nets were continuously monitored, and each bat was extracted from the net immediately after entanglement. Personal protective equipment was worn during capture and sampling, which included dedicated long-sleeve outerwear or Tyvek suits, P100 respirators (3M), safety glasses, nitrile gloves, and leather welding gloves for bat restraint. Bats were placed into cotton pillowcases and held for a maximum of 6 h before being released at the site of capture. Bats were sampled at the site of capture using a field laboratory setup. Bats were anesthetized by using isoflurane gas (103), and blood, urine, oropharyngeal swabs, and wing-membrane biopsies (for genetic studies) were collected. For some sampling periods, rectal swabs were collected, but due to resource constraints, these samples were deemed to likely be lower-yield than saliva and urine for NiV and were discontinued during the study. For each bat sampled, we recorded age, weight, sex, physiologic and reproductive status, and morphometric measurements, as described (27). Bats were classified as either juveniles (approximately 4 to 6 mo—the age by which most pups are weaned) to 2 y old (the age when most *Pteropus* species reach sexual maturity) or adults (sexually mature) based on body size and the presence of secondary sexual characteristics, pregnancy, or lactation—indicating reproductive maturity (27, 104).

Up to 3.0 mL of blood was collected from the brachial vein and placed into serum tubes with serum clot activator (Vaccutainer). Blood tubes were stored vertically on ice packs in a cold box, and serum was allowed to separate overnight. Serum was drawn from the tube after 24 h, placed in a screw-top cryovial (Corning), and stored in a liquid nitrogen dewar (Princeton Cryogenics). Sterile pediatric swabs with polyester tips and aluminum shafts were used to collect urogenital and rectal samples, and larger polyester swabs with plastic shafts (Fisher) were used to collect oropharyngeal samples. All swabs were collected in duplicate, with one set being placed individually in cryotubes containing lysis buffer (either trireagent or NucliSENS Lysis buffer;

BIOMERIEUX) and the second set in viral transport medium (VTM). All tubes were stored in liquid nitrogen in the field and then transferred to a −80 °C freezer.

During each sampling event, pooled urine samples were collected beneath bat roosts using polyethylene sheets (2′ × 3′) distributed evenly under the colony between 3 AM and 6 AM. Urine was collected from each sheet either by using a sterile swab to soak up droplets or a sterile disposable pipette. Swabs or syringed urine from a single sheet were combined to represent a pooled sample. Each urine sample was divided in half and aliquoted into lysis buffer or VTM at an approximate ratio of one part sample to two parts preservative.

Serological and Molecular Assays. Sera from the cross-sectional survey were heat-inactivated at 56 °C for 30 min, as described (105), prior to shipment to the Center for Infection and Immunity at Columbia University for analysis. Samples were screened for anti-NiV IgG antibodies using an ELISA, as described in ref. 27. Sera from the longitudinal studies were sent to the Australian Animal Health Laboratory and were gamma-irradiated upon receipt. Because of the large sample size and development of a high-throughput multiplex assay of comparable specificity and sensitivity, for these samples, we used a Luminex-based microsphere binding assay to detect anti-Nipah G IgG antibodies reactive to a purified NiV-soluble G-protein reagent, as described (106, 107). Samples resulting in an MFI value of 250 and below are considered negative for other bat species, and previous studies have reported using a threshold of at least three times the mean MFI of negative sera to determine the cutoff (47, 108–110). For this study, MFI values of over 1,000 were considered positive for NiV antibodies, an approach considered appropriate for research purposes for bats.

Total nucleic acids from swabs and urine samples were extracted and complementary DNA was synthesized by using SuperScript III (Invitrogen) according to manufacturer's instructions. A nested RT-PCR and a real-time assay targeting the N gene were used to detect NiV RNA in samples (111). An RT-qPCR designed to detect the nucleocapsid gene of all known NiV isolates was also utilized (112). Oligonucleotide primers and probe were as described (112). Assays were performed by using AgPath-ID One-StepRT-PCR Reagents (Thermofisher) with 250 nM probe, 50 nM forward, and 900 nM reverse primers. Thermal cycling was 45 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 45 s. Cutoff values were cycle threshold (C_T) ≤ 40 for positive and C_T ≥ 45 for negative. Results with C_T values between 40 and 45 were deemed indeterminate, i.e., not conclusively positive or negative. Samples with NiV RNA detected by real-time PCR were confirmed by gel electrophoresis and product sequencing.

A subset of NiV-positive samples was processed by high-throughput sequencing (HTS) on the Ion Torrent PGM platform in order to obtain additional NiV genomic sequence. Libraries were prepared according to the manufacturer's instructions, and 1 million reads were allocated per sample. HTS reads were aligned against host reference databases to remove host background by using the bowtie2 mapper, and host-subtracted reads were primer-trimmed and filtered based on quality, GC content, and sequence complexity. The remaining reads were de novo assembled by using Newbler (Version 2.6) and mapped to the full-length NiV genome. Contigs and unique singletons were also subjected to homology search by using MegaBlast against the GenBank nucleotide database, in case variance in parts of the genome precluded efficient mapping. From these data, N-gene consensus sequences were constructed by using Geneious (Version 7.1) and were used for phylogenetic analyses.

Phylogenetic Analysis. All *P. medius* NiV sequences have been submitted to GenBank, and accession numbers are included in Fig. 6. Sequence alignments were constructed by using ClustalW in Geneious Prime software (113). Phylogenetic trees of NiV N-gene sequences were constructed by using neighbor-joining algorithms, and figures were constructed in FigTree (Version 1.4.2).

Satellite Telemetry and Home-Range Analysis. We developed a collar system to attach 12g solar-powered Platform Terminal Transmitters (PTTs) (Microwave Telemetry) to adult bats using commercial nylon feline collars with the buckle removed and 0-gauge nylon suture to attach the PTT to the collar and to fasten the collar around the bat's neck. Collars were fitted to the bat such that there was enough space to allow for normal neck movement and swallowing, but so that the collar would not slip over the head of the animal (*SI Appendix*, Fig. S6). PTTs were programmed with a duty cycle of 10 h on and 48 h off. Data were accessed via the Argos online data service (argos-system.org). A total of 14 collars were deployed as follows: February 2009: three males and three females from a colony in Shuvarampur, Faridpur district; February 2011: three males and two females from the same colony; and April 2011 Cox's Bazaar, three bats from a colony in Cox's Bazaar, Chattogram district. Bats were selected based on size, such that the total weight of the collar (~21 g) was less than 3% of the bat's body mass (*SI Appendix*, Table S3).

The individual telemetry dataset was combined for each region, and its aggregate utilization distributions (UDs) were computed in R by using package "adehabitatHR" (114). Population-specific home range is represented by the *95% area enclosure of its UD's volume. The volume of intersection between the colonies quantifies the extent of home-range overlap. To evaluate the potential for contact with the Cox's Bazaar colony, we calculated the most likely distance moved ("mldm") for each sampled bat at Faridpur, where the population was more intensively monitored. Movement distance was measured in kilometers with respect to a center location (w) shared by the whole colony. This information was used to predict how likely an animal was to use the landscape.

Statistical Approach–Cross-Sectional Study. We fit a Bayesian generalized linear model with a logit link and a Bernoulli outcome to identify potential predictors which influenced a bat's serostatus. We included age, sex, age- and sex-normalized mass and forearm length, mass:forearm ratio, body condition, and whether the bat was pregnant, lactating, or carrying a pup, using weak zero-centered normal priors for all coefficients. We included location of sampling as a group effect (similar to a random effect in a frequentist context) nested within Nipah Belt or non-Nipah Belt regions. We fit the models and performed posterior predictive checks in R 3.4.3, using the **rstanarm** and **rstan** packages.

Statistical Approach–Longitudinal Study. We fit binomial GAMs (115) to the time series of adult and juvenile seroprevalence in the longitudinal study. We included annual, synchronous birthing that occurred between March and April. We assumed that pups weaned from their dams at 3 mo and became independent flyers, and that maternal antibodies waned after 6 mo, at which point pups transitioned into the "juvenile" class (30, 61). We assumed that juveniles became sexually mature at 24 mo and entered the "adult" class based on other pteropid species (30, 47, 116). For juveniles, we modeled the birth cohort of bats as separate random effects in a pooled model of juvenile seroprevalence starting from June of their birth year, June being the earliest month we sampled free-flying juveniles in any cohort. We determined the cohort year of juveniles by using cluster analysis to group individuals by weight, assuming that those in the smallest group were born in the current year and those in the larger group were born the previous year. Of juveniles captured, 92% were yearlings. For adults, we analyzed seroprevalence of adults as a single pool over the entire course of the study. We tested models with and without annual cyclic effects.

Where time series had significant temporal autocorrelation (adults only), we aggregated data by week. We determined periods of significant increase or decrease as those where the 95% CI of the GAM prediction's derivative did not overlap zero. We fit the models and performed checks in R (Version 3.4.3), using the **mgcv** package.

To examine the importance of different biological mechanisms in transmission, we fit an age-structured (adult and juvenile) maternally immune (M)–susceptible (S)–infected (I)–recovered (R) model with recrudescence (R to I) and loss of immunity (R to S) to the seroprevalence data on a weekly timescale:

$$\frac{dS_J}{dt} = -S_J(\beta_{JJ}I_J + \beta_{AJ}I_A) - \mu_J S_J + bN_A(t-5)\frac{S_A}{N_A} - bN_A(t-52)\frac{S_J}{N_J}(1-\mu_J)^{52} + \lambda M_J$$

$$\frac{dI_J}{dt} = S_J(\beta_{JJ}I_J + \beta_{AJ}I_A) - \gamma I_J - bN_A(t-52)\frac{I_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dR_J}{dt} = \gamma I_J - \mu_J S_J - \mu_J R_J - bN_A(t-52)\frac{R_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dM_J}{dt} = bA(t-5)\frac{R_A}{N_A} - \lambda M_J - \mu_J M_J - bN_A(t-52)\frac{R_A}{N_A}(1-\mu_J)^{52}\lambda^{52}$$

$$\frac{dS_A}{dt} = -S_A(\beta_{AA}I_A + \beta_{JA}I_J) - \mu_A S_A + \tau R_A + bN_A(t-52)\frac{S_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dI_A}{dt} = S_A(\beta_{AA}I_A + \beta_{JA}I_J) - \gamma I_J - \mu_A I_A + bN_A(t-52)\frac{I_J}{N_J}(1-\mu_J)^{52} + \Delta R_A$$

$$\frac{dR_A}{dt} = \gamma I_A - \mu_A R_A + bN_A(t-52)\frac{R_J}{N_J}(1-\mu_J)^{52} - \tau R_A - \Delta R_A$$

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We included a class *M* for the density of juvenile bats with maternal antibodies to allow for the biological possibility that maternal antibodies are lost at a much higher rate than antibodies acquired following infection. The subscripts *J* refer to juveniles and *A* to adults; β is the transmission rate; γ is the recovery rate; μ is the mortality rate; τ is the rate of loss of adult immunity; λ is the rate of loss of maternal antibodies (61); Δ is the adult recrudescence rate (*R* to *I*); and *b* is the birth rate (pups join the juvenile population after 5 wk). Juveniles transition to adults after 52 wk. We included terms for loss of antibody in adults (τ, *S* to *R*) and viral recrudescence (Δ, *R* to *I*) based on previous studies on captive bats that demonstrated the existence of these processes without providing enough data to characterize them precisely (64, 65). We fit this deterministic model to the seroprevalence data by maximum likelihood, which assumes that deviations from the model are due to observation error. We estimated the CIs around maximum-likelihood parameter estimates using likelihood profiles using the *profile* function in package *bbmle* in R (Version 3.2.2).

We used model fitting and model comparison to examine the need for several of the biological processes in the model above that could influence NiV dynamics. First, we examined both density- and frequency-dependent transmission by comparing the fit of the model above to one with transmission terms that have population size (N_A or N_J) in the denominator. Second, we examined the CIs of the parameters describing viral recrudescence, loss of antibodies in adult bats, and loss of antibodies in juvenile bats. If the confidence bounds for these parameters included zero, then these biological processes are not needed to explain the serological dynamics. Finally, we examined the confidence bounds for parameters describing the loss of maternal and nonmaternal antibodies (τ and λ) to determine if the rate of loss of these two types of immunity were different. We note that this model structure has similarities to a susceptible–infected–latently infected (*L*)–infected (SILI) model (if latently infected individuals are seropositive), but the model above differs in allowing for the possibility of individuals to transition from the *R* class back to the *S* class.

Code Availability. SIR model code written in R is available upon request.

Data Availability. All molecular sequences are available via GenBank (GenBank accession nos. MK995284–MK995302). The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. Q:32 Q:33 Q:34 Q:35

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From:	Jon Epstein on behalf of Jon Epstein <epstein@ecohealthalliance.org>
To:	Anthony, Simon J.; Ariful Islam; A. Marm Kilpatrick; Shahneaz Ali Khan; Maria Sanchez Leon; Noam Ross; ina.smith@csiro.au; Carlos M. Zambrana-Torrelio MSc; Yun Tao; Ausraful Islam; Phenix-Lan Quan; Kevin Olival, PhD; Salah Uddin Khan; Emily Gurley; Jahangir Hossain; Hume Field; Fielder, Mark; Thomas Briese; Mahmud Rahman; Christopher Broder; Gary Crameri; Linfa Wang; Stephen Luby; Lipkin, W. I.; Peter Daszak
Cc:	Alison Andre
Subject:	PROOFS: PNAS paper - please respond by Friday 10 AM.
Date:	Wednesday, September 30, 2020 5:42:06 PM
Attachments:	ProofPDF_PNAS_202000429.pdf

All,
Attached are the author proofs for our PNAS paper. Please check that your name and affiliation, including department and postal code, are correctly written.

I'll address the other questions listed.

Please send me any necessary corrections by Friday morning, 10 AM EDT.

Cheers,
Jon

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EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation

Year 5: Task 4. Conduct targeted serological surveys in people and livestock *Description and execution:* As for Y4. *Subtasks*: 5.4.1 Test samples and analyze results *Resources:* As for Y4 *Metrics of success:* Successfully test human serum and livestock samples from South Africa and analyze results *Deliverables:* Obtain serological results from human and livestock serum at bat-human-livestock interphases in South Africa to determine potential spillover.

Year 5: Task 5. Reporting and communication strategy *Description and execution:* As for Y1-5. *Subtasks*: 5.5.1. Discuss results and compile an annual report, including all stakeholders 5.5.2. Develop a communication plan and implement it 5.5.3. Present at scientific conferences and prepare manuscripts for publications 5.5.4. Annual report to governmental partners 5.5.5. Annual report to DTRA. 5.5.6. Attend DTRA technical review. *Resources:* As for Y1-4 *Metrics of success:* All stakeholders partake in analyses and reporting of results and development of a communication strategy *Deliverables:* Final reporting to stakeholders and DTRA, scientific publications and conference contributions, communication to the general public, risk and threat mitigation.

SUMMARY OF PERFORMANCE SCHEDULE

Table: Summary of the tasks that will be performed for the duration of the project (Also in project narrative).

TASK	Y1	Y2	Y3	OY4	OY5	TASK	Y1	Y2	Y3	OY4	OY5
1. Build and enhance capacity in Southern Africa to conduct biosurveillance for filo-, henipa- and coronaviruses						**3. Conduct targeted observational and human behavior studies**					
1.1.Project stakeholder workshop						3.1.Design the study and questionnaires					
1.2.Establishment and sustaining a Southern African Bat Research network (SABRENET)						3.2.Apply for necessary ethical approvals and obtain community permissions					
1.3.Modeling and data analysis workshop						3.3.Collect and analyze data					
1.4.Introduction to bat biology and taxonomy						**4. Conduct targeted serological surveys in people and livestock**					
1.5.Bat field sampling training						4.1.Scoping visits to potential study sites					
1.6.Establishment of Luminex serology technology at UP and NICD						4.2.Apply for necessary permits and ethical approvals					
1.7.Laboratory diagnostic training						4.3.Collection of samples					
1.8.Data interpretation workshop						4.4.Test and analyze data					
2. Implement biosurveillance in bats in Southern Africa and testing of samples						**5. Reporting and Communication**					
2.1.Cross-sectional and longitudinal surveillance in bat species in South Africa						5.1.Discuss results, finalize and compile an annual report including all stakeholders					
2.2 Nucleic acid testing of samples and DNA sequencing of positives.						5.2. Develop a communication plan and implement it					
2.3. Serological testing of serum samples collected from bats and data analysis						5.3. Present at scientific conferences and prepare manuscripts for publication					
2.4. Characterization of additional genes or genome regions of positive samples, verify positive serological results						5.4. Annual report to all governmental partners and stakeholders					
2.5. Enter all data and results in a database and analyze						5.5. Annual report to DTRA					
2.6.Once off surveillance of bats in Mozambique and Zimbabwe						5.6. Attend DTRA technical review					

and analyses of results. Human serum samples will be tested by NICD and livestock at UP. *Subtasks*: 4.4.1 Test samples and analyze results *Resources:* UP: one research scientists, one postdoc, one postgraduate student, and research assistants (testing of samples and analyses of results). NICD: two research scientists, one postgraduate student, and a postdoc (testing of samples and analyzes of results). EHA: two research scientists (analyzes of results). nDOH, RSA (analyzes of results). Department of Agriculture and Rural development Limpopo and KwaZulu Natal, DLRRD (analyses of results). *Metrics of success:* Successfully testing of human and livestock serum samples from South Africa and analyses of results *Deliverables:* Obtain serological results from human and livestock serum at bat-human-livestock interphases in South Africa to determine potential spillover.

Year 4: Task 5. Reporting and communication strategy *Description and execution:* As for Y1-Y3. *Subtasks:* 4.5.1. Discuss results and compile an annual report, including all stakeholders 4.5.2. Develop a communication plan and implement it 4.5.3. Present at scientific conferences and prepare manuscripts for publications 4.5.4. Annual report to governmental partners 4.5.5. Annual report to DTRA. 4.5.6. Attend DTRA technical review. *Resources:* As for Y1-3 with four additional postgraduate students from Moz and Zim included. *Metrics of success:* As for Y1-Y3 with reporting of results for Y4 included. *Deliverables:* Annual reporting to stakeholders and DTRA, scientific publications and conference contributions, communication to the public, risk, and threat mitigation.

Year 5 (Optional): Task 1. Continued: Build and enhance capacity in Southern Africa to conduct biosurveillance for filo-, henipa- and coronaviruses *Description and execution:* As in Y4 but with no new enrollments of students and no field training since all fieldwork activities end in Y4. Diagnostic training will continue. All students will complete degrees in Y5. The stakeholder workshop will be in South Africa (3 days) and will involve finalizing results and reporting, mitigation strategies and policies for threat reduction, finalize a sustainable biosurveillance plan for the region, and preparation of a final report and publications. It will also include a final data interpretation workshop (5 days) in New York, USA. *Subtasks:* 5.1.1. Project stakeholder workshop 5.1.2. Sustain the Southern African Bat Research Network (SABRENET) 5.1.3. Continuous use of the Luminex serology technology at UP and NICD 5.1.4. Laboratory diagnostic training 5.1.5. Data interpretation workshop. 5.1.6. Attend DTRA technical review. *Resources:* As for Y4. *Metrics of success:* As for Y4 with all students completing degrees (additional six students in Y5), final report, and finalization of threat reduction and mitigation strategies. *Deliverables:* Surveillance and communication capacity enhanced with specific results reported, development of risk mitigation strategies and finalization of a biosurveillance plan for the region, completion of training of all partners, completion of student degrees.

Year 5: Task 2. Implement biosurveillance in bats in Southern Africa and testing samples *Description and execution:* No more sampling will be done, sample testing will be completed, including characterization and follow up of serological positives and analyses of results *Subtasks:* 5.2.1 Nucleic acid testing of samples and DNA sequencing of positives 5.2.2. Serological testing of serum samples collected from bats and data analysis 5.2.3 Characterization of additional genes or genome regions of positive samples and verify serological positives 5.2.4. Enter all data and results in a database and analyze. *Resources:* As for Y 4. *Metrics of success:* Complete testing of all samples and analyses of results. *Deliverables*: Completion of all sample testing and analyses of results, data of nucleic acid, and seroprevalence for several bat species in Southern Africa, longitudinal data analyzed together with environmental and ecological factors to determine factors that increase spillover risk.

Year 5: Task 3. Conduct targeted observational and human behavior studies *Description and execution:* As for Y3 and Y4 with only data analyzes to be performed and no additional data collection. *Subtasks:* 5.3.1. Analyses of data collected in South Africa in Y3 and in Moz and Zim in Y4. *Resources*: As for Y 4. *Metrics of success:* Analyzes of all results *Deliverables:* Completed analyses of results and obtain data on risk activities for spillover

publications and conference contributions, communication to the public, risk and mitigation strategies.

Year 4 (Optional): Task 1. Continued: Build and enhance capacity in Southern Africa to conduct biosurveillance for filo-, henipa- and coronaviruses *Description and execution:* As for Y1, Y2, and Y3 with a stakeholder workshop (1 day) using video calling and African partners meeting in South Africa and USA partners dialing in. The data interpretation workshop will be in South Africa (5 days). No more field sampling will take place in South Africa in Y4, but sampling will commence in Zim, and Moz and field training will, therefore, be done at these sites. Diagnostic training visits in South Africa will continue as in previous years. Four additional postgraduate students will be identified from Moz and Zim (biosurveillance and human behavior studies in Moz and Zim). *Subtasks:* 4.1.1. Project stakeholder workshop 4.1.2. Sustain the Southern African Bat Research Network (SABRENET) 4.1.3. Bat field sampling training 4.1.4. Continuous use of the Luminex serology technology at UP and NICD 4.1.5. Laboratory diagnostic training 4.1.6. Data interpretation workshop. *Resources:* As for Y1-Y3 with additional postgraduate students from Moz and Zim enrolling. *Metrics of success:* As for Y1-Y3 with five additional postgraduate students graduating (enrolled in Y2). *Deliverables:* As for Y1-Y3 with additional students graduating, biosurveillance data from Moz and Zim will be included in analysis.

Year 4: Task 2. Implement biosurveillance in bats in Southern Africa and testing samples *Description and execution:* In Y4, sampling will only be done at selected sites in Moz and Zim (twice/year n=2400) and all diagnostic testing will still be done in South Africa. The testing of samples collected in South Africa in Y1-Y3 will be completed. *Subtasks:* 4.2.1. Cross-sectional and longitudinal surveillance in bat species in South Africa (only testing) 4.2.2. Nucleic acid testing of samples and DNA sequencing of positives 4.2.3. Serological testing of serum samples collected from bats and data analysis 4.2.4. Characterization of additional genes or genome regions of positive samples and verify serological positives 4.2.5. Enter all data and results in a database and analyze 4.2.6. Once of surveillance of bats in Mozambique and Zimbabwe. *Resources:* As for Y3 with an additional four post graduate students from Moz and Zim being involved in fieldwork, testing of samples in South Africa, and analyzes of results. *Metrics of success:* Participation in fieldwork and diagnostic training of at least one representative from each partner, supervision of students and two students completing degrees (enrolled in Y3), testing of samples collected in Y1-Y4, DNA sequencing of all positive molecular results and identify samples for further genomic characterization and serological follow up, environmental, ecological and movement data collected, all data entered into the database, analyzed and communicated to the relevant stakeholders. *Deliverables:* Perform all fieldwork collections as specified in the grant schedule, entering all data into the database, testing of samples and analyzing results for Y1-Y4, ecological and movement data on bats, longitudinal data for Y1-Y3.

Year 4: Task 3. Conduct targeted observational and human behavior studies *Description and execution:* In Y4, no additional data will be collected in South Africa, but we will collect data at two sites in Moz and Zim (n=150/site), and data analysis for South Africa will continue. These studies will be coordinated by UP (Markotter and postdoc), EHA (Epstein and Hagan), NICD (Weyer and postdoc) and Ministry of Health of Zim and Moz. *Subtasks:* 4.3.1. Collection and analysis of data at Zim and Moz sites 4.3.2. Analysis of data collected at South African sites. *Resources:* UP: one research scientist and one postdoc (data collection and analysis). NICD: 1 research scientist and a postdoc (data collection and analysis). EHA: two research scientists (data collection and analysis). Ministry of Health Moz and Zim and postgraduate student (data collection and analyses). *Metrics of success:* Obtain 150 completed questionnaires per site and analyze results. *Deliverables:* Completed questionnaires and analyses of results for South Africa, Zim and Moz. Identify high-risk behavior.

Year 4: Task 4. Conduct targeted serological surveys in people and livestock *Description and execution:* No additional samples will be collected, and the focus will be on testing of samples

samples and DNA sequencing of positives 3.2.3. Serological testing of serum samples collected from bats and data analysis 3.2.4. Characterization of additional genes or genome regions of positive samples and verify serological positives 3.2.5. Enter all data and results in a database and analyze 3.2.6. Obtain ethical clearance and permits for sampling in Moz and Zim in Y4. *Resources:* As for Y2 with two additional postgraduate students (UP) enrolling and CVL Moz and Zim coordinating and obtaining permits and ethical clearances for sampling. *Metrics of success:* Participation in fieldwork and diagnostic training of at least one representative from each partner, supervision of students and four students completing degrees, testing of samples collected in Y1, Y2 and Y3, DNA sequencing of all positive molecular results and identify samples for further genomic characterization and serological follow up, environmental, ecological and movement data collected, all data entered into the database, analyzed and communicated to the relevant stakeholders. *Deliverables:* Perform all fieldwork collections as specified in the grant schedule, entering all data into the database, testing of samples and analyzing results for Y1, Y2 and Y3, ecological and movement data on bats, preliminary longitudinal data for sites where sampling started in Y1.

Year 3: Task 3. Conduct targeted observational and human behavior studies *Description and execution:* In Y3, data will be collected at three sites in South Africa (n=150/site) where potential human and livestock contact is a possibility as described in the narrative. These studies will be coordinated by UP (Markotter and postdoc), EHA (Epstein and Hagan), NICD (Weyer and postdoc), nDOH, RSA. We will also obtain ethical clearance for studies to be conducted in Moz and Zim in Y4. *Subtasks:* 3.3.1. Collection and analysis of data at South African sites. 3.3.2. Obtain ethical clearances for studies in Moz and Zim *Resources:* UP: one research scientist and one postdoc (data collection and analysis), NICD: one research scientist, and a postdoc (data collection and analysis). EHA: two research scientists (data collection and analysis). nDOH, RSA (data collection and analysis), Ministry of Health Moz and Zim (ethical clearance for studies in Y4). *Metrics of success:* Obtain 150 completed questionnaires per site, obtain ethical clearances for studies in Y4 in Moz and Zim. *Deliverables:* Completed questionnaires and analyses of results for South Africa and ethical clearances for studies in Moz and Zim. Identify high-risk behavior.

Year 3: Task 4. Conduct targeted serological surveys in people and livestock *Description and execution:* Collect serum samples from humans and livestock at three sites in South Africa [human serum samples (n=150/site) and livestock are 150/samples per site/species for cattle, donkeys, goats, and pigs]. The collection of human serum samples will be done in collaboration with nDOH and livestock in collaboration with the Department of Agriculture and Rural Development of the different provinces. Human serum samples will be tested at NICD and livestock at UP. *Subtasks*: 3.4.1 Collection of samples 3.4.2. Test and analyze samples *Resources:* UP: one research scientists, one postdoc, one postgraduate student (collection and testing of livestock samples). NICD: two research scientists, postdoc, one postgraduate student (sample collection and testing of human serum samples). EHA: two research scientists (analyzes of results). nDOH, RSA (facilitating collection of human serum samples and analyzes of results). Department of Agriculture and Rural Development Limpopo and KwaZulu Natal, state veterinarians (collection of livestock samples and analyzes of results). *Metrics of success:* Successfully collect human serum and livestock samples from all three sites, test and analyze results *Deliverables:* Obtain serological results from human and livestock serum at bat-human-livestock interphases in South Africa to determine potential spillover.

Year 3: Task 5. Reporting and communication strategy *Description and execution:* As for Y1 and Y2. *Subtasks:* 3.5.1. Discuss results and compile an annual report, including all stakeholders 3.5.2. Develop a communication plan and implement it 3.5.3. Present at scientific conferences and prepare manuscripts for publications 3.5.4. Annual report to governmental partners 3.5.5. Annual report to DTRA. 3.5.6. Attend DTRA technical review. *Resources:* As for Y1 and Y2 with two additional postgraduate students (UP) enrolling. *Metrics of success:* As for Y1 and Y2, with reporting of results for Y3 included. *Deliverables:* Annual reporting to stakeholders and DTRA, scientific

Obtain ethical clearance and community permission, design of questionnaires. *Deliverables:* Ethical clearance, design a questionnaire.

Year 2: Task 4. Conduct targeted serological surveys in people and livestock *Description and execution:* Obtain ethical clearance and permits to conduct sampling in Y3 at three sites in South Africa. The collection of human serum samples will be done in collaboration with nDOH and livestock in collaboration with the Department of Agriculture and Rural Development of the different provinces. *Subtasks:* 2.4.1 Scoping visits to potential study sites 2.4.2. Apply for necessary permits and ethical approval. *Resources:* UP: one research scientist and one postdoc (involved in study design and obtaining ethical clearances), NICD: two research scientists and a postdoc (involved in design of the study and obtaining ethical clearances). EHA: two research scientists (assist with planning and development of the study). nDOH, RSA (Involve in planning of the study). Department of Agriculture and Rural Development Limpopo and KwaZulu Natal, state veterinarians (planning of the study and obtaining permits). *Metrics of success:* Obtain ethical clearance and permits *Deliverables:* Ethical clearance and permits.

Year 2: Task 5. Reporting and communication strategy *Description and execution:* As for Y1. *Subtasks:* 2.5.1. Discuss results and compile an annual report, including all stakeholders, 2.5.2. Develop a communication plan and implement it 2.5.3. Present at scientific conferences and prepare manuscripts for publications 2.5.4. Annual report to governmental partners 2.5.5. Annual report to DTRA. 2.5.6. Attend DTRA technical review. *Resources:* UP: one research scientist, three postdocs, eight postgraduate students and four research assistants (data analysis, reporting, and communication of results. NICD: three research scientists, one postdoc (data analysis, reporting, and communication of results). EHA: five research scientists, one postdoc, and one PhD (data analysis, reporting, and communication of results). USU: Two research scientists and three research assistants (data analysis, reporting, and communication of results). AfricanBats: One consultant and one research scientist (data analysis, reporting, and communication of results). DNMNH: One research scientist (data analysis, reporting, and communication of results). nDOH, DEFF, DLRRD, Ministries and CVL, Moz and Zim (data analysis, reporting, and communication of results). *Metrics of success:* All stakeholders partake in analyses and reporting of results (Y1 and Y2), development of a communication strategy and implementation, risk and mitigation strategies. *Deliverables:* Annual reporting to stakeholders and DTRA, scientific publications and conference contributions, communication to the public, risk and mitigation strategies.

Year 3: Task 1. Continued: Build and enhance capacity in Southern Africa to conduct biosurveillance for filo-, henipa- and coronaviruses *Description and execution:* As in Y2 except for the stakeholder workshop (3 days) and data interpretation workshop that will be in New York, USA (5 days). Enrollment of two additional students (MSc/PhD) focusing on human behavioral studies and human and livestock serology. *Subtasks:* 3.1.1. Project stakeholder workshop 3.1.2. Sustain the Southern African Bat Research Network (SABRENET) 3.1.3. Bat field sampling training, 3.1.4. Continuous use of the Luminex serology technology at UP and NICD 3.1.5. Laboratory diagnostic training 3.1.6. Data interpretation workshop. *Resources:* As for Y2 with two additional postgraduate students (UP). *Metrics of success:* As for Y2 with four postgraduate students completing degrees (enrolled in Y1) and two new students enrolled. *Deliverables:* Surveillance and communication capacity enhanced with specific results reported, development of risk mitigation strategies and improvement on a draft biosurveillance plan for the region, training individuals of all partners, continuous diagnostic capacity at two South African laboratories, student training and completion of student degrees, sustain the regional network.

Year 3: Task 2. Implement biosurveillance in bats in Southern Africa and testing of samples Biosurveillance activities for South Africa as for Y2 (n=7200). Planning, obtaining ethical clearance, and permits for sampling in MOZ and Zim in Y4 will commence. *Subtasks:* 3.2.1. Cross-sectional and longitudinal surveillance in bat species in South Africa 3.2.2. Nucleic acid testing of

capacity in two South African laboratories, perform training, generation of Luminex reagents, attend data interpretation workshop). AfricanBats: one consultant (attend workshops, present fieldwork training). DNMNH: one research scientist (attend workshops, present fieldwork training). DALLRD, nDOH, DEFF, CVL and Ministries of Health, Moz and Zim: (Attend all workshops and training, analyzes of results). *Metrics of success:* Present all training and workshops as indicated in the grant schedule, training of students, fellows, researchers, and technologists representative of all partners, enrollment, and supervision of postgraduate students, sustain SABRENET, support Luminex technology and continuous supply of reagents to UP and NICD. *Deliverables:* Surveillance, reporting, and communication capacity enhanced by attendance of workshop and training by all partners and reporting of results, sustain a regional research network, serological diagnostic capacity continued in two South African laboratories and continuous support, enrollment, and supervision of students.

Year 2: Task 2. Implement biosurveillance in bats in Southern Africa and testing of samples As for Y1 with the addition of two sites (Black Rock and Table Mountain) for South Africa (n=7200). Sample testing will also commence in the NICD laboratory in Y2. DNA sequencing, NGS characterization of positive samples (UP and NICD) as well as follow up serological testing (NICD) will be performed. *Subtasks:* 2.2.1. Cross-sectional and longitudinal surveillance in bat species in South Africa, 2.2.2. Nucleic acid testing of samples and DNA sequencing of positives 2.2.3. Serological testing of serum samples collected from bats and data analysis 2.2.4. Characterization of additional genes or genome regions of positive samples and verify serological positives 2.2.5. Enter all data and results into a database. *Resources:* UP: one research scientist, three postdocs, four research assistants, eight postgraduate students (See task 1) (conduct fieldwork, laboratory diagnostic testing, enter all data into a database, analyze results). NICD: two research scientists, one postdoc (conduct fieldwork and build diagnostic capacity). EHA: two research scientists, one postdoc, one PhD student (perform limited fieldwork, movement tracking, disease ecology). USU: one research scientist (establish diagnostic Luminex capacity and analyze results), three research assistants (assist with training, analyses of results and generation of Luminex reagents for testing). AfricanBats: one consultant and one research scientist (fieldwork, specifically the *Miniopterus* studies and movement tracking). DNMNH: one research scientist (fieldwork). Department of Agriculture and Rural Development Limpopo: state veterinarians (assist in sample collection). nDOH, DLRRD, DEFF, Moz and Zim partners (attend field and diagnostic training (task 1). *Metrics of success:* Participation in fieldwork and diagnostic training of at least one representative from each partner, supervision of students, testing of samples collected in Y1 and Y2, DNA sequencing of all positive molecular results and identify samples for further genomic characterization and serological follow up, environmental, ecological and movement data collected, all data entered into the database, analyzed and communicated to the relevant stakeholders. *Deliverables:* Perform all fieldwork collections as specified in the grant schedule, entering all data into the database, testing of samples and analyzing results for Y1 and Y2, ecological and movement data on bats.

Year 2: Task 3. Conduct targeted observational and human behavior studies *Description and execution:* In Y1, only training on conducting human behavior was conducted. In Y2 planning, design of questionnaires and obtaining ethical clearance and community permissions will commence for three sites in South Africa where potential human and livestock contact is a possibility as described in the narrative. These studies will be coordinated by UP (Markotter and postdoc), EHA (Epstein and Hagan), NICD (Weyer and postdoc), nDOH, RSA. *Subtasks:* 2.3.1. Design the study and questionnaires 2.3.2. Apply for necessary ethical approvals and obtain community permissions. *Resources:* UP: one research scientist and one postdoc (study design and developing of questionnaires). NICD: two research scientists and a postdoc (design of the study and questionnaires, obtaining ethical clearances). EHA: two research scientists (assist with planning and development of questionnaires). nDOH, RSA (Involve in planning and design of questionnaires). *Metrics of success:*

of samples in collaboration with nDOH and the Department of Agriculture and Rural Development of the different provinces. Testing of human serum samples with the Luminex will be done at NICD and livestock samples at UP. *Subtasks:* This activity will only start in Y2.

Year 1: Task 5. Reporting and communication *Description and execution:* The grantee will synthesize all data collected and analyses of results, including outputs on capacity building activities. This

will be discussed at the annual stakeholder workshop and finalized (task 1). A final report will be prepared for all partners and an annual report to DTRA. Results will also be communicated to all relevant governmental agencies, the public and relevant communities using different forums. Scientific results will be published and presented at conferences and scientific meetings. *Subtasks:* 1.5.1. Discuss results and compile an annual report including all stakeholders 1.5.2. Develop a communication plan and implement it 1.5.3. Present at scientific conferences and prepare manuscripts for publications 1.5.4. Annual report to governmental partners 1.5.5. Annual report to DTRA. 1.5.6. Attend DTRA technical review. *Resources:* UP: one research scientists, three postdocs, four post graduate students and four research assistants to be involved in data analysis, reporting and communication of results. NICD: three research scientists to be involved in data analysis, reporting, and communication of results. EHA: five research scientists to be involved in data analysis, reporting, and communication of results. USU: Two research scientists and three research assistants to be involved in data analysis, reporting, and communication of results. AfricanBats: One consultant and one research scientist to be involved in data analysis, reporting, and communication of results. DNMNH: one research scientists to be involved in data analysis, reporting, and communication of results. nDOH, DEFF, DLRRD, Ministries and CVL laboratories in Moz and Zim (to be involved in data analysis, reporting, and communication of results). *Metrics of success:* All stakeholders partake in analyses and reporting of results (Y1), development and implementation of a communication strategy, risk and mitigations strategies. *Deliverables:* Annual reporting to stakeholders and DTRA, scientific publications and conference contributions, communication to the public, risk and mitigation strategies.

Year 2: Task 1. Continued: Build and enhance capacity in Southern Africa to conduct biosurveillance for filo-, henipa- and coronaviruses *Description and execution*: No additional training workshops are scheduled for Y2, except a stakeholder and data interpretation workshop. The stakeholder workshop will be held using video conferencing with all the African partners meeting in South Africa and interacting with USU and EHA in New York and Washington, respectively (1 day). It will focus on sharing results and progress, planning of activities for the year, reporting, identifying trainees and students, develop mitigation and threat reduction strategies, and development of a draft plan for sustainability of biosurveillance in the region. A data interpretation workshop is planned in South Africa (5 days), and training at field sites and diagnostic laboratories will continue as in Y1. An additional PhD student (disease modeling, EHA), two MSc/PhD and two honors students [corona and paramyxovirus surveillance (UP)] will be identified. USU will continue to be involved in training, troubleshooting and data analysis of the Luminex technology and will continue to supply reagents. *Subtasks*: 2.1.1. Project stakeholder workshop 2.1.2. Sustain the Southern African Bat Research Network (SABRENET) 2.1.3. Bat field sampling training 2.1.4. Continuous use of the Luminex serology technology at UP and NICD 2.1.5. Laboratory diagnostic training 2.1.6. Data interpretation workshop. *Resources:* UP: one research scientists and three postdocs (attend workshops, conduct field, and laboratory diagnostic training and facilitate diagnostic capacity development and testing, attend data interpretation workshop), eight postgraduate students (attend training activities). NICD: two research scientists, one postdoc (attend workshops, establish diagnostic capacity and facilitate diagnostic training). EHA: five research scientists, one postdoc (attend workshops, facilitate disease modelling, training in fieldwork, sustain regional networks, attend data interpretation workshop), USU: one research scientist and three research assistants (attend workshops, support Luminex

the following; *Rousettus aegyptiacus* (n=7040), *Miniopterus* spp. (n=8480) and mollosids and *Rhinolophus* spp. (n=5600) with a total of 21 120 bats sampled over five years and the total per year varies, depending on the sample sites included. Y1 will include Maplapitsi, Madimatle and *Miniopterus* sites (n=4320) in South Africa including RFID and radio tracking technologies to investigate bat movement. Samples will be collected in duplicate wth one sample set pulled for testing and another kept for future follow up. Inactivated samples will be transported to UP and tested using molecular and serological methods (postdocs, research assistants). NICD will also assist with sample testing from Y2 onwards when technologies have been established. Amendments to current ethics and DAFF section 20 approvals will be made in Y1, and current permits will be extended (UP, Markotter; DLRRD) *Subtasks:* 1.2.1. Cross-sectional and longitudinal surveillance in bat species in South Africa 1.2.2. Nucleic acid testing of samples and DNA sequencing of positives 1.2.3. Serological testing of serum samples collected from bats and data analysis 1.2.4. Characterization of additional genes or genome regions of positive samples and verify serological positives 1.2.5. Enter all data and results in a database and analyze. *Resources:* UP: one research scientists, three postdocs, four research assistants, four postgraduate students (See task 1) (conduct fieldwork, laboratory diagnostic testing, enter all data into a database, analyze results), NICD: two research scientists (conduct fieldwork and build diagnostic capacity). EHA: two research scientists (perform limited fieldwork, movement tracking), USU: one research scientists (establish diagnostic Luminex capacity and analyze results), three research assistants (assist with training, analyses of results and generation of Luminex reagents for testing). AfricanBats: one consultant and one research scientist (fieldwork, specifically the *Miniopterus* studies and movement tracking). DNMNH: one research scientist (fieldwork). Department of Agriculture and Rural Development Limpopo: state veterinarians (assist in sample collection). nDOH, DLRRD, DEFF, Moz and Zim partners (attend field and diagnostic training (task 1). *Metrics of success:* Participation in fieldwork and diagnostic training of at least one representative from each partner, supervision of students, testing of samples collected in Y1, DNA sequencing of all positive molecular results and identify samples for further genomic characterization and serological follow up, environmental, ecological and movement data collected, all data entered into the database, analyzed and communicated to the relevant stakeholders. *Deliverables:* Perform all fieldwork collections as specified in the grant schedule, entering all data into the database, testing of samples and analyzing results for Y1, preliminary ecological and movement data on bat species that can inform subsequent movement studies for Y2 and Y3.

Year 1: Task 3. Conduct targeted observational and human behavior studies *Description and execution:* In Y1, only training on how to conduct human behavior studies will be presented as part of the annual stakeholder workshop (See task 1). Y2 will be used for planning and obtaining ethical and community permissions, Y 3 for data collection in RSA and planning and ethical clearance for studies in Zim and Moz, Y4 for Moz, and Zim data collection and Y5 for analysis. Human behavior, KAP analysis, will be implemented at three sites in South Africa in Y3 where potential human and livestock contact is a possibility as described in the narrative. It will include serum collection at South African sites and only questionnaire-based human studies in Moz and Zim. These studies will be coordinated by UP (Markotter), EHA (Epstein and Hagan), NICD (Weyer), nDOH and Ministries of Health in Moz and Zim. *Subtasks:* 1.3.1 Training to do human behavioral studies. *Resources:* UP: one research scientist and one postdoc (Attend training). NICD: two research scientists (attend training), EHA: two research scientists (present training). nDOH, Ministries of Health Moz, and Zim (attend training). *Metrics of success:* Build capacity to perform human behavioral studies *Deliverables:* Build capacity to perform human behavioral studies.

Year 1: Task 4. Conduct targeted serological surveys in people and livestock *Description and execution:* Human and livestock samples, including cattle, goats, pigs, and donkeys (n=150/species/site), will be collected in Y3 at South African sites only. Sampling more than 100 animals/species/site will allow us to detect seropositivity with a 95% confidence at a seroprevalence of 3% assuming populations of 500. UP (Markotter) and NICD (Weyer) will coordinate the collection

insectivorous bats. *Subtasks:* 1.1.1. Project stakeholder workshop1.1.2. Establishment of a Southern African Bat Research Network (SABRENET) 1.1.3. Modeling and data analysis workshop 1.1.4. Introduction to bat biology and taxonomy 1.1.5. Bat field sampling training 1.1.6. Establishment of Luminex serology technology at UP and NICD 1.1.7. Laboratory diagnostic training 1.1.8. Data interpretation workshop. *Resources:* UP: one research scientist and three postdocs (attend workshops, conduct field and laboratory diagnostic training and facilitate diagnostic capacity development and testing, attend data interpretation workshop), one administrative assistant, (coordinate project including financial and grant management and communication between partners), four postgraduate students (attend training activities). NICD: two research scientists (attend workshops, establish diagnostic capacity and facilitate diagnostic training). EHA: five research scientists (attend workshops, facilitate training in disease modeling, risk modeling, human behavior and communication, fieldwork and development of SOPs, establishment of SABRENET, attend data interpretation workshop), one part-time administrative assistant (facilitate communication and management between the Co-PIs and partners). USU: one research scientist and three research assistants (attend workshops, establish Luminex capacity in two South African laboratories, perform training, generation of Luminex reagents, attend data interpretation workshop). AfricanBats: one consultant (attend workshops, present bat biology and taxonomy, and fieldwork training). DNMNH: one research scientist (attend workshops, present bat biology, taxonomy, and fieldwork training). DALLRD, nDOH, DEFF, CVL and Ministries of Health, Moz and Zim: (Attend all workshops and training). *Metrics of success:* Present all training and workshops as indicated in the grant schedule, development of standardized SOPs, standardized reporting and communication strategies, training of students, fellows, researchers and technologists representative of all partners, enrollment of postgraduate students, establishment of SABRENET, establishment of Luminex technology and supply of reagents to UP and NICD. *Deliverables:* Surveillance, reporting and communication capacity enhanced by attendance of workshop and training by all partners, establishment of a regional research network, serological diagnostic capacity established in two South African laboratories, enrollment of students

Year 1: Task 2. Implement biosurveillance in bats in Southern Africa and testing of samples *Description and execution:* Biosurveillance activities will be focused in South Africa (Y1-Y3) with limited fieldwork collections in Moz and Zim (Y4). Cross-sectional studies will focus on *Rousettus*, *Rhinolophus*, *Miniopterus* and Molossid bat species at several sites in South Africa [Matlapitsi (Y1-3), Madimatle (Y1-3), Table mountain (Y2-3), Black Rock (Y2-3), *Miniopterus* roosts (Y1-3)] to determine the nucleic acid and seroprevalence of filo, henipa- and potentially zoonotic coronaviruses. Sampling in bats will be non-destructive where blood, swabs (oral and rectal) and urine samples will be collected. In addition, we will implement longitudinal surveillance in both *Rousettus* and *Miniopterus* spp. (implicated as filo- and henipavirus hosts), including during birthing periods to investigate seasonality of virus shedding. Once-off sampling in Moz and Zim will focus on the cave-dwelling *R. aegyptiacus* and insectivorous bats co-roosting, including know roosts such as Chinoyi caves. Environmental (weather, rainfall and humidity) and ecological data of the bats (age, reproductive status, sex and measurements) will be collected, as well as population size estimates. We will employ marking and tracking technologies such as tattooing, pit tags (RFID), and telemetry systems to monitor virological status in individuals recaptured over time but also to determine foraging patterns (home ranges, distances traveled and habitat selection) and estimate population sizes. Fieldwork activities will be coordinated by UP (Markotter and postdocs) in collaboration with EHA (Epstein and Phelps), NICD (Weyer), AfricanBats (Seamark and Keith), DNMNH (Kearney) and Department of Agriculture and Rural Development of the relevant provinces (State veterinarians). Ecological and environmental data will also be collected. Testing for viral antibodies and nucleic acids from all bats samples collected will be performed at UP and NICD, RSA, including follow up DNA Sanger sequencing of all positive amplicons and bioinformatics analysis and follow up of serological positives. Detailed sample numbers are indicated in the project narrative but include

Year 5 (optional): Task 1. Continued: Build and enhance capacity in Southern Africa to conduct biosurveillance for filo-, henipa- and coronaviruses – Stakeholder workshop, data interpretation workshop, and diagnostic training, Sustain SABRENET. **Task 2.** Continued: Implement biosurveillance in bats in Southern Africa and testing of samples – No more sampling, complete testing of samples **Task 3.** Continued: Conduct targeted observational and human behavior studies – No more data collection, only analyze data **Task 4.** Conduct targeted serological surveys in people and livestock –No more sampling, only testing of samples **Task 5.** Continued: Reporting and Communication – All reporting, develop a communication plan and implement, publications

DETAILED TASKS

Year 1: Task 1. Build and enhance capacity in Southern Africa to conduct biosurveillance for filo-, henipa- and coronaviruses *Description and execution:* Capacity building will focus on South Africa, Mozambique, and Zimbabwe and will follow a One Health approach. It will include development of effective surveillance strategies, developing standard operating protocols, training in biosafety, data analysis, basic epidemiological principles, geographic information systems (GIS), disease modeling, design of human behavioral studies, effective reporting and communication, bat biology and taxonomy and continuous field and laboratory diagnostic training. Analysis of results, reporting, and development of mitigation and threat reduction strategies will involve all stakeholders. Training workshops will be in Y1 and coordinated by UP (Markotter) and EcoHealth Alliance (EHA) (Epstein), after which annual practical training in fieldwork (Y1-Y4), diagnostics (Y1-Y5) and data analysis (Y1-Y5) will continue. Trainees representing animal, human, environmental health and academia will be selected from each partner and country and identified at annual stakeholder workshops. In Y1, a stakeholder workshop (3 days) will be held in Pretoria, RSA, chaired by Markotter (UP) and Epstein (EHA). At least one representative of each partner will attend. This workshop will introduce the projects, roles, responsibilities, timelines, finalize and plan surveillance strategies, development and agreement of standard operating protocols and decide on a standardized reporting policy. General training on biosafety, including sample shipment, human behavioral studies and communication strategies (UP (Markotter; EHA, Epstein and Hagan) will be presented to the larger group after which specific workshops on modeling, data analysis (2 days) (EHA, Olival, Ross) and bat biology and taxonomy (4 days) (AfricanBats, Seamark, DNMNH (Kearney) will commence. Bat field sampling (UP, Markotter and postdocs; EHA, Phelps) and diagnostic training (UP, Markotter and postdocs; NICD, Weyer and Moolla) will be scheduled throughout Y1 with trainees joining field visits (See task 2) and training visits to diagnostic laboratories, UP and NICD (21 days/visit). This will include individuals of the Central Veterinary Laboratories (CVL) from Mozambique (Moz) and Zimbabwe (Zim); postgraduate students involved in research projects and well as technical staff of the partners. A data interpretation workshop is scheduled annually (Y1 in New York, USA for five days). The Co-PIs (Markotter, Epstein), Uniformed University Services (USU, Laing) and postdocs (UP, NICD and EHA) will do in-depth data analysis that can be presented and discussed at stakeholder workshops. Diagnostic technologies to detect filo-, henipa- and coronaviruses for the region at central facilities in South Africa (UP and NICD) will be established. Since molecular technologies are already well established, the focus will be on building serological capacity specifically Luminex technology, in collaboration with USU. USU will present on-site training in South Africa (after the stakeholder workshop), provide reagents (Y1-Y5), and data analysis support (Laing, research assistants x 3). Reagents will be expressed proteins to detect the diversity of filo and paramyxoviruses and positive control antibodies. UP has access to a Bioplex system, but an additional instrument will be purchased in Y1 to accommodate the high throughput of samples collected and tested in this project. Four postgraduate students (PhD/MSc) will be identified in Y1 that will be co-supervised by Markotter (UP), Epstein (EHA), Liang (USU) and NICD (Weyer) respectively (Y1-Y3). Student projects will focus on Luminex filovirus serology, filovirus nucleic acid detection, and movement tracking of frugivorous and

sampling, viral detection techniques, developing standardized protocols, ecological and environmental data collection and observational and human behavior studies.
2) Establishment of serological and nucleic acid detection platforms in key partner laboratories in human and animal health in South Africa that will sustainably build and enhance regional diagnostic capacity and will generate comparable data across the region.
3) Targeted serological and molecular surveillance and screening of samples in bats, as well as additional serological surveillance in potential spillover hosts (livestock and humans) at selected bat-human-animal interphases.
4) Assessing human behavioral risk for exposure to these agents at selected bat-human-animal interphases.
5) Multi-stakeholder workshops to develop a sustainable regional biosurveillance strategy, analyze results and develop mitigation strategies and threat reduction policies for the region.

DETAILED DESCRIPTION OF TASKS

SUMMARY

Year 1: **Task 1.** Build and enhance capacity in Southern Africa to conduct biosurveillance for filo-, henipa- and coronaviruses – Stakeholder workshop and training, data interpretation workshops, the establishment of SABRENET, transfer Luminex technology. **Task 2.** Implement biosurveillance in bats in Southern Africa and testing of samples – Cross-sectional and longitudinal at three sites in South Africa. **Task 3.** Conduct targeted observational and human behavior studies – Only theoretical training in Y1. **Task 4.** Conduct targeted serological surveys in people and livestock – Only start in year 2. **Task 5.** Reporting and Communication – All reporting, develop a communication plan and implement, publications

Year 2: **Task 1.** Continued: Build and enhance capacity in Southern Africa to conduct biosurveillance for filo-, henipa- and coronaviruses – Stakeholder workshop, data interpretation workshop, field sampling, and diagnostic training, Sustain SABRENET. **Task 2.** Continued**:** Implement biosurveillance in bats in Southern Africa and testing of samples - Cross-sectional and longitudinal at five sites in South Africa. **Task 3.** Continued**:** Conduct targeted observational and human behavior studies – Design and plan South African studies, ethical clearances. **Task 4.** Conduct targeted serological surveys in people and livestock – Site visits, planning, and ethical clearances, Only in South Africa. **Task 5.** Continued**:** Reporting and Communication – All reporting, develop a communication plan and implement, publications

Year 3: **Task 1.** Continued: Build and enhance capacity in Southern Africa to conduct biosurveillance for filo-, henipa- and coronaviruses – Stakeholder workshop, data interpretation workshop, field sampling, and diagnostic training, Sustain SABRENET. **Task 2.** Continued: Implement biosurveillance in bats in Southern Africa and testing of samples - Cross-sectional and longitudinal at five sites in South Africa. **Task 3.** Continued: Conduct targeted observational and human behavior studies – Collect data in South Africa, plan studies and obtain ethical clearances for Mozambique and Zimbabwe. **Task 4.** Conduct targeted serological surveys in people and livestock in South Africa. **Task 5.** Continued: Reporting and Communication – All reporting, develop a communication plan and implement, publications

Year 4 (optional): **Task 1.** Continued: Build and enhance capacity in Southern Africa to conduct biosurveillance for filo-, henipa- and coronaviruses – Stakeholder workshop, data interpretation workshop, field sampling, and diagnostic training, Sustain SABRENET. **Task 2.** Continued: Implement biosurveillance in bats in Southern Africa and testing of samples - Cross-sectional and longitudinal at two sites in Mozambique and Zimbabwe, testing of all samples. **Task 3.** Continued: Conduct targeted observational and human behavior studies – Analyze data from South African sites and collect data from Mozambique and Zimbabwe **Task 4.** Conduct targeted serological surveys in people and livestock –Test samples and analyze data **Task 5.** Continued: Reporting and Communication – All reporting, develop a communication plan and implement, publications

HDTRA1-14-24-FRCWMD-BAA,
CBEP-Thrust Area 6 – Cooperative Counter WMD Research with Global Partners

Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa

STATEMENT OF WORK

BACKGROUND

Over the past four decades, approximately 75% of important emerging and re-emerging diseases were of zoonotic origin and most of these originating from Africa (Chan et al., 2010) and from a wildlife origin (Daszak et al., 2000, Olival et al., 2017). This includes paramyxoviruses in the genus *Henipavirus* such as Hendra (HeV) and Nipah virus (NiV), filoviruses that include Ebola (EBOV) and Marburg virus (MARV), as well as coronaviruses like severe acute respiratory syndrome coronavirus (SARS-CoV), all select agents, causing outbreaks with high mortality in humans. Surveillance data for the Southern African region has been limited, with mostly opportunistic, once-off sampling events focused on restricted geographical regions and selected bat species (Geldenhuys et al., 2013; Ithete et al., 2013; Cronje 2017; Bourgarel et al., 2017; Paweska et al., 2018; Mortlock et al., 2019). Most of these studies were conducted by the University of Pretoria (UP) and collaborators with select agents in bats comprising only a small part of these projects. Also, this surveillance only included molecular detection of pathogens and very limited serological investigations in bat species and none in potential spillover hosts. Preliminary studies indicate that seasonal reproduction of *R. aegyptiacus* drives viral infection dynamics, and that gradual loss of passive immunity among juveniles in this bat population increases the number of susceptible individuals, creating favorable conditions for viral spread. It also results in changes in virus shedding and seroconversion patterns, which are driven by sex, age and reproductive condition of female bats. To understand infection dynamics, longitudinal studies are needed. Southern Africa possesses a rich diversity of bat fauna, including both frugivorous and insectivorous bat species (Monadjem et al., 2010) and this includes *Rousettus*, *Rhinolophus* and *Miniopterus* spp. as well as several free-tail bat species (Mollosidae) implicated as potential hosts for high consequence pathogens in other studies. Livestock such as cattle, goats, and pigs, are also present throughout Southern Africa and overlaps with bat diversity hotspots (Robinson et al., 2014; StatsSA2016). People with frequent close contact with bats due to religious, cultural or sociological reasons (Markotter, personal communication) also inhabit the same areas. Understanding contact between bats, humans and other animals, including behavior analysis, is needed to understand risk in areas where these viral pathogens are present. South Africa plays a prominent role in the Southern African region with laboratory capacity that can be expanded and shared to strengthen sustainable biosurveillance in the region. These pathogens can easily affect any country and therefore biosurveillance capacity must include regional networks and engagement with neighboring countries. This project will strengthen South Africa's, and the region's, capacity to develop and implement surveillance strategies with appropriate biosafety and biosecurity principles, detect pathogens (both molecular and serological) in bats before outbreaks occur, recognize and diagnose outbreaks if they do occur and develop appropriate reporting strategies for the region. All this information can be used collectively to assess risk, predict potential outbreaks, and develop risk mitigation strategies that can be used throughout the region.

OBJECTIVES AND SCOPE

The overall objective is to detect filo-, henipa- and zoonotic coronaviruses in bat populations in Southern Africa and evidence of spillover in humans and livestock.
Specific objectives to achieve this are:
1) Regional training and establishment of networks in Southern Africa [Southern Africa Bat Research Network (SABRENET)] focused on biosafety, bat capturing techniques, bat taxonomy, virological

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CBEP-Thrust Area 6 – Cooperative Counter WMD Research with Global Partners
Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa

TECHNICAL PROPOSAL

I. ABSTRACT

Bat species, which are widespread in the Southern African region and beyond, have been identified as hosts of a number of viral agents, including filo- and viruses related to the Middle East respiratory syndrome (MERS) corona- and paramyxo- such as the henipaviruses. These viruses have proven to cause substantial human morbidity and mortality, threaten livestock and wildlife health and have pandemic potential. However, their distribution and potential to infect people and livestock in Southern Africa are unknown. This project will focus on building capacity and enhancing surveillance, including early detection programs for henipa-, filo- and zoonotic coronaviruses in Southern Africa. The project will specifically address the following: 1) regional training, including biosafety, bat capturing techniques, clinical sampling, viral detection and serological techniques, developing standardized protocols, ecological data collection and human behavioral studies; 2) establishment of serological and nucleic acid detection technologies in South Africa to develop sustainable regional diagnostic capacity that can be shared; 3) targeted serological and molecular surveillance in bats as well as serological surveillance in potential spillover hosts (livestock and humans); 4) assessing human behavioral risk for exposure to these agents; and 5) multi-stakeholder workshops to develop a sustainable regional biosurveillance strategy, analyze results and develop mitigation strategies and threat reduction policies for the region. Planning, training, execution and reporting will use an interdisciplinary One Health approach involving local government agencies of the respective countries including the Departments of Health, Agriculture, Environment, Veterinary and Wildlife Services to ensure long-term sustainability and stakeholder preparedness. The proposed project closely aligns with the aims of the Cooperative Biological Engagement Program with regards to support for biosurveillance, capacity building and threat reduction.

II. SCOPE

A. OBJECTIVE:

The overall objective is to detect filo-, henipa- and zoonotic coronaviruses in bat populations in Southern Africa and evidence of spillover in humans and livestock.
Specific objectives to achieve this are:
1) Regional training and establishment of networks in Southern Africa [Southern Africa Bat Research Network (SABRENET)] focused on biosafety, bat capturing techniques, bat taxonomy, virological sampling, viral detection techniques, developing standardized protocols, ecological and environmental data collection and observational and human behaviour studies.
2) Establishment of serological and nucleic acid detection platforms in key partner laboratories in human and animal health in South Africa that will sustainably build and enhance regional diagnostic capacity and will generate comparable data across the region.
3) Targeted serological and molecular surveillance and screening of samples in bats, as well as additional serological surveillance in potential spillover hosts (livestock and humans) at selected bat-human-animal interphases.
4) Assessing human behavioral risk for exposure to these agents at selected bat-human-animal interphases.
5) Multi-stakeholder workshops to develop a sustainable regional biosurveillance strategy, analyze results and develop mitigation strategies and threat reduction policies for the region.
We propose to test the following **hypothesis:** A diversity of potentially zoonotic henipa-, filo- and coronaviruses circulate in bats in South Africa and neighboring Mozambique and Zimbabwe, and may previously have infected livestock and people in areas where interactions between bats and such

hosts occur. Enhancing biosurveillance and building regional capacity will reduce biothreats by improving the ability to detect, diagnose and report emerging and remerging pathogens of biosecurity concern.

B. BACKGROUND:

Over the past four decades, approximately 75% of important emerging and re-emerging diseases were of zoonotic origin and most of these originating from Africa (Chan et al., 2010) and from a wildlife origin (Daszak et al., 2000; Olival et al., 2017). This includes paramyxoviruses in the genus *Henipavirus* such as Hendra (HeV) and Nipah virus (NiV), filoviruses that include Ebola (EBOV) and Marburg virus (MARV), as well as coronaviruses like severe acute respiratory syndrome coronavirus (SARS-CoV), all select agents, causing outbreaks with high mortality in humans. Fatal zoonoses have significant financial implications, with the World Bank estimating economic losses between 1997 to 2009 of US$80 billion, and that US$6.7 billion per year could be saved globally by preventing emerging disease outbreaks (Mazet et al., 2015). International agencies such as CDC, USDA, WHO and OIE have identified zoonotic diseases as a threat to global health security with pathogens not adhering to international borders and the ability to spread extensively and rapidly.

Filoviruses: The family *Filoviridae* are negative-sense, single-stranded RNA viruses represented by six genera of which two, *Ebolavirus* and *Marburgvirus* (Kuhn et al., 2019), cause fatal hemorrhagic fever outbreaks in humans. The diversity of filoviruses have also expanded in recent years with several novel viral detections in bats including Lloviu virus (LLOV) in Schreibers' long-fingered bats (*Miniopterus cf schreibersii*) in Spain and Hungary (Kemenesi et al., 2018), Mengla virus from China in rousette fruit bats (*Rousettus* spp.) and cave nectar bats (*Eonycteris* spp.) (Yang et al., 2019), as well as Bombali virus from Sierra Leone and Kenya associated with molossid bats [Angolan free-tailed bat (*Mops condylurus*) and the little free-tailed bat (*Chaerephon pumilus*)] (Goldstein et al., 2018; Forbes *et al.*, 2019). MARV was first identified in 1967 in green monkeys imported into Europe from Uganda after laboratory workers became infected (Olival and Hayman, 2014) and has since been detected and isolated in several African countries. Substantial evidence now exists that the Egyptian rousette fruit bat (*Rousettus aegyptiacus*) is a reservoir of MARV (Towner et al., 2009; Amman et al., 2012), and this species is widespread throughout Africa and into the Middle East. Historically, detections have been from central and eastern Africa, but recently MARV was also detected in *R. aegyptiacus* in the northern regions of South Africa (Paweska et al., 2018) and Sierra Leone. Detections have been opportunistic and sporadic, and not always corresponding to where human outbreaks have been identified or representative of the geographic range of the reservoir, which highlights a lack of surveillance. Longitudinal studies identified distinct viral pulses in juvenile bats that corresponded to the timing of human outbreaks, implicating birthing pulses as a driver of infection (Amman et al., 2012). Experimental infection studies of MARV virus in captive-bred Egyptian fruit bats reported viral RNA in oral and vaginal secretions, as well as excreta implicating these as potential routes of transmission (Swanepoel et al., 1996; Leroy et al., 2005; Amman et al., 2015). The first *Ebolavirus* spp. was discovered in 1976 and the genus now consists of six species: *Zaire ebolavirus*, Ebola virus (EBOV), *Bundibugyo ebolavirus*, Bundibugyo virus (BDBV), *Sudan ebolavirus*, Sudan virus (SUDV), *Tai Forest ebolavirus*, Taï Forest virus (TAFV), *Bombali ebolavirus*, Bombali virus (BOMV) and *Reston ebolavirus*, Reston virus (RESTV) (Kuhn et al., 2019) with potentially unique ecological niches. More than 25 human outbreaks have occurred with the most significant being the 2013-2016 outbreak in Guinea, Sierra Leone and Liberia where more than 11000 people died (Spengler et al., 2016). Index cases were reported to have had contact with dead wildlife including non-human primates and antelope, however, these species are not considered viral reservoirs since they also succumbed to the disease. Viral RNA for EBOV has only been detected in bat species with distributions restricted to West Africa and not occurring in Southern Africa, including the hammer-headed bat *(Hypsignathus monstrosus)*, Franquet's epauletted fruit bat (*Epomops franqueti)* and little collared fruit bat (*Myonycteris torquata)* (Leroy et al., 2005). However, a recent detection of EBOV has been reported in a Greater long-fingered bat (*Miniopterus*

cf inflatus) in Liberia (Epstein personal communication) expanding the potential geographic range for EBOV. Ebolavirus reactive antibodies are present in an even wider range of bat species, including *Epomophorus, Rousettus* and *Mops* spp. that do occur in Southern Africa (Olival and Hayman, 2014). RESTV had been detected in *Miniopterus cf schreibersii* in the Philippines (Jayme et al., 2015) again implicating the *Miniopterus* genus. Pigs are currently the only species of livestock known to be at risk of infection by an ebolavirus species as demonstrated by studies in the Philippines and China indicating that they are naturally infected with RESTV (Marsh et al., 2011). The reservoir and geographical distribution of EBOV and other Ebola virus species are still not clear with limited surveillance performed in the Southern African region in wildlife species due to the historical absence of human outbreaks.

***Paramyxoviruses*:** This is negative-sense single-stranded RNA viruses capable of infecting a diverse host range including mammals, birds, reptiles and fish (Virtue et al., 2009). HeV and NiV were first detected in the 1990s following outbreaks of fatal respiratory and encephalitic illness in Australia and Malaysia, respectively (Murray et al., 1995; Chua et al., 1999). During the initial emergence, both viruses spilled over into the human population from pteropid bats by means of intermediate hosts, i.e. horses and pigs respectively. Multiple NiV outbreaks have subsequently been reported from Bangladesh and India linked to repeated spillover events from flying foxes of the *Pteropus* genus directly to humans followed by a high rate of human-to-human transmission and high mortality (Gurley et al., 2007a; Chadha et al., 2006; Arunkumar et al., 2018). These viruses have been shown to be excreted in bat urine, saliva and feces (Chua et al., 2002; Smith et al., 2011). In Bangladesh, contamination of date palm sap by bats while feeding overnight, is the primary route of spillover, though other mechanisms of spillover may exist elsewhere (Gurley et al., 2007b). In Malaysia, pigs were infected by pteropid bats after eating dropped fruit contaminated with saliva, and subsequently infected farmers and abattoir workers (Chua et al., 1999; Paton et al., 1999). Initially, the distribution of henipaviruses was believed to be restricted to the geographical distribution of *Pteropus* spp. in Australia and South-East Asia, however, detections of henipavirus-related virus antibodies and nucleic acids in in African fruit bat species has expanded the geographical range (Hayman et al., 2008; Drexler et al., 2009). Serological evidence of these viruses in bat populations in several African counties including Zambia, Tanzania, Malawi, Madagascar, and Cameroon, as well as several islands in the Gulf of Guinea were reported (Peel et al., 2012; Peel et al., 2013; Brook et al., 2019). In addition, serological evidence in pigs (Hayman et al., 2011) and antibodies in Cameroonian locals were also identified (Pernet et al., 2014), highlighting the potential of spillover. A number of studies also reported the detection of henipa- and rubulavirus-related viral RNA in several bat species on the African continent (Drexler et al., 2012; Baker et al., 2012; Mortlock et al. 2015) including *Rousettus* and the Straw-coloured fruit bat (*Eidolon helvum)* (Peel et al., 2013). Sosuga virus (*Pararubulavirus* genus) has been the only paramyxovirus associated with human disease on the African continent, following the identification of the virus as the causative agent of a non-fatal febrile disease in a single human infection (Albarino et al., 2014), believed to have spilled over from *R. aegyptiacus* in Uganda (Amman et al., 2015). Various research studies, mostly targeted towards HeV and NiV, have reported strong seasonality associated with dry winters, correlations with bat densities, nutritional stress, and various events during the bat reproductive period (Plowright et al., 2008; Dietrich et al., 2015; Paez et al., 2017; Mortlock et al., 2019) as drivers of infection. The diversity and viral dynamics of henipa- and other potentially zoonotic paramyxoviruses in African bat species are poorly understood with only limited sporadic surveillance.

Coronaviruses: These are positive-sense RNA viruses divided into four genera, namely, *Alphacoronavirus, Betacoronavirus, Gammacoronavirus,* and *Deltacoronavirus*. A number of subgenera were recently assigned to each of these genera (Wong et al., 2019) due to an expansion of global diversity. These viruses can undergo recombination, creating new variants that may result in spillover infections and manifest as respiratory pathogens in humans that are transmitted through aerosols. Bat coronaviruses are predominantly excreted in faecal material, though oral swabs, and

urine have also tested positive implicating several routes of transmission. Severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) coronaviruses emerged in 2002 and 2012 respectively, and both pandemics resulted in significant morbidity and mortality (10% and 35%, respectively) in humans, which was exacerbated by global travel (Berry et al., 2015). Global coronavirus surveillance increased significantly after the detection of SARS. In the subsequent search for the reservoir, serological and partial nucleic acid detection in 2005 linked SARS-CoV to horseshoe bats (*Rhinolophus*) in China by identifying a closely related virus. Subsequent studies identified an even larger diversity of SARS-CoV-related viruses in horseshoe bats worldwide (Hu et al., 2017; Lau et al., 2010), however, the virus protein responsible for receptor-binding that mediates cellular entry (spike protein) (Li et al., 2005) was unable to interact with the SARS-CoV virus receptor. Thus, it is unlikely that any of these identified SARS-CoV related viruses were the source of the SARS-CoV human outbreak (Li et al., 2005). Recently a bat SARS-related CoV was found to possess a spike protein that facilitated binding to the same virus receptor as SARS-CoV (Ge et al. 2013; Yang et al., 2016), indicating the potential to cross species barriers and infect humans directly. In 2012, Middle East Respiratory syndrome virus (MERS) was identified in the Arabian Peninsula (Zaki et al., 2012), and a partial nucleic acid sequence was detected in an Egyptian tomb bat (*Taphozous perforates*) from Saudi Arabia (Memish et al., 2013). It has subsequently been shown that detected bat MERS-related CoVs are only distantly related to MERS-CoV and dromedary camels are considered the reservoir species transmitting the virus to people. Diverse coronavirus sequences were found in many different bat species worldwide, leading to the hypothesis that bats host the genetic diversity of the *Alphacoronavirus* and *Betacoronavirus* mammalian infecting genera (Woo et al., 2012), however, the risks and factors important to spillover is unknown (Hu et al., 2017; Anthony et al., 2017a). The diverse SARS-related coronaviruses circulating within the horseshoe bat (*Rhinolophus*) genus throughout their Asian, European or African distribution make this an important host genus to monitor for viral diversity and novel emergences.

Surveillance data for the Southern African region has been limited, with mostly opportunistic, once off sampling events focused on restricted geographical regions and selected bat species (Geldenhuys et al., 2013; Ithete et al., 2013; Cronje 2017; Bourgarel et al., 2018; Paweska et al., 2018; Mortlock et al., 2019). Southern Africa possesses a rich diversity of bat fauna including both frugivorous and insectivorous bat species (Monadjem et al., 2010) distributed across several biotic zones, but mostly concentrated in the eastern region of the continent, including areas in South Africa, Mozambique, Zimbabwe, Malawi and Zambia (Cooper-Bohannon et al., 2016). In addition to the taxonomic diversity, these bat species also exemplify a diversity of roosting behavior and site selection including trees, rock crevices and man-made structures. This include *Rousettus, Rhinolophus* and *Miniopterus* spp. as well as several free-tail bat species (Molossidae) implicated as potential hosts for high consequence pathogens in other studies. If pathogens are present, several factors play a role in spillover to other species and very few studies investigated this. This include host ecology, movement of bats (both foraging and long term movement), viral shedding and routes of transmission. These factors may be seasonal, emphasizing the need for longitudinal studies. Livestock such as cattle, goats, and pigs, are also present throughout Southern Africa and overlaps with bat diversity hotspots (Robinson et al., 2014; StatsSA2016). People with frequent close contact with bats due to religious, cultural or sociological reasons (Markotter, personal communication) also inhabit the same areas. This bat-human-animal interphase and risk for spillover has not been studied in the region. Habitat loss, primarily through deforestation has been increasing in the Southern African region (Brink and Eva, 2009), and there is evidence of increased bushmeat consumption (Regan et al., 2015; Lindsey et al., 2013). Understanding contact between bats, humans and other animals, including behavior analysis, are needed to understand risk in areas where these viral pathogens are present.

Several factors need to be considered when designing biosurveillance strategies in bats. Individuals do not generally exhibit signs of disease, populations can be large with low viral

prevalence and seasonality affect prevalence and transmission. Detection of viral nucleic acids can therefore be challenging and should be paired with **serological surveys**, which take advantage of long-term antibody persistence. Serology has some limitations including inherent antibody cross reactivity between target and related viruses, statistical interpretation of seropositive cut-offs, limited bat specific assays and control sera, and not being able to directly characterize the infectious agent involved. As a result, greater value can be achieved when combining serological surveys with molecular detection assays and subsequent viral characterization. When investigating potential spillover in non-bat hosts, such as domestic animals and humans, serology can be a more comprehensive strategy given the commercially available reagents that can be used to detect both past/convalescent (immunoglobulin G) and recent/acute (immunoglobulin M) infections. Serological assays for emerging zoonotic pathogens are limited and mostly based on in-house assays using different methodologies including virus neutralization, ELISA, western blots and indirect fluorescent assays (IFAs) and can only detect evidence of exposure to a single virus target. Multiplex serological assays using Lumenix xMAP-based technology offer the advantage to simultaneously detect virus specific and cross-reactive antibodies in a single test, saving time, resources and cost. Since the volume of bat sera collected in surveillance is limited, this is a major advantage. The Luminex system is based on the principles of flow cytometry, allows for multiplexing at least 100 analytes in a single microplate well and uses very small sample volumes (2 µl). This assay uses inactivated serum and recombinant reagents, which means the analysis can be performed in a Biosafety Level 2 laboratory. This technology has been successfully used in surveillance studies in bats and spillover hosts including detection of antibodies against paramyxo and filoviruses (Brook et al., 2019; Laing et al. 2018; Hayman et al., 2011; Peel et al., 2012; Chowdhury et al., 2014;) (See preliminary results for more detail). Broadly reactive **molecular RT-PCR assays** based on the conserved regions of viral genomes at family or genus level have been successfully used in surveillance studies for the viral families targeted in this proposal (See work plan for detail). These assays target short genomic regions that can be sufficient for determining phylogenetic relationships, but not formal genus or viral species demarcation that requires longer gene and/or genome sequences. For this, additional sequencing data is needed including targeting regions that may be important in spillover potential such as the receptor-binding and virulence factor encoding regions.

C. RELEVANCE; SCIENTIFIC AND THREAT REDUCTION IMPACT:

South Africa plays a prominent role in the Southern African region with laboratory capacity that can be expanded and shared to strengthen sustainable biosurveillance in the region. These pathogens can easily affect any country and therefore biosurveillance capacity must include regional networks and engagement with neighboring countries. Surveillance data will identify presence but also shedding of high consequence viruses in different seasons, bat species and biomes. Serological investigations will identify if spillover in livestock and humans occurred in the past and behavioral analysis will investigate risk factors for potential spillovers that may happen in the future. This project will strengthen South Africa's, and the region's, capacity to develop and implement surveillance strategies with appropriate biosafety and biosecurity principles, detect pathogens (both molecular and serological) in bats before outbreaks occur, recognize and diagnose outbreaks if they do occur and develop appropriate reporting strategies for the region. All this information can be used collectively to assess risk, predict potential outbreaks, and develop risk mitigation strategies that can be used throughout the region. It follows a One Health approach involving multi-sectoral stakeholders including government engagement and local communities to enhance communication and threat reduction activities. A regional network, Southern African Bat Research Network (SABRENET), will be established to improve communication and collaboration between countries and between disciplines. Sustainability and threat reduction will be specifically addressed by workshops involving all stakeholders from multiple Southern African countries and to align with the Global Health Security Agenda, and comply with the International Health Regulations (IHR) and other reporting guidelines. This project is closely aligned with the goals of the Cooperative Biological Engagement

Program (CBEP) and align with several of the Biological Threat Reduction Programme (BTRP) Lines of effort (LOE) including National Regulatory Frameworks (LOE3), Disease detection (LOE4), Laboratory diagnosis (LOE5), Epidemiological Analysis and investigation (LOE6) and Reporting and Communication (LOE7).

D. PRELIMINARY WORK AND ESTABLISHED COLLABORATIONS (See table 1 for explanation of abbreviations for institutions):

The two major partners, UP, South Africa (Markotter) and EHA, USA (Epstein) will combine their expertise in implementing surveillance programs and diagnostic sample testing. The major partners will also establish regional networks, and link with international networks including the Bat One Health Network (BOHRN), EcoHealthNet (an NSF-funded disease ecology research training program led by EHA), and the Western Asia Bat Research Network (WABNet). UP and EHA initiated discussions about possible collaborations five years ago and both UP and EHA currently collaborate with CEZPD-NICD. UP also collaborated with AfricanBats NPC (Seamark) and DNMNH (Kearney) since 2004. This includes training activities, joint fieldwork, co-supervision of students, as well as several joint publications. UP already has DAFF section 20 approval to research zoonotic pathogens in bats in Southern Africa and regularly communicate surveillance data to DAFF and NDoH. UP has been collaborating with USU (Broder) since 2017, and has initiated acquisition of Luminex reagents for filo- and henipavirus testing including a signed Material transfer agreement. EHA also have a successful collaboration with USU. EHA and CEZPD-NICD have a joint DTRA project on Rift Valley fever virus since 2014. UP has previously collaborated with the CVL laboratories in Mozambique and Zimbabwe, with joint publications on rabies as an output. Preliminary surveillance activities in small mammals were also conducted in Mozambique in 2017, and a preliminary visit to the CVL laboratory in Zimbabwe was conducted in 2018 to discuss future collaborations. Previous studies led by the South African team, including UP, CEZPD-NICD, DNMNH and AfricanBats have reported detections of several potential zoonotic pathogens in bats (Figure 1). This work was performed under the South African Research Chair of Prof. Markotter funded by the South African Department of Science and Innovation (ongoing) and the Global Disease Detection Programme of the Centers for Disease Control and Prevention (USA) 2016-2019. Biosurveillance on select agents in bats was only a small part of these projects, and therefore surveillance studies were sporadic and limited in scope to only specific bat species and limited

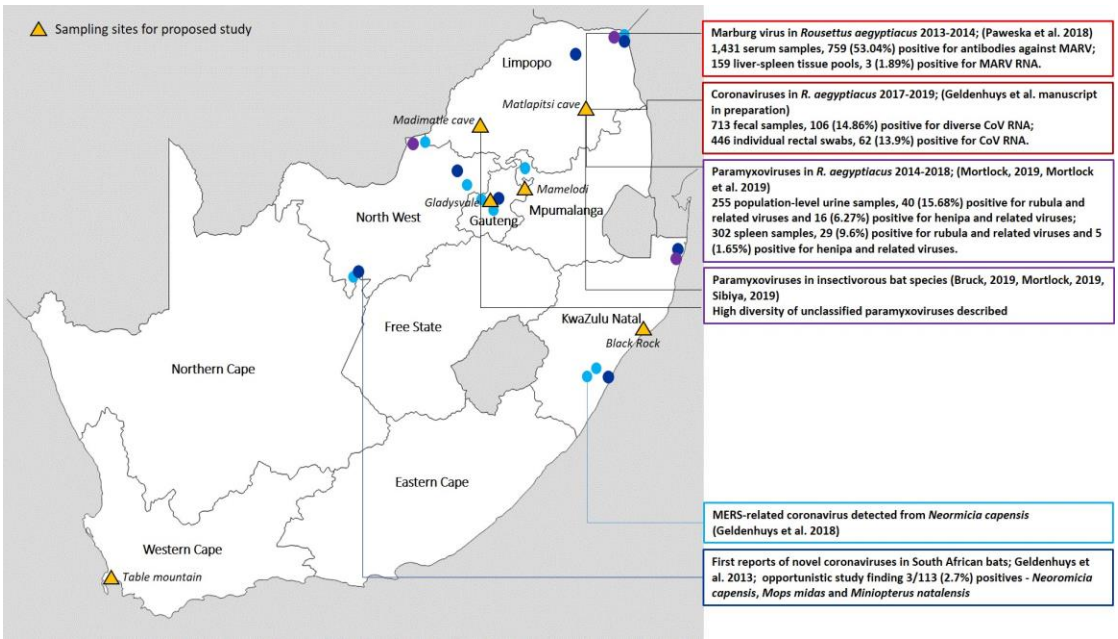

Fig 1: Summary of filo, corona and paramyxovirus nucleic acid detections in South Africa. Previous and proposed samples sites for this project are indicated (Unpublished).

geographical locations in South Africa. In addition, this surveillance only included molecular detection of pathogens and very limited serological investigations in bat species and none in potential spillover hosts. Preliminary studies indicate that seasonal reproduction of *R. aegyptiacus* drives viral infection dynamics, and that gradual loss of passive immunity among juveniles in this bat population increases the number of susceptible individuals, creating favorable conditions for viral spread (Figure 2). It also results in changes in virus shedding and seroconversion patterns, which are driven by sex, age and reproductive condition of female bats (Figure 2 and 3). Molecular assays to detect paramyxo-, corona- and filoviruses are established in the South African laboratories (UP and NICD) with some improvements needed (see detail under Work plan). Serology was not part of previous studies, with the exception of targeted Marburg virus serological studies. At the time, we had no feasible serological option available that could accommodate a multiplex platform suitable for low amounts of sera collected from bats and we initiated discussions to obtain luminex reagents from USU. Purified oligomeric, receptor-binding protein (G) of henipaviruses (e.g. HeV and NiV) have been used by the USU lab and collaborators for several years in antigen-based immunoassays to differentiate antibodies specific to HeV or NiV (Bossart et al., 2007), and in biosurveillance studies to examine exposure to these high priority viruses (Chowdhury et al., 2014; Peel et al., 2012) (Figure 4). With the isolation of new viruses including Cedar virus (CedV), Ghana virus (GhV) and Mojiang virus (MojV), the lab has continued to generate tetrameric henipavirus G for all presently described henipaviruses, and expand the HeV/NiV immunoassay into a pan- henipavirus multiplex assay capable of simultaneously

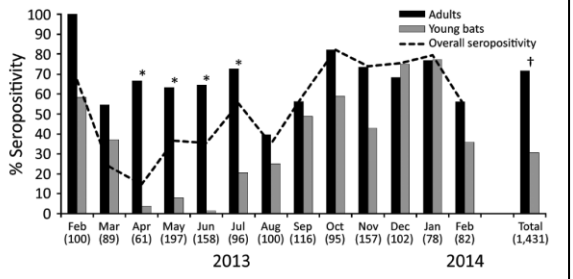

Fig 2: Seropositivity per month for Marburg virus as reported in Paweska et al., 2018

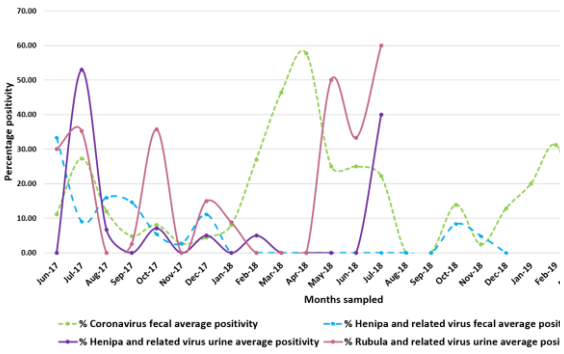

Fig 3: Detected viral RNA excretion dynamics in urine and fecal from the *R. aegyptiacus* bat at Matlapitsi cave

detecting and differentiating henipavirus-specific antibodies. Furthermore, USU constructed native-like glycoprotein antigens of all presently described filoviruses to generate a pan-filovirus antigen-based immunoassay that can be simultaneously used with the pan-henipavirus immunoassay to detect antibodies reactive with four of the WHO priority pathogens with pandemic potential. This pan-filovirus immunoassay was used by Laing et al. to detect serologic evidence of Asiatic filoviruses, antigenically-like ebolaviruses, in three fruit bat species collected in Singapore (Laing et al., 2018) (Figure 5).

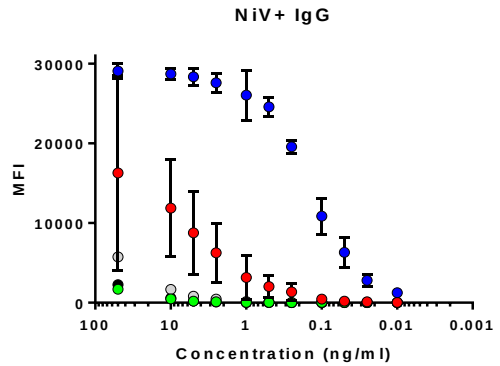

Figure 4: Pan-henipavirus immunoassay can specifically differentiate between antibodies reactive with heterologous viruses. Rabbits were immunized with virus specific G antigens. Antisera were screened with multiplex pan-henipavirus immunoassay in two independent experiments in technical triplicates; bars indicate 95% confidence intervals.

This assay was also used at the National Centre for Biological Sciences, Bangalore, India in a DTRA BTRP supported project to Duke-NUS researcher Dr. Ian Mendenhall, to detect exposure to Asiatic filoviruses in bats and humans hunting these bats for food, implicating likely cross-species transmission of novel filoviruses at this wildlife-human interface (Figure 6) (Dovih et al., 2019). This pan-filovirus/henipavirus immunoassay has been transferred to three laboratories in Peninsular Malaysia where it is being utilized in serology-based biosurveillance of wildlife (e.g. bats), livestock and indigenous human communities, funded by DTRA BTRP to Co-PI J. Epstein and contributed further evidence that a diversity of novel Asiatic

filoviruses, distantly related to Reston virus, but with unknown pathogenesis, circulate in certain species of bats.

In collaboration with DNMNH, a taxonomic database for bat species, including voucher specimens with morphological classification and genetic sequence confirmation, has been generated for most South African bat species, and for some species in Zimbabwe and Mozambique. UP, USU and EHA have developed several standard operation procedures and training material for past studies including

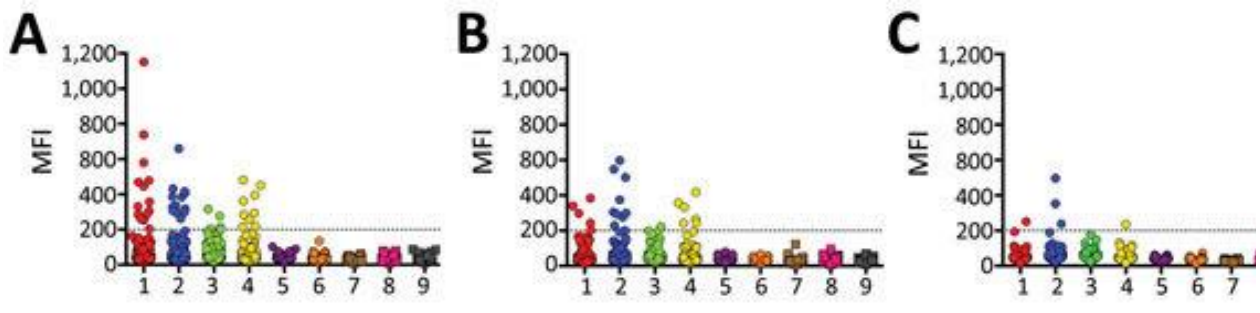

Figure 5. Serologic evidence of exposure to Asiatic filoviruses in 3 fruit bat species. (A) *Eonycteris spelaea* (B) *Cynopterus brachyotis* (C) *Penthetor lucasi*. Mean fluorescence intensity (MFI) values obtained from Bio-Plex assay (Bio-Rad, Hercules, CA, USA) screening of individual serum samples from bats of 3 species with soluble filovirus glycoproteins. Dashed line indicates the cutoff value at 200 MFI. 1, *Zaire ebolavirus*; 2, *Bundibugyo ebolavirus*; 3, *Taï Forest ebolavirus*; 4, *Sudan ebolavirus*; 5, *Reston ebolavirus*–monkey; 6, *Reston ebolavirus*–pig; 7, Marburg virus–Musoke; 8, Marburg virus–Angola; 9, Ravn virus.

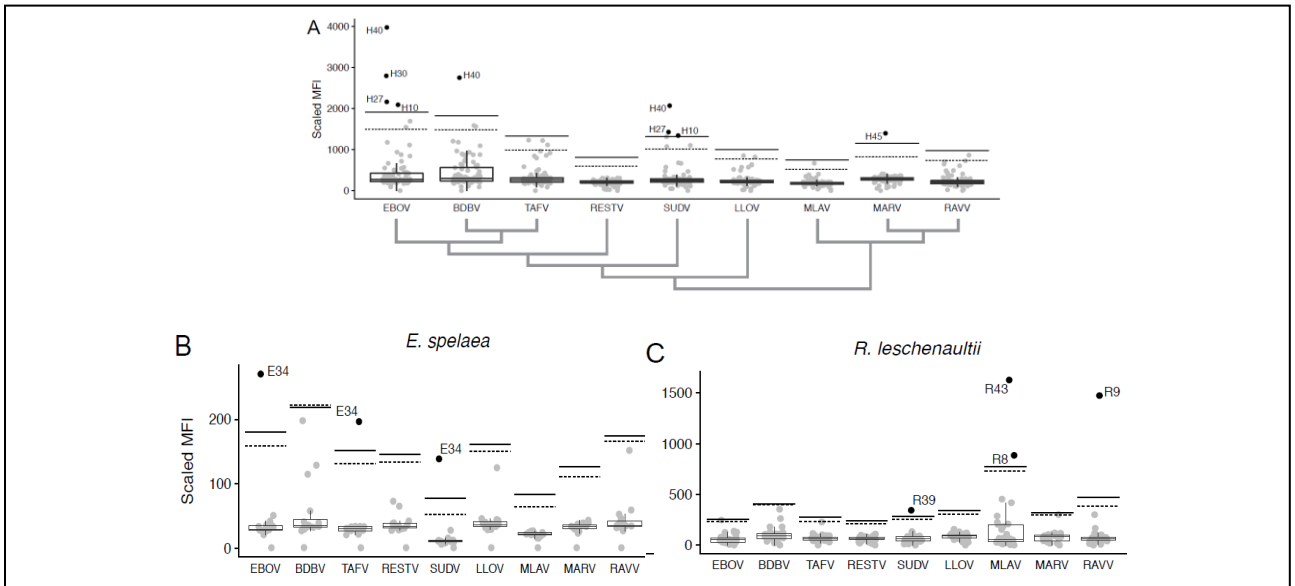

Figure 6. Serologic evidence of exposure to Asiatic filoviruses in humans and fruit bats, Nagaland, India. (A) Humans sampled; phylogenetic tree under X-axis is used to orient antigen-antigen relatedness (B-C) Fruit bat species indicated. (MFI) values obtained from MAGPIX assay (Bio-Rad, Hercules, CA, USA) screening of individual serum samples

biosafety, collection of bat samples, RT-PCR testing, DNA sequencing, bioinformatics analysis, luminex serological testing and behavioral risk assessment tools. This will be revised in Y1 and improved where necessary.

E. INSTITUTIONS AND ROLES (Summarized in table 1):

University of Pretoria (UP; prime (PI)), Pretoria RSA, Will finalize and implement the study design, coordinate overall project, coordinate and present training and workshops, develop and finalize SOPs, supervise Hons, MSc, PhD and post-doctoral students, obtain relevant regulatory and

ethical approvals in coordination with relevant government departments; coordinate and perform all field work, coordinate sample testing and testing of all animal samples, coordinate behavioural studies in coordination with relevant government agencies and partners; build diagnostic capacity in the UP laboratory (focused on animal sample testing) that can be shared including hosting and training of partners from neighbouring countries; data analyses, report and publish data; disease modelling and assessing risk mitigation strategies; and development of threat reduction policies. UP will be responsible for all contractual obligations with DTRA including reporting. **Staff**: Wanda Markotter (PI, Overall coordination – involved in all activities listed above), Jessica Coertse (Postdoctoral fellow, Bat filovirus surveillance), Marike Geldenhuys (Postdoctoral fellow, Bat coronavirus surveillance), Marinda Mortlock (Postdoctoral fellow, Bat paramyxovirus surveillance), Liz Basson (Grant administrator), Research assistants x 4 (fieldwork, processing and testing of samples (molecular and serology), database management) (To be hired), students to be identified (PhD, MSc and Honours),

EcoHealth Alliance (EHA; sub (Co-PI), NY USA, Will co-develop and implement the study design, coordinate and present training workshops, develop and finalize SOPs, co-supervise students, develop and implement human behavioural studies in coordination with relevant government agencies; fieldwork training and sampling (limited), data analyses, report and publish data; disease modelling and assess risk mitigation strategies; and development of threat reduction policies, develop awareness material. **Staff:** Jonathan Epstein (Co-PI – Involved in all activities listed above), Noam Ross (Disease modelling), Kendra Phelps (Fieldwork training and finalizing SOPs), Kevin Olival (Risk mapping, Developing SOPs & participating in training workshops, linkage with other bat networks e.g. WABNet), Emily Hagan (Develop and direct behavioural risk tools and analysis, assist in developing and implementing risk reduction strategies, community engagement and outreach material development), 1 postdoc and 1 PhD student who will work on disease modelling in bats and spatial analysis of movement data, respectively (To be identified).

Uniformed Services University (USU, sub CO-I), Maryland USA, Will develop and transfer Luminex reagents and SOPs to UP and NICD; train laboratory staff; co-supervise MSc and PhD students involved in serological projects; analysis and interpretation of filo-, henipa-, and coronavirus serological data; standardization of multiplex immunoassay and data interpretation. Assess risk mitigation strategies and development of threat reduction policies. **Staff:** Eric Laing (Co-investigator – Involved in all activities listed above), Christopher Broder (Head of the research group, scientific expertise and guidance), Laboratory technicians x 3 (Preparation of Luminex reagents at USU and assistance with training and data analysis).

National Institute for Communicable Diseases (NICD) Centre for Emerging Zoonotic and Parasitic Diseases (CEZPD) (CO-I), RSA, Develop SOPs specifically for laboratory diagnostics, fieldwork and reporting, co-supervise Hons, MSc, PhD and post-doctoral students, obtain relevant regulatory and ethical approvals pertaining to human studies in coordination with relevant government departments; coordinate and perform field work, involved in human behavioural and serological studies in coordination with relevant government agencies; build diagnostic capacity in the NICD laboratory that can be shared including hosting and training of partners, provide serological positive and negative serum panels, verify positive serology results obtained from the luminex, analyse results, report and publish data. Assess risk mitigation strategies; and development of threat reduction policies **Staff:** Jacqueline Weyer (Co-investigator – Involved in all of the above), Janusz Paweska (Head of the research group, scientific expertise and guidance), Naazneen Moolla (Medical scientist – Assistance and verification of luminex serological results), Postdoctoral fellow (Human and livestock serology) to be identified.

Table 1: Prime and partner institutions and summarized roles

	Study design and coordination	Contractual obligation with DTRA and in-country lead	Regulatory and ethical requirements	Coordinate and/or attend training	Development of SOPs	Collection of bat samples	Collection of host, environmental and ecological data	Collection of livestock samples	Collection of human samples	Molecular testing of samples	Transfer of Luminex technology and testing of samples	Human behavioral studies	Disease modelling	Co-analyze and reporting of data. Risk mitigation strategies and development of threat reduction policies
University of Pretoria (UP), Centre for Viral Zoonoses, South Africa (PI: Prof. Markotter)	■	■	■	■	■	■	■	■	■	■	■	■	■	■
EcoHealth Alliance (EHA), USA (Co-PI: Dr Epstein)	■			■	■	■	■					■	■	■
Uniformed Services University (USU), USA (CO-I: Dr Laing)				■	■						■			■
National Institute for Communicable Diseases (NICD), Centre for Emerging Zoonotic and Parasitic Diseases (CEZPD), South Africa (Co-I: Dr. Weyer)			■	■	■	■	■	■	■	■	■	■	■	■
Department of Agriculture, Land Reform and Rural development (DALRRD previously DAFF), South Africa together with KwaZulu-Natal, Limpopo and Western Cape Department of Agriculture and Rural Development				■	■	■		■						■
National Department of Health (NDoH), South Africa				■	■				■			■		■
National Department of Environment, Forestry and Fisheries (DEFF), South Africa				■	■									■
Central Veterinary laboratories (CVL), Agrarian Research Institute of Mozambique, Ministry of Agriculture and Food Security. Department of Veterinary Services and Wildlife services, National Ministries of Health, Mozambique and Zimbabwe				■	■	■	■					■		■
AfricanBats NPC, South Africa, Mr. Seamark				■	■	■	■							■
Ditsong National Museum of Natural History (DNMNH), South Africa: Dr Kearney				■	■	■	■							■

Department of Agriculture, Land Reform and Rural Development (DALRRD previously DAFF) together with KwaZulu, Limpopo and Western Cape Departments of Agriculture and Rural development, RSA, Identify representatives and attend training, meetings and workshops from the animal health sector, assistance with obtaining local regulatory documents and import permits, take part in and assist with field studies for collection of bat and livestock samples in South Africa, co-analyze data, report and publish, assess risk mitigation strategies and development of threat reduction policies. **Staff:** Grietjie de Klerk, DVM (Director - Involve in all of the above), Alicia Cloete (Epidemiologist, Involve in all of the above), State veterinarians for sampling areas; Limpopo, Western Cape and KwaZulu Natal RSA (assist and facilitate collection of bat and livestock samples and reporting).

National Department of Health (nDoH), RSA, Identify representatives and attend training, meetings and workshops from the human health sector, assist with collection of human samples and behavioral questionnaires in South Africa, co-analyze data, report and publish. Assess risk mitigation strategies and development of threat reduction policies. **Staff:** Wayne Ramkrishna (Epidemiologist - Involve in all of the above)

National Department of Environment, Forestry and Fishery (DEFF) RSA, Identify representatives and attend training, meetings and workshops from the environmental health sector, co-analyze data, report and publish, assess risk mitigation strategies and development of threat reduction policies. **Staff:** Abednego Baker - Involve in all of the above.

Central Veterinary laboratories (CVL), Agrarian Research Institute of Mozambique, Ministry of Agriculture and Food Security, Mozambique, Identify relevant trainees in animal health, attend training and workshops, coordinate field studies in Mozambique, collect and test samples collected in Mozambique in South Africa, assistance with obtaining local regulatory documents, co-analyze data, report and publish, assess risk mitigation strategies and development of threat reduction policies. **Staff:** Sara Achá (Veterinarian and researcher – Head of laboratory), Lourenço Mapaco (Veterinarian and researcher – Involved in training, fieldwork and testing of samples), Iolanda Vieira Anahory Monjane (Veterinarian, researcher and country coordinator for Mozambique study, involved in training, fieldwork and testing of samples).

Central Veterinary laboratories (CVL), Agrarian Research Institute of Zimbabwe, Ministry of Agriculture and Food Security Department of Veterinary Services and Wildlife services, Zimbabwe, Identify relevant trainees in animal health, attend training and workshops, coordinate field studies in Zimbabwe, collect and test samples collected in Zimbabwe in South Africa, assistance with obtaining local regulatory documents, co-analyze data, report and publish, assess risk mitigation strategies and development of threat reduction policies. **Staff:** Pious Makaya (Veterinarian – Laboratory Head), Babra Bhebhe (Veterinarian and coordinator for Zimbabwe study, involved in training, fieldwork and testing of samples)

National Ministries of Health, Mozambique, Identify relevant trainees in human health, attend training and workshops, coordinate human behavior studies in Mozambique, assistance with obtaining local regulatory documents, co-analyze data, report and publish, assess risk mitigation strategies and develop threat reduction policies. **Staff:** Inocêncio Salvador Chongo (focal point of One Health group at the Ministry of Health - Involve in all of the above)

National Ministries of Health, Zimbabwe, Identify relevant trainees in human health, attend training and workshops, coordinate human behavior studies in Zimbabwe, assistance with obtaining local regulatory documents, co-analyze data, report and publish, assess risk mitigation strategies and develop of threat reduction policies. **Staff:** To be identified

AfricanBats NPC, RSA, Coordinate and present training and workshops focused on bat biology and taxonomy, develop SOPs focused on these topics, coordinate all field studies focused on *Miniopterus* spp. and assistance with other fieldwork, assistance with RFID tracking and environmental monitoring at bat roosts, echolocation data analyses co-analyze data, report and publish data. Assess risk mitigation strategies and development of threat reduction policies. **Staff:** Ernest Seamark (Chief Executive Officer) and Dr. Mark Keith (Board of Directors) (Involvement of all of the above).

Ditsong National Museum of Natural History (DNMNH), RSA, Coordinate and present training and workshops focused on bat biology and taxonomy, developing SOPs focused on these topics, Involved in fieldwork, taxonomic identification of bats collected and administration of museum vouchers, comparison of morphological and genetic identification of bat species, co-analyze data, report and publish data. Co-supervision of students, assess risk mitigation strategies and development of threat reduction policies. **Staff:** Teresa Kearney (Research scientist/taxonomist – Involvement in all of the above).

F. CREDENTIALS (See detailed bio sketches of key staff for additional information):

University of Pretoria (UP) is a leading research-intensive university and is recognized nationally and internationally for the quality of its research and the extent of its research outputs. UP is also ideally situated in the executive capital of South Africa, Pretoria, in close proximity to the International airport (O.R Tambo) ensuring ease of travel to enhance collaborations and student exchange. One Health and zoonotic diseases is an important research theme and the Centre for Viral Zoonoses was established in 2016 to enhance multidisciplinary collaboration between the faculty of

Health Sciences, Natural and Agricultural Sciences and the only Veterinary Science faculty in South Africa. The UP has solid strength in the zoonosis research field and has in the last number of years made a series of significant financial and other contributions to support this research. Recently the Future Africa Institute was established to facilitate interdisciplinary research and this include state of the art training and meeting facilities. UP is also ideally situated close to several important collaborative networks including NICD, Onderstepoort Veterinary Institute (ARC-OVI), NDOH, DALRRD (DAFF) and DEFF. UP has a financial and grant management infrastructure and has successfully managed USA federal funding. Infrastructure include a biosafety level 3 laboratory, ultra-low temperature freezers in a biobank with back-up generator power, molecular and serological laboratories and Sanger DNA sequencing and next generation sequencing facilities on site. It also include information technology and bioinformatics infrastructure. Several established field sites exist as well as basic infrastructure and equipment to sample bats. **PI: Prof. Wanda Markotter** is the Director of the Centre for Viral Zoonoses and since January 2016 she is also occupying a South African Research Chair in "Infectious Diseases of Animals (Zoonoses)" funded by the South African government. She is a virologist who is involved in a multidisciplinary research programme on disease ecology in bat species in South Africa and other African countries leading to several high impact research outputs. Experience include molecular and serological diagnostics and fieldwork to collect samples. Her research group consist of support staff, postdoctoral fellows and several post graduate students that also have extensive experience in this field. She plays a key role in national governmental committees including the National Rabies Advisory Group and National One Health forum steering committee.

EcoHealth Alliance (EHA) is a scientific organization, working with local partners in over 30 countries at the nexus of health, biodiversity conservation and international development. EHA has a staff of 35 in New York, including scientists (e.g. social scientists, veterinarians, ecologists, analysts, IT experts, and economists), administration, and communications staff. EHA has an extensive record of publishing high quality, peer-reviewed papers, journals, briefing documents and reports, including seminal work on emerging infectious diseases and bat borne viral zoonoses with particular expertise in modelling of host-virus dynamics and human behavioural analyses. EHA's ability to produce highly utilized and understandable science-based outputs will contribute significantly to achieving project goals and provide objective methods for tracking project utilization of project findings. In 2014, EHA became the first foreign NGO to sign a Memorandum of Agreement (MOA) with the Government of Malaysia. The MOA is to study zoonotic disease in populations exposed to wildlife and includes three sectors of government: Ministry of Health, The Department of Wildlife and National Parks, and The Department of Veterinary Services. **Co-PI: Dr. Jonathan Epstein** is a veterinary epidemiologist and the Associate Vice President at EHA. He is a technical lead for surveillance and outbreak response under the USAID Emerging Pandemic Threats: PREDICT program, a $100 million effort focused on predicting and preventing pandemic diseases. Dr. Epstein is recognized internationally for his expertise on the ecology of emerging zoonotic viruses and currently directs research and surveillance programs in West Africa, South and Southeast Asia, and China. He has led investigations of NiV, EBOV, SARS CoV and MERS CoV in Asia, the Middle East, and Africa. He has also served as a consultant for WHO, FAO, OIE, and the Institute of Medicine.

Uniformed Services University (USU) is a US federal government health science university that trains military physicians through medical school, military masters of public health and military & civilian doctoral candidates in varying disciplines including emerging infectious diseases, and microbiology. USU hosts the Center for Global Health Engagement chapter, which has a mission to support the Department of Defense Global Health Engagement. USU has a world renowned health science faculty who have close ties with the proximally located Walter Reed Army Institute of Research and Naval Medical Research Centers. The Department of Microbiology and Immunology includes 12 full-time Faculty members, and has an overall focus on mechanisms of infectious diseases

and the host response/immunology. Faculty interests and active research programs at USU are diverse, with many nationally- and internationally-known investigators researching areas of viral immunology, vaccine and antiviral therapeutics, and animal model development. USU is also physically located directly across from the main NIH campus in Bethesda, Maryland. The overall broad scientific environment at both USU and the NIH is highly conducive to productive collaborations.

National Institute for Communicable Diseases (NICD), Centre for Emerging Zoonotic and Parasitic Diseases (CEZPD) The NICD is a national public health institute of South Africa, providing reference microbiology, virology, epidemiology, surveillance and public health research to support the government's response to communicable disease threats. The NICD serves as a resource of knowledge and expertise of communicable diseases to the South African Government, Southern African Development Community countries and the African continent. The institution assists in the planning of policies and programmes to support and respond to communicable diseases. CEZPD was established by the amalgamation of six former NICD sections: Special Bacterial Pathogens Reference Laboratory, Special Viral Pathogens Reference Laboratory, Arbovirus Reference Laboratory, Electron Microscopy, Parasitology Reference Laboratory and Vector Control Reference Laboratory. The CEZPD operates the only suit biosafety level 4 (BSL4) facility on the African continent, which places it both strategically and critically in a position to assist in the response of highly dangerous emerging and re-emerging zoonotic pathogens.

Department of Agriculture, Land Reform and Rural Development (DALRRD previously DAFF), RSA together with KwaZulu Natal, Limpopo and Western Cape Department of Agriculture and Rural Development and specifically the Animal Health section aim to manage risk associated with animal health by promoting, preventing and controlling animal diseases including zoonoses. This includes formulating policies and rendering epidemiological services for early warning and monitoring of animal diseases. Provincial departments and specifically the state veterinarians are involved in sampling animals.

National Department of Health (NDoH), RSA aim to improve the health status through the prevention of illnesses and the promotion of healthy lifestyles and to consistently improve the healthcare delivery system by focusing on access, equity, efficiency, quality and sustainability. The Directorate of Communicable Diseases include zoonoses and include multisector committees to report surveillance data and mobilize outbreak responses. This also include a National One Health forum including all stakeholders.

National Department of Environment, Forestry and Fisheries (DEFF), RSA aim to ensure an environment that is not harmful to people's health or wellbeing, and to have the environment protected for the benefit of present and future generations. To this end, the department provides leadership in environmental management, conservation and protection towards sustainability for the benefit of South Africans and the global community.

Central Veterinary laboratories (CVL), Mozambique, CVL is a technical department of the Directorate of Animal Science (DCA), of the Agrarian Research of Institute of Mozambique (IIAM) and is the national reference laboratory. The role is to perform animal diseases diagnostics and epidemiological studies; to identify and propose research area on animal diseases; to develop research methodologies and standardized techniques for animal diseases and zoonotic diseases; to guarantee the specialized training and follow-up of laboratory technicians and to provide technical assistance to the regional and provincial laboratories. There is a One Health Technical Working Group (TWG) established which includes different stakeholders: Ministries of Health, Agriculture and Food Security, Fisheries, Environmental and Wildlife.

Central Veterinary laboratories (CVL), Department of Veterinary Services and Wildlife services Zimbabwe, CVL is a branch under the Directorate of the Division of Veterinary Technical Services of the Department of Veterinary Services. Veterinary Services falls under the Ministry of Lands, Agriculture, Water, Climate, and Rural Resettlement. The role is to provide diagnostic support

for zoonotic diseases and diseases of economic interest in animals, epidemiological disease investigations and surveillance of diseases as well as Food safety monitoring and Port Health Services.

AfricanBats NPC, is registered within South Africa as a Not for Profit Company. The vision is to have a functioning, integrated program for education, capacity building, research and management for the conservation of bat populations in Africa. Activities include the publication of African Bat Conservation News and the African Chiroptera Report. AfricanBats NPC has been doing capacity building through workshops and training courses, attended by post-graduate students and emerging scientists undertaking research on bats. Research projects have focused on bat monitoring, bat cave conservation, bat systematics, taxonomy and life histories, bat in protected areas and anthropocene bats.

Ditsong National Museum of Natural History (DNMNH), formerly known as the Transvaal Museum was founded in 1892 and it has, since acted as custodian and documentation centre of South Africa's natural heritage. The Museum's collections and exhibits include fossils, skeletons, skins and mounted specimens of amphibians, fish, invertebrates, reptiles and mammals. It is one of the largest in South Africa and the largest collection of bat specimens in South Africa and extensive taxonomic expertise.

III. WORK TO BE PERFORMED

The project will focus on capacity building in the Southern African region (Table 2) and biosurveillance (Table 3) as summarized and 3 and explained below.

Capacity building will focus on South Africa and neighboring countries. Pathogens do not respect borders and a regional approach is needed for South Africa to effectively detect, predict and respond to infectious disease threats. The main activities will be: 1) training representatives from Southern African countries in development of effective surveillance strategies, standard operating protocols, biosafety, data analysis, disease modelling, field and laboratory investigations, effective reporting and communication following a One Health approach; 2) establish diagnostic technologies to detect filo-, henipa- and coronaviruses for the region at a central facilities in South Africa; and 3) build capacity between stakeholders including governmental representatives through joined meetings including development of mitigation and threat reduction strategies to inform policy. Although the biosurveillance part of the project will be focused on South Africa, capacity building will include training of colleagues from Mozambique and Zimbabwe. Trainees, representing animal, human, environmental health and academia, will be selected from each country in line with a One Health focus by the different stakeholders. Theoretical and practical training will take place in South Africa through workshops, at field sites and diagnostic laboratories. In addition to developing emerging researchers and postdoctoral fellows, projects will be developed for at least 4 PhD, 4 MSc and 4 honors students, including students from neighboring countries, with co-supervision between partners. Fellows and students will spend time with different team members to learn laboratory and analytical techniques. By including colleagues from neighboring countries, we will develop a workforce that can then initiate long-term surveillance programs in the respective countries. These projects will be supported in the long-term by the regional diagnostic capacity developed in South Africa in two institutions and access to these facilities for testing to the region. Both these institutions invest in staff and infrastructure independent of this project and funding. The South African Research Chair of Prof. Markotter is also a long-term commitment from the South African government [Department of Science and Innovation (DSI)] and the academic institution (UP). To also ensure sustainability there is close collaboration with all ministries and capacity building is focused on emerging researchers to ensure long-term continuity

Table 2: Workshops and training activities:

Activity	Location	Month and year	Duration in days	Chairs/ Trainers	Attendees	Proposed activities
Project Stakeholder meeting	UP, Pretoria, RSA UP, Pretoria, RSA Set up a video conference, Moz and Zim representatives will meet with South African colleagues in South Africa and USU and EHA will dial in from the USA New York, USA UP, Pretoria, RSA Set up a video conference, Moz and Zim representatives will meet with South African colleagues in South Africa and USU and EHA will dial in from the USA UP, Pretoria, RSA	June Y1 June Y2 June Y3 June Y4 June Y5	3 1 3 1 3	Markotter and Epstein	**~ 30 attendees** **At least one representative of each stakeholders mentioned below** South Africa: *UP, NICD, DALRRD, nDOH, DEFF, AfricanBats, DNMNH* Mozambique *CVL Moz, Ministry of Health), Academia, Ministry of Enviromental and Wildlife* Zimbabwe *CVL Zim, Ministry of Health* USA *EHA, USU, DTRA representatives*	**Y 1** Introduction to the project, roles and responsibilities, timelines Planning of surveillance strategies, standardized SOPs , standardize reporting policy Reporting of results and engagements with government (including between countries) Planning and design of human behavioral studies, communication strategy and awareness Biosafety training including packaging and shipping of samples Establishment of SABRENET **Y 2, 3 and 4** Share and discuss results and progress, reporting, publications Agree on activities for the next year Identify trainees and student involvement Develop mitigation and prevention policies, threat reductions based on preliminary results Draft a plan for sustainability of biosurveillance for the region and decide on implementation timelines **Y 5** Analyze and discuss all results, finalize mitigation strategies and policies for threat reduction Finalize the plan for sustainable biosurveillance in the region. Prepare final report and publications
Modelling and data analysis workshop	UP, Pretoria, RSA	June Y1	2	Olival, Ross, (EHA)	**~ 30 attendees** **At least one representative of each stakeholders mentioned above**	Epidemiology Risk analysis GIS Hotspot mapping Niche modelling Introduction to disease modelling
Introduction to bat biology and taxonomy training	UP, Pretoria, RSA	June Y1	4	Kearney (DNMNH), Seamark (African Bats), Phelps (EHA), Markotter (UP)	**~ 15 attendees** **At least one representative of each stakeholders mentioned above**	Introduction to bat biology, physiology, ecology, taxonomy, echolocation and environmental monitoring. The focus will be on Southern African bat species. Will include practical taxonomic exercises and sampling at both insectivorous and frugivorous bat roosts.
Bat field sampling training	Field sites in South Africa, Mozambique and Zimbabwe	Y1-Y4 various months	14	Markotter (UP), Postdocs (UP), Kearney (DNMNH) Seamark (African Bats), Phelps (EHA)	**At least one representative of each stakeholders mentioned above** A strong focus on training students and staff that will be involved in fieldwork	There will be continuous field training. This will consist of each trainee (Identified by the stakeholders) joining at least 2 field visits a year (part of the biosurveillance activity).
Laboratory diagnostic training	UP and NICD laboratories, RSA	Y1-Y5 various months	21	Markotter (UP), Weyer (NICD), Moolla (NICD) Laing (USU) Postdocs (UP and NICD)	**At least one representative of each stakeholders mentioned above** Focus will be on training students and staff involved in diagnostic testing	There will be continuous laboratory diagnostic training. This will consist of trainees visiting the diagnostic laboratories (NICD and UP), receive training and perform sample processing, testing and analyses of results.

Activity	Location	Month and year	Days	Chairs/ Trainers	Attendees	Proposed activities
Data interpretation workshop *	EHA, New York UP, RSA EHA, New York UP, RSA EHA, New York	Feb Y1 Feb Y2 Feb Y3 Feb Y4 Feb Y5	5	Markotter (UP), Epstein (EHA)	Markotter (UP), Epstein (EHA) E. Laing (USU) Postdocs UP, NICD, EHA	A smaller team that will do in depth data analysis that can then be discussed and analyzed by the broader group (Stakeholder meeting)

*Depending on data collected, additional people will attend the data analysis workshops.

Biosurveillance activities will be focused in South Africa with limited fieldwork collections in Mozambique and Zimbabwe (Y4). Activities will already start at the beginning of Y1 since ethical approvals and Department of Agriculture and Rural Development (Previously DAFF) Section 20 to perform biosurveillance in Southern Africa is already approved. Minor amendments will be applied for. Due to the proposed project start date (June 2020) no retrospective sample testing will be performed but previous results generated will be part of data analysis.

Sampling strategy: Targeted longitudinal surveillance in bats, as well as sampling in potential spillover hosts (cattle, goats, donkeys, and pigs), will be implemented in South Africa and once-off biosurveillance of bats in neighboring countries. The surveillance strategy has been designed based on species previously associated with high consequence pathogens in the literature as well as host and other ecological factors that may play a role in spillover (See background for detail). Cross sectional studies will focus on *Rousettus*, *Rhinolophu*s, *Miniopterus* and molossid bat species at several sites in South Africa (Figure 1) to determine the nucleic acid and seroprevalence of filo, henipa- and potentially zoonotic coronaviruses (Table 3). Prevalence depends on many factors, including sample type, seasonality, specific species targeted, target virus and sensitivity of the assay with studies reporting prevalence ranging from 0-100% and the prevalence of nucleic acids is usually much lower that the presence of antibodies. We assumed a nucleic acid and seroprevalence of 10% and sample sizes of individual bats were selected to detect positives with a 95% confidence interval based on estimated population sizes (Markotter, personal communication) and seasonality. Sampling in bats will be non-destructive where blood, swabs (oral and rectal) and urine samples will be collected and this sample strategy has proven to be successful in previous studies (Anthony et al., 2017a). Limited voucher specimens will be collected to confirm species identification where necessary. In addition, will we implement longitudinal surveillance in both *Rousettus* and *Miniopterus* spp. (implicated as filo- and henipavirus hosts), including during birthing periods to investigate seasonality of virus shedding. *Miniopterus* spp. move between Highveld roosts in urban areas during winter months and maternity summer roosts, and therefore several roosts are included to sample representative individuals over the study period. *Rousettus* roosts occur in different biomes in South Africa, and representative roosts are included specifically Black Rock and Table Mountain. Roost type differs and therefore caves, tree roosts, crevices and man-made structures are included in the selected sites and chosen because of a potential bat-animal human interphase. Matlapitsi is in a rural savanna area with free roaming livestock, vegetable and fruit farming. People visit the cave for cultural reasons, and there are fruit trees in-between human dwellings where fruit bats feed. Movement of *R. aegyptiacus* between Matlapitsi and Black Rock has been reported (>500 km) (Jacobsen and Du Plessis, 1976); Black Rock is in a tropical rainfall area, located inside a protected conservation area (Isimangaliso Wetland park), with human settlements and livestock within the foraging distance of the bats; The Table Mountain roost is located in the Table Mountain National Park, representing the fynbos biome and a winter rainfall with tourists visiting the area; Madimatle cave, Limpopo, South Africa is a maternity roost for *Miniopterus* spp., co-roosting with *Rhinolophus* spp. It is an important cultural site frequently visited by people travelling back and forth between this cave and major metropolitan cities in the most populated province in South Africa, Gauteng. It is also surrounded by wildlife farming areas. One-off sampling in Mozambique and Zimbabwe will focus on the cave dwelling *R. aegyptiacus* (implicated as a filo- and henipavirus host) and insectivorous bats co-roosting including know roosts such as Chinoyi caves.

Table 3: Summary of sampling sites, bat species targeted, sample numbers and sample frequencies.

	***Rousettus aegyptiacus*[#]**			***Miniopterus* spp[#]**			***Mollosid*s and *Rhinolophus* spp[#]**		
Site name (Fig 1)	**Year of sampling and frequency**	**Sample numbers**	**Estimated population sizes**	**Year of sampling and frequency**	**Sample numbers**	**Estimated population sizes**	**Year of sampling frequency**	**Sample numbers**	**Estimated population sizes**
Matlapitsi Limpopo, RSA*	Y1, Y2, Y3 Monthly	120/mo 1440/year Total=4320	Varies between 6000-20000 with lower numbers during the winter months	Y1, Y2, Y3 Seasonally (4 x/year)	120/visit 480/year Total=1440	Varies between 1000-2500 individuals	Y1, Y2, Y3 Seasonally (4 x/year)	120/visit 480/year Total=1440	Populations sizes are unknown but can be between 1000-2500 individuals
Black Rock KZN, RSA*	Y2 and Y3 Seasonally (4 x/year)	120/visit 480/year Total =960	Varies between 6000-10000 with lower numbers during the winter months	Y2 and Y3 Seasonally (4 x/year)	120/visit 480/year Total=960	Varies between 1000-2500 individuals	Y2 and Y3 Seasonally (4 x/year	120/visit 480/year Total=960	
Table Mountain Western Cape, RSA	Y2 and Y3 Seasonally (4x/year)	120/visit 480/year Total=960	A small population of about 1000-3000 individuals	Y2 and Y3 Seasonally (4 x/year)	120/visit 480/year Total=960	Varies between 1000-2500 individuals	Y2 and Y3 Seasonally (4 x/year)	120/visit 480/year Total=960	
Madimatle Limpopo RSA*	*R. aegyptiacus* not present	N/A	N/A	Y1, Y2, Y 3 Monthly for Oct – Feb	120/mo for 5 months 600/year Total=1800	Varies between 1000 in the winter months and 500 000 during birthing periods	Y1, Y2, Y3 Seasonally Only 5 month, 2 seasons	120/visit 240/year Total=720	
Other Miniopterus sites (Highveld) X 2 (Mamelodi and Gladysvale)	*R. aegyptiacus* not present	N/A	N/A	Y1, Y2, Y 3 Monthly for March – Sept	120/mo for 7 months 840/year Total=2520	Varies between 1000-2500 individuals	Y1, Y2 , Y3 Seasonally Only 7 month, 2 seasons	120/visit 240/year Total=720	
Mozambique*	Y4 Twice/year	200/visit 400/year Total=400	Unknown but *R. aegyptiacus* population estimated to be 2000-5000	Y4 Twice/year	200/trip 400/year Total=400	Unknown Varies between 1000-2500 individuals	Twice/year (Y4)	200/trip 400/year	
Zimbabwe*	Y4 Twice/year	200/visit 400/year Total=400	Unknown but *R. aegyptiacus* population estimated to be 2000-5000	Y4 Twice/year	200/trip 400/yea Total=400	Unknown Varies between 1000-2500 individuals	Twice/year (Y4)	200/trip 400/year Total=400	
Total bats sampled	**7040**			**8480**			**5600**		
	21 120								

Population densities of the bat species differ significantly and is different for the same species between sites and seasons. Estimations are indicated in the table.

*Sites that will be targeted for once of human behavior studies and collection of human and livestock serum for sero-surveillance. Only human questionnaire-based studies will be performed in Mozambique and Zimbabwe.

Human behavior (KAP analysis) and collection of human serum samples (n=150/site) will be implemented at three sites in South Africa in Y3 (Table 3) where potential human and livestock contact is a possibility. Sampling more than 100 per site will allow us to detect seropositivity with a 95% confidence at a seroprevalence of 3% assuming populations of 500. Livestock samples, including cattle, goats, pigs and donkeys (n=150/species/site), will also be collected in Y3 at South African sites. Only questionnaire-based human studies will be performed in Mozambique and Zimbabwe in Y4.

Collection of samples and testing*:* Sample collection will be performed in full personal protective equipment including disposable tyveks, double latex gloves, leather gloves, respiratory protection

(PAPRs) and gumboots. Oral and rectal swabs, urine and blood will be collected in duplicate. Onne sample set will be pooled for testing. All samples will be collected in inactivation buffer, DNA/RNA shield (ZymoResearch), at the site of collection, therefore avoiding any transport or storage of potential infectious samples (since viruses, bacteria, fungi and parasites are inactivated) and requirement of high biocontainment laboratories for processing of samples. This also protects the quality of nucleic acids by neutralizing nucleases and eliminates the need for cold-chain, if necessary. DNA/RNA shield abides by Center for Disease Control's (CDC) guidelines for pathogen inactivation and has been validated by various research groups to inactivate both enveloped and non-enveloped viruses such as parvoviruses, Chikungunya Virus, and West Nile Virus, Dengue Virus, Ebolavirus, Herpes Simplex Virus-1, Herpes Simplex Virus-2, Influenza A, Rhinoviruses, and MERS-coronavirus (Nowotny and Kolodziejek, 2014). Serum will be inactivated using 60°C for 15 min. This has been shown to be effective in inactivating viral pathogens (Van Vuren and Paweska, 2010).

Ecological and environmental data: Environmental (weather, rainfall and humidity) and ecological data of the bats (age, reproductive status, sex and measurements) will be collected, as well as population size estimates. We will employ marking and tracking technologies such as tattooing, pit tags (RFID), and telemetry systems to monitor virological status in individuals recaptured over time but also to determine foraging patterns (home ranges, distances traveled and habitat selection) and estimate population sizes. There is limited information about the habitat use and foraging patterns of *R. aegypticus*, and tracking this to estimate spillover risk will be a key component of this project. Movement data has been useful in understanding host and virus ranges and where bats may interact with people and livestock (Epstein et al., 2009; de Jong et al., 2013). EHA (Epstein) and the Movement Ecology laboratory (Hebrew University of Jerusalem) have extensive experience in using telemetry in fruit bats and will collaborate with UP on this specific part of the project.

Diagnostic testing of samples: Testing for viral antibodies and nucleic acids from all bats samples collected will be performed at UP and NICD, RSA. This will include follow up DNA Sanger sequencing of all positive amplicons. Molecular detection technologies are already established with some improvements needed (Table 4) and synthetic RNA controls will be used as positive controls and to determine sensitivity of the assays. In addition, these assays are conventional nested RT-PCRs, and the assay times are extensive. Given the scope of the proposed project and the high number of samples that will be tested, we aim to adapt these assays to use randomly primed cDNA, which can be used as template for all virus specific assays. This will save cost and processing time. Assays will be optimized on a SYBR Green real-time PCR platform, which will drastically improving throughput by reducing the processing times for sample testing, limits contamination risk and save on costs. Additional characterization of gene regions using Sanger (UP) and high throughput genome sequencing (UP) will be performed on all novel sequences identified. Luminex technology will be established in partnership with USU and will be transferred to both NICD and UP. This will include coupled antigens and controls for henipa- and filoviruses (Marburg and Ebola) and proteins are available for Hendra, Nipah, Cedar, Mojiang, Ghana henipavirus, Ebola virus, Sudan, Reston, Bundibugyo, Tai forest, Cueva, Lloviu, Marburg, Ravn, Mengla and Bombali virus. Currently, there are no antigens available for coronaviruses and cross reactivity complicates the value of such an assay and will therefore be excluded. Negative *R. aegyptiacus* sera available from previous experimental infections are available (NICD) to be used as additional controls to establish and verify cut-off values. NICD is the reference laboratory for human sample testing as well as for filovirus diagnostic testing and therefore the technologies will be established at both NICD and UP.

Data analyses, reporting and communication strategy: Data analyses will include statistics, bioinformatics, epidemiology and disease modelling. This will be done annually, including all stakeholders (Table 3). It will also include retrospective results generated before this project started. Standard operating procedures will be established for reporting and will include developing risk mitigation and prevention strategies. A communication strategy will be developed at the start of the project that will include education and awareness materials, also targeting communities.

Biobank and database: A secure and up to date database that will be shared by all partners will be established. UP has developed an in-house database that can function on a web-based system and can be adapted for this project. The PACS system is also available to manage sample information. For storage of samples the current biobank infrastructure at UP will be expanded.

Table 4: Detail of nucleic acid detection methods that will be used in this study

Paramyxoviruses						
Respiro-, *Morbilli-*, and *Henipavirus*	Hemi-nested RT-PCR (RMH assay)	Tong et al., 2008	10-100 copies of RNA (Tong et al., 2008); [Two-step protocol 10 copies of RNA (Bruck, 2019)]	Partial polymerase (L) gene	611 bp (first round); 494 bp (nested round)	The sensitivity and specificity of the assays were evaluated against a range of paramyxoviruses including several human viruses such as measles (*Morbillivirus*) and mumps virus (*Orthorubulavirus*), as well as the zoonotic bat-borne Hendra and Nipah viruses (*Henipavirus* genus) amongst others. These assays have been widely applied for biosurveillance in bats which led to the detection of viruses such as Ghanaian bat henipavirus (Drexler et al., 2012), bat mumps orthorubulavirus (Drexler et al., 2012) and viruses related to other human pathogens such as human parainfluenza virus 2 (Mortlock et al., 2019). Additionally, the family wide PAR assay has been successfully used in the USAID PREDICT program for paramyxovirus biosurveillance.
Avula- and *Rubulavirus*	Hemi-nested RT-PCR (AR assay)	Tong et al., 2008	10-100 copies of RNA (Tong et al., 2008); [Two-step protocol 300 copies of RNA (Mortlock et al., 2019)]	Partial polymerase (L) gene	272 bp (first round); 224 bp (nested round)	
Assay comment: Both the selected assays (RMH and AR) are very sensitive and capable of detecting a diversity of paramyxoviruses. The family wide assay (PAR) was is much less sensitive (500-1000 copies of RNA), hence the selection of the two more specific and sensitive assays for biosurveillance. All three assays were developed and published in 2008 and included the then known diversity of bat-borne paramyxoviruses (Hendra, Nipah, Menangle and Tioman viruses). However, these assays have not yet been updated or evaluated against more recently described bat- and wildlife-borne viruses such as Sosuga, Cedar, Mojiang and Ghanaian bat henipavirus. We aim to evaluate the sensitivity of these established assays against more recently described bat-borne viruses such as Sosuga, Cedar, Ghanaian bat henipavirus and the presumed rodent-borne Mojiang virus and update the primers if necessary.						
Filoviruses						
Diversity targeted	**Assay type**	**Reference**	**Sensitivity**	**Targeted gene region**	**Size of amplicon**	**Potential to detect zoonotic viruses**
Ebola- and Marburg virus and new diversity (Bombali and Mengla)	Qualitative real-time RT-PCR (probe based)	Panning et al., 2007; Rieger et al., 2016 (RealStar® Filovirus Screen RT-PCR Kit)	Panning et al., 2007: Virus dependent (500-500 RNA copies); RealStar kit: ~ 10 RNA copies	Partial polymerase (L) gene	293 bp	Capable of detection of all Ebola- and Marburvirus strains, limited for detection of other viruses/strains (Rieger et al., 2016; Emperador et al., 2019)
	Hemi-nested RT-PCR	Yang et al. 2019, 2017; He et al. 2015	Not determined	Partial polymerase (L) gene	214-478 bp	Capable of detection of Ebola virus strains and Mengla virus (not evaluated for detection of other filoviruses)
	Qualitative real-time RT-PCR (probe based)	Kemenesi et al. 2018	Not determined	Partial polymerase (L) gene	73 bp	Capable of detection of Lloviu virus (not evaluated for detection of other filoviruses)
	RT-PCR	Goldstein et al. 2018	Not determined	Partial polymerase (L) gene	680 bp	Capable of detection of Bombali virus (not evaluated for detection of other filoviruses)
	Quantitative real-time PCR (probe based)	Forbes et al. 2019; Jääskeläinem et al. 2015	6 RNA copies for *Zaire ebolavirus*	Partial polymerase (L) gene	108 bp	Capable of detection of *Zaire ebolavirus* and Bombali virus (not evaluated for detection of other filoviruses)
Assay comment: Most diagnostic assays target the *Ebola-* and *Marburgvirus* genera and to allow for detection and differentiation, most of these assays target the more variable genes i.e. glycoprotein (GP) and the RNA-dependent RNA polymerase (L) (reviewed in Emperador et al., 2019). Genomic sequences of filovirues of different genera differ from each other by more than 55% complicating assay selection for use in family-wide targeted surveillance and a combination of several assays (as listed) will be required to allow detection of the complete diversity. Although established assays have been reported to be successful for detection of novel filoviruses (for example Forbes et al. 2019), the majority has not been fully validated or evaluated for detection of all members of the *Filoviridae* family with important information regarding specificity and sensitivity lacking. Therefore, several assays will be required for surveillance; the Panning/kit assay will be used for detection of Ebola- and Marburgvirus strains in combination with the other assays listed to cover the entire diversity (i.e. Bombali-, Lloviu- and Menglavirus).						

Coronaviruses						
Diversity targeted	**Assay type**	**Reference**	**Sensitivity**	**Targeted gene region**	**Size of amplicon**	**Potential to detect zoonotic viruses**
Coronaviruses - focused on the mammalian infecting *Alpha-* and *Betacoronavirus* (includes SARS coronavirus) genera	Hemi-nested RT-PCR	Geldenhuys et al., 2018	The nested assay is capable of detecting 50 copies of synthetic control RNA (Nkambule, 2019)	Partial region of the RNA dependent RNA polymerase (RdRp) gene	443 bp (first round); 268 bp (nested round)	Capable of detecting the range of human coronaviruses (synthetic controls); detected a MERS-related coronavirus from an insectivorous bat species (Geldenhuys et al., 2018); SARS-related coronaviruses from Horseshoe bats in Rwanda (Markotter et al., 2019)
Assay comment: Members of the *Coronaviridae* family are highly diverse, which complicates sequence detection of all species with one nucleic acid detection assay. The RNA dependent RNA polymerase (RdRp) gene region (or nsp 12) is situated within ORF 1b of the genome, and is highly conserved and a number of nucleic acid detection assays (Stephensen et al., 1999; Poon et al., 2005; Woo et al., 2005; Watanabe et al., 2010; de Souza Luna et al., 2007; Tong et al., 2009; Gouilh et al., 2011; Geldenhuys et al., 2013; Geldenhuys et al., 2018) use a region located between approximately 14,800 and 15,600 nt depending on the genome (in reference to SARS coronavirus AY714217) for coronavirus RNA detection. Since the majority of assays use the same region, it allows overlapping sequence regions to be compared when investigating partial genes. Assays reported in Watanabe et al., 2010 and Quan et al., 2010 are used in the PREDICT project (Anthony et al., 2017a; Anthony et al., 2017b; Wacharapluesadee et al., 2015; Lacroix et al., 2017), though the sensitivity of these assays have not been reported in literature. The Geldenhuys et al., 2018 assay is a hemi-nested RT-PCR assay that is a multiplexed assay focused on diversity of alpha- and betacoronaviruses and correspond to the same regions targeted in these assays. The primers of the Geldenhuys et al., 2018 assay was updated in 2017, and included the latest diversity of coronaviruses (including human and major bat coronavirus lineages).						

IV. SUMMARY OF TASKS AND PERFORMANCE SCHEDULE

Table 5: Summary of the tasks that will be performed for the duration of the project. Detailed tasks are indicated in the attached Statement of work (SOW).

TASK	**Y1**	**Y2**	**Y3**	**OY4**	**OY5**	**TASK**	**Y1**	**Y2**	**Y3**	**OY4**	**OY5**
1. Build and enhance capacity in Southern Africa to conduct biosurveillance for filo-, henipa- and coronaviruses						**3. Conduct targeted observational and human behavior studies**					
1.1.Project stakeholder workshop						3.1.Design the study and questionnaires					
1.2.Establishment and sustaining a Southern African Bat Research network (SABRENET)						3.2.Apply for necessary ethical approvals and obtain community permissions					
1.3.Modelling and data analysis workshop						3.3.Collect and analyze data					
1.4.Introduction to bat biology and taxonomy						**4. Conduct targeted serological surveys in people and livestock**					
1.5.Bat field sampling training						4.1.Scoping visits to potential study sites					
1.6.Establishment of luminex serology technology at UP and NICD						4.2.Apply for necessary permits and ethical approvals					
1.7.Laboratory diagnostic training						4.3.Collection of samples					
1.8.Data interpretation workshop						4.4.Test and analyze data					
2. Implement biosurveillance in bats in Southern Africa and testing of samples						**5. Reporting and Communication**					
2.1.Cross sectional and longitudinal surveillance in bat species in South Africa						5.1.Discuss results, finalize and compile an annual report including all stakeholders					
2.2 Nucleic acid testing of samples and DNA sequencing of positives.						5.2. Develop a communication plan and implement it					
2.3. Serological testing of serum samples collected from bats and data analysis						5.3. Present at scientific conferences and prepare manuscripts for publication					
2.4.Characterization of additional genes or genome regions of positive samples, verify serological positive results						5.4. Annual report to all governmental partners and stakeholders					
2.5. Enter all data and results into a database and analyze						5.5. Annual report to DTRA					
2.6.Once off surveillance of bats in Mozambique and Zimbabwe						5.6. Attend DTRA technical review					

From:	Wanda Markotter on behalf of Wanda Markotter <wanda.markotter@up.ac.za>
To:	Christopher Broder; epstein@ecohealthalliance.org; Eric Laing; Marike Geldenhuys <geldenhuysmarike@gmail.com>; Marinda Mortlock <marinda.moller@gmail.com>; PhD Kevin Olival; Spencer Sterling
Subject:	DTRA documents
Date:	Wednesday, September 2, 2020 9:57:15 AM
Attachments:	DTRA Markotter Epstein Statement of Work.pdf DTRA Markotter Epstein Technical proposal.pdf

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Dear all

See attached the DTRA technical proposal and Work statement that will form part of our meeting tomorrow.

Talk soon.

Wanda

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Nipah virus dynamics in bats and implications for spillover to humans

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Keywords: bats, henipavirus, Nipah virus, *Pteropus medius, Pteropus giganteus,* satellite telemetry, viral phylogeny, disease dynamics, modeling

Author Contributions: Study design: JHE, AMK, EG, MJH, HEF, TB, MR, SPL,WIL, and PD. Data collection/generation and analysis: JHE, SJA, AI, AMK, SAK, MS, NR, IS, CZT, YT, AuI, PLQ, KJO, MSK, TB, CCB, GC, LFW, WIL, and PD. Manuscript writing: JHE, SJA, AI, AMK, CZT, YT, KJO, EG, HEF, MDF, TB, GC, LFW, SPL, WIL, and PD.

This PDF includes: Main text, Figures 1-6; Table 1; Supporting Information Figures S1-S6; Tables S1-S2.

Abstract

Nipah virus (NiV) is an emerging bat-borne zoonotic virus that causes near-annual outbreaks of fatal encephalitis in South Asia – one of the most populous regions on Earth. In Bangladesh, infection occurs when people drink date palm sap contaminated with bat excreta. Outbreaks are sporadic and the influence of viral dynamics in bats on their temporal and spatial distribution is poorly understood. We analyzed data on host ecology, molecular epidemiology, serological dynamics, and viral genetics to characterize spatio-temporal patterns of NiV dynamics in its wildlife reservoir, *Pteropus medius* bats, in Bangladesh. We found that NiV transmission occurred throughout the country and throughout the year. Model results indicated that local transmission dynamics were modulated by density-dependent transmission, acquired immunity that is lost over time, and recrudescence. Increased transmission followed multi-year periods of declining seroprevalence due to bat population turnover and individual loss of humoral immunity. Individual bats had smaller host ranges than other *Pteropus* spp., although movement data and the discovery of a Malaysia-clade NiV strain in eastern Bangladesh suggest connectivity with bats east of Bangladesh. These data suggest that discrete multi-annual local epidemics in bat populations contribute to the sporadic nature of Nipah virus outbreaks in South Asia. At the same time, the broad spatial and temporal extent of NiV transmission, including the recent outbreak in Kerala, India, highlights the continued risk of spillover to humans wherever they may interact with pteropid bats, and the importance of limiting opportunities for spillover throughout *Pteropus*'s range.

Significance

Nipah virus (NiV) is a zoonotic virus and WHO priority pathogen that causes near-annual outbreaks in Bangladesh and India with >75% mortality. This work advances our understanding of transmission of NiV in its natural bat reservoir by analyzing data from a 6-year multidisciplinary study of serology, viral phylogenetics, bat ecology and immunology. We show for the first time that outbreaks in *Pteropus* bats are driven by increased population density, loss of immunity over time and viral recrudescence, resulting in multi-year inter-epidemic periods. Incidence is low, but bats carry NiV across Bangladesh and can shed virus at any time of year, highlighting the importance of routes of transmission to the timing and location of human Nipah virus outbreaks.

Introduction.

Outbreaks of zoonotic diseases are often sporadic, rare events that are difficult to predict, but can have devastating consequences (1). Emerging viral zoonoses of wildlife that have become pandemic include HIV/AIDS, 1918 H1N1 influenza virus, SARS coronavirus, and the current COVID-19 pandemic caused by SARS-CoV-2 (2-5). Bats are important hosts for many zoonotic viruses including Ebola virus, SARS-CoV, SARS-CoV-2 and Nipah virus; the ecological drivers and transmission dynamics of these viruses in their reservoir hosts are poorly understood (6-12). A better understanding of the transmission dynamics of zoonotic pathogens in their natural reservoirs may help anticipate and prevent outbreaks (10, 13).

Nipah virus (NiV) is an emerging zoonotic paramyxovirus (genus *Henipavirus*) that has repeatedly spilled over from bats to cause outbreaks in people and livestock with high case fatality rates across a broad geographic range. To date, human Nipah virus infections have been identified in India, Bangladesh, Malaysia, Singapore, and the Philippines (14-18). It has caused repeated outbreaks in Bangladesh and India, with a mean case fatality rate greater than 70% (14, 19, 20). A single genus of frugivorous bats (*Pteropus*) appears to be the main reservoir for henipaviruses throughout Asia and Australia (21-25). This includes *Pteropus medius* (formerly *Pteropus giganteus* (26)), the only pteropid bat present in Bangladesh and India (16, 27-30). Nipah virus has several characteristics that make it a significant threat to human and animal health: 1) its bat reservoir hosts are widely distributed throughout Asia, and occur within dense human and livestock populations, leading to widespread frequent spillover events and outbreaks; 2) it can be transmitted directly to humans by bats or via domestic animals; 3) it can be transmitted from person to person; 4) spillover has repeatedly occurred in highly populous and internationally connected regions, giving it pandemic potential; 5) it is associated with high mortality rates in people; and 6) there are currently no commercially available vaccines to prevent infection or drugs to mitigate disease (31-33). As a result, the World Health Organization has listed Nipah virus among the ten most significant threats to global health (34). In May 2018, an outbreak of Nipah virus encephalitis with a 91% mortality rate occurred in a new location - Kerala, India - more than 1,200 km southwest of previous Indian and Bangladeshi outbreaks (35). A single case was subsequently reported in Kerala in 2019, and while local *P. medius* populations have been implicated as the local source of infection, the route of spillover in both instances remains unknown (35, 36).

In Malaysia and Bangladesh, consumption of cultivated food resources contaminated with bat excreta such as mangoes in Malaysia and date palm sap in Bangladesh and northeastern India have been identified as the predominant cause of spillover to pigs and people, respectively (37). Human outbreaks occur almost annually in Bangladesh and the seasonal timing (November-April) and spatial distribution of outbreaks coincide with patterns of raw date palm sap consumption in a region termed the “Nipah belt” (38). However, there is variability in the geographic locations and number of spillover events, as well as the number and magnitude of human outbreaks that occur each year (39, 40). Spillover has also occurred outside the predominant season and region of date-palm sap consumption (41). Whereas no human outbreaks have been reported in eastern Bangladesh despite date palm sap harvesting and consumption, human outbreaks have been reported in Kerala, India where date palm sap is not cultivated (38). These observations suggest an alternate route of spillover in certain locations, and a critical need to understand the mechanisms of underlying viral infection dynamics in bats and the extent

of genetic diversity within the virus – each of which may influence the timing, location and epidemiology of human outbreaks (38).

Previous research on the transmission dynamics of Nipah and Hendra viruses in *Pteropus* spp. bats has produced mixed and sometimes contradictory findings. Nipah virus, like Ebola, Marburg, Hendra and some bat coronaviruses, is associated with seasonal spikes in infection that coincide with annual or semi-annual synchronous birth pulses (21, 42-48). Seasonal periods of Nipah virus shedding were observed in *P. lylei* in Thailand and seasonal spikes in NiV (or a related henipavirus) seroprevalence coinciding with pregnancy periods were observed in *Eidolon dupreanum* in Madagascar (49, 50), but not in *P. vampyrus* or *P. hypomelanus* in Peninsular Malaysia (25). Hendra virus prevalence in Australian pteropid bats has shown multi-year inter-epidemic periods during which little virus can be detected, followed by periods of markedly increased viral shedding (51-53). It has been hypothesized that multi-year periodicity in the incidence of henipavirus infections could arise from a build-up and waning of herd immunity in the reservoir host, with re-introduction of virus via immigration or recrudescence or viral persistence (11, 54-56).

There is a paucity of data related to henipavirus-associated immune dynamics in free-ranging pteropid bats, including the duration of immunity in adults and juveniles, which limits our understanding of population-level viral dynamics. Experimental infections of *Pteropus* bats with Hendra and Nipah virus show that bats mount an antibody response following infection with Hendra and Nipah virus (57-59). Waning of anti-Nipah virus antibodies was observed in recaptured wild *Eidolon helvum*, a bat related to *Pteropus* spp., in Madagascar (60). Passive transfer and waning of maternal antibodies also occurred in captive *Pteropus* species, and along with loss of immunity in adults, could contribute to the loss of herd immunity in wild populations (61). Some pteropid bat species are migratory and interconnected colonies form a metapopulation which could allow for viral re-introductions into susceptible colonies (10, 25, 62, 63). In addition, NiV recrudescence has been observed in wild-caught *P. vampyrus* and possibly also in *Eidolon helvum* (64-66). Either of these phenomena could allow NiV to persist regionally during periods of high local immunity. However, no study has yet shown evidence in open, free-ranging bat populations that examines the influence of these factors on NiV transmission dynamics.

Here we examine the distribution, dynamics, genetic diversity, and underlying drivers of NiV infection in *Pteropus medius* in Bangladesh to improve our understanding of human outbreak patterns. Specifically, we analyze the spatial, temporal and demographic variation in serological dynamics and viral shedding in bats over a six-year period to determine the spatio-temporal drivers and dynamics of virus transmission. We also analyze the movement patterns of individual bats and analyze NiV phlyogenetics to understand patterns of spatial mixing and virus strain diversity.

Results

Comparative Nipah seroprevalence and virus infection study in bats inside and outside the Nipah belt and concurrent longitudinal bat study inside the Nipah belt.

In a cross-sectional spatial study conducted between January 2006 and July 2012, we caught and tested 883 *P. medius* (~100 per district) from eight colonies in different districts across Bangladesh. We detected anti-Nipah IgG antibodies in all colonies (**Figure 1**). Seroprevalence varied by location (χ^2 =

55.61, p<.001). In most locations adult seroprevalence exceeded juvenile seroprevalence; in Tangail and Rajbari seroprevalence was similar across ages. Viral detection in individuals was rare; overall, we detected NiV RNA in 11/2088 individuals and in three pooled oropharyngeal samples (representing five bats, but which could not be resolved to an individual) (**Table 1**). We detected viral RNA in individual bats in Faridpur and Rajbari and from pooled samples from Thakurgaon and roost urine samples from Cumilla. Of the 11 PCR positive individuals, three had IgG antibodies (**Table S1**). We also detected virus in pooled urine collected from tarps placed below bats at roosts associated with human outbreaks in Bhanga and Joypurhat. The viral prevalence in Rajbari in January 2006 was 3.8% (95% CI: 0% -11%; n=78). In Faridpur, which is adjacent to Rajbari and where we conducted an intensive longitudinal study (see below), viral prevalence estimates ranged from 0% to 3% (95% CI: 0%-10%; n=100 at each of 18 sampling times) (**Table 1**). Nipah virus RNA was detected in bats from inside (Rajbari, Thakurgaon, and Faridpur) and outside (Cumilla) the Nipah Belt. There was no significant difference between NiV detection rates from individual bats by the two main sample types: urine/urogenital swabs, 0.37% (n=2,126) and oropharyngeal swabs, 0.15% (n=1973) (χ^2= 1.92 p=0.17). The estimated detection rate from pooled urine samples, collected from tarps placed underneath roosts) across the entire study was 2.5% (n=829), which was significantly higher than either sample type collected from individual bats (χ^2 = 55.6, p < 0.001).

Factors associated with NiV IgG *serostatus in* P. medius

There was no statistical difference between seroprevalence in bats inside the Nipah Belt and outside (95% odds ratio (OR) 1.2, highest posterior density interval (HDPI) 0.47-3.1)**.** Adults had higher seropositivity than juveniles (OR 2.4, 1.7-3.6 HDPI), and males greater than females (OR 1.6, 1.0-2.4 HDPI) (**Figure 2**). There was weak evidence that seroprevalence was higher in pup-carrying (OR 4.0, HDPI 0.6-34) and pregnant (1.5 times, HDPI 0.85-2.8) individuals than other females. Neither mass, forearm length nor the M:FA ratio (a proxy for age) consistently correlated with seropositivity. However, bats with poor body condition (an assessment of pectoral muscle mass by palpation) were less likely to be seropositive (Poor/Fair body condition OR = 0.69, HDPI 0.49-0.96). Finally, serostatus was strongly correlated in mother-pup pairs; 39 of 41 pups with seropositive mothers were seropositive, and 32 of 39 pups with seronegative mothers were seronegative.

Longitudinal NiV serodynamics in P. medius*, Faridpur district (2006-2012)*

We sampled bats quarterly from a single population in the Faridpur district. We also microchipped a total of 2,345 bats between 2007 and 2012. We used generalized additive models (GAMs) to characterize changes in NiV seroprevalence over time. There were significant fluctuations in adult (>24 mo.) and juvenile (6 – 24 mo.) seroprevalence over the six-year study period (**Figure 3A**). Juvenile seroprevalence ranged from 0% to 44% (95%CI 37%-51%), and decreased over the first year of life for bats born in each year ("yearlings"), consistent with loss of maternal antibodies in juveniles. A more pronounced decrease occurred from mid-October to mid-December than other parts of the year. However, the GAM indicating this had only slightly better fit (ΔAIC < 1), than one with a linear decrease over the whole year (**Figure 3B**).

Adult seroprevalence ranged from 31% (95% CI: 20%-46%) to 82% (95% CI: 77%-87%) with three cycles of clear variability over the course of the study (**Figure 3A**). We found no evidence of regular

seasonal fluctuations; a GAM with annual cyclic terms fit worse than one without (ΔAIC > 10). Viral RNA was detected during periods of increasing, decreasing, and stable seroprevalence.

We fitted a series of age-stratified compartmental susceptible-infected-recovered models to examine different biological processes influencing serodynamics, including density- vs. frequency-dependent transmission, recrudescence vs. immigration of infected individuals, and seroreversion (loss of antibodies) in both juveniles and adults (see Methods and (**Figure 4**)). Density-dependent models were a far better fit to the data than frequency-dependent models (difference in log-likelihood 10.0; ΔAIC = 20.0), suggesting that movements of bats and fluctuations in colony size alter spatio-temporal variation in the risk of NiV infection in bats. In Faridpur (see "Domrakhandi/Khaderdi" in **Figure S1**) during the period of sampling, the roost population declined from approximately 300 bats to 185, which decreased transmission potential in the fitted model: R_0 in adult bats was estimated to decrease from 3.5 to 2.1 as the number of bats in the colony decreased. As a result, over the six-year study period, the fitted model predicted that the threshold for herd immunity (i.e. the seroprevalence below which the reproductive ratio R_t >1) for adults fell from 72% (when bat counts were highest - in 2006) to 52% (when bat counts were lowest).

The fitted model suggests that serodynamics in juveniles were strongly driven by inheritance and loss of maternal antibodies. The estimated duration of maternal antibodies was 17.6 weeks (95% CI: 13.7-25.0), which was much quicker than the loss of antibodies in adults (290.8 weeks, 95% CI: 245.0-476.4) (**Table S2**). Finally, models with recrudescence fit the data better than models without recrudescence (**Table S2;** difference in log-likelihood 32.6; ΔAIC = 65.1), and models with recrudescence fit the data better than models with immigration (ΔAIC = 3.76).

Mark-recapture and seroconversion/seroreversion

There were 56 recapture events over the study period (**Table S3**). Thirty-one bats were recaptured at a nearby roost other than the original capture location. This network of roosts or "roost complex" formed a polygon covering approximately 80 Km^2 and included 15 roosts sampled during the longitudinal study (**Figure S2A and S2B**). Ten instances of seroconversion (change from IgG negative to IgG positive) and nine instances of seroreversion (positive to negative) were observed (**Table S3**). The mean time between positive and negative tests in *adults* (excluding juveniles with maternal antibodies) was 588 days (n=6) (range: 124-1,082 days).

Home range and inter-colony connectivity analysis

Home range analysis of satellite telemetry data from 14 bats (mean duration of collar data transmission = 6.25mo; range = 1-25mo; **Table S4**) showed that the majority of bats roosted within 10 km of where the bats were originally collared, in the Faridpur (Nipah belt) colony, and within 7 km from where the bats in the Cox's Bazaar colony were originally collared (315km east of Faridpur). The average foraging radius was 18.7 km (s.d. 21.5 km) for the Faridpur bats and 10.8 km (s.d. 11.9 km) for the Cox's Bazaar bats (**Figure S2**). Home range analysis suggests that bats in Faridpur and Cox's Bazar (separated by approx. 310km) would have a <5% probability of intermingling (**Figure 5**). Home-range size was larger during the wet season than the dry season (2,746 km^2 vs. 618 km^2) (**Figures S3 & S4**).

NiV phylogenetic analysis.

Phylogenetic analysis of NiV sequences from a 224nt section of the N gene (nt position 1290-1509 [position ref gb|FJ513078.1| India]) suggests that strains from both India and Malaysia clades are present in bats in Bangladesh (**Figure 6**). This finding is supported by an additional analysis of near-whole N gene sequences (~1720 nt) from bats, pigs, and humans, including those from a subset of *P. medius* from this and a more recent study by our group (**Figure S5**) (67). Eleven 224nt N gene sequences obtained from bats between 2006 and 2012 (all from the Faridpur population) were identical. Overall, the N gene sequences identified from the Faridpur, Rajbari and Bhanga colonies between 2006 and 2011 had 98.21%-100% shared nucleotide identity. Sequences from Rajbari district obtained five years apart (January 2006 and January 2011) had only a single nucleotide difference resulting in a synonymous substitution (G to A) at position 1304, which was found in four other bat NiV sequences from this study, as well as in the NiV isolate from *P. vampyrus* in Malaysia. Five human NiV N gene sequences from various locations within the Nipah belt over the same time period as our bat study show more nucleotide diversity than those from the Faridpur *P. medius* population. Human sequences throughout Bangladesh and from Kerala, India, all nested within the diversity found in *P. medius* (**Figure 6**). By contrast, the sequences found in *P. medius* from Cumilla, a location 150Km to the east of Faridpur, showed 80.8%-82.59% shared nucleotide identity with sequences from *P. medius* in Faridpur and clustered within the Malaysia group of NiV sequences. The two Cumilla sequences were identical to each other, and had up to 87.95% shared nucleotide identity to sequences from *P. lylei* in Thailand.

Discussion

Our findings suggest Nipah virus (NiV) circulation occurs in bat populations throughout the country. We observed that virus can be shed by bats at any time of year, and that viral dynamics are cyclical but not annual or seasonal. Our models fit to serological data suggest that these cycles may be driven by demographic and immunological factors; the waning of herd immunity through turnover or individual waning in bat populations allows heightened viral transmission when seroprevalence passes below a critical threshold. Previous studies from Bangladesh suggested that human Nipah virus (NiV) outbreaks occur primarily within a defined region in western Bangladesh, termed the “Nipah belt,” during a defined season (Nov-Apr) (41, 68). These observations raised the question of whether the timing and location of human infections are due solely to differences in the frequency and intensity of date palm sap consumption, or whether ecological factors such as the distribution and timing of bat viral infection also play a role (19, 38, 69). Our extensive survey of *Pteropus medius*, which is common across Bangladesh and throughout the Indian subcontinent, demonstrates that viral circulation within their populations is not limited to the Nipah belt (16, 27, 30).

A number of mechanisms have been proposed for the maintenance of acute viral infections in bat populations which are often formed of interconnected colonies, including synchronous birthing and subsequent loss of maternal antibodies (11, 43, 45), lowered immunity within pregnant females due to stress, nutritional stress and other factors (47), immigration of infected individuals from other colonies (62, 70, 71), and recrudescence within previously-infected individuals (11, 64, 72). However, little is known about how henipaviruses are transmitted among wild bats. *Pteropus* species are typically gregarious and their roosts, often comprising multiple hardwood trees, are highly socially structured, with individuals segregated by age, sex, and social dominance (69, 73, 74). Interactions among

individuals are often dependent on their grouping and the intensity of social interactions varies with specific behaviors such as mother-pup interactions, play (juveniles), territorial fighting (adult males), and mating (adults) (74). Our data and previous experiments show that henipaviruses can be shed orally, urogenitally, in feces, and in birthing fluids (59, 75). This suggests multiple mechanisms for transmission are possible, including mutual grooming, fighting, mating, exposure to excreta or birthing fluids, and ingestion of food contaminated by saliva. Roost size also increases seasonally during mating and birthing periods, which the fitted models suggest would increase transmission, if seroprevalence is below the herd immunity threshold (30, 73). While *P. medius* does not roost with other bat species, it does feed with other frugivorous bats, and it's possible that inter-species viral transmission occurs during feeding (76, 77). In Madagascar, henipavirus antibodies have been detected in multiple species of frugivorous bats, though it is unknown whether the same virus or antigenically related viruses was shared among them (60). While serological evidence suggests it is possible henipaviruses circulate in other frugivorous bat species, our findings as well as others (59, 78) suggest that in Bangladesh, *P. medius* is the main natural reservoir for Nipah virus. Henipaviruses other than Nipah may be circulating in *P. medius (28)*. We assumed that the anti-IgG antibodies detected by the serological assays used in this study were specific to Nipah virus, but it is possible that the ELISA used in the cross-sectional study may have detected antibodies against unknown henipaviruses, which could elevate NiV seroprevalence estimates. An advantage of the Luminex assay used in the longitudinal study is that we could compare MFI values to multiple specific henipaviruses (Nipah, Hendra and Cedar) and differentiate between specific reactions to NiV and reactions to the other viruses, which could indicate antibodies against an unknown henipavirus. Hendra and Cedar viruses are enzootic in Australian *Pteropus spp.* and are not known to occur in Bangladesh, so we considered reactions to these viruses NiV-negative results.

Our modeling indicates that NiV is primarily driven by immunity and density-dependent transmission dynamics among bats, with cycles of higher seroprevalence dampening intra-colony transmission followed by waning of antibody titers within individuals and death of seropositive individuals. Waning humoral immunity against NiV has been consistently shown in henipavirus studies of African pteropodid bats (56, 60). Our recapture data provided the first reported evidence of the loss of detectable NiV IgG antibodies in individual free-ranging bats, which supports the fitted model suggesting limited duration individual immunity and the importance of population-level waning immunity. The consistently decreasing seroprevalence that we observed in juveniles suggests that they lose maternal antibodies over their first year (the fitted model estimates after 3-5 months), consistent with other studies of maternal antibodies against henipaviruses in pteropodid bats (47, 56, 61, 65). Our analysis do not support the hypothesis (45) that seasonal pulses of these new seronegative individuals are sufficient to drive new outbreaks because high seroprevalence in adults limited transmission in several years (Figure 4).

Nipah virus reintroduction into a colony may occur from a persistently infected individual (e.g. via recrudescence) or immigration of an infected individual. Our analyses suggested that recrudescence was a more important driver of transmission dynamics than immigration. Recrudescence of henipavirus infection has been observed for NiV in captive *P. vampyrus* (64), for henipavirus in captive *E. helvum* (56, 66), and humans infected by NiV (79) and Hendra virus (80). It is difficult to know from serology alone whether wild-caught seronegative bats had been previously infected. Experimental infections comparing naïve to previously infected *Pteropus medius* that have sero-reverted would provide a better

understanding of how humoral immunity influences individual susceptibility to infection, and inform dynamics models attempting to explain viral maintenance within bat populations (60).

Our longitudinal study was limited to a single population of interacting subcolonies and bat populations across Bangladesh likely represent a dynamic metapopulation. Our roost count data and recapture data from microchipped bats showed how roost sizes can fluctuate, and that bats shift among local roosts. The fitted model strongly suggested that decreases in local roost counts substantially reduced local transmission potential of NiV. However, a larger study across multiple regional populations would be needed to understand how local shifts in bat colonies impact broader fluctuations in regional populations and spatial patterns of NiV transmission.

Understanding how bat populations connect across landscapes is important for understanding viral maintenance, and studying local and migratory bat movements can provide important ecological information related to viral transmission, including how bats move between different colonies (62, 81). Our satellite telemetry data suggest that *P. medius* exists as a metapopulation, like other pteropid species (11, 71). The numbers of individuals we collared represents a small sample size, however they are comparable to other bat satellite telemetry studies of related species, and our data suggest that bat dispersal in Bangladesh may currently be more localized than other species elsewhere. *Pteropus medius* appear to travel shorter distances and remain within a smaller home range (321.46km^2 and 2,865.27km^2 for two groups) than *P. vampyrus* in Malaysia (64,000 km^2 and 128,000 km^2) and *Acerodon jubatus* in the Philippines, both of which are similarly sized fruit bats (62, 82). Pteropodid bat migration is primarily driven by seasonal food resource availability (63, 83-85). In Bangladesh, *P. medius* prefer to roost in human-dominated environments in highly fragmented forests as opposed to less populated, intact forested areas such as in national parks (86). The conversion of land to villages and farmland over recent human history has likely led to increased food availability for *P. medius* and may have reduced the impetus for long-distance migration (37). This may reflect a similar adaptation to anthropogenic food resources as observed over the last few decades in Australian *Pteropus* species (71). Home ranges were significantly smaller during the dry season which corresponds to winter months and the time when most female bats are pregnant, likely resulting in them flying shorter distances to conserve energy. Genetic analysis of *P. medius* across Bangladesh has shown that historically, there has been extensive gene flow and intermixing among populations, and we did observe a few instances of longer distance flights, however, the movement data indicated that overall these bats had much smaller home ranges (81). Less connectivity among bat populations across Bangladesh may influence NiV transmission by creating longer inter-epidemic periods and larger amplitude fluctuations in population level immunity in *Pteropus medius* compared to more migratory species (71). Bat movement and population connectivity may also influence the genetic diversity of NiV found in different locations.

The potential existence of a more transmissible or pathogenic strain of NiV already circulating in bats further underscores the need to strengthen efforts to prevent spillover. While the overall strain diversity among NiV has not been well characterized due to a dearth of isolates, two distinct NiV clades have been described: A Bangladesh clade, that includes sequences identified in India and Bangladesh; and a Malaysian clade, that comprises sequences from Malaysia, Cambodia, The Philippines and Thailand (18, 67, 87). Our findings of substantially different NiV sequences in Faridpur and Cumilla suggest that viruses from both clades are circulating in Bangladesh. Strains of NiV from these two clades

359 are associated with differences in pathogenesis, epidemiological and clinical profiles in humans and
360 animal models and observed shedding patterns in bats (49, 88-92). Phenotypic variation in Nipah virus
361 could influence human outbreak patterns by altering transmission to, or pathogenesis in, humans, and
362 the likelihood of smaller outbreaks being identified or reported (93). Human-to-human NiV transmission
363 via contact with respiratory and other secretions has been regularly observed in Bangladesh and India,
364 including the recent 2018 outbreak in Kerala (14, 68, 94), whereas transmission among people was not a
365 common feature of the Malaysia outbreak, despite close contact between cases and health care
366 providers (95, 96). Nipah virus cases in Bangladesh have shown more strain diversity than in the
367 Malaysia outbreak, which could be due to greater virus diversity in *P. medius* (97).
368 Until now, there have been very few Nipah sequences obtained from *P. medius.* We found that
369 Nipah N-gene sequence from bats from the Faridpur population were nearly identical over time,
370 compared to variation in N-gene sequences from bats and humans from other locations observed over
371 the same time period (2006-2010). This suggests that there may be locally prevalent and stable NiV
372 genotypes that persist within bat colonies. Human NiV genotype diversity is likely a reflection of the
373 diversity of the NiV strains in the local bats that seed outbreaks (10). This is also supported by viral
374 sequences obtained from human and bats associated with the 2018 NiV outbreak in Kerala, India, where
375 human NiV sequences were most closely related to local *P. medius* sequences *(98)*.
376 Connectivity of pteropid bats in Bangladesh with those in Southeast Asia could explain the
377 observed strain diversity in our study. Historical interbreeding of *P. medius* with pteropid species found
378 in Myanmar, Thailand, and Malaysia and our telemetry findings showing bats are capable of flying
379 hundreds of kilometers, could explain the presence of a Malaysia clade NiV sequence in bats from
380 Cumilla (81). NiV Bangladesh strains have also been found in *P. lylei* in Thailand (99). The N gene of the
381 Cumilla NiV strain differs from those reported from bats in the Nipah belt by 20%, whereas NiV Malaysia
382 and NiV Bangladesh differ by only 6-9% and are associated with different clinical profiles. Whole
383 genome sequence would have allowed for better characterization of the Cumilla strain, however this
384 was not obtained. Despite the short sequences used in our analysis, the N gene is generally conserved
385 relative to other genes and is representative of the diversity across henipavirus genomes (87). We would
386 expect the rest of the Cumilla viral genome to also be highly divergent, potentially even qualifying it as a
387 different henipavirus species. It is therefore plausible that the clinical profile of a 20% divergent Nipah-
388 related virus differs significantly from known strains. Sequence information from an isolated human NiV
389 case in Cumilla has not been reported, so comparison to sequence we found in bats was not possible
390 (41). Studies linking viral genotype to clinical outcomes in people would provide additional insight into
391 the effect of strain diversity in bats on the potential for larger-scale human outbreaks.
392 Our study sheds light on the sporadic nature of human NiV outbreaks, with multi-year inter-
393 epidemic periods in South Asia. PCR results show that overall NiV incidence in *P. medius* is low,
394 consistent with previous studies of Hendra and Nipah virus (47, 52, 100, 101). The fitted model suggests
395 that transmission increases when bat populations that have become susceptible through waning
396 immunity (11). In the current study, observed seroprevalence patterns and the fitted model suggest that
397 three periods of transmission occurred over the 6 years of sampling, each of which followed periods of
398 low adult and juvenile seroprevalence. Viral detection in bats has coincided with some human
399 outbreaks, supporting the hypothesis that spillover is a sporadic event (67, 98). In our study periods of
400 low seroprevalence in bats were not always followed by outbreaks in humans. We detected NiV RNA

during periods of both increasing and high seroprevalence, consistent with recrudescence which was strongly supported by the fitted model and has been demonstrated in captive animals (64-66). This likely contributes to the sporadic variation in human outbreaks (e.g. spillover events) from year-to year in Bangladesh.

Overall, our results suggest that NiV outbreaks in humans stem from an interaction of four factors: 1) multi-year fluctuations in transmission intensity among bats driven by immunity and colony size/ density-dependent transmission; 2) relatively localized bat movements creating spatially variable transmission dynamics; 3) occasional shedding by previously infected bats due to recrudescence; and 4) highly seasonal contact between bats and humans via consumption of raw date-palm sap. The timing of multiple factors involved in driving transmission dynamics needs to align for intra-colony NiV transmission events and further align with human behavior and availability of a route of spillover for human outbreaks to occur, as previously hypothesized (102). We further conclude that NiV dynamics in bats combined with the seasonality and specific geography of date palm sap consumption in Bangladesh likely explains the sporadic nature of human outbreaks in the region (38).

These findings suggest that human NiV outbreaks in other regions of Bangladesh (and Asia) where *Pteropus* bats occur, are also likely to be sporadic and rare, leading to under-reporting or a lack of reporting. This is probably exacerbated by the fact that the clinical syndrome is similar to that of other common infections, such as Japanese encephalitis, malaria and measles (103). Understanding whether some NiV strains are capable of causing mild or asymptomatic cases will provide important insights about why outbreaks have not been detected in areas such as eastern Bangladesh or other parts of Asia, where host, virus, and potential routes of spillover exist. One reason is that mild or asymptomatic cases would be unlikely to be detected by current surveillance systems. About half of all Nipah outbreaks in Bangladesh between 2007 and 2014 were unreported, suggesting that many cryptic spillover events have occurred (104). The 2018 and 2019 spillover events in Kerala, India, which were linked to local *P. medius* colonies and which occurred in an area that does not cultivate date palm sap, further emphasize the point, but raise questions about the mechanism of spillover.

In the last two decades, the world has experienced large epidemics of bat-associated viruses, including Ebola in West Africa and DRC; SARS coronavirus, and SARS CoV2. The World Health Organization has listed Nipah virus and other henipaviruses as priority pathogens for vaccine and therapeutic research and development, along with Ebola viruses and coronaviruses. Surveillance for henipaviruses and antibodies in bats and people where they are in close contact will help determine spillover risk; characterize henipavirus genetic diversity; and understand the genetic determinants of NiV transmissibility and pathogenicity among humans. These measures may help target interventions that reduce spillover, substantially improving our ability to reduce the risk of a more transmissible strain of NiV emerging and causing a large-scale epidemic with significant human and animal mortality.

Methods

The study period was between January 2006 and November 2012. The study was conducted under Tufts University IACUC protocol #G929-07 and icddr,b AEEC protocol 2006-012 with permission from the Forest Department, Government of Bangladesh. Locations were selected based on whether the district had any previously recorded human NiV encephalitis clusters at the time of this study and was therefore inside the Nipah Belt (e.g. Rajbari, Tangail, Thakurgaon, and Kushtia) or whether they had not and were outside the Nipah Belt (e.g. Cumilla, Khulna, Sylhet, and Chattogram). The Thakurgaon study was conducted as part of an NiV outbreak investigation, and coincided with ongoing human transmission (105). Between 2006-2012, three different studies of *Pteropus medius*, with similar bat sampling protocols were performed: 1) a cross-sectional spatial study with a single sampling event in each of the eight locations listed above; 2) a longitudinal study of a Faripur bat colony with repeated sampling approximately every 3 months from July 2007 to November 2012; and 3) a longitudinal study of the Rajbari colony with repeated sampling at a monthly interval between 12 month period between April 2010 and May 2011. Opportunistic sampling of *Pteropus medius* was also performed during this time period during NiV outbreak investigations [Bangha, Faridpur (Feb 2010), Joypurhat (Jan 2012), Rajbari (Dec 2009), West Algi, Faridpur (Jan 2010)]. Bats were captured using mist nets at locations within eight different districts across Bangladesh between January 2006 and December 2012 (**Figure 1**).

Capture and sample collection

For the country-wide cross-sectional and Faridpur longitudinal study, approximately 100 bats were sampled at each sampling event, which lasted 7-10 days. This sample size allowed us to detect at least one exposed bat (IgG antibody-positive) given a seroprevalence of 10% with 95% confidence. Bats were captured using a custom-made mist net of approximately 10 m x 15 m suspended between bamboo poles which were mounted atop trees close to the target bat roost. Catching occurred between 11 pm and 5 am as bats returned from foraging. To minimize bat stress and chance of injury, nets were continuously monitored and each bat was extracted from the net immediately after entanglement. Personal protective equipment was worn during capture and sampling, which included dedicated long-sleeve outerwear or Tyvek suits, P100 respirators (3M, USA), safety glasses, nitrile gloves, and leather welding gloves for bat restraint. Bats were placed into cotton pillowcases and held for a maximum of 6 hours before being released at the site of capture. Bats were sampled at the site of capture using a field lab setup. Bats were anesthetized using isoflurane gas (106) and blood, urine, oropharyngeal swabs, and wing membrane biopsies (for genetic studies) were collected. For some sampling periods, rectal swabs were collected but due to resource constraints, these samples were deemed to likely be lower yield than saliva and urine for NiV, and were discontinued during the study. For each bat sampled we recorded age, weight, sex, physiologic and reproductive status, and morphometric measurements as described previously (27). Bats were classified as either juveniles (approximately four to six months - the age by which most pups are weaned) to two years old (the age when most *Pteropus* species reach sexual maturity) or adults (sexually mature) based on body size and the presence of secondary sexual characteristics, pregnancy, or lactation - indicating reproductive maturity (27, 107).

Up to 3.0 mL of blood were collected from the brachial vein and placed into serum tubes with serum clot activator (Vaccutainer, USA). Blood tubes were stored vertically on ice packs in a cold box and serum was allowed to separate overnight. Serum was drawn from the tube after 24 hours and

placed in a screw-top cryovial (Corning, USA) and stored in a liquid nitrogen dewar (Princeton Cryogenics, NJ, USA). Sterile pediatric swabs with polyester tips and aluminum shafts were used to collect urogenital and rectal samples, and larger polyester swabs with plastic shafts (Fisher, USA) were used to collect oropharyngeal samples. All swabs were collected in duplicate, with one set being placed individually in cryotubes containing lysis buffer (either tri-reagent or NucliSENS Lysis buffer, BIOMERIEUX, France) and the second set in viral transport medium. All tubes were stored in liquid nitrogen in the field then transferred to a -80C freezer.

During each sampling event, pooled urine samples were collected beneath bat roosts using polyethylene sheets (2' x 3') distributed evenly under the colony between 3 am and 6 am. Urine was collected from each sheet either using a sterile swab to soak up droplets, or a sterile disposable pipette. Swabs or syringed urine from a single sheet were combined to represent a pooled sample. Each urine sample was divided in half and aliquoted into lysis buffer or VTM at an approximate ratio of one part sample to two parts preservative.

Serological and molecular assays

Sera from the cross-sectional survey were heat inactivated at 56^{o}C for 30 minutes, as described (108) prior to shipment to the Center for Infection and Immunity at Columbia University (New York, USA) for analysis. Samples were screened for anti-NiV IgG antibodies using an enzyme linked immunosorbent assay (ELISA) as described in (27). Sera from the longitudinal studies were sent to the Australian Animal Health Laboratory and were gamma irradiated upon receipt. Because of the large sample size and development of a high throughput multiplex assay of comparable specificity and sensitivity, for these samples we used a Luminex®-based microsphere binding assay to detect anti-Nipah G IgG antibodies reactive to a purified NiV soluble G protein reagent, as described previously (109, 110). Samples resulting in a Median Fluorescent Intensity (MFI) value of 250 and below is considered negative for other bat species and previous studies have reported using a threshold of at least three times the mean MFI of negative sera to determine the cutoff (47, 111-113). For this study, MFI values of over 1000 were considered positive for NiV antibodies, an approach considered appropriate for research purposes for bats.

Total nucleic acids from swabs and urine samples were extracted and cDNA synthesized using Superscript III (Invitrogen) according to manufacturer's instructions. A nested RT-PCR and a real-time assay targeting the N gene were used to detect NiV RNA in samples (114). A RT-qPCR designed to detect the nucleocapsid gene of all known NiV isolates was also utilized (115). Oligonucleotide primers and probe were as described (115). Assays were performed using AgPath-ID One-StepRT-PCR Reagents (Thermofisher) with 250 nM probe, 50 nM forward and 900 nM reverse primers. Thermal cycling was 45◦C for 10 min, followed by 45 cycles of 95◦C for 15 s and 60◦C for 45 s. Cut-off values were cycle threshold (C_T) ≤40 for positive and CT ≥45 for negative. Results with C_T values between 40 and 45 were deemed indeterminate, i.e. not conclusively positive or negative. Samples with NiV RNA detected by real time PCR were confirmed by gel electrophoresis and product sequencing.

A subset of NiV-positive samples was processed by high-throughput sequencing (HTS) on the Ion Torrent PGM platform in order to obtain additional NiV genomic sequence. Libraries were prepared according to the manufacturer's instructions, and 1 million reads allocated per sample. HTS reads were aligned against host reference databases to remove host background using bowtie2 mapper, and host-

subtracted reads primer trimmed and filtered based on quality, GC content and sequence complexity. The remaining reads were de novo assembled using Newbler (v2.6) and mapped to the full length NiV genome. Contigs and unique singletons were also subjected to homology search using MegaBlast against the GenBank nucleotide database, in case variance in parts of the genome precluded efficient mapping. From these data, N gene consensus sequences were constructed using Geneious v 7.1, and used for phylogenetic analyses.

Phylogenetic analysis

All *P. medius* NiV sequences have been submitted to Genbank and accession numbers are included in **Figure 6**. Sequence alignments were constructed using ClustalW in Geneious Prime software (116). Phylogenetic trees of NiV N-gene sequences were constructed using Neighbor-Joining algorithms and figures constructed in FigTree 1.4.2.

Satellite telemetry and home range analysis

We developed a collar system to attach 12g solar-powered Platform Terminal Transmitters (PTT) (Microwave Telemetry, Colombia, MD, USA) to adult bats using commercial nylon feline collars with the buckle removed and 0-gauge nylon suture to attach the PTT to the collar and to fasten the collar around the bat's neck. Collars were fitted to the bat such that there was enough space to allow for normal neck movement and swallowing, but so that the collar would not slip over the head of the animal (**Figure S6**). PTTs were programmed with a duty cycle of 10 hours on and 48 hours off. Data was accessed via the Argos online data service (argos-system.org; France). A total of 14 collars were deployed as follows: Feb 2009: 3 males, 3 females from a colony in Shuvarampur, Faridpur district; Feb 2011: 3 males, 2 females from the same colony; and April 2011 Cox's Bazaar, 3 bats from a colony in Cox's Bazaar, Chattogram district. Bats were selected based on size such that the total weight of the collar (~21g) was less than 3% of the bat's body mass (Table S3).

The individual telemetry dataset was combined for each region and its aggregate utilization distributions (UD) computed in R using package 'adehabitatHR' (117). Population-specific home range is represented by the *95% area enclosure of its UD's volume. The volume of intersection between the colonies quantifies the extent of home range overlap. To evaluate the potential for contact with the Cox's Bazaar colony, we calculated the most likely distance moved ('mldm') for each sampled bat at Faridpur where the population was more intensively monitored. Movement distance was measured in kilometers with respect to a center location (w) shared by the whole colony. This information was used to predict how likely an animal was to use the landscape.

Statistical approach – cross-sectional study

We fit a Bayesian generalized linear model with a logit link and a Bernoulli outcome to identify potential predictors which influenced a bat's serostatus. We included age, sex, age- and sex-normalized mass and forearm length, mass:forearm ratio, body condition, and whether the bat was pregnant, lactating, or carrying a pup, using weak zero-centered normal priors for all coefficients. We included location of sampling as a group effect (similar to a random effect in a frequentist context) nested within Nipah Belt or non-Nipah Belt regions. We fit the models and performed posterior predictive checks in R 3.4.3, using the **rstanarm** and **rstan** packages.

563
564 *Statistical approach – longitudinal study*
565 We fit binomial general additive models (GAMs) (118) to the time series of adult and juvenile
566 seroprevalence in the longitudinal study. We included annual, synchronous birthing that occurred
567 between March and April. We assumed that pups weaned from their dams at 3 months, and became
568 independent flyers, and that maternal antibodies waned after 6 months at which point pups
569 transitioned into the "juvenile" class (30, 61). We assumed that juveniles became sexually mature at 24
570 mo., and entered the "adult" class based on other pteropid species (30, 47, 119). For juveniles, we
571 modeled the birth cohort of bats as separate random effects in a pooled model of juvenile
572 seroprevalence starting from June of their birth year; June being the earliest month we sampled free-
573 flying juveniles in any cohort. We determined the cohort year of juveniles by using cluster analysis to
574 group individuals by weight, assuming those in the smallest group were born in the current year and
575 those in the larger group were born the previous year. 92% of juveniles captured were yearlings. For
576 adults, we analyzed seroprevalence of adults as a single pool over the entire course of the study. We
577 tested models with and without annual cyclic effects.
578 Where time series had significant temporal autocorrelation (adults only), we aggregated data by
579 week. We determined periods of significant increase or decrease as those where the 95% confidence
580 interval of the GAM prediction's derivative did not overlap zero. We fit the models and performed
581 checks in R 3.4.3, using the **mgcv** package.
582 To examine the importance of different biological mechanisms in transmission, we fit an age-
583 structured (adult and juvenile) maternally immune (M)-susceptible (S)-infected (I)-recovered (R) model
584 with recrudescence (R to I) and loss of immunity (R to S) to the seroprevalence data on a weekly
585 timescale:

$$\frac{dS_J}{dt} = -S_J(\beta_{JJ}I_J + \beta_{AJ}I_A) - \mu_J S_J + bN_A(t-5)\frac{S_A}{N_A} - bN_A(t-52)\frac{S_J}{N_J}(1-\mu_J)^{52} + \lambda M_J$$

$$\frac{dI_J}{dt} = S_J(\beta_{JJ}I_J + \beta_{AJ}I_A) - \gamma I_J - bN_A(t-52)\frac{I_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dR_J}{dt} = \gamma I_J - \mu_J S_J - \mu_J R_J - bN_A(t-52)\frac{R_J}{N_J}(1-\mu_J)^{52}$$

586 $$\frac{dM_J}{dt} = bA(t-5)\frac{R_A}{N_A} - \lambda M_J - \mu_J M_J - bN_A(t-52)\frac{R_A}{N_A}(1-\mu_J)^{52}\lambda^{52}$$

$$\frac{dS_A}{dt} = -S_A(\beta_{AA}I_A + \beta_{JA}I_J) - \mu_A S_A + \tau R_A + bN_A(t-52)\frac{S_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dI_A}{dt} = S_A(\beta_{AA}I_A + \beta_{JA}I_J) - \gamma I_J - \mu_A I_A + bN_A(t-52)\frac{I_J}{N_J}(1-\mu_J)^{52} + \Delta R_A$$

$$\frac{dR_A}{dt} = \gamma I_A - \mu_A R_A + bN_A(t-52)\frac{R_J}{N_J}(1-\mu_J)^{52} - \tau R_A - \Delta R_A$$

587 We included a class M for the density of juvenile bats with maternal antibodies to allow for the biological
588 possibility that maternal antibodies are lost at a much higher rate than antibodies acquired following
589 infection. The subscripts J refer to juveniles and A to adults, β is the transmission rate, γ is the recovery

rate, μ is the mortality rate, τ is the rate of loss of adult immunity, λ is the rate of loss of maternal antibodies(61), Δ is the adult recrudescence rate (R to I), b is the birth rate (pups join the juvenile population after 5 weeks). Juveniles transition to adults after 52 weeks. We included terms for loss of antibody in adults (τ, S to R), and viral recrudescence (Δ, R to I) based on previous studies on captive bats that demonstrated the existence of these processes without providing enough data to characterize them precisely (64, 65). We fit this deterministic model to the seroprevalence data by maximum likelihood, which assumes that deviations from the model are due to observation error. We estimated the confidence intervals around maximum likelihood parameter estimates using likelihood profiles using the *profile* function in package *bbmle* in R v3.2.2.

We used model fitting and model comparison to examine the need for several of the biological processes in the model above that could influence NiV dynamics. First, we examined both density and frequency-dependent transmission by comparing the fit of the model above to one with transmission terms that have population size (N_A or N_J) in the denominator. Second, we examined the confidence intervals of the parameters describing viral recrudescence, loss of antibodies in adult bats, and loss of antibodies in juvenile bats. If the confidence bounds for these parameters included zero, then these biological processes are not needed to explain the serological dynamics. Finally, we examined the confidence bounds for parameters describing the loss of maternal and non-maternal antibodies (τ and λ), to determine if the rate of loss of these two types of immunity were different. We note that this model structure has similarities to a susceptible-infected-latently infected(L)-infected (SILI) model (if latently infected individuals are seropositive), but the model above differs in allowing for the possibility of individuals to transition from the R class back to the S class.

Code availability

SIR model code written in R is available upon request.

Data availability

All molecular sequences are available via Genbank. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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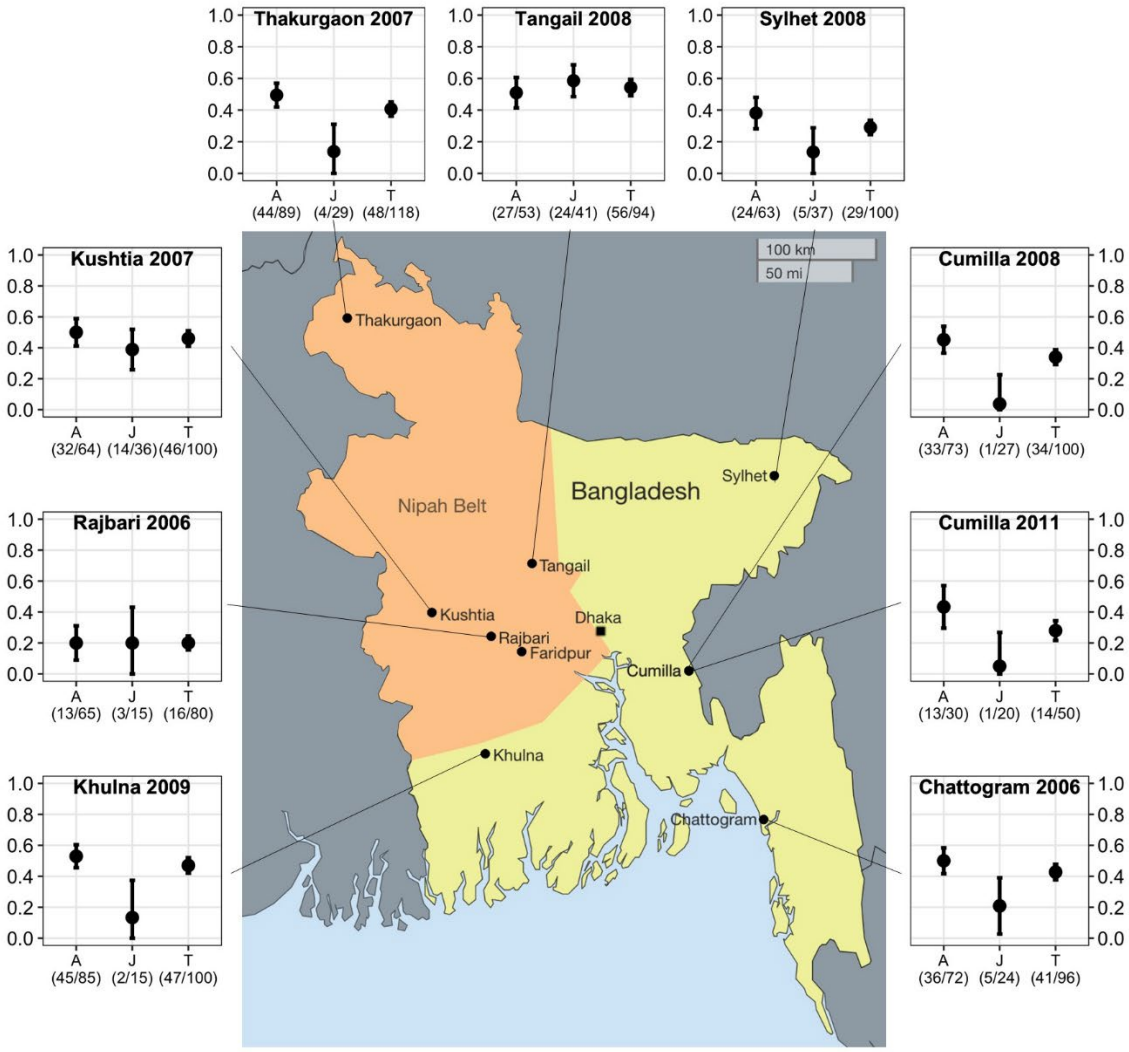
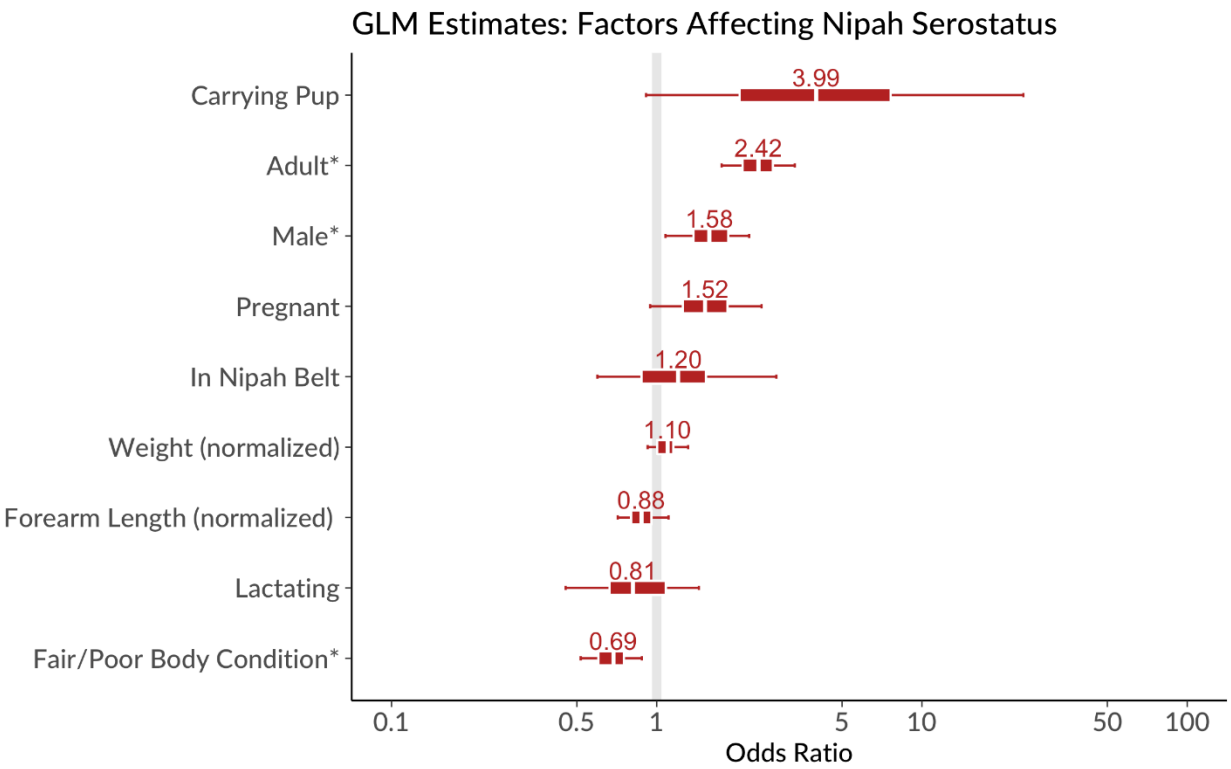


Figure 1. Map showing age-stratified seroprevalence in *Pteropus medius* colonies, Bangladesh. Bats from eight colonies were sampled and tested for anti-NiV IgG antibodies: four within the "Nipah belt" (orange shaded) and four outside. Seroprevalence of adults (A), juveniles (J) and total seroprevalence (T) are shown with 95% CI error bars. The shaded region represents the "Nipah belt" where previous human NiV outbreaks have been reported.



641
642 **Figure 2.** Results of Bayesian GLM of factors affecting Nipah serostatus in bats in cross-sectional study. Bars
643 indicate odds ratios and 50% (inner) and 95% (outer) credible intervals for model parameters. Factors with
644 asterisks (*) have 95% CIs that do not overlap 1. Model intercept (predicted probability of seropositivity for a
645 juvenile, female, bat outside the Nipah belt of mean size and good body condition) was 0.26, (95% CI 0.12-0.56).
646 Random effect of location (not shown) had an estimated SD of 0.63 (95% CI 0.29-1.27)
647

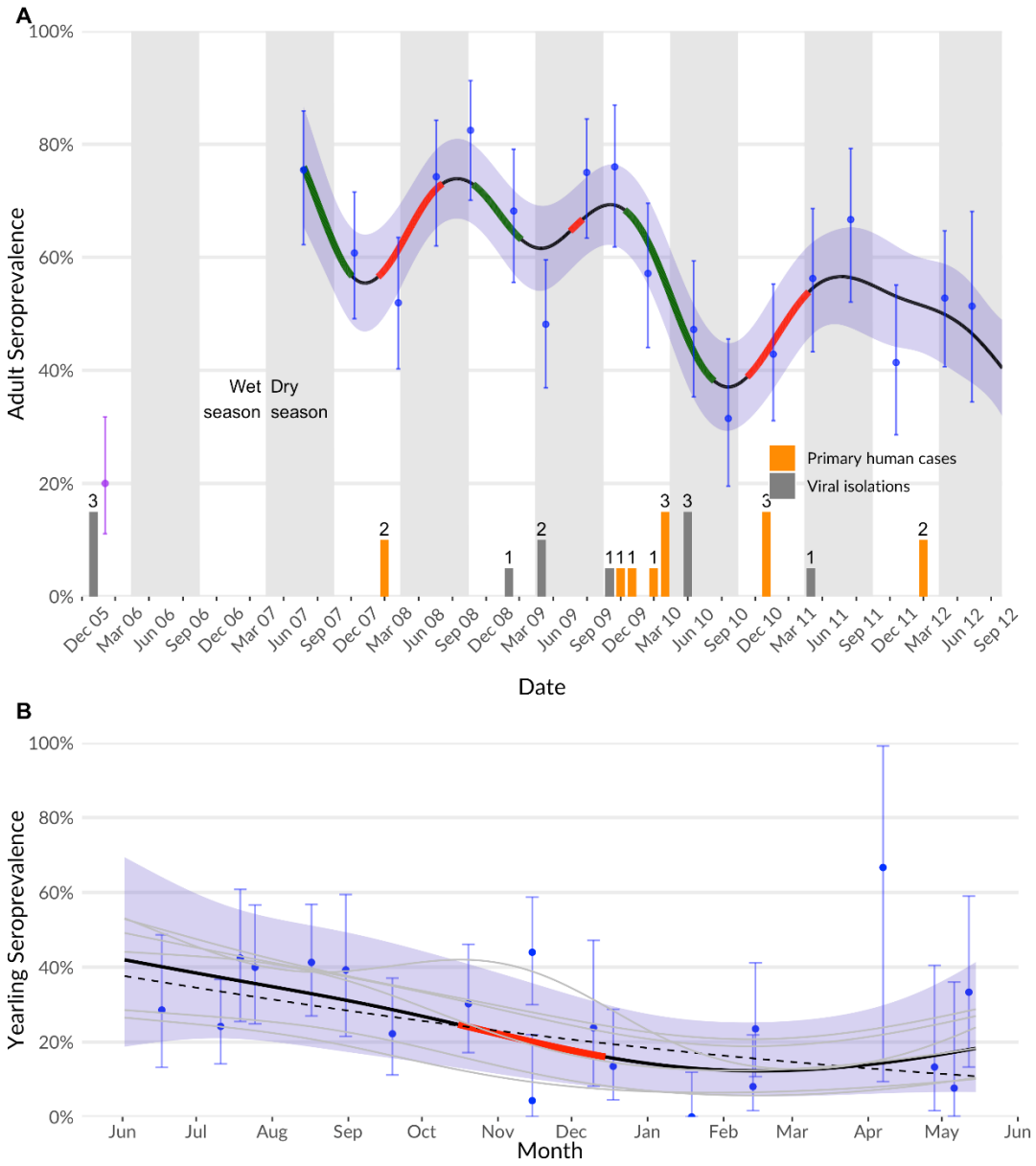


Figure 3 A & B. Serodynamics of the Faridpur bat population, measured and fit to a GAM. (A) Adult seroprevalence over time, with measured values and 95% CI in blue, and mean GAM prediction and 95% shown with line and surrounding shaded areas. Point from Feb 2006 (purple) shown separately due to ELISA vs Luminex measure. Periods of significant change (where GAM derivative 95% does not overlap zero) are shown in red (increasing) and green (decreasing). Periods of increase indicate viral circulation events in the adult population; these do not occur with consistent periodicity or seasonality. Counts of primary human cases from local district (dark gray), and bat viral viral detections (orange, see Table 1) are shown on bottom for comparison. (B) Juvenile seropravelnce during the first year of life ("yearlings"). All years' measurements are collapsed onto the scale of a single year overlain to show yearling dynamics. Measured values and 95% CI shown in blue, and mean and 95% CI for the GAM model pooled across cohorts shown with line and surrounded shaded areas. GAM realizations for individual years in grey, overall effect in black. The period of significant decline in the GAM is shown in red. Juvenile seroprevalence decreases over the course of the year, and is not distinguishable from a simple linear decrease (ΔAIC < 1, dotted line).

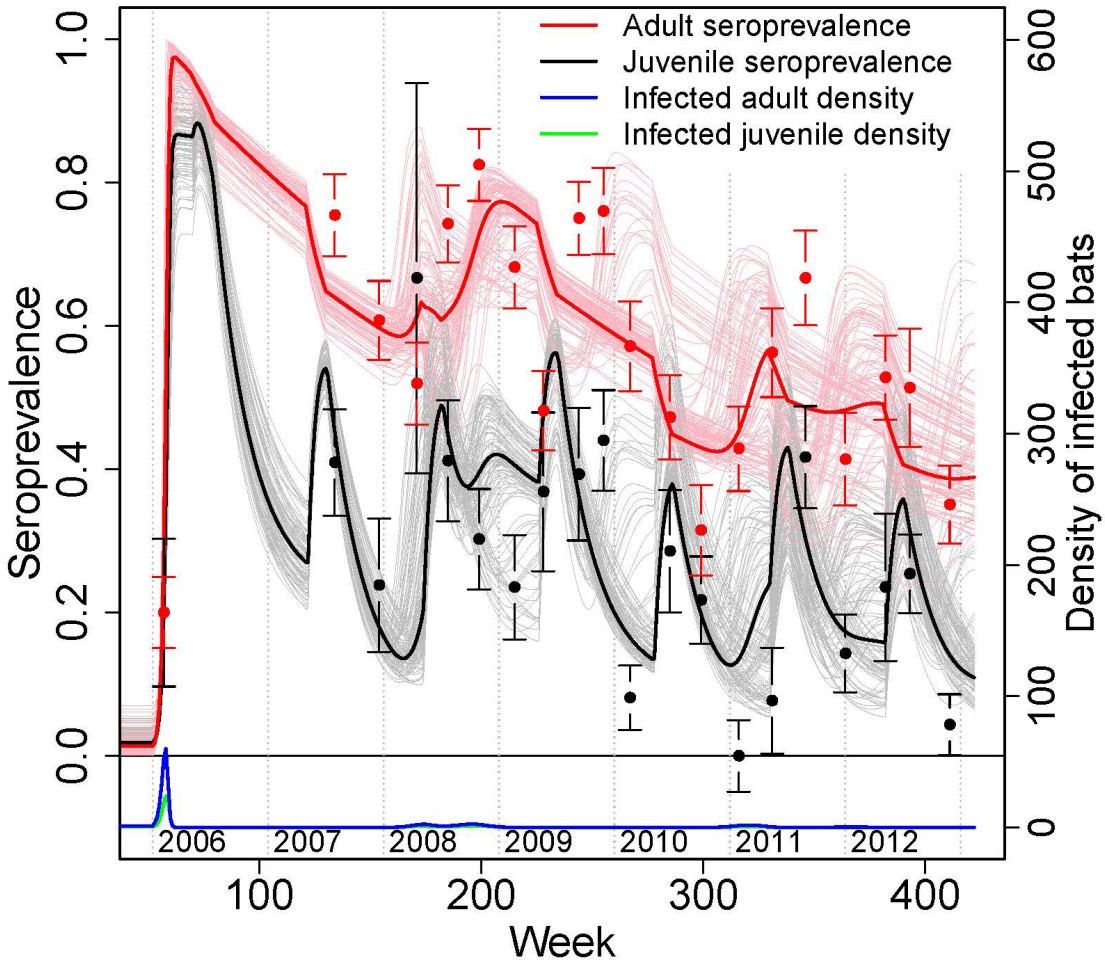


Figure 4. Longitudinal data and fitted model for NiV serological dynamics in adult and juvenile bats**.** Red and black points show observed data (±1 SE) and solid lines show the fitted model (thick lines show the trajectory for the model with maximum likelihood parameter estimates; thin lines show realizations for parameter estimates drawn from the estimated distributions) for the fraction of adults and juveniles seropositive for NiV (left axis), and the model-estimated number of infected adult and juvenile bats (bottom panel and right axis). See Methods for details of model structure.

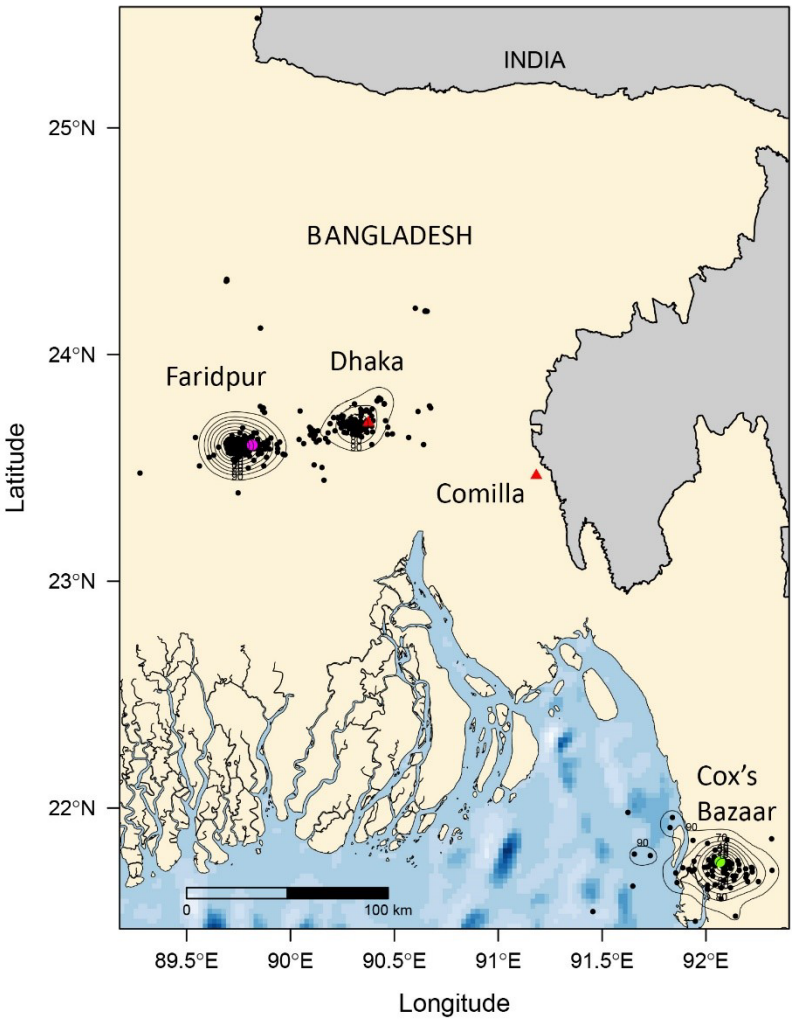
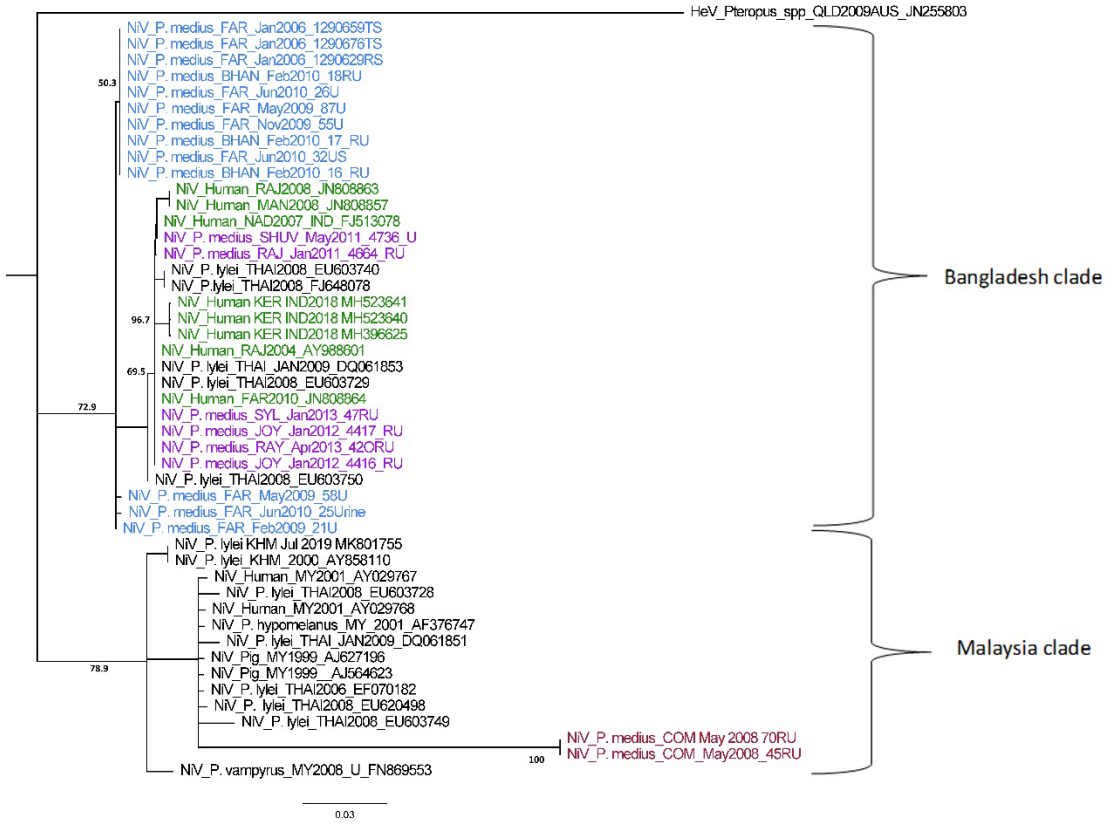


Figure 5. Satellite telemetry and home range analysis. Location data from satellite collars (n=14) placed on 11 bats from Faridpur and 3 bats from Cox's Bazaar, Chattogram collected between 2009 and 2011, were used to calculate local and long-range movement patterns and home range for these two groups.



677

678 **Figure 6**. **Nipah Virus partial N gene phylogeny (224nt).** Phylogenetic Neighbor-joining tree created in Geneious
679 Prime 2019 using a Tamura-Nei model with 1,000 bootstrap replicates, and Hendra virus as an outgroup (120).
680 Branch lengths shown as the number of substitutions per site. Sample collection date, location and Genbank
681 accession numbers are included in the label for each sequence except *P. medius* sequences we collected: Genbank
682 accession numbers MK995284 – MK995302. Blue labels indicate bat sequences from Faridpur and Bhanga (an
683 outbreak response in Faridpur). Purple sequences are from *P. medius* from other roosts sampled during the
684 longitudinal study. Red sequences are from *P. medius* in Cumilla. Green sequences are human NiV sequences from
685 Bangladesh and India.

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120. Anonymous (2019) Geneious Prime 2019.

EcoHealth Alliance

7/21/2020

Dear Editor,

We are pleased to provide a revised manuscript entitled: *Nipah virus dynamics in bats and implications for spillover to humans* and a detailed response to reviewers' comments. We thank the reviewers for their overall enthusiasm for the study and their constructive comments which have allowed us to significantly improve the manuscript. We have responded to each of their comments, as detailed below. Our manuscript has even more relevance at a time when the world is experiencing a severe pandemic due to what is likely a bat-origin zoonotic virus, and hope that this study provides insight that will inform future ecological studies of zoonotic viruses, including coronaviruses, in bats.

We look forward to the review of our revised manuscript and hope that it is now considered acceptable for publication in PNAS.

Sincerely,

(b) (6)

Dr. Jonathan Epstein
EcoHealth Alliance

Specific responses to reviewers:
Reviewer 1

1) **The methods section uses jargon** –We agree with Reviewer 1 that the methods included many technical terms. We have tried to reduce jargon where possible.

2) **What is the nature of recrudescence and of antibody disappearance in bats? Some of these questions could be answered by experimental studies.** We have added background describing what is known about antibody response to NiV infection in bats and the loss of antibodies in adults and juveniles, based on experimental data. Lines 124 - 131

3) **Are colonies species specific?** In Bangladesh, where this study took place, *Pteropus medius* is the only endemic species of *Pteropus* bat and they do not co-roost with other bat species. We have clarified this in lines 85-86. We also discuss *Pteropus* ecology in more detail in lines 273-285, including interspecies interactions.

4) **In the abstract the term "epidemic" is used rather than "epizootic".**
While epizootic is the technical term for circulation among animals, readers may be more familiar with the term epidemic, so in our effort to reduce jargon, we have stayed with this term and made sure that there is appropriate context to clarify whether we're discussing an outbreak among bats or in people. (see lines 398-404).

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5) **Lines 91-92, regarding pandemic potential, I would not use this term because some observers, probably most, think the pandemic potential is low. This might be a controversy well avoided.** We removed the word pandemic from the abstract, but left it in the introduction. S. Luby describes the pandemic potential of henipaviruses in (Luby. Antiviral Research (100) 1. 2003.). Given that we know so little about other henipaviruses in bats, and there is already evidence for variation in transmissibility among humans between Bangladesh and Malaysia strains, we believe that the possibility that there are or could be more transmissible strains is valid.

6) **Explain the movement data more and in general expand on speculation in the discussion / strengthen the conclusion.** We have added more discussion of the localized movement findings and speculation in the discussion. We have also strengthened the language in the conclusion.

7) **Figures: Figure 1. Improve coloring and visibility of error bars; Figure 3 needs a clearer explanation; Figure 4 is difficult to read. Do peaks of juvenile prevalence follow declines of adult prevalence? Fig. 5 expand on discussion of movement data.** We have updated figures 1-4 to improve readability and captions for 3 and 4 have been updated for clarity. Peaks of juvenile prevalence reflect seasonal birthing where pups are born with maternal antibodies then lose them after about 5-6 months. Our longitudinal data and mechanistic model shown in Figure 4 show that adults lose seroprevalence over time, but spikes in adult seroprevalence are independent of spikes in juveniles, which appear to be due to maternal antibodies. We address this in the discussion section in lines 201-310. We have expanded our discussion of movement data.

Reviewer 2

1) **Statistical support for higher adult seroprevalence? And, could test be non-specific?** Figure 3 shows that adults had a statistically significant greater seroprevalence than juveniles. The ELISA used is specific to Nipah virus and the Luminex multiplex assay contained specific antigens from other henipaviruses (Nipah, Hendra, and Cedar virus). We agree that it's possible for either assay to cross-react with antibodies against other unknown henipaviruses circulating in *P. medius*. The Luminex assay allows us to determine whether there may be reactivity to non-Nipah henipaviruses based on the ability to compare quantitative outputs for multiple henipavirus antigens. We added this to the discussion in lines 289-297.

2) Fig 4: **Are spikes in juveniles observed before spikes in adults reflective of juveniles transitioning into adults?** We don't think so, because adults showed independent patterns of spikes in seroprevalence not associated with annual seasonal spikes in juveniles. We believe the spikes in juveniles are consistent with synchronous birthing patterns where pups are born with then lose maternal antibodies.

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3) Figure 5: **Is sample size adequate to represent full range? Do P. medius interact with other bats? Does range change with season? Could other species serve as reservoirs?**
We discuss the small but illustrative sample size in lines 331-2, and that these sample sizes are comparable to other satellite telemetry studies in pteropodid bats. We compare them to range data from pteropid species in Malaysia which showed greater range of movement. *P. medius* do not roost with other bat species, however they do feed in the same locations as two other frugivorous bats: *Rousettus leischenaulti* and *Cynopterus horesefldi*. These bats have been screened for henipaviruses under different studies, and have not been found to be hosts. We cannot rule it out, but the majority of evidence (experimental, virus isolation, PCR detection and serology) globally is that *Pteropus* bats are the primary reservoir for henipaviruses, which we discuss. Seasonal data is presented in the results section (lines 229-230). Host range does contract in the dry season, as we show in suppl. Fig S3 & S4 and added a line in the discussion to suggest a reason for the finding (lines 343-4).

4) **Fig 6. Is the 224nt fragment representative of the whole genome?** The Nucleocapsid gene was used because it is among the most conserved regions of the henipavirus genome, and past published data indicate that N gene genetic tree has a high concordance with whole genome phylogeny. This was also confirmed in our current study. While we, unfortunately, could not get whole genome sequences from our positive samples, we were able to obtain whole and near-whole N gene sequences (1,592 nt) from a subset of the samples in this study and from the isolates described in (Anderson et al. 2019), and we generated a phylogenetic tree using these sequences (Fig. S5). This tree supports the relationships we see in the tree we use in Figure 6. Further, in Anderson et al., 2019, we constructed a tree using whole NiV genomes from bat and human isolates. Some of the bat isolates are included in our N gene trees. We see consistent relationships among the bat and human whole genome sequences in Anderson et al., and the N gene sequences in Fig 6. in this paper. We are therefore confident that the N gene reflects the relative phylogenetic relationships that would be seen among whole genome sequences. Finally, it should be noted that recombination has not been observed in paramyxovirus genomes and this also explains the concordance of phylogeny between individual genes (except the most variable regions) and the whole genome sequences.

The question of how divergent the Comilla strain would be across its whole genome is an excellent one. The sequence we have from the N gene suggests this virus is more closely related to viruses in the Malaysia clade than those from Bangladesh. The serological assays used in our study would not differentiate between Nipah virus strains from the Malaysia or Bangladesh clade.

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To:	Anthony, Simon J.; Ariful Islam; A. Marm Kilpatrick; Shahneaz Ali Khan; Maria Sanchez Leon; Noam Ross; ina.smith@csiro.au; Carlos M. Zambrana-Torrelio MSc; Yun Tao; Ausraful Islam; Phenix-Lan Quan; Kevin Olival, PhD; Salah Uddin Khan; Emily Gurley; Jahangir Hossain; Hume Field; Fielder, Mark; Thomas Briese; Mahmud Rahman; Christopher Broder; Gary Crameri; Linfa Wang; Stephen Luby; Lipkin, W. I.; Peter Daszak
Subject:	Update on PNAS Nipah paper: resubmitted
Date:	Tuesday, July 21, 2020 4:27:06 PM
Attachments:	Nipah dynamics in bats_Epstein et al 2020_revised w figs.docx Nipah dynamics in bats_Epstein et al 2020_response letter_final.pdf

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Hi everyone,
I hope everyone is staying safe and healthy. I wanted to update you on the status of our PNAS paper. I was finally able to finish responding to the reviewers' substantive though positive comments, and have resubmitted the revised manuscript. Thank you to those of you who helped with specific questions the reviewers had. I've attached the revised manuscript and the response letter to the Editor, for your records.

I don't know what the timing of review will be given that PNAS says they're backed up and prioritizing COVID-19 papers, but hopefully it won't be too long. Mostly, thank you all for your continued patience, and here's hoping for a positive response.

Cheers.
Jon

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PROJECT SUMMARY/ABSTRACT

Southeast Asia is one of the world's highest-risk EID hotspots, the origin of the SARS pandemic, Nipah virus, and repeated outbreaks of influenza. This is driven by high diversity of wildlife and rapidly expanding demography that brings human and wildlife populations closer. This proposal will launch the **E**merging **I**nfectious **D**iseases - **S**outh **E**ast **A**sia **R**esearch **C**ollaboration **H**ub **(EID-SEARCH)**, a collaboration among leaders in emerging disease research in the USA, Thailand, Singapore and the 3 major Malaysian administrative regions. These researchers have networks that span >50 clinics, laboratories and research institutions across almost all SE Asian countries and will use the EID-SEARCH as an early warning system for outbreaks involving exchange of information, reagents, samples and technology, and a collaborative power-house for fundamental and translational research. The EID-SEARCH will also act as **a significant asset to scale-up and deploy resources in the case of an outbreak in the region.** This EIDRC will conduct research to: **1)** Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife, by analyzing previously-archived wildlife samples, conducting targeted wildlife surveillance, and using serology & PCR assays to identify novel viruses. These will be characterized to assess risk of spillover to people, and a series of *in vitro* (receptor binding, cell culture) and *in vivo* (humanized mouse and collaborative cross models) assays used to assess their potential to infect people and cause disease; **2)** Collect samples and questionnaire data from human communities that live in EID hotspots and have high cultural and behavioral risk of animal exposure (e.g. wildlife hunting, bat guano collection). These will be tested with serological assays to identify evidence of novel virus spillover, and analyzed against metadata to identify key risk pathways for transmission; **3)** Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts. We will conduct syndromic surveillance at clinics serving the populations in Aim 2, enroll patients with undiagnosed illness and symptoms consistent with emerging viral pathogens, and test samples with molecular and follow-up serological assays to identify causal links between these syndromes and novel viruses.

This research will advance our understanding of the risk of novel viral emergence in a uniquely important region. **It will also strengthen in-country research capacity** by linking local infectious disease scientists **with an international collaborative network that has proven capacity to conduct this work and produce significant findings.** The large body of high impact collaborative research from this EIDRC leadership team provides proof-of-concept that EID-SEARCH has the background, collaborative network, experience, and skillset to act as a **unique early warning system for novel EIDs of any etiology threatening to emerge in this hottest of the EID hotspots.**

II. Research Strategy:

1. Significance: Southeast Asia is a critical hotspot for emerging diseases due to its high biodiversity of wildlife and their viruses, and the presence of key ecological and socioeconomic drivers of emergence **(Fig. 1)** (*1*). These include dense human populations living in networks of rural and urban communities, with strong cultural and occupational connection to wildlife and livestock farming and trading, and intimate connection to global travel networks (*2*). The region is undergoing rapid environmental and demographic change, both of which increase the risk of disease emergence and spread. Pathogens that emerge in this region often spread and sometimes enter the USA (e.g. prior influenza pandemics, SARS) and threaten global health security.

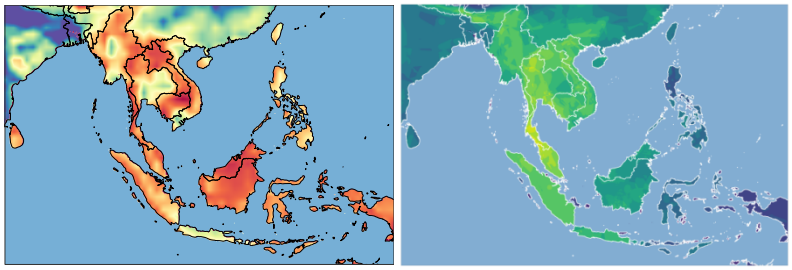


Fig. 1: Southeast Asia, and in particular, parts of Thailand and Malaysia are hotspots of risk for disease emergence **(left)**, and predicted high diversity of 'missing' or as-yet undiscovered viruses, yellow = highest diversity **(left)**. From (*1-3*).

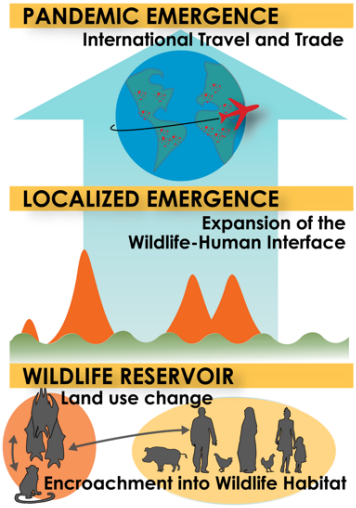
Not surprisingly novel viruses, including near-neighbors of known agents, have emerged in the region leading to often unusual clinical presentations **(Table 1)**.This adds to a significant background burden of repeated Dengue virus outbreaks in the region (*20*), and over 30 known *Flavivirus* species circulating throughout S. and SE Asia (*21*). The events in **Table 1** are dominated by three viral families: coronaviruses (CoVs), paramyxoviruses (PMVs - particularly henipaviruses) and filoviruses (FVs), which are initial examples of our team's research focus and capabilities. These viral groups have led to globally important emerging zoonoses (*4, 5, 22-31*), causing tens of thousands of deaths, and costing billions of dollars (*32-34*). Furthermore, near-neighbors of known pathogens in these families have been reported throughout the region, including by members of our consortium: henipaviruses in bats in Thailand, Cambodia, Philippines, Laos, Indonesia, China, Malaysia, Bangladesh and India (*35-41*); a novel henipavirus, Mòjiāng virus, in wild rats in Yunnan (*14*); serological evidence of FVs in bats in Bangladesh (*42*) and Singapore (*43*); Nipah- and Ebola-like viruses in *Hipposideros*, *Cynopterus* and *Rhinolophus* species in Malaysia (Hughes *et al.*, in prep.); evidence of novel FVs in bats in China (*44-46*), including Měnglà virus that appears capable of infecting human cells (*47*); novel PMVs in bats and rodents consumed as bushmeat in Vietnam (*48*); a lineage C β-CoV in bats in China using the same cell receptor as MERS-CoV to infect human cells *in vitro* (*49*); MERSr-CoVs in bat guano harvested as fertilizer in Thailand (*50*), and directly in bats (Wacharapluesadee *et al.*, in prep.); 172

Viral agent	Site, date	Impact	Novelty of event	Ref.
Nipah virus	Malaysia, Singapore 1998-9	~246 human cases, ~40% fatal	2nd emergence of a zoonotic henipavirus, 1st large outbreak	(*4-6*)
Melaka & Kampar virus	Malaysia 2006	SARI in family group, individual	1st disease due to bat-origin reoviruses, also Singapore, Vietnam etc.	(*7-10*)
Reston filovirus	Philippines 2008	Seropositive people killed pigs,	No prior FVs in pigs	(*11*)
Thrombocytopenia Syndrome virus	E. Asia 2009	100s of deaths in people	Novel tick-borne zoonosis with large caseload	(*12*)
Reston filovirus	Shanghai 2011	PCR positive pigs	Further evidence of pig RESTV infection	(*13*)
Mòjiāng virus	Yunnan 2012	Death of 3 mineworkers	1st evidence of rodent origin henipavirus in people	(*14*)
Nipah-like virus	Philippines 2015	Killed horses	No prior horse infection for NiV, known for HeV	(*15*)
SARSr-CoV & HKU10-CoV	S. China 2015	Seropositive people	1st evidence human infection HKU10 & SARSr-CoV	(*16*)
SADS-CoV	China 2017	>25,000 pig deaths seronegative people	Novel emergence of bat-origin CoV	(*17*)
Nipah virus	Kerala 2018, 2019	Killed 17/19 people 2018, 1 infected 2019	1st outbreak of NiV outside Bangladesh, W. Bengal focus	(*18, 19*)

Table 1: Recent emergence events in SE Asia indicating potential for novel pathways of emergence, or unusual presentations for known or related viruses.

novel β-CoVs (52 novel SARSr-CoVs) and a new β-CoV clade ("lineage E") in bats in S. China that occur throughout the region (*24, 49, 51, 52*); 9 novel wildlife-origin CoVs and 27 PMVs in Thailand, and 9 novel CoVs in Malaysia reported by us from USAID-PREDICT funding (*51, 53*). These discoveries underscore the clear and present danger of zoonotic events in the region. The wide diversity of potentially pathogenic viral strains also has significant potential use in broadly active vaccine, immunotherapeutic and drug development (*49*).

Most high-impact zoonotic viruses originate in wildlife reservoirs, sometimes spilling over first into livestock 'amplifier' hosts, or directly into localized human populations with high levels of animal contact **(Fig. 2)**. Efforts to prevent emerging zoonoses have targeted these high-risk populations in regions prone to disease emergence (*31, 54*). However, these regions tend to be in the developing world, where nations often lack the resources to investigate novel zoonotic events, or wildlife reservoirs of zoonoses, and prioritize more immediate public health concerns such as Dengue virus or NTDs (*20*). Zoonotic disease surveillance and control is also hampered by inadequate information on basic disease ecology (e.g. range of wildlife reservoirs, risk factors for spillover) and pathogen biology (viral diversity, pathogenesis), and by logistical challenges sharing novel diagnostics, animal models, samples and reagents at sites where outbreaks begin (*55*). Countermeasure and vaccine development is challenged by the small number of isolates available, and the presence of closely-related strains, some with evidence of transmission to people but with unknown pathogenicity. For example, using NIH and other funding, we found serological evidence of exposure to African henipaviruses in 3-4% of a sample of people in Cameroon, with a significant risk factor being butchering of bats; and serological responses to bat SARSr-CoVs in 6/209 (2.87%) and bat HKU10-CoV in 2/412 (0.5%) people living close to a bat cave in Yunnan, all novel viruses with unknown clinical impact (*16, 56*). We discovered two novel bat-borne reoviruses, Melaka and Pulau viruses, and showed that 14/109 (13%) of people living in close proximity to bat roosts on Tioman Island, Malaysia were exposed to these viruses (*7*) as well as 12/856 (1.4%) of a random sample screened in Singapore (*8*). Previous studies in the Philippines found famers and butchers who had contact with sick pigs tested positive for Reston ebolavirus antibodies but had not experienced illness (*11*), and it was our team that discovered Philippine bats as natural reservoir hosts for Reston virus (*57*). In preliminary screening in Malaysia with funding from DTRA we found serological evidence of exposure to henipaviruses (Hendra, Nipah and Cedar) in 14 bats and 6 human samples, and Ebola (Zaire and Sudan)-related viruses in 11 bats, 2 non-human primates (NHP) and 3 human samples (Hughes, in prep.).



Even when spillover leads to outbreaks of illness and mortality, they are often unreported, undiagnosed or misdiagnosed. We initiated targeted syndromic surveillance of encephalitis patients in Bangladesh clinics and showed that Nipah virus (NiV) causes repeated annual outbreaks with an overall mortality rate of ~70% (*58*). NiV has been reported in people in West Bengal, India, which borders Bangladesh (*28*), in bats in Central North India (*59*), and now in people in Kerala, South India in 2018 and 2019, raising the specter of future spillover at other sites in the region (*18, 60*).

Fig. 2: Our Early Warning Approach: Most zoonotic viruses emerge from wildlife hosts, sometimes via livestock **(lower panel)**, to cause small scale outbreaks (green) or larger chains (red) of human-to-human transmission **(middle)**. In some cases, these spread more widely via air travel **(upper)**. Our EIDRC proposal targets each stage: **SA1** examines the diversity and spillover potential of viral threats in targeted SE Asian wildlife; **SA2** seeks evidence of their spillover into focused high-risk human populations; **SA3** identifies their capacity to cause illness and to spread more widely. Figure from (*54*).

Under a current NIAID R01, we have shown that we can use analytical approaches to target surveillance of wildlife and people and identify likely sites of spillover. We used phylogenetic analysis of bat hosts and their CoVs to identify a key cave complex in SW Yunnan Province that harbors all the genetic elements of SARS-CoV (*61*), as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics (*24, 49, 51, 52, 62-64*). We conducted risk factor surveys and biological sampling of human populations living close to this cave and found serological evidence of spillover in people highly exposed to wildlife (*16*). **This work provides proof-of-**

concept for an early warning approach that we will expand in this EIDRC to target other viral groups in one of the world's most high-risk EID hotspots.

The overall premise of this EIDRC proposal is that there is substantial evidence that: 1) diverse CoVs, henipaviruses and FVs related to known human pathogens are circulating in wildlife reservoirs in Southeast Asia; **2)** these viruses likely spillover regularly to people, are often unreported or misdiagnosed, and their clinical manifestations and potential to cause pandemics are unknown and underestimated; and **3)** our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence. To scale-up this approach, we propose to launch **EID-SEARCH** (the **E**merging **I**nfectious **D**iseases - **S**outh **E**ast **A**sia **R**esearch **C**ollaboration **H**ub), to better understand, and respond to, the risk of zoonotic viral emergence in Southeast Asia. We will initially focus on the diversity of known and novel CoVs, henipaviruses, and FVs in wildlife reservoirs. We will use this work to build our center's interactive network throughout the region, bridge barriers, provide early focal point for basic and translational studies, develop sample handling and storage routines, communication networks, cohort management, infrastructure development, and cross-training in surveillance and novel diagnostic platforms. We will develop and transfer technology to test these viruses' capacity to infect human cells and mouse models, develop new specific and sensitive serological and molecular diagnostic tools, conduct surveillance of human communities with high risk of exposure to wildlife, and clinical cohorts to identify evidence of spillover of viruses causing previously 'cryptic' clinical syndromes in people. We will test the capacity of novel viruses to infect human cells and/or mouse models, and thus asses potential for spillover and spread which will feed into our risk models. Our group also has extensive experience in other viruses, including vector-borne flaviviruses, and alphaviruses, and will use this experience to provide robust long-term surveillance, diagnostics, and basic science opportunities. While the current proposal focuses its basic and applied research agendas on this group of viral EID, we note that the EID-SEARCH is poised to rapidly respond to any outbreak. **Thus, FVs, henipaviruses and CoVs are examples of sentinel surveillance systems in place that illustrate EID-SEARCH's capacity to identify and initiate a rapid response targeting any novel microbial pathogen that emerges in this region.**

2. Innovation: Previous work by our consortium has developed strategies to target surveillance in wildlife and people to better anticipate and identify early spillover events and pre-empt outbreaks of emerging viruses. In the EID-SEARCH we scale up this approach in a central research "hub" made up of leading laboratories, clinics and surveillance sites in three critically high-risk EID hotspot countries in SE Asia. EID-SEARCH's reach is expanded through its regional network of collaborators that covers nearly all of the greater SE Asia region. The innovation of the EID-SEARCH is in: **1)** Its multidisciplinary approach that combines modeling to target geographic and taxonomic sampling targets with on-the-ground zoonotic disease surveillance in human and wildlife populations; **2)** the biological characterization approach that identifies how likely viruses are to be able to infect people, and enables evaluation of existing countermeasure technologies; an approach that is grounded in our successful work on SARSr-CoVs; and **3)** the strategic sampling of both high-risk communities with extensive animal contact at the front-lines of viral spillover *and* syndromic clinical cohorts that present with signs of novel viral emergence, to identify risk of viral spillover in an early warning system approach **(Fig. 2)**. **In Aim 1, we will target viruses in archived and newly collected wildlife samples, and characterize their risk of spillover to people**. We will use spatial and phylogeographic analyses to identify the geography and species of wildlife to test samples from, and to further characterize viruses we have recently discovered. We will use *in silico* methods (novel ecological-phylogenetic risk ranking and mapping of receptor binding domains), cell culture, and animal models to assess their potential for spillover into high-risk human populations. **In Aim 2, we will conduct targeted cross-sectional serological surveys of human communities with high levels of animal contact to find evidence of viral spillover, and identify occupational and other risk factors for zoonotic virus transmission.** We will use serological tests targeting viruses identified in Aim 1 to identify the baseline spillover of known or novel CoVs, henipaviruses and FVs in these populations. Serological findings will be analyzed together with subject metadata (including a simple animal-contact survey, location data, viral sequence/isolates etc.) to identify zoonotic risk factors and routes of exposure from specific wildlife or vector species. **In Aim 3, we will use syndromic surveillance of clinical human cohorts to identify evidence of viral etiology for otherwise 'cryptic' outbreaks.** We will enroll and

collect biological samples from patients in local clinics and hospitals who present with syndromes previously linked to known viral agents and live within communities at high risk for disease emergence. We will conduct molecular and follow-up serological diagnostic assays to identify likely viral etiology of syndromes that may represent otherwise cryptic events. Where viruses are identified, we will attempt to isolate or molecularly re-derive them, and use survey data, ecological and phylogenetic analysis to identify likely reservoir hosts and inform intervention programs. Our US partner BSL-4 laboratory, NEIDL, will attempt isolation and characterization of viruses requiring BSL-4 of containment (e.g. novel filoviruses, close relatives of Hev/NiV).

3. Approach: 3.1. Research team: The EID-SEARCH builds on long-term collaboration among world leaders in emerging virus research with proven experience collaborating internationally on field, lab and human surveillance research **(Fig. 3)**. Over the past two decades, our consortium partners have conducted high profile research on EIDs within the region and globally, including identifying the wildlife reservoirs for NiV and Hendra virus (HeV), MERS- and SARS-CoVs (*24, 27, 59, 61, 65-67*), discovering SADS-CoV (*17*), and developing an array of serological, molecular, *in vitro* and *in vivo* approaches to characterizing high-risk CoVs, henipaviruses, and FVs (*62-64, 68-81*). Our team has substantial experience conducting human surveillance in the community and during outbreaks **(Sections 2.2.a., 2.2.b., 2.2.c, 3.2.a, 3.2.b)**, including: USAID-PREDICT, and DTRA-funded viral surveillance of indigenous communities in Peninsular Malaysia and syndromic surveillance of hospital patients in Sabah (Co-I Hughes); collection of >2,000 serum samples from healthy blood donors and sanitation workers, febrile and influenza like illness (ILI), and diarrheal stool samples from children under 5 years of age across Borneo Malaysia (Co-I Kamruddin); collaboration on the MONKEYBAR project with the London School of Hygiene and Tropical Medicine to analyze risk and prevalence of the zoonotic malaria *Plasmodium knowlesi* through case control and cross-sectional studies in Sabah villagers (n=~800, 10,000 resp.) and clinical observational studies to determine the etiology of central nervous system infections and acute undifferentiated febrile illness in Sabah. (Co-Is William, Rajahram, Yeo); and community-based surveillance of hundreds of bat-exposed villagers in Thailand (Co-Is Wacharapluesedee, Hemachudha). As a proof-of-concept of how our team can rapidly scale up resources for diagnostics and surveillance in the face of a new EID, in 2016, we discovered a novel bat-origin HKU-2 CoV (*82, 83*) killing >20,000 pigs in S. China, designed PCR and serological assays, surveyed pig farmers for evidence of exposure, and conducted experimental infections, pathogenesis and molecular studies, **all in the span of 3 months** (*17, 84*).

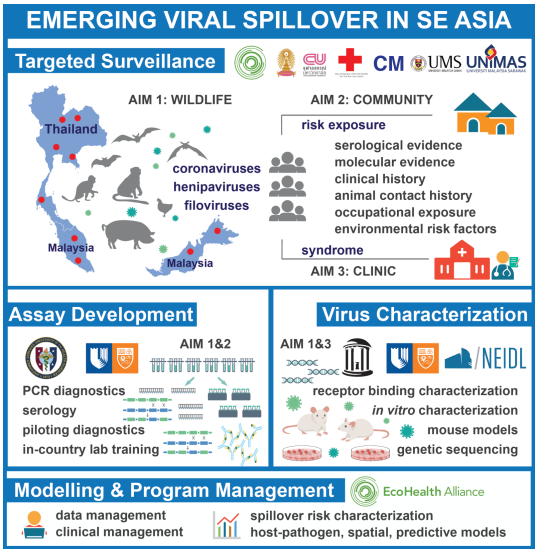


Fig. 3: EID-SEARCH scope, core institutions, and roles.

The administration of this center **(Section 4.1.)** will be centralized at EcoHealth Alliance (EHA) led by PI Daszak, who has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5-20 yrs on NIAID- and USAID-funded research. He will oversee all activities in this EIDRC, supported by Deputy lead, Dr. Olival – who has managed human and wildlife disease surveillance projects in SE Asia for >10 years, and a **Core Executive Committee (Section 4.1.a)**. Co-Is Olival and Zambrana are leaders infectious disease analytics, and head the modeling and analytics work for USAID-PREDICT. Co-Is Wang, Baric, Broder, Anderson, and Laing have developed a unique array of *in vitro* cell culture, serological and molecular reagents to identify and characterize emerging viruses in people and animals (henipaviruses and other PMVs, CoVs, FVs and many others). Co-Is Wacharapluesedee, Hemachudha, and Hughes and collaborators have coordinated clinical and community surveillance of people, and of wildlife and livestock within Thailand and Malaysia for the past 15 years, in collaboration with Ministries of Health, Agriculture and Environment. Finally, this consortium has extensive external funding from NIH, DoD, USAID, DHS and other USG agencies supporting laboratory, analytical and field studies directly related to the current proposal, and will bring substantial leverage to our EIDRC.

3.2. Geographical focus: The three core (hub) countries for this proposed EIDRC are Thailand, Malaysia and Singapore, three contiguous EID hotspot countries that stretch through one of the most significant foci of EID origins globally. Our proposed work includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sarawak and Sabah, and thus contains overlapping wildlife biogeography with India, Bangladesh, Myanmar, SW China, Laos, Cambodia, Vietnam, Philippines, and Indonesia. It encompasses diverse cultural and socioeconomic backgrounds which have correspondingly diverse and intensive interactions with wildlife reservoirs and the global travel network. We have already begun sampling of wildlife hunters, livestock workers, wildlife farmers and market workers, rural villagers living close to high risk wildlife species, people otherwise highly exposed to animals in these countries, and clinical cohorts, as part of current/prior NIH, DTRA, and USAID funding. Our core member's extensive network of collaborators in clinics, research institutes and public health laboratories in almost every other Southeast Asian country positions the EID-SEARCH within a much larger catchment area for emerging zoonoses and will allow us to rapidly scale up to include additional sites in response to an outbreak or shift in research priorities. We will conduct regular meetings with key research, public health and community leaders, including >50 leading laboratories/institutions in 10 countries **(Section 4.2)** to maintain these collaborative relationships with the core members of our consortium (**Fig. 4**). We will use these, and our annual meetings, to share information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks across the region.

Fig.4 : Map of Southeast Asia indicating three hub countries for this proposed EIDRC (**Red**: Thailand, Singapore, Malaysia) and countries in which Key Personnel have existing collaborating partners via other funded work (**Green**), indicating that EID-SEARCH provides access to key collaborators and sites throughout the region.

3.3. Capacity for linkage with other NIAID studies, and scaling up to respond to outbreaks: EID-SEARCH US-based partners include senior scientists with significant experience on NIAID-funded projects, extensive collaborations with other NIAID-funded scientists, and previous experience working with NIAID leadership through workshops, review of programs (e.g. PI Daszak's role in NIAID CEIRS external review), and work on study sections. These contacts ensure that the EID-SEARCH senior leadership will be ready and able to rapidly set up data, sample and reagent/assay sharing protocols with NIAID researchers, the other EIDRCs, the EIDRC CC and other NIAID research centers. EID-SEARCH in-country partners include senior medical doctors and infectious disease specialists at national and regional hospitals in each country and administrative state in this proposal. On news of potential outbreaks, **EID-SEARCH will rapidly mobilize its core staff and their extensive collaborative network across almost all SE Asian countries (Fig. 4) (Sections 4.1.a, 4.2. 4.3).** The core analytical approach can be used to rapidly identify sampling sites and targets to harvest vectors, vector borne arboviruses, birds and mammalian species that harbor most other viral threats. Through existing projects such as the USAID PREDICT Project and DTRA-funded biosurveillance projects, EHA is currently partnered with hospitals and researchers across South and Western Asia, West and Central Africa, and Latin America. Our partners already have capacity to conduct human and wildlife sampling and testing for novel viral agents, so expanding in geography or scale is readily achievable given sufficient resources.

Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife.

1.1 Rationale/Innovation: The vast majority of emerging zoonotic pathogens are linked directly or indirectly to wildlife host, particularly from important mammalian groups like bats, rodents and NHPs (*3*). Our previous work on wildlife origin viruses demonstrates that zoonotic viral emergence tends to occur in specific regions with high diversity of wildlife that harbor viruses of zoonotic potential, and human populations that have close contact to these wildlife **(Fig. 1)** (*2, 3*). In Aim 1 **(see Fig. 9 for overview)**, we will strategically conduct EID

surveillance by applying risk analytics to prioritize the regions, wildlife species, and human populations that have a particularly high risk of viral spillover. We will target high-risk zoonotic host reservoirs (bats, rodents and NHPs) in these regions and apply novel predictive models to estimate viral diversity and host range to identify wildlife species that have been ignored or under-sampled by previous zoonotic disease surveillance efforts. We will strategically collect specimens from these species and screen them, together with some of the tens of thousands of recently collected samples we have archived, for known and novel agents. We will begin screening using conserved PCR assays to identify known and novel viruses in high risk RNA viral families, *Coronaviridae*, *Paramyxoviridae*, and *Filoviridae*. We will expand to other groups of pathogens, including arboviruses, pending available resources and priorities established with the EIDRC coordinating center (EIDRC-CC) and other regional EIDRCs. Using risk ranking algorithms and a series of ecological and virological risk factors, we will triage the wildlife viruses we discover for further genomic characterization and viral isolation. On this subset of viruses, we will then use *in vitro* and *in vivo* approaches (viral rescue, cell lines, gene edited humanized transgenic mice and the collaborative cross genetic reference mouse population) previously developed and widely used by our team to predict capacity of novel viruses to infect people and spillover (*63, 85-88*). These high-risk viruses and their close relatives will be targets for human community serosurveys and clinical sampling in Aims 2 and 3, respectively.

1.2 Preliminary data: 1.2.a. Geographic targeting: EHA has led the field in analyzing where the highest risk of zoonotic spillover is across the globe, identifying SE Asia as a major EID hotspot (*1, 2*). These hotspots are sites where high wildlife biodiversity, human population density and rapid ecologic change collide **(Fig 1)**. Using host and ICTV viral data for all known mammalian viruses, and generalized additive models to correct for sampling bias, we are able to predict the relative number of yet-to-be-described viruses that a species harbors (*3*). This demonstrates substantial undiscovered viral diversity in mammals across Southeast Asia, and particularly in regions of Thailand, Singapore and Malaysia **(Fig. 1)**. Separately, we found that factors driving spillover (host diversity, climate) differ from those driving spread in human populations (population density, bushmeat hunting, livestock production) (*89*). We therefore analyze capacity for viral spread as a separate risk factor, using our unique demographic and air travel and flight data modeling software (*90, 91*). In the current proposed work, we will use all the above approaches to target wildlife sampling (archival and new field collections) in Aim 1, to inform sites for human sampling in Aim 2, and to assess risk of spread in Aim 3.

1.2.b. Reservoir host species targeting: Our analysis of all known mammalian host-viral associations demonstrates that bats, rodents and NHPs represent the three most important reservoirs for zoonoses, responsible for the highest proportion of known and predicted viral diversity of zoonotic potential (*3*). Thus, the bulk of our proposed wildlife sampling and testing will consist of these three taxa with a focus on bats, because this group is the known or hypothesized reservoir for the majority of high-impact zoonotic CoVs, PMVs and FVs (*50, 92-94*). Bats have the highest proportion of predicted zoonotic viruses in any mammalian group (*2*), and are exceedingly diverse in Southeast Asia (~500 species). To further prioritize taxonomic sampling targets within these diverse geographic hotspots, we used a novel Bayesian phylogeographic algorithm to identify host genera and species that are the most important centers of evolutionary diversity for a given viral group, e.g. bat genera for β-CoVs **(Fig. 5)** (*51*). This approach will be extended to other mammal taxa and viral families of interest, allowing us to identify the reservoir hosts of specific zoonotic viral groups, where viral diversity is likely highest.

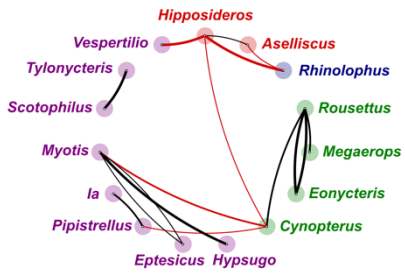


Fig 5: Preliminary analysis of sequence data collected under prior NIH funding, identifying SE Asian bat genera with the highest β-CoV evolutionary diversity. We will apply this Bayesian discrete phylogeographic model to identify taxonomic and geographic centers of viral diversification and sharing for relevant PMV, CoV, FV and other virus clades. Line thickness proportional to probability of virus sharing between two genera. Inter-family switches in red.

Finally, we have designed a strategy to estimate sampling gaps, using a viral 'mark-recapture' approach we previously published (*95, 96*) **(Fig. 6)**. Using prior data for bat, rodent and NHP viral discovery vs. sampling rates, we can identify the likely total viral diversity for specific host groups in a region, and use this to estimate sample sizes to discovering the majority of unknown viruses within a specific family or genus. **In the current**

proposal, we will use these analyses to estimate geographic and species-specific sampling targets **to more effectively identify new strains to support experimental infection studies and risk assessment.**

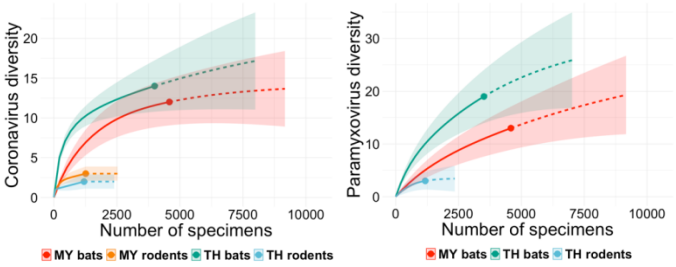


Fig. 6: Estimated CoV **(left)** and PMV **(right)** diversity in bats and rodents from Thailand and Malaysia, using data from PCR screening and RdRp sequences from >10,000 specimens in bats and 4,500 in rodents. Bats have 4X more viral species than rodents, controlling for sampling effort. We estimate that additional collection of 5k-9k bat specimens and testing of our archived bat and rodent specimens alone will identify >80% of remaining CoV and PMV viral species in these key reservoirs, yielding >800 unique viral strains.

1.2.c. Sample testing to identify known and novel viruses: We have conducted extensive wildlife surveillance for novel viruses across Southeast Asia in prior NIAID funded work and as part of the USAID PREDICT project. Under the 10-year PREDICT program globally, we identified 912 novel and 210 known viruses, more than the total number of viruses previously recognized in mammals by the ICTV (*97*). This work included collecting nearly 300,000 individual mammal samples from 14 EHA-led PREDICT countries, and PCR-screening of more than 60,000 individual specimens (*53*). In southern China alone, we identified 178 β-CoVs, of which 172 were novel, discovered a new β-CoV clade, "lineage E" (41), diverse HKU3r-CoVs (179 sequences) within a 'sister' clade to the SARS-CoV lineage, and a new bat-origin SADS-CoV, responsible for killing >25,000 pigs in Guangdong Province (*17*). We have collected 28,957 samples from bats, rodents and NHPs in Thailand and 47,178 in Malaysia, **but have only tested a minority of these using PCR.** We have identified 100 novel viruses in Thailand and 77 in Malaysia. **Furthermore, we have only sequenced a small segment of one gene for all novel viruses found. We have archived duplicate samples which are now available for use in this project**, including fecal, oral, urogenital, serum samples, and biopsies from bats, rodents, and NHPs. In the current proposed work, we will attempt to sequence, isolate and characterize those viruses that our analyses below suggest are most likely to be able to infect humans.

1.2.d. In vitro & in vivo *characterization of viral potential for human infection:* Using Coronaviridae as an example, we have conducted *in vitro* and *in vivo* experiments to characterize the pathogenic potential of novel SARSr-CoVs from bats. We isolated three SARSr-CoVs from bat feces: WIV1, WIV16 and Rs4874, with Spike (S) protein sequences that diverged from SARS-CoV by 3-7% (*24, 61, 98*). We conducted full-length genome sequencing of 12 other novel SARSr-CoVs, some highly similar to outbreak SARS-CoV in the most variable genes (*61*). Using our reverse genetics system we constructed chimeric viruses and rederived full length recombinant SARSr-CoV from in silco sequence. All 3 SARSr-CoV full length isolates and the two chimeric viruses replicated efficiently in Vero E6 cells and in HeLa cells expressing hACE2, civet and bat ACE2, but not in those without ACE2 (*24, 61, 98*). We used the SARS-CoV reverse genetics system (*72*) to generate a chimeric virus with a mouse-adapted SARS-CoV backbone expressing SHC014 S protein with 10% sequence divergence from SARS-CoV S. This and the other full length and chimera viruses replicated in primary human airway epithelium, using the human ACE2 receptor to enter into cells (*62*). Thus, **SARSr-CoVs with diverse variants of SL-CoV S protein without deletions in their RBD can use human ACE2 as receptor for cell entry.** The Baric lab has a well-established hACE2 and DPP4 transgenic mouse model that **we used to assess the capacity of novel SARSr-CoVs and MERS-CoV to infect humans and cause disease** (*85, 99*). Infection of bat SARSr-SHC014 or WIV1 caused SARS-like clinical signs in the transgenic hACE2 mouse model **that weren't reduced by immune-therapeutic monoclonals that attenuate SARS-CoV pathogenicity, nor after challenge following vaccination against SARS-CoV** (*62*) **(Fig. 7)**. We repeated this virus characterization approach with chimeras using HKU3r-CoV S proteins that are ~25% divergent from SARS-CoV S, **and found that they are unable to use the ACE2 receptor**. Additionally, we were unable to culture HKU3r-CoVs in Vero E6, or human cell lines.

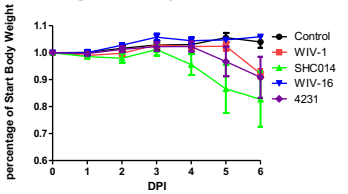


Fig. 7: Body weight change in hACE2 transgenic mice after SARSr-CoV infection, demonstrating that bat-origin SARSr-CoVs cause similar clinical signs to outbreak SARS-CoV strain (4231) (*62, 63*).

A similar approach to the above pipeline for CoVs will be applied to novel henipaviruses we discover during our research. The broad mammalian tropism of HeV and NiV (*68*) is likely mediated by their usage of highly conserved ephrin ligands for cell entry (*19, 100*). A third isolated henipavirus, Cedar virus does not cause pathogenesis in animal models. **Co-Is Broder and Laing** have developed a reverse genetics system and rescued a recombinant CedV to test ephrin receptor usage/tropism and pathogenicity during henipavirus infections (*77*). CedV is unable to use the Ephrin-B3 receptor (*77, 101*) which is found in spinal cord and may underlie NiV encephalitis (*102*) and that pathogenesis also involves virulence factors V and W proteins. The putative henipaviruses, Ghana virus and Mòjiāng virus, predict V and W protein expression, with GhV able to bind to ephrin-B2, but not -B2 (*103*), and the receptor for MoJV remains unknown (*104*) but is likely ephrin-B2 (*105*). Our group has also used similar methods to test the hypothesis that novel Filoviruses discovered in wildlife are able to infect people. NiV, EBOV and MERS-CoV all infect primary microvascular endothelial cells, which are available at Co-I Baric's lab for EID-SEARCH collaborators (*71, 106*). Primary human lung endothelial cells are highly susceptible to Ebolavirus infection (*107*), and Huh7 cells can be used to isolate virus from clinical samples (*108*) offering advantages for reverse genetic recovery of novel FVs (*109*). The three filovirus genera, *Ebolavirus*, *Marburgvirus* and *Cuevavirus*, use Niemann–Pick type C1 (NPC1) protein as cell entry receptor (*110*). For novel filoviruses, cell culture would be carried out under BSL-4, however they can readily be characterized following identification of their encoded GP sequences which can be pseudotyped into recombinant GP-bearing vesicular stomatitis virus (VSV) pseudovirions (*111*). These pseudovirions can be safely used to characterize the tropism and entry filoviruses mediated by GP without a need for BSL4 containment. **Co-Is Wang and Anderson** used cells originating from various mammalian species to determine spillover and/or zoonotic potential of MLAV, a newly discovered Asian filovirus (*47*). Despite the low amino acid sequence identity (22–39%) of the glycoprotein with other filoviruses, MLAV is capable of using NPC1 as entry receptor. Cells from humans, monkeys, dogs, hamsters and bats were able to be infected with a MLAV pseudovirus, implying a broad species cell tropism with a high risk of interspecies spillover transmission.

Mouse models. For novel viruses across all families, we will use the UNC collaborative cross mouse that captures 90% of the diversity within all mouse groups to assess the most suitable model for further experiments (*112*). We have used this model for CoV, FV (Ebola), Flaviviruses, and alphaviruses infections. Clinical signs such as weight loss, hemorrhage, encephalitis, and, acute or chronic arthritis were recapitulated in these mice following SARS, EBOV, West Nile virus and Chikungunya infections, respectively (*86-88, 113-116*). Note that CC OR3015 shows hemorrhagic disease phenotypes after EBOV infection **(Fig. 8)**. *Bat Models.* Duke-NUS has developed two models for bat *in vivo* studies: a colony of *Eonycteris spelaea* (cave dwelling small fruit bat) and a bat mouse model, which contains the bat immune system in an immunodeficient mouse (*117*). In a proof of concept study, **Co-Is Wang and Anderson** infected *E. spelaea* bats with MERS-CoV to demonstrate their susceptibility and suitability for infection with a bat borne virus under BSL3 containment. These bats and the bat mouse model will serve to complement and expand studies from the UNC collaborative cross mouse, pending further funding from EIDRC CC. Recombinant viruses, transgenic mouse models and experimental recombinant protein constructs described above will be made available to the EID-SEARCH consortium and other EIDRCs following standard procedures **(see Resource Sharing Plan)**.

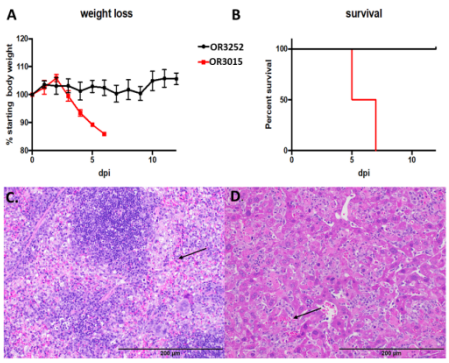


Fig. 8: EBOV Infection in Collaborative Cross Mouse. **Panel A/B:** Wt EBOV in two different CC lines demonstrate different disease patterns. **Panel C/D:** Hemorrhagic phenotypes on d. 6 (arrow) in spleen and liver, resp. From (*86*).

1.3. General Approach: We will conduct analyses to geographically- and taxonomically-target testing of samples from wild mammals that are most likely to harbor known high-profile zoonotic pathogens, or close relatives with potential to infect people. This includes selecting from tens of thousands of samples collected on recent projects and stored in freezers in our laboratories, and new sampling of wildlife in high risk locales. We will use viral family level PCR assays to identify and partially characterize viruses, then follow up sequencing of relevant receptor binding proteins to assess homology and predict binding affinity, then attempt to isolate and

biologically characterize viruses that phylogenetic and other data suggest have high spillover and pandemic potential. We will follow this with *in vitro* binding assays and cell culture experiments, and *in vivo* animal model infections to assess their potential to infect people and cause disease **(Fig. 9)**. EHA will lead the study design, targeted sampling, and data analysis; Co-Is Hughes and Wacharapluesadee will lead field sampling and in-country testing; Duke-NUS, UNC, USUS will lead the serological and PCR diagnostic development and the *in vitro* and *in vivo* analyses, as well as cross-training and tech transfer to the hub labs in Thailand and Malaysia. This will include via support of one Malaysian Ph.D student, Mei-Ho Lee (CM Ltd.) who has worked with the EID-SEARCH collaborators for 9 years. The focus of her study will be infection potential of novel CoVs and PMVs already found in bats in Malaysia under preliminary data for this proposal.

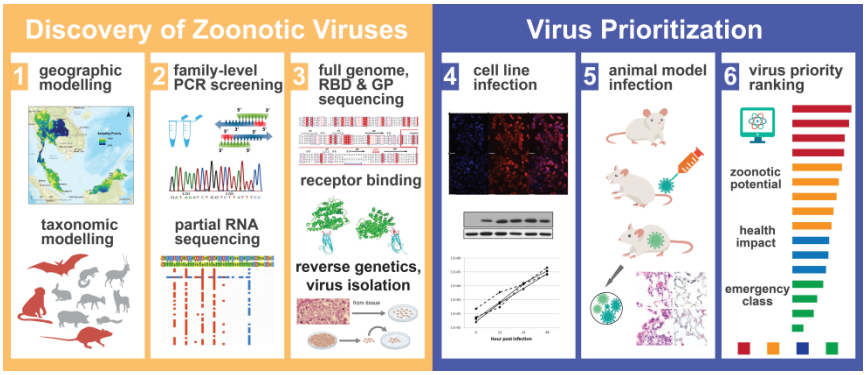


Fig. 9: Aim 1 will use analytical tools to target selection of archived and newly collected samples, conduct PCR and sequencing of novel viruses, recover by reverse genetics, and assess zoonotic potential using *in vitro* and *in vitro* models and analyses.

1.4 Wildlife samples: 1.4.a. Geographic and taxonomic targeting for newly collected wildlife samples: We will prioritize sites within each country that rank highest for both the risk of zoonotic disease emergence (*2*) and the predicted number of 'missing' zoonotic viruses (*3*). Our preliminary analysis **(Fig. 1)** suggests priority areas include: the Kra Isthmus (S. Thailand) and adjacent forests in N. Peninsular Malaysia, NW Thailand (near the Myanmar-Laos border), and lowland rainforests of Sarawak and Sabah. In each of these 'hottest of the hotspots' regions, we will identify 2-4 distinct sampling sites based on the geographic distribution of key hosts we prioritize for additional wildlife sampling. We will priority rank bat, rodent, and NHP species using host trait-based models (*3*). We will also identify species predicted to be centers of evolutionary diversity for CoVs, PMVs, and FVs using ancestral host reconstruction of available sequence data and Bayesian discrete phylogeographic models (*118-120*). We will leverage our strong collaborations with wildlife experts in Malaysia and Thailand, including many of whom are based at our core partner institutions, to ground-truth geographic and taxonomic model outputs with local expert knowledge to ensure the sampling plan is realistic based on current species distributions, population size estimates, seasonality, and accessibility to sites. Most zoonotic viruses are naturally carried by multiple wildlife host species, and there are strong, predictable relations between host phylogeny and viral taxonomy (*3*). We will use a combined network analysis and phylogeographic model to rapidly predict and prioritize new (unsampled) hosts for important, novel viruses we discover during our research. We have shown this relatively simple model (using just wildlife species range overlap and phylogenetic similarity between host species) is both generalizable to estimate host range for all mammalian and avian viruses *and* robust – accurately predicting hosts 98% of the time when cross-validated using data from known viruses (*121*).

1.4.b. Sample size justifications for testing new and archived wildlife specimens: We calculate initial sample sizes targets using our preliminary data on average PCR prevalence from screening tens of thousands of bat, rodent and NHP specimens for CoV and PMVs based on previous studies in Malaysia and Thailand. We will refine these sample size targets, in real-time, over the course of our project using viral discovery curve analyses **(Fig. 6)** to either scale-up or scale-back new sampling and testing of archived specimens for each species depending on viral capture rates. We will only collect additional specimens projected from each prioritized taxa to achieve statistically robust sample sizes and to complement our archived specimens. For example, *Rousettus* spp. bats, known FV reservoirs, were not adequately sampled under PREDICT research in Thailand. Preliminary analysis indicates that, for all viral target families, we will require ~14,000 new samples to identify 80% of remaining viral species diversity in our study region, ~5,000 samples from Thailand and ~9,000 samples from Malaysia **(Fig. 10).** Viral strain diversity from a sampling effort this size is expected to number in the hundreds of strains. For example, given ~6% prevalence of CoVs in bat species previously sampled under the PREDICT project, a target of 14,000 individuals would yield ~800 PCR positive individuals,

representing, an estimated ~600 novel sequences/strains. Similarly, for PMVs, which have an average PCR detection prevalence of ~1.5%, sampling of 14,000 individuals would yield ~200 positives and an estimated 150 unique strains. Filovirus estimates are not feasible yet due to the small number of positive samples in prior studies, but will be estimated as the project begins. From each mammal, we will collect serum, whole blood, throat swabs, urine and fecal samples or rectal swab using our previously-validated nondestructive methods. We will sample sites at different time-points throughout each year, and sample an equal number of males and females to account for seasonal or sex-specific differences viral shedding **(See Vertebrate Animals)** (*122, 123*). Dead or animals euthanized due to injury will be necropsied.

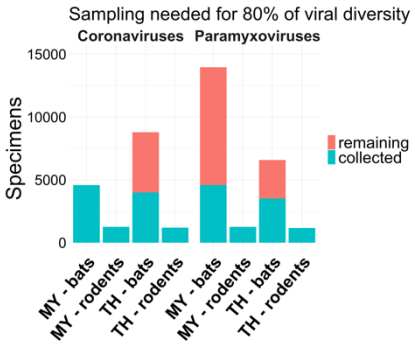


Fig. 10: Stacked bar plot showing number of currently-archived specimens **(blue)** and new specimens required **(orange)** to discover 80% of predicted number of CoVs and PMV species from high-risk bat and rodent taxa in Malaysia and Thailand.

1.4.c. Sample collection, testing, viral isolation: Wildlife will be captured and sampled using previously published techniques, by personnel wearing appropriate personal protective equipment (Tyvek suits or dedicated long external clothing, double nitrile gloves, leather gloves, an N95 or p100 respirator, and safety glasses) (*53, 93, 95, 96, 122, 124*). All samples will be placed in a vapor phase liquid nitrogen dry shipper immediately upon collection, and then transferred to a -80C freezer once back in the lab, until testing. Viral RNA will be extracted from bat fecal pellets/anal swabs. RNA will be aliquoted, and stored at -80C. PCR screening will be performed with pan-coronavirus (*125, 126*), filovirus (*127*) and paramyxovirus primers (*128*), in addition to virus specific primers where additional sensitivity is warranted in key viral reservoirs, i.e. Nipah virus or MERS virus specific primers (*129, 130*). PCR products will be gel purified and sequenced. We will attempt isolation on samples that contain viruses of interest (determined in **Aim 1.5 below**), using Vero cells and primary cell culture from the wildlife taxonomic order (bat, rodent and NHP cell lines). Over 70 bat cell lines are maintained at Duke-NUS from five different bat species. These include primary lung, brain, bone marrow, heart, kidney, intestine, liver, lymph node, muscle, spleen, PBMC and ovary/testes from *Eonycteris spelaea, Cynopterus bracyhotis, Rhinolophus Lepidus, Myotis muricola* and *Pteropus alecto* bats. In addition we have 8 immortalized cell lines, including those derived from lungs, therefore suitable for the isolation of respiratory viruses.

1.4.d. Moving beyond RNA viruses: To detect pathogens from other families, such as non-RNA viruses, we will combine conserved PCR assays with NGS. We will perform either unbiased NGS, or use a pan-viral enrichment library to sequence full-length genomes (*131*). We will conduct this work via EID-CC research projects or as part of outbreak detection/investigation in the region from DNA viruses or other agents.

1.4.e. Sequencing Spike Glycoproteins: Based on earlier studies, our working hypothesis is that zoonotic CoVs, FVs and henipaviruses encode receptor binding glycoproteins that elicit efficient infection of primary or continuous human cell lines (e.g., lung, liver, etc.) (*16, 61, 62*). Characterization these for SARSr-CoVs suggest that viruses up to 10%, but not 25%, different in the spike glycoprotein use human and bat ACE2 receptors for docking and entry. Uneven sampling gaps (e.g., no strains were found with 10-25% spike variation) prevent a thorough understanding of the transition point where the most divergent strains lose human ACE2 receptor usage. Our viral discovery curves **(Fig. 6)** suggest further sampling will reveal a rich diversity of as-yet-undiscovered SARSr-CoVs and strains of other viruses that fill in the phylogenetic tree between currently-described viruses. In this aim we will use phylogenetic and viral discovery analysis to specifically target bat species and regions that are under-sampled to allow sufficient power to identify and characterize missing strain diversity for potential zoonoses. We will sequence the S proteins of novel SARSr-CoVs to prioritize viruses for experimental work in Aim 3 to test this hypothesis. Likewise, we will test the hypothesis that phylogenetic related zoonotic receptor binding protein genes of MERS-related, Ebola-related and NiV/HeV-related viruses will also program efficient entry via human and ortholog receptors. For all novel virus strains, we will sequence the complete entry spike genes by amplifying overlapping fragments using degenerate primers as shown previously (*24, 61*). ***Reverse Genetics:*** Full-length genomes of selected CoVs, FVs or henipaviruses (representative across subclades) will be sequenced using NEBNext Ultra II DNA Library

Prep Kit for Illumina and sequenced on a MiSeq sequencer. PCR or MinIon sequencing will be performed to fill gaps in the genome. The full length spike gene sequences, including the amount of variation in the receptor binding residues that bind the appropriate human receptor will be used to select strains for Aim 3 experiments. We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-CoV cross neutralization, synthetically reconstruct a small subset (1-2) and evaluate growth in Vero, BHK and Huh7 cells (*63, 132*). From full length sequences, synthetic clones will be ordered to recover recombinant viruses using reverse genetics as described by our groups (*62, 63, 77, 109, 133, 134*). Recombinant virus growth will be accessed in Vero, BHK, Huh7 and select bat cells and recovered viruses used for downstream studies. Virus strain prioritization is described below.

1.5. Assessing risk for spillover. 1.5.a. Capacity to infect human cells: We will use a series of cell cultures to assess the capacity of target viruses to enter human cells. For CoVs, FVs and henipaviruses, we will use primary human ciliated airway epithelial cells (HAE) and lung endothelial cell cultures derived from the lungs of de-identified transplant recipients. HAE are highly differentiated and grown on an air-liquid interface for several weeks prior to use (*62, 63, 132*). We will prepare HAE and lung endothelial cultures from three different patient codes in triplicate in collaboration with the tissue procurement facility at the Cystic Fibrosis Center at UNC. Cultures will be inoculated with chimeric bat SARSr-CoVs or novel FVs or henipaviruses to assess efficient replication. At 72 hpi, cultures will be fixed for immunofluorescent staining using antisera to the SARS-CoV conserved nucleocapsid protein (N) or to structural genes of the other viruses (*64, 71*). SARSr-CoVs that differ significantly in S protein sequence (11-24%) from epidemic SARS-CoV yet replicate *in vitro*, will also be evaluated for sensitivity to neutralization in Vero cells by PRNT50 assays using broadly SARS-CoV cross reactive human mAbs S227.14, S230.15, S215.17, and S109.8 (*63, 135*) or monoclonal antibodies targeting the Ebola or Marburg E glycoprotein (Available through BEI), or NiV/HeV glycoproteins (*136*). As controls or if antisera is not available, the S genes of novel SARSr-CoV or spike entry genes of other novel viruses under investigation will be inserted into VEE 3526 replicon vectors (VRP3526-S), packaged and used to vaccinate mice (*137*). Polyclonal sera against test bat-SARS virus or other spike genes will be harvested and tested for ability to cross neutralize SARS-CoV, GD03, WIV-1, SHC014, WIV-16, other novel SARSr-CoV and HKU3-SRBD by PRNT50 assay (*63, 138, 139*). Using PRNT50 titers with sera (n=4 each) among these viruses, antigenic cartography (*140*) will allow comparison of antigenic vs. phylogenetic distance, identifying the transition at which SARSr-CoV strains escape SARS-CoV based vaccines, informing immunotherapeutic and vaccine design strategies (*141-143*). Similar approaches will be applied to novel MERS-related viruses, other CoV, FVs or henipaviruses. While most wildlife-origin viruses with unknown transmission potential or pathogenicity in people uncategorized, we use BSL-3 for isolation as standard, and cell culture for potentially BSL-4 pathogens will be undertaken at collaborating institute NEIDL. To facilitate this, duplicate samples in culture medium from animals PCR +ve for FVs and very close relatives of NiV or HeV will be shipped to NEIDL **(See letter of support)** for culture, characterization and sharing with other approved agencies including NIH Rocky Mountain Laboratories where PI Daszak and Co-Is Baric have ongoing collaborations. Similarly, some duplicate samples of animals PCR +ve for CoVs will be shipped to UNC for further characterization by Co-I Baric. Where feasible and appropriate, training opportunities at NEIDL and UNC for in-country staff will be given, thereby enriching hands-on collaboration across sites.

1.5.b. Host ACE2, DPPR, Niemann-Pick C1 (NPC1) and EphrinB2/EphrinB3 receptors: We will sequence relevant host receptors for select mammalian wildlife species we find positive for strains of high-priority CoV clades, and all new henipaviruses and FVs. Of particular interest is homology across 18 bat and human orthologue ACE2 contact interface residues that engage the SARS RBD as a potential indicator of SARSr-CoV cross species transmission potential and growth in human cells (*70*). Likewise, variation in the NPC1 receptor has been shown to alter EBOV ability to infect in humans and bats, so this can be structurally mapped for prediction and downselection of strains (*144*). Signatures of positive selection in bat NPC1 were concentrated at the virus-receptor interface, with the strongest signal at the same residue that controls EBOV infection in *Eidolon helvum* cells. Thus NPC1 is a genetic determinant of FV susceptibility in bats, and some NPC1 variations likely reflect host adaptations to reduce FV replication and virulence (*145*). NiV and HeV use ephrin B2 or ephrin B3 as a receptor, and variation affects its ability to use human vs bat receptor molecules (*103, 146*).

1.5.c. Host-virus evolution and predicting receptor binding: We will use Bayesian and Maximum Likelihood phylogenetic analyses of RNA-dependent RNA polymerase (RdRp) sequences from PCR screening, receptor binding glycoproteins, and/or full genome sequence data (when available) to reconstruct the evolutionary history of the novel bat SARSr-CoVs, bat MERSr-CoV, FVs and henipaviruses that we identify. We will run co-phylogenetic analyses to map out instances of host-switching (*147, 148*) and ancestral state reconstruction analyses **(Fig. 4)** to identify the most important species for shaping evolutionary diversity for these viruses (*51, 149*), allowing for more targeted future surveillance and spillover mitigation interventions. For receptor binding glycoprotein sequence data from characterized viruses, we will apply codon usage analyses and codon adaptation indices, as used recently on henipaviruses (*150*), to assess the relative contributions of natural selection and mutation pressure in shaping host adaptation and host range.

1.5.d. Viral strain prioritization: Of the expected 100-200 novel SARSr-CoV strains, and approximately 600 total CoV strains, we will down-select to prioritize for further characterization based on S genes that are: i) different from SHC014, WIV1, SARS-CoV with diversity ranges of 10-25%; ii) have virus S RBD that could use human/bat receptors; iii) have recombinant chimeric spikes indicative of gene flow between clade I and II strains; iv) have bat ACE2 or DPP4 receptors that might select for spike RBDs that can use human receptors for entry (15/18 conserved residues in human/bat ACE2 molecules that bind SARSs-CoV S RBD domains are likely more efficient receptors than 3/18 conserved sites). Using structural models based on the SARS S glycoprotein, the extent and location of antigenic variation will be annotated onto the structure, further resolving the locations of highly evolving and conserved structural motifs/epitopes that function in protective immunity (*135, 151-153*). For novel henipaviruses, we will examine the receptor-binding proteins (e.g. envelope attachment (G) proteins) for sequence and structural homology with particular attention to conversation of amino acids that mediate ephrin receptor binding. We will prioritize strains that are predicted to interact with ephrin-B2 and expression V/W proteins to determine whether these viruses represent zoonotic threats. Using the recombinant Cedar virus (rCedV) platform, we will be able to rescue rCedV that express heterotypic G proteins to explore receptor utilization and tissue dissemination in animal models. We will assess ability to infect human cells using a continuous lung epithelial cell line, Calu3, which our group has previously used to generate data on NiV and HeV growth curves and a variety of host expression changes after infection (*154*). We will also use primary human airway cultures and microvascular endothelial cells to assess human infection for FVs, henipaviruses and MERSr- and SARSr-CoVs (*155*) (*156*) . There is some evidence for NiV and HeV replication in mouse lung, low level titers in young animals, 10 fold higher titers in aged mice (*157, 158*). Thus it is likely these models could be improved in the collaborative cross mouse resource at UNC. We have identified CC lines that are highly vulnerable to wildtype SARS and others that develop hemorrhagic disease following mouse adapted Ebola infections (*86-88*).

1.5.e. Animal models: We will use transgenic mouse models and the UNC Collaborative Cross model to assess spillover potential of viruses. For work on CoVs, we will follow the protocols in the transgenic mouse model used by the Baric lab previously. Briefly, in the BSL3 lab, 10- to 20-week old hACE2 or hDPP4 transgenic mice will be intranasally inoculated with 5 x 10^4 PFU of full length wildtype rbat CoV, or chimeric SARSr-CoV or MERSr-CoV encoding different bat CoV spike proteins respectively, then monitored daily for weight loss, morbidity, and clinical signs of disease. Mice will be euthanized at 2, 4, and 6 dpi (endpoint of 14 dpi), organs harvested and virus quantified by plaque assay or genome (mRNA1) RT-PCR. After 7 days in formalin, tissues for histopathology will be removed from the BSL3 and stained with H&E, and for immunohistochemistry using polyclonal antibodies against the N protein. We will conduct limited evaluation of existing countermeasures using therapeutic monoclonal antibodies *in vitro*. Existing SARSr-CoV or MERS-CoV mAbs (*159, 160*) will be diluted in DMEM starting at 1:20, and serial dilutions mixed with an equal volume of 50 PFU of chimeric bat SARSr-CoVs encoding different spike proteins and RFP indicator genes, then incubated on Vero E6 cells at 37°C for 1 h. Antibody neutralization titers will be evaluated by plaque quantification at 4-5dpi. In select instances, hACE2 transgenic mice will be injected with SARS-CoV mAbs, and infected with chimeric bat SARSr-CoVs. Clinical signs and morbidity will be assessed and tissue pathology examined and compared with mice without treatment of mAbs to determine the therapeutic effect on SARSr-CoV infection, and protection of SARSr-CoV by wildtype SARS-S based vaccines assessed as described (*132, 161*). We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-

CoV cross neutralization, synthetically reconstruct a small subset (1-2), recover full length viruses and evaluate the ability of nucleoside analogues to inhibit growth in HAE cultures and/or *in vivo* (*63, 132*). For the Collaborative Cross model, we will infect six to eight mice from 6-8 Collaborative Cross mouse lines (10 weeks of age) with six test viruses/yr, intransasally or by subcutaneous infection with 1 x 10e4 virus. Animals will be followed for weight loss, morbidity, mortality and other clinical disease symptoms (e.g., hunching, hindleg paralysis). One half the animals will be sacrificed at day 3 and day 7 pi. to evaluate virus growth in various organs, and organ pathology. In highly vulnerable lines, additional experiments will be performed using more animals, evaluating virus growth, clinical disease symptoms, respiratory pathology, tropism and organ pathology through day 28 pi. We anticipate that some CC lines will prove highly vulnerable to select wildtype viruses, because of host susceptibility combinations that promote severe disease outcomes.

1.6. Potential problems/alternative approaches: Challenges with logistics or obtaining permission for wildlife sampling in sites we select. We have a >20-year track record of successful field work in Malaysia, and >10 years in Thailand, and have strong established partnerships with local wildlife authorities and communities to ensure access. We have existing permissions in place which will be renewed at the start of this project. Due to the abundance of wildlife habitats in the region, if access to a site is withdrawn, we will rapidly identify a suitable, complementary site with similar bat species composition and abundance. Finally, we have already collected tens of thousands of wildlife samples from the region and will focus on these while waiting for permission. **We may not identify novel viruses in our sample wildlife species due to seasonality of viral shedding or other factors.** We have calculated sample sizes based on realistic detection rates from our extensive preliminary data, and are confident that we will detect and identify large numbers of potentially zoonotic pathogens, particularly for CoVs and PMVs. We acknowledge that FV strain diversity may be harder to capture, given relatively low seroprevalence in bat populations – suggesting lower levels of viral circulation (*42, 43*). However, our team was the first and only group to sequence FV RNA from Asian wildlife species (*46, 47, 57*), and we are confident that with our targeted sampling and testing strategy offers the best chance globally to identify additional strains of Ebola and related viruses. The sampling regions we have selected are subtropical, and our previous data, and even published studies in temperate regions (*162*), do not suggest a strong pattern of seasonality in CoV shedding, and where seasonal patterns do occur, i.e. for henipaviruses in Thailand (*163*), or can be predicted from wildlife serology data, we will be sure to conduct sampling at different timepoints throughout the year to account for this.

Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays.

2.1 Rationale/Innovation: Rapid response requires early detection. Assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities that interface frequently with wildlife that harbor high risk emerging viral strains. To enhance the low statistical probability of identifying these rare events, In Aim 2 we will target rural human communities inhabiting regions identified in Aim 1 as having high viral diversity in wildlife, that also engage in practices that increase the risk of spillover (e.g. hunting and butchering wildlife). We will initially identify 4 subsites in each of Thailand, Peninsular Malaysia, Sarawak, and Sabah for sampling. We will design and deploy human risk factor questionnaires, and specific and sensitive serological assays to identify the baseline frequency, and causes of, spillover of known and related novel viral pathogens in these populations. **We will use the results to test a key hypothesis: that hunting wildlife increases the risk of exposure to novel viruses, compared to the rest of the community.** The alternative hypothesis is that seroprevalence in the general community is not statistically different to those who hunt and butcher wildlife, because exposure to wildlife pathogens is largely through inhabiting a biodiverse landscape where wildlife make indirect contact with people (e.g. via fomites). For participants who report symptoms related to 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever; or 6) unusual presentation of high fever with severe diarrhea; all within the last 10 days, an additional sample type will be collected, two nasal or oropharyngeal swabs. Samples will be marked for additional PCR-based assays to identify presence of known and novel CoVs, henipaviruses and FVs, and for isolation and biological characterization of potential pathogens, using the approach laid out in Aim 1.

2.2 Preliminary data on high-zoonotic risk human community surveillance: 2.2.a Biological sample collection in SE Asia: Our long-term collaboration in the region has included qualitative and quantitative survey data, and collecting large archives of banked human sera and other biological samples from high zoonotic-risk populations. For example, under the USAID-PREDICT project, EHA has collected 1,400 and 678 specimens in Malaysia and Thailand respectively from people living in zoonotic disease interfaces, all of which will be available for retrospective serological testing by EID-SEARCH which we have ensured is compliant with our existing IRB. Our core group has collected and tested many thousand additional specimens from other important community cohorts **(Table 2)**, and some of these will continue under EID-SEARCH **(Section 2.4)**.

Country, site	Lead	# enrolled, focus	Findings
Peninsular Malaysia	Hughes	9,800+ samples, Orang Asli indigenous pop., for PCR/serol.	25+ novel CoVs, influenza, Nipah ab+ve, FV ab+ve
Malaysia Sabah	Kamruddin	1,283 for serology	40% JE, 5% ZIKV ab+ve
Malaysia Sabah	William	10,800 for zoonotic malaria study	High burden of macaque-origin malaria
Malaysia Sabah	Hughes	150 bat cave workers	Ongoing
Malaysia Sarawak	Siang	500 Bidayuh and Iban people	8% PCR prevalence HPV in women
Malaysia PREDICT	Hughes	1,400 high zoonotic-risk communities for viral PCR, serology	Ongoing. Multiple known and novel CoVs, PMVs, others.
Thailand	Wacharaplu-esadee	100s of bat guano harvesters/villagers	Novel HKU1-CoV assoc. clinical findings
Thailand PREDICT	Wacharaplu-esadee	678 high zoonotic-risk communities for viral PCR, serology	Ongoing. Multiple known and novel CoVs, PMVs, others.
Singapore	Wang	856, for Melaka virus	7-11% MELV ab+ve

Table 2: Biological sample collection from healthy populations conducted by members of **EID-SEARCH** in our hub countries.

Of particular interest is the access to indigenous communities in Peninsular Malaysia (Orang Asli) and Sarawak (Bidayuh, Iban people), and the bat guano harvesters in Thailand. These communities hunt, butcher and eat wildlife daily, and work in supremely high-risk zoonotic interfaces (e.g. bat caves). One of our indigenous community sites is experiencing an ongoing outbreak of unknown etiology that we are investigating currently **(Section 3.2.a)**.

2.2.b Human Contact Risk Factors: EHA is the lead organization across all 27 USAID-PREDICT project countries for collecting data on, and analyzing the human aspects of, zoonotic spillover risk (*164, 165*). We conducted exploratory studies using standardized one-on-one semi-structured interviews and site observation data with people living in hotspot areas or engaged in high-zoonotic spillover risk activities, using these data to assess local social and cultural norms underlying wildlife contact (*165*). We then developed a risk factor survey to accompany human biological sampling to evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. We conducted cross-sectional studies in high risk populations in 27 countries, and study participants provided biological samples (oral/oropharyngeal swab, nasal/nasopharyngeal swab, urine sample/urogenital swab, fecal sample/rectal swab, whole blood, serum, plasma). For example, survey and biological samples were collected from: 1,585 participants from 7 sites in Yunnan, Guangxi, and Guangdong provinces, China; 1,400 participants from 4 sites in Malaysia; and 678 participants from 4 sites in Thailand. Samples were tested with novel serological assays and results analyzed against risk factors from survey data (*16*). In China, CoV-seropositive individuals were mostly farmers/peasants (8/9), living in Yunnan province (7/9), 41-60 yrs old (7/9), with domestic contact with rodents (6/9), that are male (6/9). Although these results are preliminary and don't provide detailed information on routes of exposure, they identify key subpopulations to target that would likely increase the opportunity for identifying evidence of spillover for other CoVs, PMVs and FVs. Data from PREDICT surveys have been used to help identify target populations in Aim 2, identify strategies to better target at-risk people, and conduct focused **human risk factor surveys and serosurveys to produce statistically significant findings for henipaviruses, CoVs and FVs.** In Aim 2, we will use combined biological sampling and risk factor surveillance in targeted populations within the community to 1) test the hypothesis that occupational (i.e. wildlife hunting) risk factors correlate with seropositivity to henipaviruses, FVs and CoVs; 2) assess possible health effects of infection in people; and 3) where recent

illness is reported, obtain biological samples for PCR testing and potential viral isolation and characterization. Obtaining this information could be a significant step in understanding the likelihood of recent 'hidden' spillover events and their public health impacts, as well as the risk of future emergence of viral disease.

2.2.c. Risk factors for illness: Our preliminary data in Thailand, Malaysia and other SE Asian countries revealed that frequent contact with wild (e.g. bats, rodents, NHPs) and domestic animals (e.g. poultry, swine) among local communities was associated with a high percentage of self-reported severe/acute respiratory illness (SARI/ARI) and Influenza-like illness (ILI) symptoms **(Section 3.2.b)**. In addition, people involved in crop production, hunting and butchering of wildlife reported statistically significantly higher SARI and ILI symptoms (Li *et al.*, in prep.). **These preliminary data suggest a target population and sites for current proposal with the correct targets for serological surveillance and risk factor surveys in Aim 2 to produce statistically significant findings**. Coupled with better serological tools from our team **(Section 2.2.d)**, we will be able to identify the likely routes of exposure to known or novel coronaviruses, henipaviruses, and FVs in the study region, as well as build data on the illnesses they cause in people.

2.2.d. Serological platform development: Most emerging viruses produced a short-lived viremia in people so that large samples sizes are required to provide enough PCR-positive individuals to analyze infection patterns. For Aim 2, we will focus on serological sampling in the community, because antibodies are typically long-lived and can be used to assess prior virus exposure or infection with much smaller samples sizes (*123*). Most serological assays target a single protein, and for emerging viruses, it's often unclear which viral antigen will be immunodominant during human infection, and therefore which to target in developing serological assays. Co-Is Wang, Anderson (Duke-NUS) and Broder, Laing (USUS) have developed a series of approaches to deal with this problem, and which will be used in testing human biological samples in Aims 2 and 3. Henipaviruses, FV and CoVs express envelope receptor-binding proteins that are the target of protective antibody responses. Co-I Broder has led efforts to express and purify henipavirus native-like virus surface proteins for immunoassay application (*166, 167*), developing monoclonal antibodies (*168, 169*) and as subunit vaccines (*170, 171*), has developed a novel, multiplex microsphere immunoassay (MMIA) allowing serum samples to be simultaneously tested for antibodies reactive with henipaviruses, FVs and CoVs. This MMIA uses a Luminex-based multiplex platform and custom designed virus receptor-binding proteins expressed in a mammalian cell-culture system and purified as native-like oligomeric proteins. These oligomeric antigens retain post-translational modifications and quaternary structures, allowing capture of conformational-dependent antibodies raised during a natural infection, which represent the majority of the protective humoral response. The known diversity of henipaviruses includes five presently described viruses, NiV, HeV, CedV, GhV and MojV and this assay has been expanded to include all described species **(Fig. 11)**. In the past, our collaborators have used NiV and HeV G protein antigens in a MMIA assay to identify a serological footprint of NiV-related African henipaviruses and exposure to virus(es) antigenically-related to NiV in livestock sampled in Bangladesh (*78, 79*). To strengthen serological data interpretation, the G protein from each virus is integrated into the MMIA so that virus-specific vs. henipavirus cross-reactive antibodies can be detected and address specificity of past virus exposure. Extensive cross-reactivity exists among ebolaviruses and between ebolaviruses and marburgviruses (*172-174*). We are validating the MMIA pan-FV assay for specificity, and as part of this, in collaboration with Duke-NUS, we have found serological evidence of past exposure to a filovirus(es) most antigenically-related to ebolaviruses (e.g. Ebola virus, Bundibugyo virus), but unreactive with Reston virus GP in three under-sampled fruit bat species, suggesting that novel FVs circulate within bat hosts in SE Asia (*43*). **This platform has now been set up for use in Malaysia and Thailand and will be available for use in this proposed work.** Co-Is Hughes, Broder, Laing have conducted initial screening of human, wildlife and livestock samples at high-risk interfaces in Malaysia, finding serological evidence of past exposure to viruses antigenically-related to NiV and EBOV in humans, bats and non-human primates (NHPs).

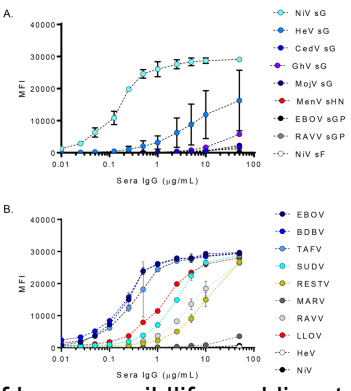


Fig. 11: Validation of multiplex microsphere immunoassay (MMIA) specificity and identification of immunologically cross-reactive viruses for A) NiV B) EBOV.

Co-Is Wang and Anderson (Duke-NUS) have developed a phage-display method to screen antibodies for all known and related bat-borne viruses, including henipaviruses, FVs and CoVs and have set up a similar Luminex-based assay. Additionally the Duke-NUS team has demonstrated capacity for rapid and cost-effective development of LIPS serological assays against novel viral agents **(Sections 2.6.a, 3.2.a)**. These will be used for verification and expanded surveillance. For molecular investigation (Aim 2 & 3), we will combine targeted PCR with a discovery-driven enrichment NGS strategy to not only survey currently known viruses, but also to discover potential novel viruses. Both the phage-display serological and enrichment platforms can be rapidly updated to include new viruses identified.

2.3 General Approach/Innovation: Our human sampling approach uses two approaches in Aims 1 & 2 (Fig. 12). In Aim 2 we will conduct community-based surveillance using a brief, focused questionnaire to identify the prevalence and frequency of risk-factors for viral spillover in tandem with collection of high quality biological samples to determine the seroprevalence of spillover viruses in at-risk human populations. We will use generalized linear models to analyze correlation between biological results and risk-factors for viral spillover. **In Aim 3**, we will conduct clinic-based syndromic surveillance and biological sampling at clinics/hospitals that are the catchment healthcare facilities for the communities sampled in Aim 2. Both community-based and clinic-based syndromic surveillance programs are cross sectional case-control studies designed with the sample sizes necessary to statistically quantify (with a power of 80%) risk factors and health impacts for viral spillover, linked to serological status and PCR prevalence in symptomatic patients. All human sampling conducted under Aims 2 and 3 will adhere to strict criteria for inclusion and recruitment and protection of human subjects from research risk **(see Human Subjects and Clinical Trials Information)**.

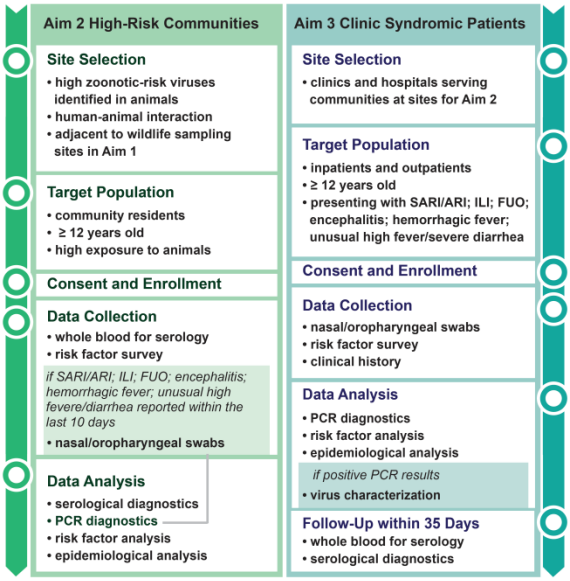


Fig. 12: Human sampling approach. Aim 2 **(left)** focuses on serology in high-risk communities to identify evidence of population exposure to novel zoonotic viruses. Aim 3 **(right)** sets up clinical cohorts at hospitals serving these high-risk communities to conduct syndromic surveillance and identify the etiology of new viral syndromes

2.4 Target population & sample sizes: We will target populations in the same geographic regions as those identified in Aim 1 that harbor wildlife with high potential zoonotic viral diversity, that are EID hotspots, and that are well-connected through regional travel and trade hubs to the global travel network **(Fig 13)**. Within each geographic region we will identify populations at four sub-sites, building on our preliminary data **(Table 2)**. We will target specific communities based on our analysis of previously collected behavioral questionnaire data, and other publicly available data (e.g. wildlife hunting licenses sold in a region, number of guano caves accessed for fertilizer etc.) to identify key at-risk populations and occupations with high exposure to wildlife. We will conduct concurrent sampling trips at visits to sub-sites during which members of the community will be enrolled and wildlife surveillance activities conducted. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock, or slaughtering wildlife will be considered for enrollment. Participants will complete a consent form and questionnaire in addition to having biological samples collected. During these community trips after data collection the field research staff will conduct community meetings to help inform the public health risk of zoonoses. **Target populations:** *Thailand (Co-I Wacharapluesadee):* 100 subjects of healthy high-risk community from 2 study sites, Chonburi (NiV, other PMVs and CoVs found by PCR in bats in our previous work), and Ratchaburi (bat guano harvester villages where we found a MERSr-CoV in bat guano (*50, 175*) and serological evidence of α-CoVs, HKU1-CoV in people (*175*). *Peninsular Malaysia (Co-I Hughes, CM Ltd.):* We will expand our sampling of the Orang Asli indigenous communities to include sub-sites in communities in Districts we have already sampled and

additional Districts in the States of Kelantan Perak, Pahang, and Kelantan all close to bat caves. Samples will be stored and tested at NPHL. *Sarawak (Co-I Tan, Fac. Med. Hlth. Sci. UNIMAS):* We will expand our sampling of the Bidayuh and Iban indigenous people to include the Dayak and Orang Ulu ("people of the interior") because this group has a more intimate connection to remote wildlife-rich areas than the other two, albeit all are hunters. Human sampling will be led by UniMAS in collaboration with CM Ltd / EHA. *Sabah:* We will conduct sampling at the Madai bat and bird cave that has 200 permanent residents who harvest bird's nests for soup, and >1,000 during the harvesting season and at Gomantong cave – one of the largest bat and cave swiftlet nesting sites in the world. *Sabah: (Co-I Hughes):* We will conduct sampling at the Madai bat and bird cave that has 200 permanent residents who harvest bird's nests for soup, and >1,000 during the harvesting season and at Gomantong cave – one of the largest bat and cave swiftlet nesting sites in the world. Protocols follow those for Peninsular Malaysia *Singapore: (Co-I Wang, Duke-NUS):* Active human surveillance will be not be conducted in Singapore, but archived human specimens e.g. from our Melaka virus serosurvey (*8*), will be made available to conduct retrospective serosurveys for specific pathogens that are identified as high interest to the EIDRC, NIH, and EID-CC.

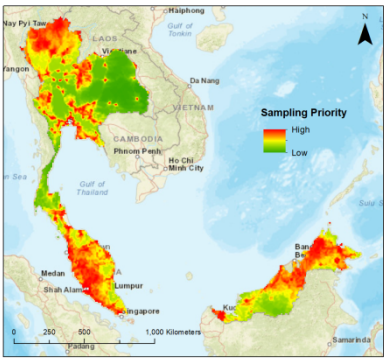


Fig. 13: Targeting high-risk sites for human zoonotic disease surveillance in Thailand and Malaysia. This map plots areas with high numbers of expected viruses in wildlife (*3*), greater spillover probability based on EID risk factors (*2*), and higher levels of connectivity (measured by road density data) that may facilitate person-person spread.

Sample sizes: From our previous work we conservatively anticipate that 10-30% of the community population will have had exposure to wildlife allowing us to capture highly exposed and non-exposure individuals at each site. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock farming, transportation, selling, or slaughtering wildlife in the surveyed areas will be targeted so that they make up ≥30% of the sampled population in each community. We will stratify sampling to ensure appropriate representation of sex, demographic, and socio-economic factors at each community site. In each of our regions, we will conduct sampling at four sub-sites in populations with high prevalence of high occupational and environmental risk of exposure to wildlife populations, sampling 175 persons at each sub-site. We will target populations with, on average, 30% or higher prevalence of occupational or environmental exposures to wildlife based on data from previous studies. This design will give us >80% power to detect 5 percentage point differences in seroprevalence against any of our viral groups between baseline and high-risk subgroups. We conservatively assume 5% base seroprevalence, as well as assuming high spatio-temporal variance amongst sub-sites, with baseline varying 0-25% amongst sub-sties and the fraction of the population at risk varying from 20-40%. (Power calculation conducted by 500 simulations of a generalized linear mixed model (GLMM)). For community-based surveillance, we will enroll 700 individuals per study region, allowing us to make county/province-level comparisons of differing effects; the total sample will be 3,500 participants over 5 years.

2.5 Data & sample collection: Following enrollment, biological specimens (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max 500 µL of whole blood and two 500 µL serum samples) will be collected by locally certified healthcare professional proficient in phlebotomy techniques and a short questionnaire will be administered by trained project staff that speak appropriate local language to all participants. Through the serological findings and the survey we will investigate the details of five risk factors related to high risk wildlife exposure, to maximize the power of the analyses, based on continuing analysis of our previous work, and will focus on: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. For participants who report the symptoms relating to 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever within the last 10 days, an additional sample type will be collected, nasal or oropharyngeal swab (2x). Blood of participants whose serum tests positive for emerging viral pathogens will be taken and Peripheral Blood Mononuclear Cells (PBMCs) frozen for future use.

These will be used for future harvesting of polyclonal and monoclonal antibodies as potential therapeutics/ diagnostics **(see Letter of Support NEIDL)**.

2.6: Laboratory analysis: 2.6.a Serological testing: We will screen all human specimens for henipaviruses, FVs and CoVs using our novel, multiplex microsphere immunoassay (MMIA) **(Section 2.2.d)**. We will use ELISA and serum neutralization tests (SNT) as confirmatory, where feasible and appropriate for the biocontainment level given sensitivity and specificity variation, and the need for live virus for SNTs **(See Select Agent Research)**. Serologic evidence of local spillover will be used to prioritize zoonotic strains for recombinant virus recovery from full length sequences. Preliminary data suggest ELISA will be effective for some viral groups. We previously developed a SARSr-CoV specific ELISA for serosurveillance using the purified NP of a bat SARSr-CoV (Rp3). The specificity of this assay was evaluated using polyclonal antibodies against HKU1, OC43, 229E, NL63, MERS-CoV and EBOV and no significant cross-activity detected (*16*). **This suggests that if we can expand our NP serology tests to cover other bat CoVs, and that doing so may identify many more seropositive individuals.** For CoVs, we will therefore use two serological testing approaches: 1) testing human sera collected from both community- and clinic- based sampling for a panel of bat CoVs that will include SARS-CoV, MERS-CoV, SADS-CoV and a range of bat SARSr-CoVs (*16*); 2) testing for antibodies to common human CoVs (HCoV NL63, OC43 – **Section 2.8**). We will test panels of NP and G recombinant proteins to assess if this approach will act as a comparison to MMIA for FVs and PMVs.

2.6.b RT-PCR testing. Specimens from individuals in the community who reported being symptomatic within the last 10 days **(Section 2.5)** will be screened using RT-PCR initially for CoVs, PMVs, and FVs following published protocols **(Section 1.4.c)**. Positive samples will be subjected to full genome sequencing and RT-PCR amplification of the glycoprotein genes. Samples from the clinic-based syndromic surveillance will also be tested using RT-PCR for Influenza A & B, HCoV NL63, OC43, HKU1, SARS-CoV & 229E, NiV, HeV, Ebola (WHO PCR protocol) based on the symptom as rule-outs.

2.6.c Biological characterization of viruses identified: Novel viruses identified in humans will be recovered by cultivation or viruses re-derived from full length genome sequences using reverse genetic approaches. Viruses will be characterized for growth in primary human cell lines and in mouse models, including the Collaborative Cross Mice or humanized mice expressing human receptors from related strains. The approaches are discussed in more detail under Aim 1.

2.7 Epidemiological analysis: We will conduct a cross sectional case-control study to identify risk factors for Henipavirus, FVs, and CoVs spillover. “Cases” are defined as participants whose samples tested positive for Henipavirus, FVs, or CoVs by serological tests. “Controls” will be selected from the pool of participants admitted to the studies and whose samples tested negative for Henipavirus, FVs, or CoVs. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models and least absolute shrinkage and selection operator (LASSO) regression analyses (*176*) to analyze correlation between biological results, serological/PCR status, and risk factors related to high risk wildlife exposure including: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) home or work proximity to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms lifetime and past 12mo. Additionally, we will use this procedure to determine how clinical presentation differs between Henipavirus, FVs, or CoVs in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms.

2.8 Potential problems/alternative approaches: Rarity of spillover events means it may be difficult to identify sufficient seropositives to statistically analyze human risk profiles. First, we will be targeting our community-based surveillance to subpopulations with high-levels of wildlife exposure, at sites selected for diverse and prevalent viruses to increase likelihood of finding positive individuals. Second, our serology testing will include a multi-plex panel of assays for a large diversity of viruses, increasing likelihood of positives. For example, in previous work our overall bat CoV PCR prevalence in the community was, 11.8%; β-CoV, 3.4%; α-CoV, 9.1%). Thus, using this broad serological panel to screen individuals prone to contact with species of higher zoonotic risk increases the potential for detecting spillover with enough power for statistical analyses,

and will shed light on behaviors that predispose to spillover. Third, we will include common human CoVs, PMVs, and influenza viruses in our panel, so that even if low prevalence of wildlife viruses is found, we will be able to conduct a valuable cross-sectional study of the seroprevalence of more common human viruses. Finally, we will be able to assess relative measures of human-wildlife contact from our survey work. We will analyze intensity of contact with high-risk taxa against other risk factors to provide useful proxy information for spillover risk. **Serological testing may be difficult to interpret in the presence of known and novel viruses.** We will use the three-tiered serological testing system outline in **2.6.a** to try to identify exposure to these 'novel' viruses. However, we know exposure to a range of related viruses generates antisera that are cross-reactive with known, related virus antigens - a serious challenge to serological surveillance and diagnostics. Our collaborations have already demonstrated that SE Asia bat sera samples screened with our pan-filovirus MMIA have been exposed to an Ebola-like virus, that is antigenically-distinct from known Asiatic filoviruses, Reston virus and Měnglà virus (*43*). Using these three serological platforms we will address inherent challenges of indirect virus surveillance through antibody capture by testing multiple binding assay platforms and antigens, using positive control samples to establish serological profiles against known viruses that will aid in our interpretations of cross-reactive antisera responses likely representative of 'novel' viruses.

Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts.

3.1 Rationale/Innovation: Our previous research **(Table 1)** and other data suggests that there are substantial numbers of outbreaks or clinical cases for which the etiologic agent is not diagnosed, e.g. (*177, 178*). To investigate this in SE. Asia, we have assembled a multi-disciplinary team that includes senior infectious disease doctors and clinicians and will work directly with local clinics and regional hospitals to identify the 'cryptic' outbreaks or cases that occur in the region. **In Aim 2, we will conduct serology in high zoonotic-risk communities to find evidence of prior exposure in people,** i.e. evidence of viral spillover into the community. In Aim 3 we identify members of these communities presenting at clinic with undiagnosed illness that may have high-impact zoonotic viral etiology. **Therefore, in Aim 3 we focus on PCR to identify the putative viruses that are replicating in these actively-infected patients (Fig. 12).** We will enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and who present at regional clinics or hospitals with symptoms typical of high impact zoonotic viruses. We will collect detailed survey data to assess their contact type and frequency with wildlife and livestock and conduct molecular and serological diagnostic assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. We will conduct molecular viral discovery assays on patients who present with symptoms indicative of a high-risk zoonotic virus. If novel pathogens are identified by PCR, we will attempt to isolate, obtain full genome sequence and/or biologically characterize the wildtype or molecularly re-derived pathogen, using the *in vitro* and *in vivo* strategy laid out in Aim 1.

3.2 Preliminary data clinical surveillance: 3.2.a. Clinical surveys and outbreak investigations: Our longterm collaboration in the region has included the following activities investigating clinical syndromes: *Peninsular Malaysia*: **At the time of writing, an outbreak of suspected undiagnosed viral illness in the indigenous Batek Orang Asli ("people of the forest") community living at Gua Musang ("civet cat cave") district, in Kelantan, Malaysia, close to Taman Negara – a National Park containing the oldest known rainforest**. This community is one where we have conducted prior routine surveillance and biological sampling, demonstrating proof-of-concept that our two-pronged high-risk community/clinic approach will work. Dozens of people are affected and the outbreak is currently being investigated by our group in collaboration with the Malaysian NPHL. The Orang Asli continue to practice traditional subsistence hunting of wild animals (bats, rodents, primates). These communities are remotely located, in heavily forested areas, with limited access to medical services. This outbreak, and others that will continue to emerge just like it, underscore the critical need for our EIDRC - to improve the technological tools, training, and human capacity for rapid EID surveillance, diagnosis, and response at the frontlines of disease emergence in SE Asia. **Investigating this outbreak is a key priority if EID-SEARCH is funded.** *Sabah*: Co-I Kamruddin (BMHRC) is leading an asymptomatic leptospirosis study on sanitation workers, an ILI study in a Kota Kinabalu clinic and diarrheal study in children under 5 yrs. Co-Is William, Tan and others have conducted clinical observational studies to determine the etiology of central nervous system infections and acute undifferentiated febrile illness in Sabah.

Co-Is William and consultant Yeo have collaborated with Co-I Wang to enroll 90 adult and over 100 pediatric participants with central nervous system infections, negative tests for malaria and dengue from 3 district hospitals in Sabah to determine the various etiologies. While a proportion tested positive for rickettsial agents, the majority had no clear diagnosis. Co-Is William, Rajahram, and Yeo have conducted clinical studies of over 200 inpatients with acute febrile illness with negative tests for malaria and dengue from 3 district hospitals in Sabah to determine the various etiologies. While a high proportion tested positive for rickettsial agents, the majority had no clear diagnosis. *Sarawak:* Co-I Siang (UNIMAS) is conducting a survey of high-risk female Kelabit indigenous people and has found 8% with HPV52 as the dominant strain. He is working on clinical cohorts of hand, foot and mouth disease and will recruit patients from that study in the current proposed work. Finally, Co-Is Siang, Kamruddin are conducting a long-term study of rotavirus and other viral etiologies of acute gastroenteritis in Kuching, Sarawak that will include swine herders and farmers. The Baric lab is a global leader in norovirus VLP diagnostic reagents to detect serologic evidence of norovirus gastroenteritis, as well as RT-PCR detection of novel noroviruses in humans, and has identified novel bat noroviruses, suggesting further opportunities for expansion of this work (*142, 179-181*). This work is particularly relevant in Malaysia because pig farms are shunned by the government and local villagers to they are placed far away from town centers, deep in the forest, and it is this scenario that led to the emergence of Nipah virus in 1998-9 (*6, 22*). *Thailand:* Co-Is Hemachudha, Wacharapluesedee (TRC-EID, Chulalongkorn Univ. Hosp.) work in coordination with the Thailand Ministry of Health to investigate outbreak etiology, including the first case of imported MERS-CoV in Thailand, **for which we produced sequence confirmation within 24 hours from acquiring the specimen**. Three imported MERS cases have been detected in Thailand since 2015, and all cases were confirmed at the TRC-EID. We worked during the Zika outbreak to identify > 500 PCR-positive Zika patients with sequence confirmation. We have also worked on returning and suspected Ebola travelers, syndromic surveillance for SARI, encephalitis, Hand Foot and Mouth disease in children and viral diarrhea. Consultant Hickey (US CDC, Thailand), screened specimens collected between 1998 and 2002 from a large cohort of children from Kamphaeng Phet Province to examine Dengue virus and JE epidemiology (*182, 183*). Among the 784/2,574 convalescent sera henipavirus-specific antibodies were detected in 13 serum samples (1.7% seroprevalence), suggesting prior exposure to endemic henipaviruses or a related PMV in rural Thailand. *Singapore:* Duke-NUS has worked with the Ministry of Health to investigate Zika cases (*184*), and other EIDs. With EHA and others, Duke-NUS has developed novel CoV assays for clinical surveillance, and for expedited analysis during outbreaks. Our pipeline of novel virus detection, reservoir host identification, serological assay development, and serological testing is as rapid as several months, with the development of novel serological assays from sequence data in 7 days (*17*). **For example, with the discovery of SADS-CoV, we identified and fully sequenced a new virus, screened archived wildlife specimens to identify the natural reservoir host species (*Rhinolophus* spp. bats), developed LIPS serological assays, and tested human and swine samples to assess seroprevelance in less than 3 months (*17*).**

3.2.b Analysis of self-reported illness: We have developed a standardized approach for analyzing data from study participants on self-reported symptoms related to target illnesses: fever with cough and shortness of breath or difficulty breathing (severe acute respiratory illness - SARI); fever with muscle aches (fevers of unknown origin (FUO); and, cough or sore throat (influenza-like illness - ILI). For all of our prior human clinical surveillance in 20+ countries, including in Malaysia and Thailand, we used a least absolute shrinkage and selection operator (LASSO) regression to identify associations between participants who reported ILI and/or SARI symptoms and demographics, occupation, and contact with animals in the last year. Results have clear biological relevance. For example, in Yunnan, China, factors strongly associated with prior disease involve animal hunting and consumption **(Fig. 14).** We will expand this approach for all clinical syndromes in Aim 3, and use targeted questions to assess patient's exposure to wildlife in terms that are relevant to each specific country.

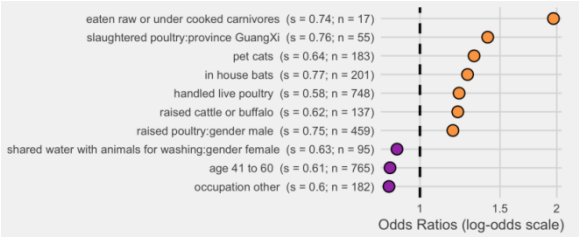


Fig. 14: Factors associated with self-reported ILI & SARI in prior 12 months (s = bootstrap support; n = #+ve out of 1,585 respondents). **Orange circles** = odds ratios > 1 (+ve association); **purple** = odds ratios <1 (-ve association).

3.3 General Approach: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent has not been identified. To capture these, we will expand on our current syndromic surveillance to enroll, and collect high quality biological samples from patients who present at regional clinics and live in rural communities linked to Aim 2. Case definitions and enrollment criteria include: 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever; or 6) high fever/diarrhea with unusual presentation. We will collect survey data to assess contact with wildlife, and occupational and environmental exposures, conduct molecular and serological assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. If novel pathogens are identified by PCR, we will attempt to isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work. We will then use behavioral survey data, ecological and phylogeographic analyses to assess the likely reservoir hosts to inform intervention programs.

3.4 Clinical cohorts. 3.4.a Cohort selection: We will initiate active clinic-based syndromic surveillance at 2 town-level level clinics and 2 provincial-level hospitals in each Thailand, Peninsular Malaysia, Sabah and Sarawak. These serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2 and are inclusive of wildlife sampling sites in Aim 1. Patients ≥ 12 years old presenting at the health facility who meet the syndromic and clinical case definitions above will be recruited into the study after completing a consent form. We will aim to enroll 300 participants per hospital, with that we will have a 95% probability of detecting live virus in patients in each hospital assuming a population prevalence of 1%. This sampling effort will total of 4,800 individuals for clinical syndromic surveillance. We assume up to 40% loss from follow-up, therefore yielding 2880 participants that will be available for follow-up blood sampling **(Section 3.4.b)**. While enrollment is limited by the number of patients that come to the healthcare facility presenting with relevant clinical symptoms, in our work in the PREDICT project we enrolled an average of 100 participants per year, so we are confident this is an achievable enrollment target. Study data will be pooled across country regions, as clinical patients are limited by the number of individuals presenting at hospitals. "Cases" will be determined as participants whose samples tested positive for either CoVs, henipavirus, or FVs, PCR tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. Sites: *Thailand:* We will work with two primary hospital, Phanat Nikhom Hosp., Chonburi district (Key Pers. Dr. P. Hemachudha) & Photharam Hosp., Ratchaburi Province, and a large tertiary hospital, King Chulalongkorn Memorial Hospital, Bangkok Thailand (Co-I Dr. T. Hemachudha). *Peninsular Malaysia:* **Focusing immediately on the current outbreak in Orang Asli, we will work closely with the Malaysian Ministry of Health to support their investigation, and with 6 Orang Asli clinics which focus solely on this indigenous community.** Co-I Sellaran (Lintang Clinic, Kuala Kangsar) refers patients to Sungai Siput Hospital and Gen. Hospital, Ipoh. Key Pers. I Lotfi (Pos Betau clinic, Kuala Lipis) will continue collaboration with Co-I Hughes in the communities at that site. *Sarawak:* Key Pers. Diyana (Director, Bario Clinic) refers patients to Miri Hospital (Co-I Utap) and serves people from the Kelabit, and nomadic tribes in the region, as well as populations within a number of large towns. *Sabah:* We will continue our collaboration with Queen Elizabeth Hospital, QEH (Key Pers. Lee, Rajahram) and Hospital University Malaysia Sabah, HUMS (Co-I Lasimbang, Director). Both clinics include our targeted sites for **Aim 2** in their catchment, and both have ongoing collaboration with the Borneo Medical Health Ctr (Co-I Kamruddin, Director). *Singapore:* Currently, there are no plans to conduct clinic surveillance based on budget constraints. However, in the event that that is an evolving need for clinic surveillance, enrollment, and sampling, we have close working relationships with all hospitals in the country, and the central infectious disease reference lab (key clinicians at which are aware of our proposal). This would allow us to rapidly on-board a hospital based site.

3.4.b Clinic enrollment and follow-up: We will recruit inpatients and outpatients to participate in the study after initial screening to determine if they meet our clinical case definitions. Once consented and enrolled, biological samples will be collected by trained and locally certified hospital staff and a questionnaire administered by trained hospital or research staff that speak appropriate local language. Every effort will be made to take samples concurrently when collecting samples for normative diagnostics. For both inpatients and outpatients, samples will be collected no more than 10 days of reported onset of illness to increase the chance

of PCR detection of viruses (*185*). Where possible, we will follow up 35 days after enrollment to collect an additional two 500 µL serum samples conduct a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. This gives adequate time for development of IgG, which occurs <28 days after onset of symptoms for SARS patients (*186-188*). Serum samples will be used to screen panels of high risk human and local zoonotic Filovirus, Henipavirus and Coronavirus strains as described in Aim 2. These samples may prove valuable to collaborators in that the PBMC could be used in production of therapeutic antibodies.

3.4.c Sampling and clinical interview: Biological specimens whole blood (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples) and two nasal or oropharyngeal swabs will be collected from all participants, and a questionnaire will be administered. We will investigate five risk factors, to maximize the power of the analyses, all related to high risk wildlife exposure, based on continuing analysis of our previous work, and will include: 11) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With additional consent considerations for participants enrolled from clinical settings, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology. Blood will be taken for future harvesting of Peripheral Blood Mononuclear Cells (PBMCs), as per **Section 2.5**.

3.5 Sample testing: The standard syndromic diagnostic PCR assays for common pathogens will be conducted and the results will be shared with the hospital with 48 hours. Specimens will be further tested for 3 viral families; CoV, PMV and filovirus by consensus PCR **(Section 1.4.c)**. Necropsy samples from deceased patients will be further characterized by NGS if previous PCRs are negative and unable to identify the cause of infection. Co-Is Wang, Anderson will conduct a VirScan serological assay using paired serum samples at Duke-NUS that identifies antibodies with a four-fold rise in titer in the convalescent sample compared with the acute sample, thereby indicating a possible etiological agent. A PCR test with the specific primer from each virus antibody positive case will be further tested from acute specimens to confirm infection.

3.6 Viral risk characterization and potential for pandemic spread: We will use statistical models built from collated biological, ecological, and genetic data to assess the likelihood of pathogenicity for the range of diverse viruses we will characterize. The aim is to quickly identify and assess which viruses pose the most significant risk to human health and thus prioritize additional resources to more fully characterize, better understand pathogenicity, and invest in our animal model pipeline and ultimately therapeutic development for those pathogens. We will combine two independent but related statistical models to predict pathogen spillover risk and pathogenicity. First, we will expand a simple generalized additive modeling approach (*3*) to assess spillover risk probability to a new virus that is currently not known to infect people, when we have only limited genetic data from initial PCR screening and sequencing. Additional data from receptor binding protein homology and cell line infection experiments will be incorporated into the model framework as available. Second, given high likelihood to spillover and infect humans, or direct evidence of human infection from our clinical surveillance, we will then assess the likelihood of pathogenicity based on traits for phylogenetically related nearest neighbor viruses as a first approximation and incorporating more detailed data from genomic characterization and animal model experiments (Aim 1). Epidemiological trait data for ~300 viral species known to infect people (e.g. case fatality rate, global estimates of number of human cases per year or decade, R_0, infectious period, and primary symptoms) have been collated by recent studies and will be used as predictor variables (*189*). Thirdly, we will apply tools already developed, and being refined by EHA under DTRA and DHS supported research, to predict pandemic spread for viruses using global flight models, as well as additional datasets on human movement and connectivity across Southeast Asia (*90, 91*) **(Fig. 15)**.

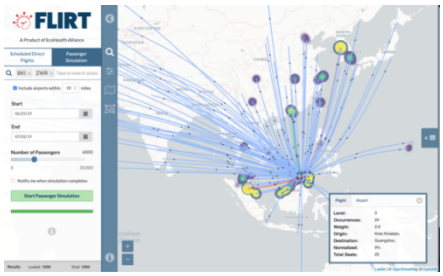


Fig. 15: Probability feed from EHA's Flight Risk Tracker tool (FLIRT). This image is of the likely pathways of spread and establishment for a Nipah-like virus emerging from Sabah, based on human movement and airline flight data (*90, 91*).

3.7 Potential problems/alternative approaches: Patients visiting clinics may have cleared virus, but not yet developed IgG antibodies, reducing seropositive cases. Our 35 day follow-up sampling should account for this because the maximum lag time between SARS infection and IgG development is ~28 days and Ebola virus infection and IgG approximately 20 days (*185-188, 190*). We also expect that patients in rural communities will only visit clinics when symptoms have progressed, likely coinciding with late illness and onset of IgG. We will also have data from our community study that can be analyzed in conjunction with, or in the absence of, clinical data from the hospital data to identify PCR- or seropositives. Finally, the risk of limited detection is outweighed by the potential public health importance of discovering active spillover of new henipaviruses, FVs or CoVs infection.

4. Administrative Plan

4.1. Project management: 4.1.a. Administrative core: The EID-SEARCH organizational partner roles are laid out by Specific Aim above **(see Fig. 3)**. EcoHealth Alliance (EHA) will lead the administrative core of the EID-SEARCH, including: coordination and management of all human and animal research; communication with consortium partners, NIH, other EIDRCs, and the EIDRC-CC; data management and quality assurance; modeling and analysis; and compliance with all US, international, and institutional rules and regulations. The administration will be led by PI Daszak, who has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5-20 yrs on NIAID- and USAID-funded research **(Fig. 16)**. He also has direct experience as one of two external reviewers of one of the CEIRS partners for NIAID (Program officer Diane Post). PI Daszak will be assisted by Deputy Lead, co-I Olival, who has overseen zoonotic disease research in Southeast Asia for >10 years and working directly with our partners in Thailand and Malaysia for 15 years. Senior program manager (Chmura) and full-time program assistant (Hamel) will provide administrative support. The EID-SEARCH data management, epidemiological analysis, and modeling activities will be led by co-Is Olival and Zambrana who have led all modeling and analytics on USAID USAID PREDICT for the last 10 years. They will overse a programmer/data scientist (Mendelsohn), evolutionary biologist/bioinformatician (Latinne), and human epidemiologists (Dawson, Li, Martinez). Our human epidemiology team will work directly with country teams in Malaysia and Thailand to manage human surveillance, IRB applications and approvals, and ensure compliance with all human subjects work. EHA's senior veterinarian (Field) and wildlife field biologist (Phelps) have extensive experience working that bats, rodents, and primates in SE Asia and will coordinate all training and field research in Malaysia and Thailand and ensure compliance with IACUCs.

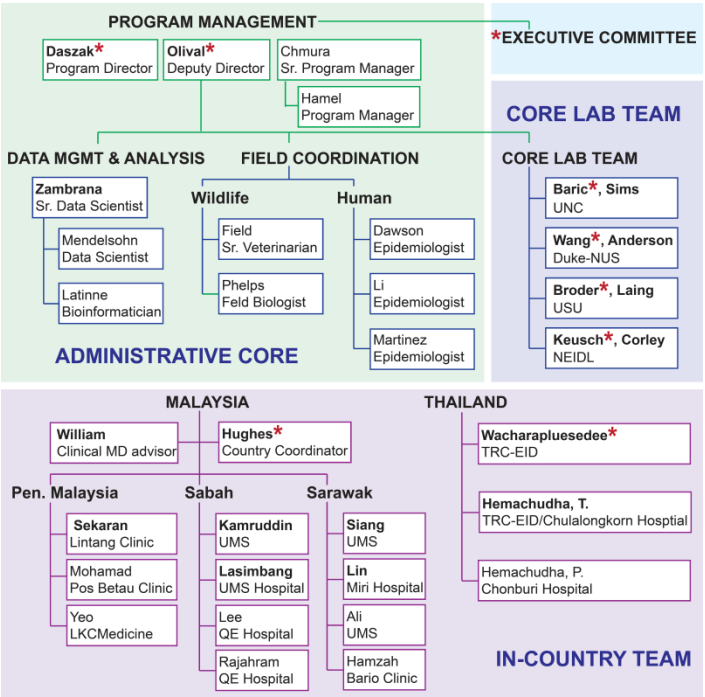


Fig. 16: Administration and program management for the **EID-SEARCH**

The EID-SEARCH core laboratory team includes global experts in virology, molecular pathogenesis, and the development of assays, reagents, and therapeutics for high consequence viral zoonoses (Co-Is Wang, Anderson at Duke-NUS; Baric, Sims at UNC; Broder, Laing at USU; Keusch, Corley at NEIDL). The core laboratory team will work with in-country diagnostic labs in Thailand (Wacharapluesedee, Hemachudha at the TRC-EID, Chulalongkorn University) and Malaysia (Hughes, Lee, CM Ltd.). Wacharapluesedee and Hughes will additionally serve as country coordinators for the EID-SEARCH, liaising directly with EHA on weekly conference calls, and working with in-country partners to ensure the smooth operation and coordination of clinical and community surveillance and wildlife research in Thailand and Malaysia, respectively. **A Core Executive Committee** comprising PI Daszak, Deputy Lead and Co-I Olival, and leads from each core partner and country: Co-Is Hughes, Wacharapleusadee, Baric, Wang, Broder, Keusch (or alternates), will conduct

regular conference calls and in-person meetings to facilitate rapid decision making within the EID-SEARCH. **This committee will also convene to manage EID-SEARCH response to outbreaks.**

4.1.b Project Management in Thailand and Malaysia: Wacharapluesedee and Hughes have collaborated directly with EHA for >15 years, including acting as country coordinators on the USAID PREDICT project for the last 10 years (project end date Sept. 2019). They maintain strong ties with Ministries of Health (MOH), Agriculture and Environment, multiple universities and research institutions, clinics, and hospitals, in their respective countries and across the region. The EID-SEARCH will use these connections to disseminate results, obtain permissions to conduct sampling, and also rapidly respond to and assist with outbreaks as they happen. Peninsular Malaysia, Sarawak, and Sabah are the three main Malaysian administrative regions, and effectively operate as three separate countries, with different regulations and government structures. We therefore provide specific details on the management of EID-SEARCH activities in each:

Coordination among Peninsular Malaysia, Sabah and Sarawak will be led by co-I Hughes (Conservation Medicine Ltd), and follow a successful model we implemented under USAID-PREDICT. **On Peninsular Malaysia** this project will be administered through the Zoonosis Technical Working Committee (ZTWC) established under the PREDICT project with a binding MOU among EHA, CM Ltd. and ZTWC, and including officers from MOH, Dept. of Veterinary Services, and PERHILITAN (the Govt. wildlife agency). EHA will communicate weekly with Co-I Hughes to coordinate and monitor implementation of research and reporting to ZTWC. Co-I Hughes will coordinate activities at all other Peninsular Malaysia institutions: NPHL, the National reference laboratory for diagnostic confirmation of pathogens, will manage molecular and serological screening (BioPlex) of Orang Asli samples, and serological screening of syndromic samples from Sabah and Sarawak; the PERHILITAN molecular zoonosis laboratory will store and conduct molecular and serological screening on wildlife samples; and Universiti Putra Malaysia (UPM) Faculty of Veterinary Medicine will conduct molecular and serological screening (BioPlex) of livestock samples, should these be required. **For Sabah & Sarawak**, work will be administered through the Sabah Zoonotic Diseases Committee (SZDC), a working technical committee comprising appointed and authorized officers from Sabah State Health Dept., Department of Veterinary Services, Sabah Wildlife Dept. (SWD), Universiti Malaysia Sabah (UMS) and EHA, all of which are also committed through a signed MOU. Co-I Hughes will oversee work at all other partners in Sabah, including: the Kota Kinabalu Public Health Lab (KKPHL) for molecular screening of syndromic samples from Sabah and Sarawak; the SWD Wildlife Health and Genetic and Forensics Lab for molecular screening of Sabah wildlife samples; The Borneo Medical Health Research Center (BMHRC) for screening some Sabah wildlife and livestock samples, if required, and human syndromic samples from Sabah and Sarawak. **In Thailand** all human community and wildlife research and testing will be coordinated by co-I Wacharapluesedee from the TRC-EID center. Clinical surveillance will be overseen by senior clinical physician and co-I T. Hemachudha.

4.1.c. Approval and release of results: In our experience, it is critical when working in resource-poor countries, on potentially important pathogens, to strictly adhere to protocols for release of results. EID-SEARCH will liaise with existing points of contact in the Ministries of Health, Environment, and Agriculture in each our administrative areas to approve and release project findings publicly. Results from human screening will be shared with participants when they become available, as per our IRB agreements ensuring no violations to anonymize data requirements (**see Protection of Human Subjects**).

4.2. Flexibility to extend the EID-SEARCH to new sites as needed: The EID-SEARCH consortium partners maintain extensive working relationships with leaders in EID outbreak control, clinical investigations and research at over 50 clinics, research institutes and public health laboratories across Southeast Asia. Due to space constraints, we haven't listed each of these, nor have we solicited >50 Letters of Support for this project. However, each core EID-SEARCH partner has contacted their networks and obtained permission for inclusion in the broader goals of the EIDRC. As examples of these contacts, our core partner, the Thai Red Cross Emerging Infectious Disease Health Science Centre (TRC-EID) at Chulalongkorn University, also serves as the WHO Collaborating Centre for Research and Training on Viral Zoonoses and has ongoing research collaborations across WHO SEARO countries including Bangladesh, Bhutan, Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, (Thailand), Timor-Leste; and has recently served as a training hub for scientists from Malaysia, Myanmar, Laos, the Philippines, and China to learn methods of wildlife sampling and diagnostic screening. Our Thai clinicians (Co-I T. Hemachudha and KP P.

Hemachudha) provide regular case consultations and clinical trainings for doctors across SEARO countries, including with Yangoon General Hospital and the National Health Lab in Myanmar, 2018. To maximize leverage of this broad network, EHA has budgeted for annual meetings in SE Asia, in addition to regular smaller network meetings, with our core team and key public health experts from network labs in each of the 10 SE Asian countries**.** Additionally, we will set up a listserv and an internal communication network to facilitate collaboration and information exchange, including on the first reports of new disease outbreaks. Our annual and smaller network meetings will critically allow face-to-face meetings of the EID-SEARCH that will foster greater sharing of information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks in the region, guided by the PI, Deputy and the Executive Committee.

4.3. Outbreak response: EHA collaboration with expert networks around the world allows us to mobilize and enhance effective One Health response to disease emergencies (*191*), ranging from real-time situation updates and risk analyses to on-the-ground investigations (*192-194*). We will adopt management tools from Emergency Operating Center (EOCs) (*195*) and Incident Management Systems (IMS) (*196*), to shift resources where necessary to help respond to novel zoonotic outbreak events and other public health emergencies. EHA has extensive experience working with governments in low and middle income countries (LMIC) applying these principles of epidemic preparedness during outbreak responses we've been involved with under the USAID-PREDICT project. For example, at the request of the government of Bangladesh, we provided technical field and laboratory support for Nipah virus and avian influenza outbreak investigations, assisting with wildlife sampling as part of the outbreak response alongside human and domestic animal sampling. In India, we provided technical assistance in response to the Nipah virus outbreak in Kerala in 2018. Last month in Indonesia were assisted the Ministry of Health's Center for Health Laboratory in Makassar to provide technical assistance in a mysterious outbreak in a small village in South Sulawesi that killed 4 villagers and infected 72. Our network partners include the key government and govt. approved laboratories that would be directly involved in public health emergency response in their respective countries. The serological and PCR platforms that EID-SEARCH develops will be made available to the main government outbreak investigation teams for clinical work and research during the outbreak. EID-SEARCH will also offer assistance training and conducting animal sampling during an outbreak, epidemiological analysis and modeling to help identify likely reservoirs or likely pathways to spread. Technical and material support for lab, field and analytical activities during an outbreak will be provided by EHA, UNC, USU, Duke-NUS, and NEIDL, as well as in-country partners. Any clinical samples, viral isolates and sequence data will be shared among partners to promote the rapid development of new diagnostic assays, reagents, and therapeutics that can be deployed to the region or other regions as part of the larger NIH EIDRC network.

Finally, while the initial pathogen focus of our group is on CoVs, PMVs and FVs, our broad collaborative group has multidisciplinary expertise on a number of virus-host systems. For example: PI Daszak was PI on a subaward from PI Laura Kramer's U01 on Poxviruses and Flaviviruses, managing a multidisciplinary research project on West Nile virus ecology. He was also co-I on a 5-year NSF-funded project to understand West Nile virus dynamics and risk in the USA (*197-201*); Co-I Baric is a global leader in Norovirus research leading to the development of vaccines and therapeutics (*202-205*); Co-I Wang has conducted significant work on bat immunology, therapeutic, and reagent development, as well as being involved in a range of outbreak investigations, viral discovery programs and other research on a wide diversity of viral groups (*206-215*). Additionally, the serological and PCR-based diagnostic platforms being developed by Co-Is Wang and Broder are adaptable to other viral targets. The modeling tools developed by Co-Is Olival and Zambrana-Torrelio can be used to predict the emergence and spread of diverse viral targets, including influenza, antimicrobial resistance, and vector-borne diseases (*216-221*). Our clinicians working in Thailand and Malaysia have a wide range of infectious disease investigations to adapt to any outbreak situation.

4.4. Communications: EHA will coordinate communication among all co-Is and key personnel, including:

- Multiple meetings per week with PI, Deputy Lead, Senior Program Manager (on project and task status)
- Weekly web/phone meetings between Program Manager and subawardee admin. staff
- Monthly web/phone conferences between EHA PIs and all subawardee PIs.

- Monthly web conferences between key personnel (research presentations/coordination)
- In-person Annual meetings with partner leads, key personnel at EHA and two in-person partner meetings annually between subawardees.
- Annual in-person meeting among all key personnel

4.5. Problem identification and resolution: Regular planning, monitoring, and evaluation meetings will be the primary mechanisms for problem identification. Minor problems (e.g. delays in sample availability or test results) will be dealt with internally through appropriate action and resolution monitored by PI Daszak and co-I Olival, and our Senior Program Manager. In the event of significant problems, such as prolonged poor productivity, inadequate scientific collaboration, or major disputes regarding research direction or resource allocation, EHA will assist with resolving the problem through negotiation with relevant co-Is and consultation with the Executive Committee. Should a resolution not be forthcoming, consultation with the EIDRC-CC, additional external technical advisors, and NIH staff may be warranted.

4.6. Adaptive management and risk mitigation: Maintaining a timeline and meeting milestones will require strict and continuous oversight of all project phases, frequent and regularly scheduled communication, and the ability to make decisions and implement strategies. To maintain our timeline on all projects, including the EID-SEARCH, we use an adaptive management approach to continually evaluate these trade-offs, to make decisions about when iteration is appropriate and when it is necessary to move forward with current information. Our ethos is that regular, scheduled communication among all staff, partners and collaborators will go a long way towards mitigating risks, especially if the process is collaborative and transparent.

5. Data Management Plan

EHA will house the Data Management and Analysis (DMA) team for EID-SEARCH, led by Co-PIs Olival and Zambrana-Torreilo and include Key Personnel Latinne and Mendelsohn. EHA has served as the data and analysis hub for numerous multi-institutional, multi-sectoral, international disease research groups, including acting as Modeling and Analytics lead for the PREDICT project (*122*), the Western Asia Bat Research Network (*222*) and EHA's Rift Valley Fever Consortium. We will leverage our experience and infrastructure from those projects to benefit the EID-SEARCH. **5.1. Project Database:** We will create a dedicated, centralized EID-SEARCH database to ingest, store, link, and provide for analysis all data associated with the proposed study and other expanded projects associated with the research network. The database will be SQL-based and use encrypted, secure cloud hosting services and enable export to archival and platform-independent formats. It will ensure data and metadata compatibility between project components, track data versioning and annotations. The system will be designed to work with both with both paper- and tablet-based field data entry and with the Lockbox laboratory information management systems **(Section 5.2)** in place in individual partner labs. The database will use existing metadata standards, including NCBI standards for genetic and molecular data and Ecological Metadata Language (EML) for field and wildlife data, as well as other standards and formats designated by the EIRDC CC. This will enable rapid publication and deposition of data. Granular security and privacy controls will be applied so that specific expansion projects undertaken in the network may be managed while maintaining data confidentiality as needed.

5.2. Biological Specimen Management: Project laboratories will use the Lockbox Laboratory Information Management System (LIMS), to manage the security, traceability, and quality of biological specimens. The LIMS will support sample barcoding at creation, tracking through transport, storage/inventory, and use via portable scanners. Lockbox supports CLIA and ISO 17025 as well as direct export to NCBI formats such as Sequence Read Archive. We will use the Lockbox LIMS application programming interface (API) to link to the central project database and associated samples with field and ecological data. We note that the project focuses on highly pathogenic viruses, including select agents; Lockbox LIMS supports sample tracking and movement compliant with US Select Agent Regulations and US Department of Commerce Pathogen Import and Export Control Regulations, and includes all necessary encryption, security, and backup protocols.

5.3. Training: Members of the DMA team will team will develop documentation and provide training for field and laboratory teams at all partner institutions in data management, metadata standards and data hygiene best practices. The DMA team will act as trainers and consultants for partner institutions in experimental

design, power analysis, data analysis, and computational and reproducibility issues, and visit each partner institution and/or field team base for training workshops and analysis consultations.

5.4. Data Identification and Privacy: For human clinical data and questionnaires, data will be identified by a unique identification code assigned to each individual and only this, de-identified code will be accessible in the project database, and destroyed at the end of the project - as per details provided in the Clinical Management Plan and Protection of Human Subjects forms.

5.5. Computing Resources: EHA operates a cluster of high-performance servers for data analysis activities, as well as infrastructure to launch cloud-based computing environments (**see EHA Facilities**). Our servers host all necessary software for statistical and bioninformatics work that is available to the DMA and partners anywhere in the world. We use a mixture of cloud services (AWS, Azure, Backblaze, GitHub) to provide redundancy, backup, version control, and rapid post-disaster recovery, and will be available to all project partners for analysis and training.

5.6. Data and Code Sharing: See details provided in the **Resource Sharing Plan.**

6. Clinical Management Plan

6.1. Clinical site selection: Our consortium partners have been conducting lab and human surveillance research, including during outbreaks, for >20 years and have developed strong relationships with local clinical facilities and processes in SE Asia and in LMIC globally. The plan for selection of clinical sites will begin in the same geographic regions as those identified in Aim 1 with high zoonotic viral diversity. Clinical sites will additionally serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2. We have already developed successful working relationships with the major healthcare facilities in Thailand and Malaysia and will use these established partners to rapidly gain appropriate permits and begin data collection quickly. Focusing on these EID hotspots in select biogeographic areas (see **Fig 13**) also reduces the number of additional sites needed to meet our goals within our geographic range. The capacity and capability requirements for clinical sites are fairly minimal, and include ability to enroll patients that meet the clinical case definitions of interest, collect and temporarily store biological samples, and follow standards for data management and subject protection with locked filing cabinets to store all paper records and an encrypted computer. We will be enrolling inpatient and outpatient participants within 10 days of the presentation of symptoms to increase the chance of PCR detection of viruses so we will not wait for advanced normative diagnostic tests, that are likely unavailable in the more remote sites, to be completed. We will work with clinical sites to determine the best process for project staff, in most cases supporting the time of currently hired staff at each site. We will recruit and train hospital staff in project-specific procedures including enrollment of patients, sample types to be collected, storage of samples, administration of the questionnaire, and data management plans. During the development and onboarding of new clinical sites we will assess the physical needs of the site and what supplies will be required for collection of data.

6.2. Standardized approach, oversight, and implementation: Management and oversite for all study sites will be undertaken by the local country coordinator with support from our Core Administrative team at EHA. Our research team has over 10 years of experience building capacity on human subjects' research and has developed training resources and materials for standardized implementation of community and clinical research and SOPs for screening, enrollment, and retention of participants. The country coordinator will conduct regular site visits to the clinical sites and annual visits to observe, monitor and evaluate the research process, and conduct follow-up training if required. Through our work with clinical sites under the USAID-PREDICT project we have developed culturally appropriate screening measures for clinal sites that do not disrupt the flow or quality of medical treatment received by the patient. This was done by working collectively with clinical staff to evaluate current procedures and patient flow at the site to determine the most efficient while minimal invasive inclusion of our study into the daily working of the clinal site. Most efficiently this was done by adding minimal basic screening questions to the current clinal intake forms, which allows the clinal research officer to quickly scan charts or logs for potential patients to enroll avoiding potential enrollees from being overlooked if staff are too busy or not on duty. Patients will be enrolled following established clinical criteria (**see Section 6.3**), samples collected and brief surveys conducted to assess the participants contact with wildlife through: 1) occupational exposures; 2) animal contact; and 3) the environment. With permission

from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology. Additionally, we will be following up with clinical participants to determine how clinical presentation differs between CoV, henipavirus, or FV in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. The country coordinator will be continually monitoring the project database to ensure we hit target sample sizes. While patient's enrollment is limited by the number of individuals presenting at hospitals, in previous research we enrolled an average of 105 patients per year, ranging from 77-244.

6.3. Clinical cohort setup, recruitment, enrollment: We will recruit inpatients and outpatients from clinical sites to participate in the study after initial screening to determine if they meet the clinical case definitions for 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever, of unknown etiology or severe diarrhea with unusual presentation for symptoms to increase the chance of PCR detection of viruses. Once enrolled, biological samples will be collected by trained hospital staff. When possible, samples will be taken concurrently with samples being collected for normative diagnostics. We will collect 5mL of blood for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples and two nasal or oropharyngeal swabs will be collected. Controls who test positive for CoVs, FVs, or Henipaviruses will be selected from the pool of participants admitted to the studies and whose samples tested negative for coronaviruses, henipavirus, or filoviruses. We will follow up 35 days after enrollment to collect an additional 5mL blood draw for collection of two 500 µL serum samples and a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. Following up 35 days post initial collection gives adequate time for 1) development of IgG and IgM, which occurs <28 days after onset of symptoms to evaluate the immunological progression of disease and 2) further the risk assessment of the participants to monitor contact with wildlife, people, and to assess the likely reservoir hosts to collect information to inform potential intervention programs.

6.4. Utilization of collected data: Sampling of wildlife and the enrollment of community surveillance participants and a clinical cohort of participants give us the ability to assess the viruses that are circulating in each of the three populations in a similar geographic region. We will use phylogenetic analysis to compare the relationship between wildlife viruses found and viruses fond in human participants. Additionally, the questionnaire data will allow us to assess relative measures of human-wildlife contact that we will analyze the intensity of contact with species known to be at a higher risk for spillover against other risk to provide useful proxy information for spillover risk. The clinical cohort will be split into cases and controls. "Cases" are defined as participants whose samples tested positive for either CoV, henipavirus, or FV via PCR tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. In clinical study, we will use this procedure to determine how clinical presentation differs between virus-exposed and -unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. We will model the outcomes to analyze correlation between biological results and risk factors related to high risk wildlife exposure. With this collective data set we aim to quantify and detect novel viruses likely to spillover regularly to people, are often unreported or misdiagnosed as their clinical manifestations, and are unknown to detect cryptic outbreaks causing previously 'hidden' clinical syndromes in people. Our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence.

6.5. Development of reagents of value to the community. Members of the EID-SEARCH consortium have substantial experience producing reagents, assays, and other products that are used widely by the clinical and research community, and some of which are on a pathway to commercialization. These include: PIs Daszak and Co-I Olival have produced software for analyzing the spread of novel viral agents through air travel networks; Co-I Baric has collaborated with a Norovirus surveillance collaboration with surveillance cohort at CDC and has developed therapeutics that have reached phase 2 and 3 clinical trials, He is currently working with Takeda Sanofi Pasteur on a Dengue therapeutic and with NIH on a tetravalent vaccine; Co-I Broder

developed a Hendra virus subunit vaccine that was commercially produced by Zoetis for horses and is labeled for human use under compassionate circumstances during outbreak situations.

6.6. Potential expansion: Expansion of the research project to other clinical sites or research areas could happen as new information is gathered, either through our research, the EID-SEARCH information network, or an outbreak being identified in the region by other organizations. If expansion is required we would rapidly shift research activities towards the clinical or community sites where the outbreak is active, using the same process we used to set up initial research locations. First, by working with local stakeholders to create a working relationship and on-boarding new clinical research staff through advanced trainings of project aims, SOPs on data collection and storage, and ethical guidelines. The network of infectious disease researchers in the community and hospital settings would allow for an accelerated response to any potential infectious disease outbreaks as staff are well trained on ethical guidelines for working with human subjects, data and specimen collection, storage, and transpiration, project staff would be on the front lines ready for the call to action. Ideally, our testing for viruses in priority viral families may lead to the discovery cryptic outbreaks before it spreads in the general public. Biological results coupled with survey data on key risk factors will provided necessary information for granular understand of disease spread.

7. Statistical Analysis Plan:

7.1. Framework: Statistical analyses across the project will be conducted under a common Bayesian framework. These models provide a unified, probabilistic approach best-suited for estimating effect sizes in heterogenous populations of human clinical and wildlife subjects in observational studies. Within this Bayesian framework, we will use generalized linear mixed models to estimate population prevalences and seroprevalences, and estimate the effects of demographic, occupational and environmental factors affecting these. We will use occupancy models (*223*) to estimate total viral species and strain diversity and completeness of sampling within the human and wildlife sub-populations, and discrete phylogeographic models to identify taxonomic and geographic centers of viral diversification. All statistical analyses will be performed reproducibly using scripted, programmatic workflows (e.g., the R and Stan languages) and maintained under source code version control (git). As with data management, the DMA team will act as trainers and consultants for exploratory data analysis, power analysis, and study design with project partners, and the EHA computing cluster will be available for partners undertaking additional or expansion studies. Power analyses, current and expansion, are performed via simulation approaches allowing planning for complex, hierarchical variation in study populations. Power analyses and specific analytical components of this study are detailed under each Specific Aim.

7.2. Data Quality Control and Data Harmonization: All data will be are examined at entry by field and lab teams upon data entry, followed by examination by DMA team members at upload and integration, for complete de-identification, completeness, accuracy, and logical consistency. The DMA will provide field and lab teams with reports, produced automatically, of data summaries, including aggregates, distribution, detected outliers and possible mis-entries. On a regular basis (quarterly or as-needed during data collection), DMA team members will review reports with field and lab teams to identify errors and update collection and entry procedures as necessary.

7.3. Statistical Considerations for Behavioral Questionnaires and Clinical Metadata: The data collected from the questionnaire will be analyzed to assess the reported measures of contact for each risk group under study, related to1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, rodents, and primates in/around house and other livestock animals (e.g. farming, butchering, slaughtering); 3) proximity of residence or workplace to environments of increased risks (e.g. nearby bat roosts); 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12 months and lifetime. Specific measures we are interested in are the proportion of respondents indicating they consume wildlife, where wildlife is obtained for consumption, have hunted wildlife, butchered or slaughtered live animals, seen wildlife in their homes, been bitten or scratched by animals, etc. Comparisons of measures of exposure contacts and types between men and women, children and adults, different study regions will be conducted in order to explore the occupational, environmental, and demographic factors (gender, age, socioeconomic status (SES)) that influence contact with animals and to determine who is

most at-risk. Statistical analysis will be employed to identify differences between groups with a 95% probably of detecting a difference. Measures of contact related to different activities within specific groups will be compared to determine the activities that put groups at most risk. As appropriate multivariate analysis (e.g. ordinary linear regression, logistic regression, non-normal distributions of outcome, least absolute shrinkage and selection operator (LASSO) regression, etc.) will be utilized to evaluate the relationship between the outcome variables, positive biological results (PCR or serology) key measures of contact and the factors that influence frequency and types of human-animal contact.

8. Project Milestones and Timelines

8.1 Milestones: <u>End of Year 1:</u> Aim 1: Sample targeting locations, species (for wildlife), sample size justifications completed for whole project and reported to in-country teams; Sample testing, viral isolation, NGS, glycoprotein sequencing begun for all archival and some newly-collected samples; *in vitro* work begun; host-pathogen dynamic analyses; animal model work begun. **Aim 2:** Target human community populations identified and sample sizes calculated for some sites in each country; Community data collection, serological testing and RT-PCR testing begun; first epidemiological analyses of data begin in last quarter. **Aim 3:** Clinical cohort selection underway; clinical enrollment, data collection and sample analysis begun. First Annual meeting in last quarter. First publications submitted by end of year, summary overview papers or reviews.

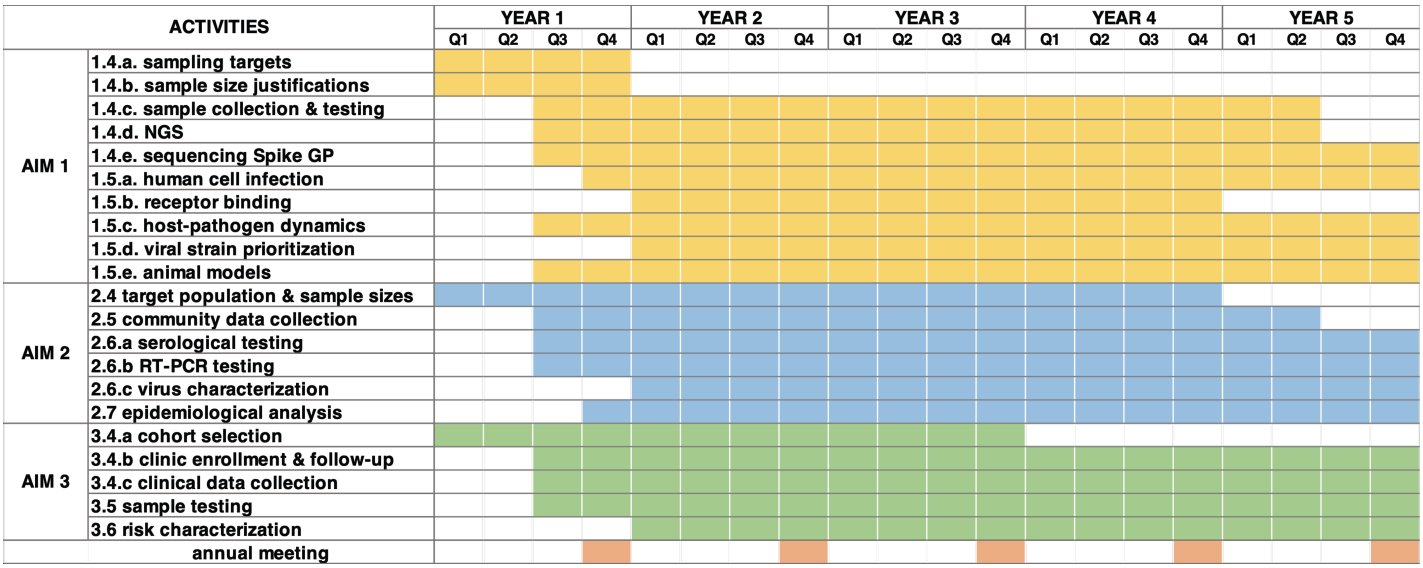
<u>End of Year 2:</u> Aim 1: No sample targeting or sample size justification analyses needed. All other aspects underway **Aim 2:** All aspects underway **Aim 3:** All sub-aims underway. Second Annual meeting in last quarter. Further 2 publications submitted by end of year, including first data papers.

<u>End of Year 3:</u> Aim 1: No sample targeting or sample size justification analyses needed. All other aspects underway **Aim 2:** All aspects underway **Aim 3:** All sub-aims underway. Third Annual meeting in last quarter. Further 3 publications submitted by end of year, largely data papers.

<u>End of Year 4:</u> Aim 1: No sample targeting or sample size justification analyses needed. All other aspects underway **Aim 2:** All aspects underway. Receptor binding work completed. **Aim 3:** No further cohort selection required; all other sub-aims underway. Fourth Annual meeting in last quarter. 3 further publications submitted, including first papers analyzing risk factors, pathogenic potential of novel viruses submitted.

<u>End of Year 5:</u> Aim 1: No sample targeting or sample size justification analyses needed. No receptor binding assays continuing. Serological and PCR testing completed end of 2nd quarter. Glycoprotein, *in vitro* and *in vivo* analyses, analysis of viral risk continue to end of project. **Aim 2:** No further community targeting or sample size work. Community data collection completed at end of 2nd quarter. All other aspects continue to end of project **Aim 3:** All sub-aims underway. Final Annual meeting in last quarter. Further 3 publications submitted.

8.2. Timeline:



CREID Network Governance
Appointments to Steering Committee and Working Groups
15-Jul-20

Research Center Steering Committee Representatives	Research Center Appointment
Primary Representative	
Secondary Representative	

Working Groups	CC Facilitator(s)	DMID Facilitator(s)	Draft Goals of WG	Date Established	Research Center Appointment
Data Capture and Harmonization	Nathan Vandergrift, Sean Hanlon	Primary: Amber Linde, Secondary Sara Woodson	(1) Develop data standards and best practices for data collection for different assays types as well as clinical data that comply with FAIR Data Principles (Wilkinson et al. 2016) (2) Develop data templates that will facilitate data sharing and dissemination to data repositories.	August/ September 2020	
Biorepository Oversight and Quality	Tony Moody	Primary: Sara Woodson, Secondary: Julie Dyall	(1) Harmonize specimen collection and processing procedures.	August/ September 2020	
Laboratory Assays Oversight and Quality	Greg Sempowski	Primary: Julie Dyall, Secondary: Sara Woodson	(1) Develop, implement, and facilitate use of harmonized SOPs for quality laboratory assays.	August/ September 2020	
Rapid Research Response	Pia MacDonald	Primary: Jean Patterson, Secondary: Sara Woodson, Candice Beaubien	(1) Identify and document roles and responsibilities for the CREID CC, the RCs and sites during an outbreak research response; (2) Review the information collected in the Inventory and Site Capacity Management system to identify gaps, potential stressors, and existing challenges; (3) Document lessons learned and best practices for rapid research response.	August/ September 2020	
Capacity Building and Country Ownership	Gretchen Van Vliet	Primary: Candice Beaubien, Secondary: Sara Woodson	(1) Define requirements for Pilot Study program, which will be implemented in Years 2-5 of the CREID Network; (2) Identify strategies for sustainability and country ownership.	December 2020/January 2021	

Direct COVID projects

- Serology of underserved, disenfranchised communities (e.g. Orang Asli, illegal immigrant communities in Borneo, migrant workers Singapore-Thailand-Malaysia)
- Thailand – first responders, people with high exposure (customs)
- Linfa – strain ID (cf. his work on SARS-like deletion)
- Serological testing across Thailand/Malaysia of archived human sample prior to COVID outbreak to see if COVID-like exposure prior to known outbreak
- Linfa's neutralization assay to see how effective vaccines are in trials.
- Ralph/Chris ideas -

COVID-related

- Linfa pseudovirus neuts to other bat-CoVs
- Tom – undiagnosed Disease X (COVID –ve)
- Tom – archived bat/intermediate host sample testing for SARS-CoV-2

From:	Hongying Li on behalf of Hongying Li <li@ecohealthalliance.org>
To:	Peter Daszak; Broder, Christopher; Kevin Olival; Sheahan, Timothy Patrick; Baric, Toni C; Emily Hagan; Aleksei Chmura; Edwards, Caitlin E; Hou, Yixuan Jacob; Gralinski, Lisa E; eric.laing.ctr@usuhs.edu
Cc:	Alison Andre
Subject:	Re: CREID Meeting - EHA, UNC, & USU_Action Items
Date:	Sunday, July 19, 2020 4:18:43 PM
Attachments:	Project Summary EIDRC SEA Daszak.pdf EIDRC SEA Research Strategy FINAL.pdf SC and WG appointments.xlsx COVID-related projects_to collect ideas.docx

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Dear Members of EID-SEARCH (Emerging Infectious Diseases - South East Asia Research Collaboration Hub) Center,

It was very nice to speak to you on Friday. Sorry, we have missed some of you on the call. (please feel free to add your team members who will work on this project into this email chain)

Attached please find the project summary and proposal in PDF if some of you haven't got a chance to read the details before.

Now we would like to ask everyone's input on two items:

1) **COVID-19 related side projects: please send paragraphs about any ideas you might be able to rapidly working on through the CREID collaborations re. COVID-19 for potential extra fundings from NIAID (by Monday, July 21).** NIAID asked about this, and Peter is talking to the program officers on Tuesday morning, so the goal is to get ~10 ideas to propose to them, to find out what they are interested to fund. Attached in the Word document are some ideas we captured from previous calls with Linfa, Tom, and Supaporn, please feel free to add on and/or flash out any of the bullet points.

2) **Working Groups: please volunteer or recommend one person from your team on the Working Groups in the attached Excel sheet (by Wednesday, July 23).** These Working Groups have been identified by DMID and the Coordinating Center as the initial Groups to establish as the Network gets up and running. The Working Groups are expected to meet monthly, and 4 of them will be convened initially if possible, in August, and will meet again in September during the NIAID-facilitated Network kickoff meeting. Each center will appoint one person for each Working Group, but we are considering a second appointment as well in case of the schedule conflicts.

Apologies for this short notice. Please let me know if you have any questions or any further information I can provide. Look forward to hearing from you.

Best regards,
Hongying

Hongying Li, MPH
Research Scientist

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www.ecohealthalliance.org

EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.

On Fri, Jul 17, 2020 at 10:50 AM Hongying Li <li@ecohealthalliance.org> wrote:

> Dear All,
>
> During the call today, we would like to talk about the CREID Working Groups.
>
> Please see below a brief description of the functions for each to help inform your appointments. The attached Excel file provides additional information on the Working Groups.
>
> **Working Groups:** These Working Groups have been identified by DMID and the Coordinating Center as the initial Groups to establish as the Network gets up and running. The Working Groups are expected to meet monthly, and 4 of them will be convened initially if possible, in August, and will meet again in September during the NIAID-facilitated Network kickoff meeting. Research Centers can appoint one person for each Working Group but can choose not to make appointments for all Working Groups.
>
> Look forward to speaking with you soon.
>
> Kind regards,
> Hongying
>
> **Hongying Li, MPH**
> *Research Scientist*
>
> EcoHealth Alliance
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> www.ecohealthalliance.org
>
> *EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.*

On Wed, Jul 8, 2020 at 7:37 AM Alison Andre <andre@ecohealthalliance.org> wrote:

> Peter Daszak is inviting you to a scheduled Zoom meeting.
>
> Topic: CREID Meeting - EHA, UNC, & USU
>
> Time: Jul 17, 2020 01:00 PM Eastern Time (US and Canada)
>
> Join Zoom Meeting

> https://zoom.us/j/91143369261
>
> Meeting ID: 911 4336 9261
>
> Password: 906755
>
> One tap mobile
>
> +16465588656,,91143369261# US (New York)

Nipah virus dynamics in bats and implications for spillover to humans

Jonathan H. Epstein[1]*, Simon J. Anthony[4], Ariful Islam[1], A. Marm Kilpatrick[2], Shahneaz Ali Khan[1,3], Maria Sanchez[4], Noam Ross[1], Ina Smith[7], Carlos Zambrana-Torrelio[1], Yun Tao[1], Ausraful Islam[5] , Phenix Lan Quan[4], Kevin J. Olival[1], Md. Salah Uddin Khan[5], Emily Gurley[5], M. Jahangir Hossein[5], Hume. E. Field[1], Mark D. Fielder[9], Thomas Briese[4], Mahmud Rahman[9], Christopher C. Broder[6], Gary Crameri[7], Lin-Fa Wang[8], Stephen P. Luby[5,11] , W. Ian Lipkin[4], and Peter Daszak[1]

1. EcoHealth Alliance, New York, New York, USA
2. University of California, Santa Cruz, California, USA
3. Chittagong Veterinary and Animal Science University, Chittagong, Bangladesh
4. Center for Infection and Immunity, Columbia University, New York, New York, USA
5. International Centre for Diarrheal Diseases Research, Bangladesh, Dhaka, Bangladesh
6. Department of Microbiology and Immunology, Uniformed Services University, Bethesda, Maryland, USA
7. CSIRO Australian Animal Health Laboratory, Geelong, VIC, AUS
8. Programme in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore
9. School of Life Sciences, Science, Engineering and Computing Faculty, Kingston University, London, UK.
10. Institute of Epidemiology, Disease Control & Research, Government of Bangladesh, Dhaka, Bangladesh
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Abstract

Nipah virus (NiV) is an emerging bat-borne zoonotic virus with pandemic potential that causes near-annual outbreaks of fatal encephalitis in South Asia – one of the most populous regions on Earth. In Bangladesh, infection occurs when people drink date palm sap contaminated with bat excreta, but a recent outbreak in India involved a different, but yet unknown, route of spillover. Outbreaks are sporadic and the influence of viral dynamics in bats over their temporal and spatial distribution poorly understood. We analyzed data on host ecology, molecular epidemiology, serological dynamics, and viral genetics to characterize spatio-temporal patterns of NiV dynamics in its wildlife reservoir, *Pteropus medius* bats, in Bangladesh. We found that NiV transmission occurred throughout the country and throughout the year. Model results indicated that local transmission dynamics were driven by density-dependent transmission, acquired immunity which is lost over time, and recrudescence. Increased transmission followed multi-year periods of declining seroprevalence likely due to bat population turnover and individual loss of humoral immunity. Individual bats had smaller host ranges than other *Pteropus* spp., although movement data and the discovery of a Malaysia-clade NiV strain in eastern Bangladesh suggest connectivity with bats east of Bangladesh. We conclude that discrete multi-annual local epidemics in bat populations contribute to the sporadic nature of Nipah virus outbreaks in South Asia. At the same time, the broad spatial and temporal extent of NiV transmission, including the recent outbreak in Kerala, India, highlights the continued risk of spillover to humans wherever they may interact with pteropid bats, and the importance of improving Nipah virus surveillance throughout *Pteropus*'s range.

Keywords: bats, henipavirus, Nipah virus, *Pteropus medius, Pteropus giganteus,* satellite telemetry, viral phylogeny, disease dynamics, modeling

Introduction.

Outbreaks of zoonotic diseases are often sporadic, rare events that are inherently difficult to predict, but can have devastating consequences (*1*). Several emerging viral zoonoses with wildlife reservoirs have become pandemics, including HIV/AIDS, SARS coronavirus, and 1918 Pandemic Influenza H1N1 (*2-4*). Bats are important hosts for many zoonotic viruses (*5*) including Ebola virus, SARS-CoV, and Nipah virus, but the ecological drivers and transmission dynamics of these viruses in their reservoir hosts are poorly understood (*6-10*). A better understanding of the transmission dynamics of zoonotic pathogens in their natural reservoirs may help anticipate and prevent outbreaks (*9, 11*).

Nipah virus (NiV) is an emerging zoonotic paramyxovirus (genus *Henipavirus*) that has repeatedly spilled over from bats to cause outbreaks in people and livestock with high case fatality rates across a broad geographic range, making it a significant threat to global health. It has caused repeated outbreaks in Bangladesh and India, with a mean case fatality rate greater than 70% (*12-14*). A single genus of frugivorous bats (*Pteropus*) appears to be the main reservoir for henipaviruses throughout Asia and Australia (*15-19*), including *Pteropus medius* (formerly *Pteropus giganteus* (*20*)) in Bangladesh and India (*21-24*). Nipah virus has several characteristics that make it a significant threat to human and animal health (*25-27*): 1) its bat reservoir hosts are widely distributed throughout Asia, overlapping dense human and livestock populations, providing broad opportunity to cause outbreaks; 2) it can be transmitted directly to humans by bats or via domestic animals; 3) it can be transmitted from person to person; 4) spillover has repeatedly occurred in highly populous and internationally connected regions, giving it pandemic potential; 5) it is associated with high mortality rates in people; and 6) there are currently no commercially available vaccines or therapeutics. As a result, the World Health Organization has listed Nipah virus among the ten most significant threats to global health (*28*). To date, human Nipah virus infections have been identified in India, Bangladesh, Malaysia, Singapore, and the Philippines (*12, 22, 29-31*). In May 2018, an outbreak of Nipah virus encephalitis associated with a 91% mortality rate, occurred in a new location - Kerala, India - more than 1,200 km southwest of previous Indian and Bangladeshi outbreaks (*32*). A single case was subsequently reported in Kerala in 2019, and while local *P. medius* populations have been implicated as the local source of infection, the route of spillover in both instances remains unknown (*32, 33*).

In Malaysia and Bangladesh, consumption of cultivated food resources contaminated with bat excreta such as mangoes in Malaysia and date palm sap in Bangladesh and northeastern India have been identified as the predominant cause of spillover to pigs and people respectively (*34*). Human outbreaks

occur almost annually in Bangladesh and the seasonal timing (November-April) and spatial distribution of outbreaks coincide with patterns of raw date palm sap consumption in a region termed the "Nipah belt" (*35*). However, there is variability in the number of spillover events and magnitude of the outbreaks that occur each year (*36*), and spillover has occasionally occurred outside the predominant season and region of date-palm sap consumption (*37*). Further, date palm sap harvesting and consumption also occurs in eastern Bangladesh, yet no human outbreaks have been reported, while date palm sap is not cultivated in Kerala at all, suggesting an alternate route of spillover (*35*). While the full range of mechanisms for zoonotic transmission remain unknown, so too are the underlying viral infection dynamics in bats and the extent of genetic diversity within the virus – each of which may influence the timing, location and epidemiology of human outbreaks (*35*).

Previous research on the transmission dynamics of Nipah and Hendra viruses in *Pteropus* spp. bats have produced mixed and sometimes contradictory findings. Nipah virus, like Ebola, Marburg, Hendra and some bat coronaviruses, might be hypothesized to have seasonal spikes in infection that coincide with annual or semi-annual synchronous birth pulses (*15, 38-44*). Seasonal periods of Nipah virus shedding were observed in *P. lylei* in Thailand and seasonal spikes in NiV (or a related henipavirus) seroprevalence coinciding with pregnancy periods were observed in *Eidolon dupreanum* in Madagascar (*45, 46*), but not in *P. vampyrus* or *P. hypomelanus* in Peninsular Malaysia (*19*). Hendra virus prevalence in Australian pteropid bats has shown multi-year inter-epidemic periods where very little virus can be detected, followed by periods of increased viral shedding, suggesting that viral dynamics are not annual (*47-49*). It has been hypothesized that multi-year periodicity in infection henipavirus dynamics could arise from a build-up and waning of herd immunity in the reservoir host, with re-introduction of virus via immigration or recrudescence or viral persistence (*10, 50-52*). Some pteropid bat species are migratory and interconnected colonies form a metapopulation which could allow for viral re-introductions (*9, 19, 53, 54*). In addition, NiV recrudescence has been observed in wild-caught *P. vampyrus* and *Eidolon helvum* and either of these phenomena could allow it to persist regionally during periods of high local immunity (*55*). However, no study has yet shown evidence in open, free-ranging bat populations that favors one or the other hypothesis in driving NiV transmission dynamics.

The goal of the current study was to understand the distribution and drivers of NiV infection dynamics and NiV diversity in *Pteropus medius* in Bangladesh to provide new insight into human outbreak patterns. We examined spatial, temporal and demographic variation in serological dynamics and viral shedding in bats over a six-year period to determine the spatio-temporal drivers and dynamics

of virus transmission. We also studied movement patterns of individual bats and analyzed NiV phlyogenetics to understand patterns of spatial mixing and virus strain diversity.

Results

Comparative Nipah virus prevalence study in bats inside and outside the Nipah Belt and concurrent longitudinal bat study inside the Nipah Belt (2006-2012)

We caught and tested 883 *P. medius* (~100 per district) from eight different districts across Bangladesh. We detected anti-Nipah IgG antibodies in all colonies (**Figure 1**). Seroprevalence varied by location (χ^2 = 55.61, p<.001), but there was no statistical difference between seroprevalence in bats inside the Nipah Belt and outside**.** In all locations except Tangail, adult seroprevalence exceeded juvenile. Viral detection in individuals was rare. Overall, we detected NiV RNA in 11 individuals as well as 3 pooled oropharyngeal samples (representing 5 bats, but which could not be resolved to an individual) and 21 pooled urine samples ("roost urine") collected from tarps underneath the roost (**Table 1**). We detected viral RNA in individual bats in Faridpur and Rajbari and from pooled samples from Thakurgaon and roost urine samples from Comilla. Of the 10 PCR positive individuals, three had detectable IgG antibodies (**Table S1**). We also detected virus in pooled urine collected from tarps placed below bats at roosts associated with human outbreaks in Bhanga and Joypurhat. The viral prevalence in Rajbari in January 2006 was 3.8% (95% CI: 0% -11%; n=78). In Faridpur, where we also conducted an intensive longitudinal study (see below), viral prevalence estimates ranged from 0% to 3% (95% CI: 0%-10%; n=100 at each of 18 sampling times) (**Table 1**). Nipah virus RNA was detected in individual bats from inside (Rajbari, Thakurgaon, and Faridpur) and outside (Comilla) the Nipah Belt. We detected viral RNA in bats both with and without detectable IgG antibodies (**Table S1**). Urine samples provided the highest NiV detection rate. Detection rates in individual bats by sample type were: urine/urogenital swab = 4% (n=2,126); oropharyngeal swab 3% (n=2,088); and rectal swab = 1.3% (n=79). The estimated detection rate from pooled urine samples across the entire study was 2.7% (+/- 1.6%; n=829).

Factors associated with NiV IgG *serostatus in* P. medius

Among adult and juvenile bats sampled in the aforementioned the cross-sectional study from which we got blood samples (844 of 883), seropositivity was 2.4 times more likely among adults than juveniles, and 1.6 times more likely among males than females (**Figure 2**). Among females, seropositivity was higher in pup-carrying (4 times) and pregnant (1.5 times) individuals. Weight or forearm length did not consistently correlate with seropositivity, however, body condition (an assessment of pectoral

muscle mass by palpation) was significantly negatively correlated (Poor/Fair body condition OR = 0.69) with serostatus. Finally, serostatus was strongly correlated in mother-pup pairs, with 71/80 pairs (89%) having matching status.

NiV serodynamics over time in a population of P. medius*, Faridpur district (2006-2012)*

We conducted an intensive longitudinal study of NiV serology in a population of bats in the Faridpur district and used flexible generalized additive models (GAMs) to characterize changes over time. There were significant fluctuations in adult (>24 mo.) and juvenile (6 – 24 mo.) seroprevalence over the six-year study period (**Figure 3A**). Juvenile seroprevalence ranged from 0% to 44% (95%CI 37%-51%), and decreased over the first year of life for bats born in each year ("yearlings"), consistent with loss of maternal antibodies in juveniles. A more pronounced decrease occurred from mid-October to mid-December. However, the GAM indicating this had only marginal better fit (ΔAIC < 1), than one with a linear decrease over the whole year (**Figure 3B**). The effect of birth cohort was significant on overall seroprevalence.

Adult seroprevalence ranged from 31% (95% CI: 20%-46%) to 82% (95% CI: 77%-87%) and went through three periods of significant decrease then increase over the course of the study (**Figure 3A**). We found no evidence of regular seasonal fluctuations; a GAM with annual cyclic terms fit worse than one without (ΔAIC > 10). Viral RNA detections occurred in periods of increasing, decreasing, and stable seroprevalence.

We fitted a series of age-stratified mechanistic models to examine different biological processes influencing serodynamics, including density- vs. frequency-dependent transmission, recrudescence, immigration of infected individuals, and seroreversion (loss of antibodies) in both juveniles and adults (**Figure 4**). We included annual, synchronous birthing, which we observed occurs between March and April. We assumed that pups weaned from their dams at 3 months, becoming independent flyers, but that maternal antibodies waned after 6 months at which point we had pups transition into the "juvenile" class (*56, 57*). We assumed that juveniles became sexually mature at 24 mo., and entered the "adult" class based on other pteropid species(*43, 56, 58*). Density-dependent models were a far better fit to the data than frequency-dependent models (difference in log-likelihood 10.0; ΔAIC = 20.0), suggesting that movements of bats and fluctuations in colony size alter spatio-temporal variation in the risk of NiV epidemic spillover to humans. In this colony (Domrakhandi/Khaderdi) during the period of sampling, the roost population declined from approximately 300 bats to 185, which decreased transmission. In the six-year study period, the number of infected bats increased when the

seroprevalence of adults fell below 72% (when bat counts were highest - in 2006) and 52% (when bat counts were lowest). R_0 in adult bats was estimated to decrease from 3.5 to 2.1 as the number of bats in the colony decreased. Serodynamics in juveniles were strongly driven by inheritance and loss of maternal antibodies. The rate of loss of maternal antibodies was 17.6 weeks (95% CI: 13.7-25.0), which was much quicker than the loss of antibodies in adults (290.8 weeks, 95% CI: 245.0-476.4) (**Table S2**). Finally, models with recrudescence fit the data better than models without recrudescence (**Table S2;** difference in log-likelihood 32.6; ΔAIC = 30.6), and models with recrudescence fit the data better than models with immigration (ΔAIC = 3.76).

Mark-recapture and seroconversion/seroreversion

A total of 2,345 bats from the Faridpur/Rajbari region were sampled and microchipped between 2007 and 2012. There were 56 recapture events (**Table S3**). Thirty-one bats were recaptured at a roost other than the original capture location. This network of roosts or "roost complex" formed a polygon covering approximately 80 Km^2 and included 15 roosts sampled during the longitudinal study (**Figure S2A and S2B**). Ten instances of seroconversion (change from IgG negative to IgG positive) and nine instances of seroreversion (positive to negative) were observed (**Table S3**). The mean time between positive and negative tests in *adults* (excluding juveniles with maternal antibodies) was 588 days (n=6) (range: 124-1,082 days).

Homerange and inter-colony connectivity analysis

Home range analysis of satellite telemetry data from 14 bats (**Table S4**) showed that the majority of bats roosted within 10 km of where the bats were originally collared, in the Faridpur (Nipah belt) colony, and within 7 km from where the bats in the Cox's Bazaar colony were originally collared (315km east of Faridpur). The average foraging radius was 18.7 km (s.d. 21.5 km) for the Faridpur bats and 10.8 km (s.d. 11.9 km) for the Cox's Bazaar bats (**Figure S2**). Homerange analysis suggests that bats in Faridpur and Cox's Bazar would have a <5% probability of intermingling (**Figure 5**). Homerange size was significantly larger during the wet season than the dry season (2,746 km^2 vs. 618 km^2) (**Figures S3 & S4**).

NiV phylogenetic analysis.

Phylogenetic analysis of NiV sequences from a 224nt section of the N gene (nt position 1290-1509 [position ref gb|FJ513078.1| India]) suggests that strains from both India and Malaysia clades are

present in Bangladesh bats (**Figure 6**). This finding is supported by an analysis of near-whole N gene sequences (~1720 nt) from bats, pigs, and humans, including those from a subset of *P. medius* from this and a more recent study by our group (**Figure S5**) (*59*). Eleven 224nt N gene sequences obtained from bats between 2006 and 2012 (all from the Faridpur population) were identical. Overall, the N gene sequences identified from the Faridpur, Rajbari and Bhanga colonies between 2006 and 2011 had 98.21%-100% shared nucleotide identity. Sequences from Rajbari district obtained five years apart (January 2006 and January 2011) had only a single nucleotide difference resulting in a synonymous substitution (G to A) at position 1304, which was found in four other bat NiV sequences from this study, as well as in the NiV isolate from *P. vampyrus* in Malaysia. Five Human NiV N gene sequences from various locations within the Nipah belt over the same time period as our bat study show more nucleotide diversity than those from the Faridpur *P. medius* population. Human sequences throughout Bangladesh and from Kerala, India, all nested within the diversity found in *P. medius* (**Figure 6**). By contrast, the sequences found in *P. medius* from Comilla, a location 150Km to the east of Faridpur, showed 80.8%-82.59% shared nucleotide identity with sequences from *P. medius* in Faridpur and clustered within the Malaysia group of NiV sequences. The two Comilla sequences were identical to each other, and had up to 87.95% shared nucleotide identity to sequences from *P. lylei* in Thailand. *Pteropus lylei* bats in Thailand were also found to carry NiV strains from both Malaysia and Bangladesh groups.

Discussion

Our study provides new insights into Nipah virus transmission dynamics, genetics and host ecology. Previous studies from Bangladesh suggested that human NiV outbreaks occur only within a defined region in western Bangladesh, termed the “Nipah belt,” and during a defined season (Nov-Apr), which generated the question of whether that observation was entirely due to date palm sap consumption, or whether ecological factors such as the distribution and timing of bat viral infection also influenced the timing and location of human cases (*13, 35, 60*). We undertook the most geographically extensive survey of *Pteropus medius* in Bangladesh to date. This study was designed to better understand Nipah virus infection patterns in its putative reservoir, *Pteropus medius*, which is common in Bangladesh and throughout the Indian subcontinent (*21, 22, 56*).

Overall, our findings suggest viral circulation is not limited to the Nipah belt, but that NiV transmission occurs in bat populations throughout the country. We observed that virus can be shed at any time of year, and that viral dynamics are not annual or seasonal, but driven by demographic and

immunological factors. Analysis of serological data from our longitudinal study suggests that the underlying mechanism driving the timing of NiV transmission in bats is the waning of herd immunity in discrete bat populations allowing heightened viral transmission.

A number of mechanisms have been proposed for the maintenance of acute viral infections in bat metapopulations, including synchronous birthing and subsequent loss of maternal antibodies (*10, 39, 41*), lowered immunity within pregnant females due to stress, nutritional stress and other factors (*43*) immigration of infected individuals from other colonies (*53, 61, 62*), and recrudescence within previously-infected individuals (*10, 55, 63*). Our modeling indicates that NiV is primarily driven by density-dependent transmission dynamics among adult bats, with cycles of higher seroprevalence that would dampen intra-colony transmission followed by waning of antibody titers within individuals and at a population level. Waning humoral immunity against Nipah virus is a consistent feature of henipavirus studies in African pteropodid bats (*52, 64*). Our recapture data has provided the first reported evidence of the loss of detectable NiV IgG antibodies in recaptured individual free-ranging bats, which supports our observation of population level waning immunity. The consistently lower and decreasing seroprevalence that we observed in juveniles suggests that they lose maternal antibodies over their first year, and likely in the first 6-7 months, consistent with other studies of maternal antibodies against henipaviruses in pteropodid bats (*43, 52, 57, 65*). However, our analysis refutes the hypothesis that seasonal pulses of these new seronegative individuals are the primary driver of new outbreaks in adults (*41*).

Our model outputs suggest that spikes in viral transmission occur after virus is reintroduced into the colony – most likely via recrudescence, though immigration is another possible factor in maintenance. Recrudescence would presumably occur in individuals with antibody titers that have waned below a neutralizing titer, if loss of humoral immunity following a primary infection is sufficient to allow a second infection. Recrudescence of henipavirus infection has been observed for NiV in captive *P. vampyrus* (*55*), for henipavirus in captive *E. helvum* (*52, 66*), and has also been observed in humans infected by NiV (*67*) and Hendra virus (*68*). It is difficult to know from serology alone whether wild-caught seronegative bats had been previously infected. Experimental infection of naïve and previously infected *Pteropus medius* that have sero-reverted would provide a better understanding of how humoral immunity influences individual susceptibility to infection, and inform dynamics models attempting to explain viral maintenance within bat populations (*64*).

Our longitudinal study is limited in that it may not necessarily reflect temporal infection dynamics in all bat populations across Bangladesh. Our roost count data and recapture data from

microchipped bats showed how roost sizes can fluctuate, and local roost shifting can occur. The observation of individual bats using multiple roost sites suggests that changes in roost count, which our models suggest impacts transmission dynamics, could reflect local shifting rather than true fluctuations in population due to wider dispersal – a limitation in our model parameterization where the latter interpretation was used.

Understanding how bat populations connect across landscapes is also important for understanding viral maintenance, and studying local and migratory bat movements can provide important ecological information related to viral transmission, including how bats move between different colonies (*53*). Our satellite telemetry data suggest that *P. medius* exists as a metapopulation, like other pteropid species (*10, 62*), though *Pteropus medius* appear to travel shorter distances and remain within a smaller home range (321.46km^2 and 2,865.27km^2 for two groups) compared to *P. vampyrus* in Malaysia (64,000 km^2 and 128,000 km^2) and *Acerodon jubatus* in the Philippines which are similarly sized fruit bats (*53, 69*). Pteropodid bat migration is primarily driven by seasonal food resource availability (*54, 70-72*). In Bangladesh, *P. medius* prefer to roost in human-dominated environments in highly fragmented forests (*73*). The anthropogenic colonization and conversion of land over recent human history has likely led to increased food availability for *P. medius* and reduced necessity for long-distance migration (*34*). This may reflect a similar adaptation to anthropogenic food resources as observed over the last few decades in Australian *Pteropus* species (*62*). Genetic analysis of *P. medius* across Bangladesh has shown that the population is panmictic – that historically, there has been interbreeding among populations across Bangladesh(*74*). If movements are generally more localized, as suggested by telemetry, then less connectivity among flying fox populations may influence Nipah transmission by creating longer inter-epidemic periods and larger amplitude fluctuations in population level immunity compared to more migratory species (*62*).

Bat movement and population connectivity may also influence the genetic diversity of Nipah virus found in different locations, and genotypic variation has been associated with different clinical outcomes in people. While the overall strain diversity among Nipah virus has not been well characterized due to a dearth of isolates, two distinct NiV clades have been described: A Bangladesh clade, which includes sequences identified in India and Bangladesh; and a Malaysian clade, which comprises sequences from Malaysia, Cambodia, The Philippines and Thailand (*31, 59, 75*). Strains of NiV from these two clades are associated with differences in pathogenesis, epidemiological and clinical profiles in humans and animal models and observed shedding patterns in bats (*45, 76-80*). Phenotypic variation in Nipah virus could influence observed human outbreak patterns by altering transmission to,

or pathogenesis in, humans, and the likelihood of smaller outbreaks being identified or reported (*81*). Human-to-human NiV transmission via contact with respiratory and other secretions has been regularly observed in Bangladesh and India, including the recent 2018 outbreak in Kerala (*12, 82, 83*), whereas transmission among people was not a common feature of the Malaysia outbreak, despite close contact between cases and health care providers (*84, 85*). Nipah virus cases in Bangladesh have shown more strain diversity than in the Malaysia outbreak (*86*).,

Until now, there have been very few Nipah sequences obtained from *P. medius.* We found that Nipah N-gene sequence from bats from the Faridpur population were nearly identical over time, compared to variation in N-gene sequences from bats and humans from other locations observed over the same time period (2006-2010). This suggests that there may be locally prevalent and stable NiV genotypes that persist within bat colonies. Human NiV genotype diversity is likely a reflection of the relative diversity of the NiV strains in the local bats that seed outbreaks (*9*). This is also supported by viral sequences obtained from human and bats associated with the 2018 NiV outbreak in Kerala, India, where human NiV sequences were most closely related to local *P. medius* sequences *(87)*. We found a significantly divergent NiV strain in Comilla, which clustered within the Malaysia NiV clade, suggesting that strains from both clades are circulating in bats in Bangladesh.

Connectivity of pteropid bats in Bangladesh with those in Southeast Asia could explain the observed strain diversity in our study. Historical interbreeding of *P. medius* with pteropid species found in Myanmar, Thailand, and Malaysia and our telemetry findings showing bats are capable of flying hundreds of kilometers, could explain our discovery of a Malaysia clade NiV sequence in bats from Comilla (*74*). NiV Bangladesh strains have also been found in *P. lylei* in Thailand (*88*). The N gene of the Comilla NiV strain differs from those reported from bats in the Nipah belt by 20%, whereas NiV Malaysia and NiV Bangladesh differ by only 6-9% and are associated with different clinical profiles. Whole genome sequence (which could not be obtained) would have allowed for better characterization of the Comilla strain, but the N gene is generally conserved relative to other genes, and suggests the rest of the genome may also be highly divergent. It is therefore plausible that the clinical profile of a 20% divergent NiV strain differs significantly from known strains. Further studies linking viral genotype to clinical phenotype would provide insight into the implications of strain diversity in bats for human outbreaks.

Finally, our study sheds light on the sporadic nature of NiV outbreaks with multi-year inter-epidemic periods in South Asia. Firstly, PCR results show that overall NiV incidence in *P. medius* is low, consistent with previous studies of Hendra and Nipah virus (*43, 48, 89, 90*). The data and our modeling suggests that PCR-positive samples are only likely to be identified during viral transmission spikes after

periodic reintroduction into populations that have become susceptible through waning population-level immunity (*10*). Vial detection in bats has also coincided with human outbreaks (*59, 87*). This is likely a rare or at least sporadic event. In the current study, observed seroprevalence patterns and the fitted model suggest that three periods of transmission occurred over the 6 years of sampling, each of which followed periods of low adult seroprevalence, though not all measurements of low seroprevalence were followed by outbreaks. We detected NiV RNA during periods of both increasing and decreasing seroprevalence, supporting the fitted model which suggested that shedding can occur at low levels in bats even in periods without sustained transmission. Our observation that not every instance of rising seroprevalence resulted in detectable viral shedding suggests that not all episodes of viral circulation in bats are of equal magnitude, and that other factors (e.g. variation in human-bat contact and exposure) may affect likelihood of spillover. Together, this evidence suggests that outbreaks can occur in bats when the population falls below a protective threshold of immunity in any season, but variability in how many bats become infected may impact the likelihood of spillover to humans, assuming a route of transmission is available. This could explain variation in the number of human outbreaks (e.g. spillover events) from year-to year in Bangladesh. Thus, the timing of multiple factors involved in driving transmission dynamics needs to align for intra-colony NiV transmission events and further align with human behavior and availability of a route of spillover for human outbreaks to occur, as previously hypothesized (*91*). This, and the seasonality and specific geography of date palm sap consumption in Bangladesh likely explains the somewhat sporadic nature of human outbreaks in the region, albeit that when spillover occurs, it is within the well-defined date palm sap collection season and geographic zone (*35*).

These findings suggest that Nipah virus outbreaks in other regions of Bangladesh where *Pteropus* spp. bats occur, are also likely to be sporadic and rare, leading to under-reporting or a lack or reporting, particularly given that human neurologic symptoms are similar to other common infections, e.g. Japanese encephalitis, malaria, measles (*92*). Understanding whether some NiV strains are capable of causing mild or asymptomatic cases will provide important insights about why outbreaks may not have been detected in areas such as eastern Bangladesh (or other parts of Asia), where host, virus, and potential routes of spillover exist. Mild or asymptomatic cases would be unlikely to be detected by current surveillance systems and it's possible that cryptic spillovers have occurred in Bangladesh, where about half of all outbreaks between 2007 and 2014 were unreported (*93*). Our work and other reports suggest that Nipah virus transmission is possible wherever *Pteropus* spp. bats and humans live in close association and at any time of year, provided there is an available route of transmission. The 2018 and

2019 spillover events in Kerala, India, which were linked to local *P. medius* colonies and which occurred in an area that does not cultivate date palm sap, further emphasize this point.

Identifying areas where high risk interfaces exist between pteropid bats and people, throughout their range, will be important for monitoring Nipah spillover events and quickly responding to outbreaks, as well as establishing interventions to prevent spillover. Raising awareness of the potential for contaminated food to be a route of Nipah virus transmission and in protecting food resources to limit human or livestock exposure, may be effective in reducing the risk of a more transmissible strain of Nipah virus from emerging and causing an epidemic with significant human and animal mortality.

Methods

The study period was between January 2006 and November 2012. The study was conducted under Tufts University IACUC protocol #G929-07. Locations were selected based on whether the district had any previously recorded human NiV encephalitis clusters at the time of this study and was therefore inside the Nipah Belt (e.g. Rajbari, Tangail, Thakurgaon, and Kushtia) or whether they had not and were outside the Nipah Belt (e.g. Comilla, Khulna, Sylhet, and Chittagong). The Thakurgaon study was conducted as part of an NiV outbreak investigation, and coincided with ongoing human transmission (*94*). Between 2006-2012, three different studies of *Pteropus medius*, with similar bat sampling protocols were performed: 1) a cross-sectional spatial study with a single sampling event in each of the eight locations listed above; 2) a longitudinal study of a Faripur bat colony with repeated sampling approximately every 3 months from July 2007 to November 2012; and 3) a longitudinal study of the Rajbari colony with repeated sampling at a monthly interval between 12 month period between April 2010 and May 2011. Opportunistic sampling of *Pteropus medius* was also performed during this time period during NiV outbreak investigations [Bangha, Faridpur (Feb 2010), Joypurhat (Jan 2012), Rajbari (Dec 2009), West Algi, Faridpur (Jan 2010)]. These data are included here because NiV testing of bat samples during these investigations supported the aims of the study. Bats were captured using mist nets at locations within eight different districts across Bangladesh between January 2006 and December 2012 (**Figure 1**).

Capture and sample collection

For the country-wide cross-sectional and Faridpur longitudinal study, on average, 100 bats were sampled at each sampling event, which lasted 7-10 days. This sample size allowed us to detect at least one exposed bat (IgG antibody-positive) given a seroprevalence of 10% with 95% confidence. Bats were captured using a custom made mist net of approximately 10 m x 15 m suspended between bamboo poles which were mounted atop trees close to the target bat roost. Catching occurred between 11 pm and 5 am as bats returned from foraging. To minimize bat stress and chance of injury, nets were continuously monitored and each bat was extracted from the net immediately after entanglement. Perosnal protective equipment was worn during capture and sampling, which included dedicated long-sleeve outerwear or Tyvek suits, P100 respirators (3M, USA), safety glasses, nitrile gloves, and leather welding gloves for bat restraint. Bats were placed into cotton pillow cases and held for a maximum of 6 hours before being released at the site of capture. Bats were sampled at the site of capture using a field lab setup. Bats were anesthetized using isoflurane gas (*95*) and blood, urine, oropharyngeal swabs, and

wing membrane biopsies (for genetic studies) were collected. For some sampling periods, rectal swabs were collected but due to resource constraints, these samples were deemed to likely be lower yield than saliva and urine for NiV, and were discontinued during the study. For each bat sampled we recorded age, weight, sex, physiologic and reproductive status, and morphometric measurements as described previously (*21*). Bats were classified as either juveniles (approximately four to six months - the age by which most pups are weaned) to two years old (the age when most *Pteropus* species reach sexual maturity) or adults (sexually mature) based on body size and the presence of secondary sexual characteristics, pregnancy, or lactation - indicating reproductive maturity (*21, 96*).

Up to 3.0 mL of blood were collected from the brachial vein and placed into serum tubes with serum clot activator (Vaccutainer, USA). Blood tubes were stored vertically on ice packs in a cold box and serum was allowed to separate overnight. Serum was drawn from the tube after 24 hours and placed in a screw-top cryovial (Corning, USA) and stored in a liquid nitrogen dewar (Princeton Cryogenics, NJ, USA). Sterile pediatric swabs with polyester tips and aluminum shafts were used to collect urogenital and rectal samples, and larger polyester swabs with plastic shafts (Fisher, USA) were used to collect oropharyngeal samples. All swabs were collected in duplicate, with one set being placed individually in cryotubes containing lysis buffer (either tri-reagent or NucliSENS Lysis buffer, BIOMERIEUX, France) and the second set in viral transport medium. All tubes were stored in liquid nitrogen in the field then transferred to a -80C freezer.

During each sampling event, pooled urine samples were collected beneath bat roosts using polyethylene sheets (2' x 3') distributed evenly under the colony between 3 am and 6 am. Urine was collected from each sheet either using a sterile swab to soak up droplets, or a sterile disposable pipette. Swabs or syringed urine from a single sheet were combined to represent a pooled sample. Each urine sample was divided in half and aliquoted into lysis buffer or VTM at an approximate ratio of one part sample to two parts preservative.

Serological and molecular assays

Sera from the cross-sectional survey were heat inactivated at 56^{o}C for 30 minutes, as described (*97*) prior to shipment to the Center for Infection and Immunity at Columbia University (New York, USA) for analysis. Samples were screened for anti-NiV IgG antibodies using an enzyme linked immunosorbent assay (ELISA) as described in (*21*). Sera from the longitudinal studies were sent to the Australian Animal Health Laboratory and were gamma irradiated upon receipt. Because of the large sample size and development of a high throughput multiplex assay of comparable specificity and sensitivity, for these

439 samples we used a Luminex-based assay to detect anti-Nipah G IgG antibodies reactive to a purified NiV
440 soluble G protein reagent (*98, 99*).
441 Total nucleic acids from swabs and urine samples were extracted and cDNA synthesized using
442 Superscript III (Invitrogen) according to manufacturer's instructions. A nested RT-PCR and a real-time
443 assay targeting the N gene were used to detect NiV RNA in samples (*100*). A RT-qPCR designed to detect
444 the nucleocapsid gene of all known NiV isolates was also utilized (*101*). Oligonucleotide primers and
445 probe were as described (*101*). Assays were performed using AgPath-ID One-StepRT-PCR Reagents
446 (Thermofisher) with 250 nM probe, 50 nM forward and 900 nM reverse primers. Thermal cycling was
447 45◦C for 10 min, followed by 45 cycles of 95◦C for 15 s and 60◦C for 45 s. Cut-off values were cycle
448 threshold (C_T) ≤40 for positive and CT ≥45 for negative. Results with C_T values between 40 and 45 were
449 deemed indeterminate, i.e. not conclusively positive or negative. Samples with NiV RNA detected by real
450 time PCR were confirmed by gel electrophoresis and product sequencing.
451 A subset of NiV-positive samples was processed by high-throughput sequencing (HTS) on the Ion
452 Torrent PGM platform in order to obtain additional NiV genomic sequence. Libraries were prepared
453 according to the manufacturer's instructions, and 1 million reads allocated per sample. HTS reads were
454 aligned against host reference databases to remove host background using bowtie2 mapper, and host-
455 subtracted reads primer trimmed and filtered based on quality, GC content and sequence complexity.
456 The remaining reads were de novo assembled using Newbler (v2.6) and mapped to the full length NiV
457 genome. Contigs and unique singletons were also subjected to homology search using MegaBlast
458 against the GenBank nucleotide database, in case variance in parts of the genome precluded efficient
459 mapping. From these data, N gene consensus sequences were constructed using Geneious v 7.1, and
460 used for phylogenetic analyses.
461
462 *Phylogenetic analysis*
463 All *P. medius* NiV sequences have been submitted to Genbank and accession numbers are
464 included in **Figure 6**. Sequence alignments were constructed using ClustalW in Geneious Prime software
465 (*102*). Phylogenetic trees of NiV N-gene sequences were constructed using Neighbor-Joining, Maximum-
466 Likelihood algorithms and figures constructed in FigTree 1.4.2.
467
468 *Satellite telemetry and homerange analysis*
469 We developed a collar system to attach 12g solar-powered Platform Terminal Transmitters (PTT)
470 (Microwave Telemetry, Colombia, MD, USA) to adult bats using commercial nylon feline collars with the

buckle removed and 0-gauge nylon suture to attach the PTT to the collar and to fasten the collar around the bat's neck. Collars were fitted to the bat such that there was enough space to allow for normal neck movement and swallowing, but so that the collar would not slip over the head of the animal (**Figure S6**). PTTs were programmed with a duty cycle of 10 hours on and 48 hours off. Data was accessed via the Argos online data service (argos-system.org; France). A total of 14 collars were deployed as follows: Feb 2009: 3 males, 3 females from a colony in Shuvarampur, Faridpur district; Feb 2011: 3 males, 2 females from the same colony; and April 2011 Cox's Bazaar, 3 bats from a colony in Cox's Bazaar, Chittagong district. Bats were selected based on size such that the total weight of the collar (~21g) was less than 3% of the bat's body mass (Table S3).

The individual telemetry dataset was combined for each region and its aggregate utilization distributions (UD) computed in R using package 'adehabitatHR' (*103*). Population-specific home range is represented by the *95% area enclosure of its UD's volume. The volume of intersection between the colonies quantifies the extent of home range overlap. To evaluate the potential for contact with the Sylhet colony, we calculated the most likely distance moved ('mldm') for each sampled bat at Faridpur where the population was more intensively monitored. Movement distance was measured in kilometers with respect to a centroid location (ω) shared by the whole colony; assuming random spatial distribution in tracking rate, we estimated its kernel density function per individual and defined mldm as the mode.

Statistical approach – cross-sectional study

We fit a full Bayesian generalized linear model with a logit link and a Bernoulli distribution to identify potential predictors which influenced a bat's serostatus. We included age, sex, age- and sex-normalized mass and forearm length, mass:forearm ratio, body condition, and whether the bat was pregnant, lactating, or carrying a pup. We included location of sampling a random effect nested within Nipah Belt or non-Nipah Belt regions. We fit the models and performed posterior predictive checks in R 3.4.3 , using the **brms** and **rstan** packages.

Statistical approach – longitudinal study

We fit binomial general additive models (GAMs) (*104*) to the time series of adult and juvenile seroprevalence in the longitudinal study. For juveniles, we modeled the birth cohort of bats as separate random effects in a pooled model of juveniles' dynamics starting from June of their birth year, June being the earliest month we sampled free-flying juveniles in any cohort. We determined the cohort year of juveniles by using cluster analysis to group individuals by weight, assuming those in the smallest

group were born in the year prior to sampling and those in the larger group were born the previous year. 92% of juveniles captured were yearlings. For adults, we modeled dynamics of adults as a single pool over the entire course of the study. We tested models with and without annual cyclic effects.

Where time series had significant temporal autocorrelation (adults only), we aggregated data by week. We determined periods of significant increase in decrease as those where the 95% confidence interval of the GAM prediction's derivative did not overlap zero. We fit the models and performed checks in R 3.4.3, using the **mgcv** package.

We fit an age-structured (adult and juvenile) maternally immune (M)-susceptible (S)-infected (I)-recovered (R) model with recrudescence (R to I) and loss of immunity (R to S) to the seroprevalence data on a weekly timescale:

$$\frac{dS_J}{dt} = -S_J(\beta_{JJ}I_J + \beta_{AJ}I_A) - \mu_J S_J + bN_A(t-5)\frac{S_A}{N_A} - bN_A(t-52)\frac{S_J}{N_J}(1-\mu_J)^{52} + \lambda M_J$$

$$\frac{dI_J}{dt} = S_J(\beta_{JJ}I_J + \beta_{AJ}I_A) - \gamma I_J - bN_A(t-52)\frac{I_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dR_J}{dt} = \gamma I_J - \mu_J S_J - \mu_J R_J - bN_A(t-52)\frac{R_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dM_J}{dt} = bA(t-5)\frac{R_A}{N_A} - \lambda M_J - \mu_J M_J - bN_A(t-52)\frac{R_A}{N_A}(1-\mu_J)^{52}\lambda^{52}$$

$$\frac{dS_A}{dt} = -S_A(\beta_{AA}I_A + \beta_{JA}I_J) - \mu_A S_A + \tau R_A + bN_A(t-52)\frac{S_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dI_A}{dt} = S_A(\beta_{AA}I_A + \beta_{JA}I_J) - \gamma I_J - \mu_A I_A + bN_A(t-52)\frac{I_J}{N_J}(1-\mu_J)^{52} + \Delta R_A$$

$$\frac{dR_A}{dt} = \gamma I_A - \mu_A R_A + bN_A(t-52)\frac{R_J}{N_J}(1-\mu_J)^{52} - \tau R_A - \Delta R_A$$

We included a class M for the density of juvenile bats with maternal antibodies to allow for the biological possibility that maternal antibodies are lost at a much higher rate than antibodies acquired following infection. The subscripts J refer to juveniles and A to adults, β is the transmission rate, γ is the recovery rate, μ is the mortality rate, τ is the rate of loss of adult immunity, λ is the rate of loss of maternal antibodies(*57*), Δ is the adult recrudescence rate (R to I), b is the birth rate (pups join the juvenile population after 5 weeks). Juveniles transition to adults after 52 weeks. We included terms for loss of antibody in adults (τ, S to R), and viral recrudescence (Δ, R to I) based on previous studies on captive bats that demonstrated the existence of these processes without providing enough data to characterize them precisely (*55, 65*). We fit this deterministic model to the seroprevalence data by maximum likelihood,

which assumes that deviations from the model are due to observation error. We estimated the confidence intervals around maximum likelihood parameter estimates using likelihood profiles using the *profile* function in package *bbmle* in R v3.2.2.

We used model fitting and model comparison to examine the need for several of the biological processes in the model above that could influence NiV dynamics. First, we examined both density and frequency-dependent transmission by comparing the fit of the model above to one with transmission terms that have population size (N_A or N_J) in the denominator. Second, we examined the confidence intervals of the parameters describing viral recrudescence, loss of antibodies in adult bats, and loss of antibodies in juvenile bats. If the confidence bounds for these parameters included zero, then these biological processes are not needed to explain the serological dynamics. Finally, we examined the confidence bounds for parameters describing the loss of maternal and non-maternal antibodies (τ and λ), to determine if the rate of loss of these two types of immunity were different. We note that this model structure has similarities to a susceptible-infected-latently infected(L)-infected (SILI) model (if latently infected individuals are seropositive), but the model above differs in allowing for the possibility of individuals to transition from the R class back to the S class.

Code availability

SIR model code written in R is available upon request.

Data availability

All molecular sequences are available via Genbank. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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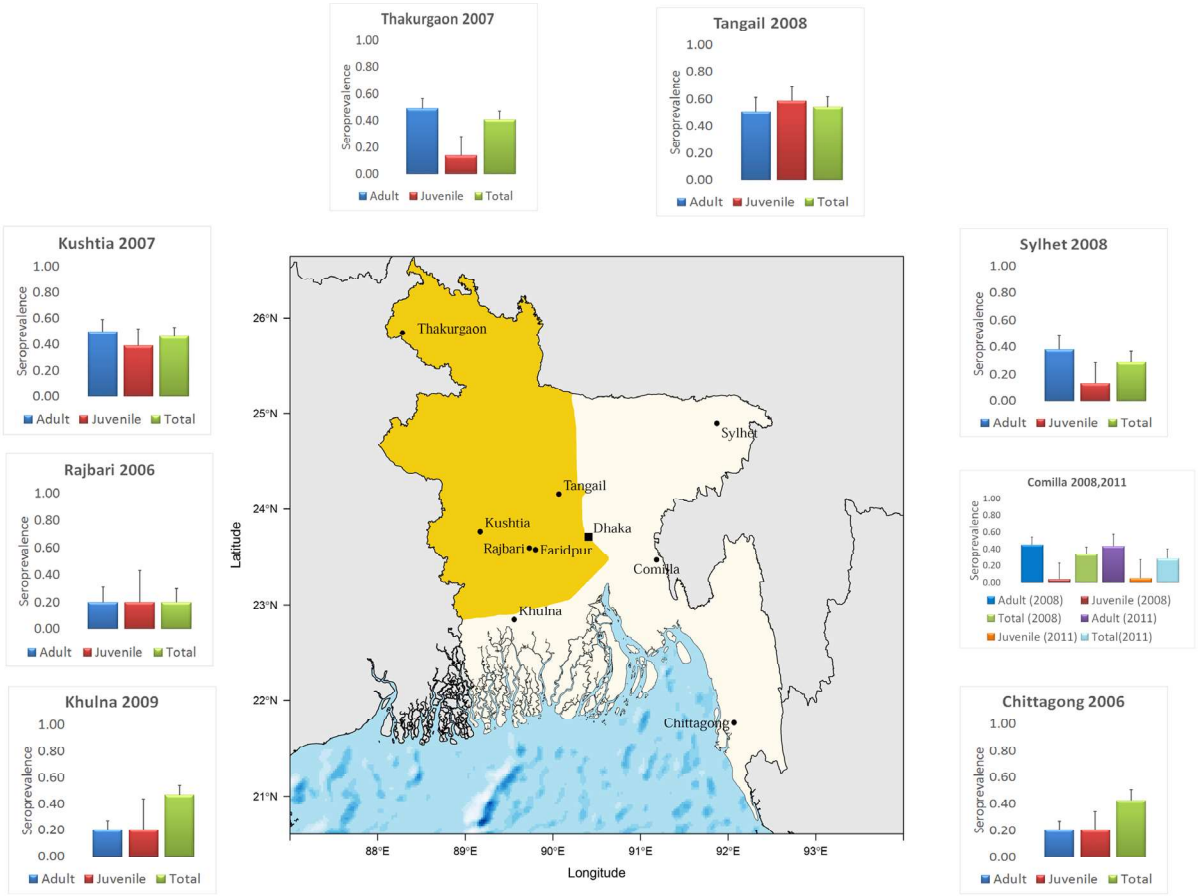

784

785 **Figure 1.** Map showing age-stratified seroprevalence in *Pteropus medius* colonies, Bangladesh. Bats from eight
786 colonies were sampled and tested for anti-NiV IgG antibodies: four within the "Nipah Belt" (orange shaded) and
787 four outside. Seroprevalence of adults (blue, purple), juveniles (red, orange) and total seroprevalence (green, light
788 blue) are shown. Number (n) of Adult, Juvenile, and Total bats sampled (clockwise): Tangail [53,41,94], Sylhet [63,
789 37, 100], Comilla 2008 [73,27,100], Comilla 2011 [30,20,50], Chittagong [72,24,96], Khulna[85,15,100], Rajbari
790 [65,15, 80], Kushtia [64,36,100], Thakurgaon [89,29,118]. The shaded region represents the "Nipah Belt" where
791 previous human NiV outbreaks have been reported.

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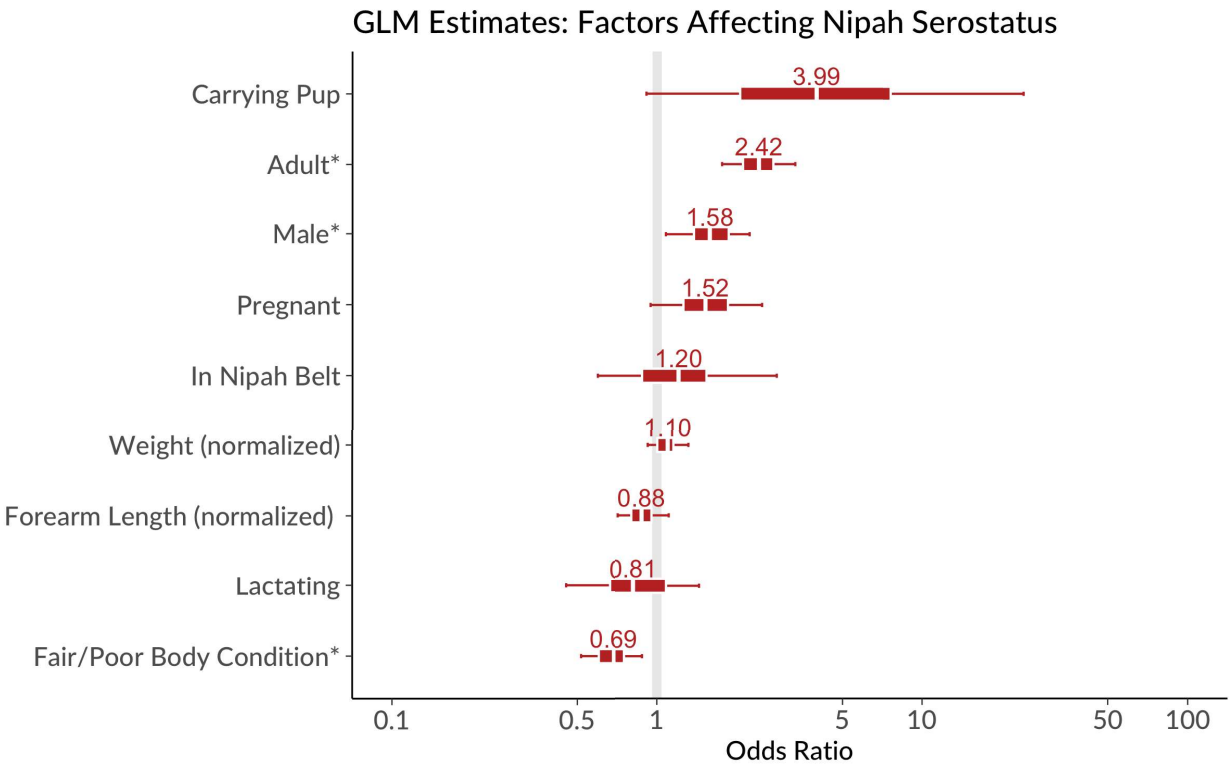

793

794 **Figure 2.** Results of Bayesian GLM of factors affecting Nipah serostatus in bats in cross-sectional study. Bars
795 indicate odds ratios and 50% (inner) and 95% (outer) credible intervals for model parameters. Factors with
796 asterisks (*) have 95% CIs that do not overlap 1. Model intercept (predicted probability of seropositivity for a
797 juvenile, female, bat outside the Nipah belt of mean size and good body condition) was 0.26, (95% CI 0.12-0.56).
798 Random effect of location (not shown) had an estimated SD of 0.63 (95% CI 0.29-1.27)

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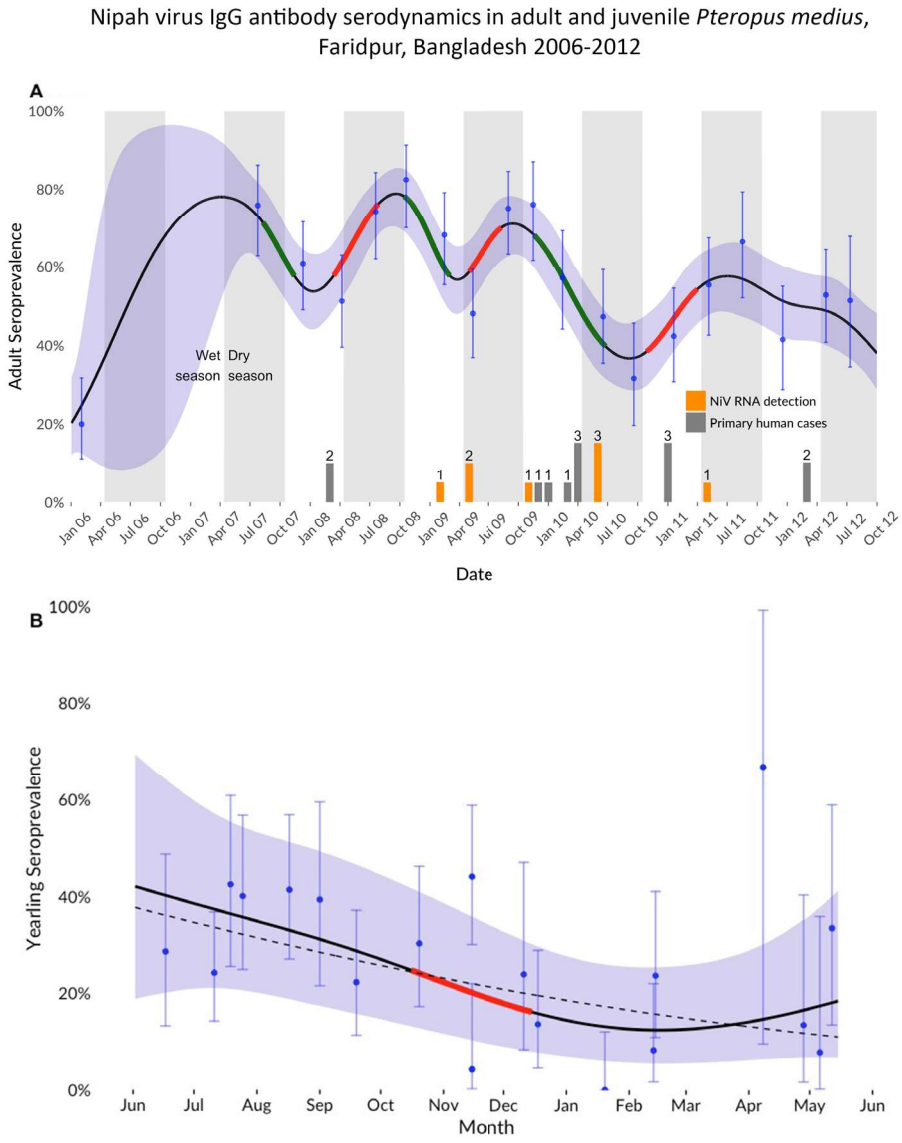

Figure 3 A & B. Serodynamics of the Faridpur bat population. (A) Adult serodynamics, with measured values and 95% CI in blue, and mean GAM prediction and 95% shown with line and surrounding shaded areas. Periods of significant increase (red) and decrease (green) shown where the GAM derivative's 95% CI does not overlap zero. Counts of primary human cases from local district (orange, and bat viral detections (dark grey, see Table 1), shown on bottom. (B) Juvenile serodynamics during the first year of life ("yearlings"), with all years' measurements overlain to show cohort-level dynamics across all study years. Measured values and 95% CI in blue, and mean and 95% CI for the GAM model pooled across cohorts shown with line and surrounded shaded areas. The period of significant decline in the GAM is shown in red. Also shown is the mean prediction of a model with only a linear mean term, with similar fit (ΔAIC < 1) as the GAM (dotted line).

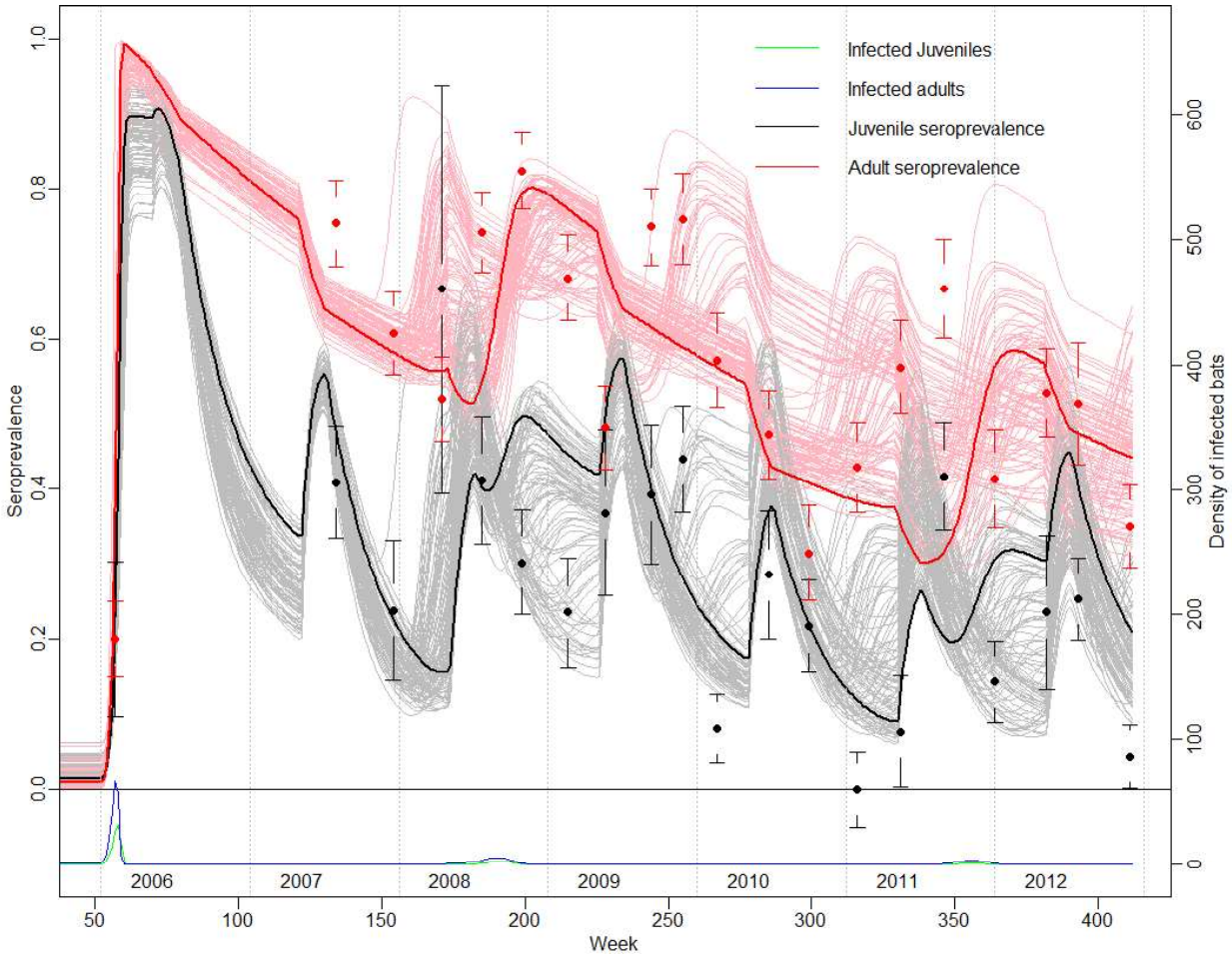

Figure 4. **NiV serological dynamics in adult and juvenile bats.** The observed data (red and black points ±1 SE) and model fit (solid lines) for the fraction of adults and juveniles seropositive for NiV (left axis), and the model estimated density of infected adult and juvenile bats (bottom panel and right axis). See Methods for details of model structure.

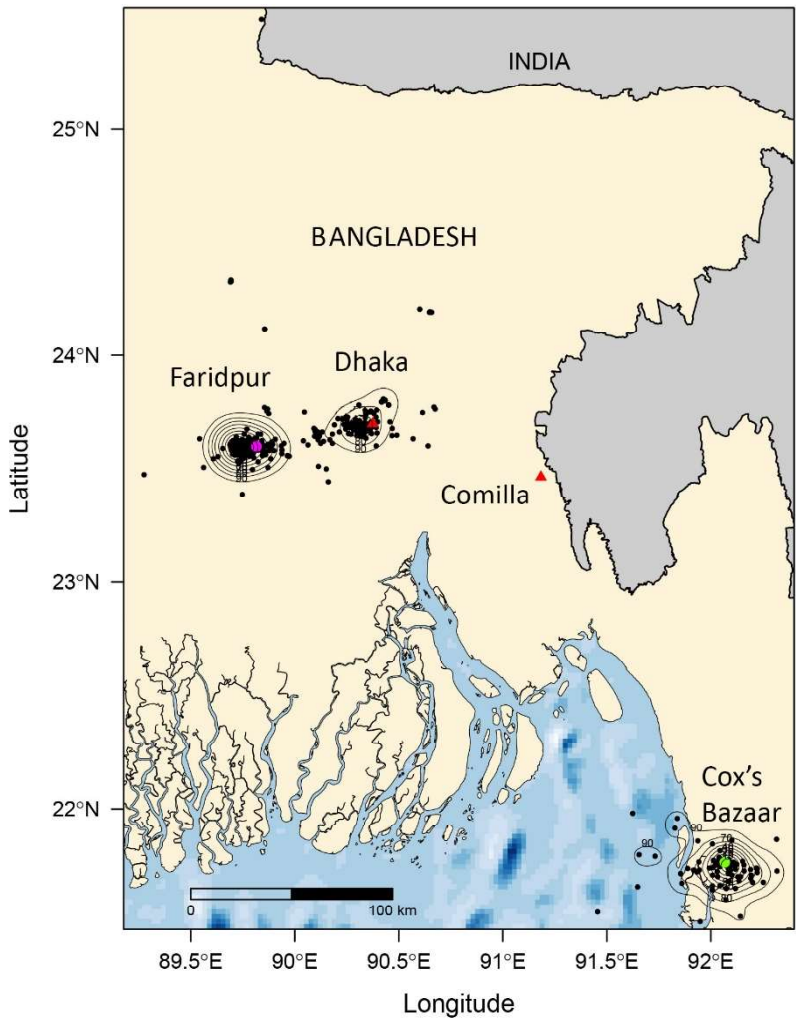

Figure 5. Satellite telemetry and homerange analysis. Location data from satellite collars (n=14) placed on 11 bats from Faridpur and 3 bats from Cox's Bazaar, Chittagong collected between 2009 and 2011, were used to calculate local and long-range movement patterns and home range for these two groups.

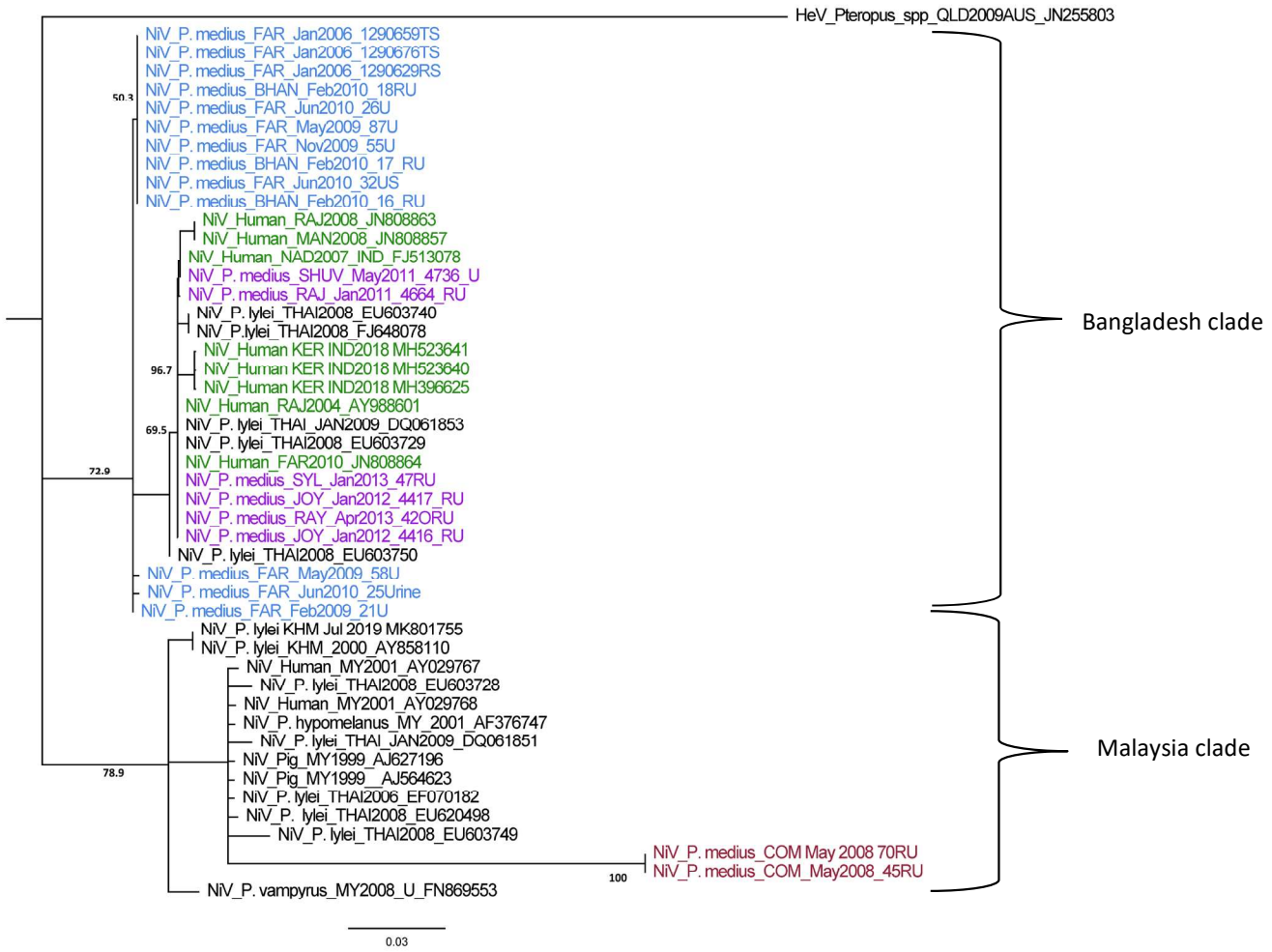

Figure 6. **Nipah Virus N gene phylogenetic tree (224nt):** Tree created in Geneious Prime 2019 using a Neighbor-joining Tamura-Nei model with 1,000 replicates (*105*). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Hendra virus was used as an outgroup. Sample collection date, location and Genbank accession numbers are included in the label for each sequence except *P. medius* sequences which are accession Nos MK995284 – MK995302. Blue labels indicate bat sequences from Faridpur and Bhanga (an outbreak response in Faridpur). Purple sequences are from *P. medius* from other roosts sampled during the longitudinal study. Red sequences are from *P. medius* in Comilla. Green sequences are human NiV sequences from Bangladesh and India.

875 **Table 1**. PCR detection of NiV RNA in *Pteropus medius* 2006-2012.

Location	date	Bats Sampled	Throat Tested	Throat Pos	Urine Tested	Urine Pos	Rectal Tested	Rectal Pos	Paired samples	Pos. Bats	Bats w multi pos samples	prev.	95% CI	Roost Urine	Roost Urine pos.
Spatial Study															
Rajbari	Jan-06	99	79	**3**	78	0	79	**1**	78	3	**1**	**0.04**	**0.11**	-	-
Thakurgaon	Mar-07	118	115	**3***	72	0	-	-	70	unk.	0	0.00	-	-	-
Kushtia	Aug-07	101	100	0	99	0	-	-	98	0	0	0.00	-	-	-
Tangail	Jun-08	100	61	0	77	0	-	-	60	0	0	0.00	-	81	0
Chittagong	Aug-06	115	19	0	-	-	-	-	-	0	-	-	-	-	0
Comilla	May-08	100	0	0	50	0	-	-	0	0	-	-	-	100	**2**
Sylhet	Sep-08	100	100	0	49	0	-	-	48	0	0	0.00	-	100	0
Khulna	Jan-09	100	50	0	80	0	-	-	32	0	0	0.00	-	50	0
Comilla	Mar-11	50	50	0	50	0	-	-	0	0	0	0.00	-	-	-
Outbreak Investigation															
Bangha	Feb-10													19	3
Joypurhat	Jan-12													19	**16**$^{\alpha}$
Rajbari	Dec-09													35	0
West Algi	Jan-10													31	0
Longitudinal Study															
Faridpur	Jul-07	102	64	0	50	0	-	-	22	0	0	0.00			
Faridpur	Dec-07	101	N/A	N/A	N/A	-	-	-		0					
Faridpur	Apr-08	100	64	0	88	0	-	-	54	0	0	0.00		51	0
Faridpur	Jul-08	100	58	0	74	0	-	-	54	0	0	0.00			
Faridpur	Oct-08	100	98	0	99	0	-	-	98	0	0	0.00			
Faridpur	Feb-09	100	50	0	100	**1**	-	-	49	**1**	0	**0.01**	**0.10**	50	0
Faripdur	May-09	101	100	0	99	**2**	-	-	99	**2**	**0**	**0.02**	**0.10**	9	0
Faridpur	Aug-09	100	100	0	99	0	-	-	95	0	0	0.00		3	0
Faridpur	Nov-09	100	100	0	82	**1**	-	-	82	**1**	**0**	**0.01**	**0.11**	50	0
Faridpur	Feb-10	100	100	0	100	0	-	-	100	0	0	0.00		45	0
Faridpur	Jun-10	100	100	0	100	**3**	-	-	100	**3**	**0**	**0.03**	**0.10**	25	0
Faridpur	Sep-10	100	100	0	100	0	-	-	-	0	-	-		20	0
Faridpur	Jan-11	100	100	0	100	0	-	-	0	0	0	0.00		15	0
Faridpur	May-11	102	102	0	102	**1**	-	-	0	**1**	0	**0.01**	**0.10**	20	0
Faridpur	Aug-11	100	100	0	100	0	-	-	-	0	-	-		10	0
Faridpur	Dec-11	100	100	0	100	0	-	-	-	0	-	-		16	0
Faridpur	Apr-12	100	78	0	78	0	-	-	-	0	-	-		50	0
Faridpur	Jul-12	100	100	0	100	0	-	-	-	0	-	-		30	0
Faridpur	Nov-12	100	100	0	100	0	-	-	-	0	0			34	0
Total		**2789**	**2088**	**6**	**2126**	**8**	**79**	**1**		**11**	**1**	**0.005**	**0.02**	**829**	

876 *NiV RNA was detected in three pooled oropharyngeal samples, confirmed by sequencing, although confirmation from individual samples could

877 not be made. These data re not used in prevalence estimates. $^{\alpha}$ Detection by qPCR, Ct ranges 20-38.

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Supplemental Data

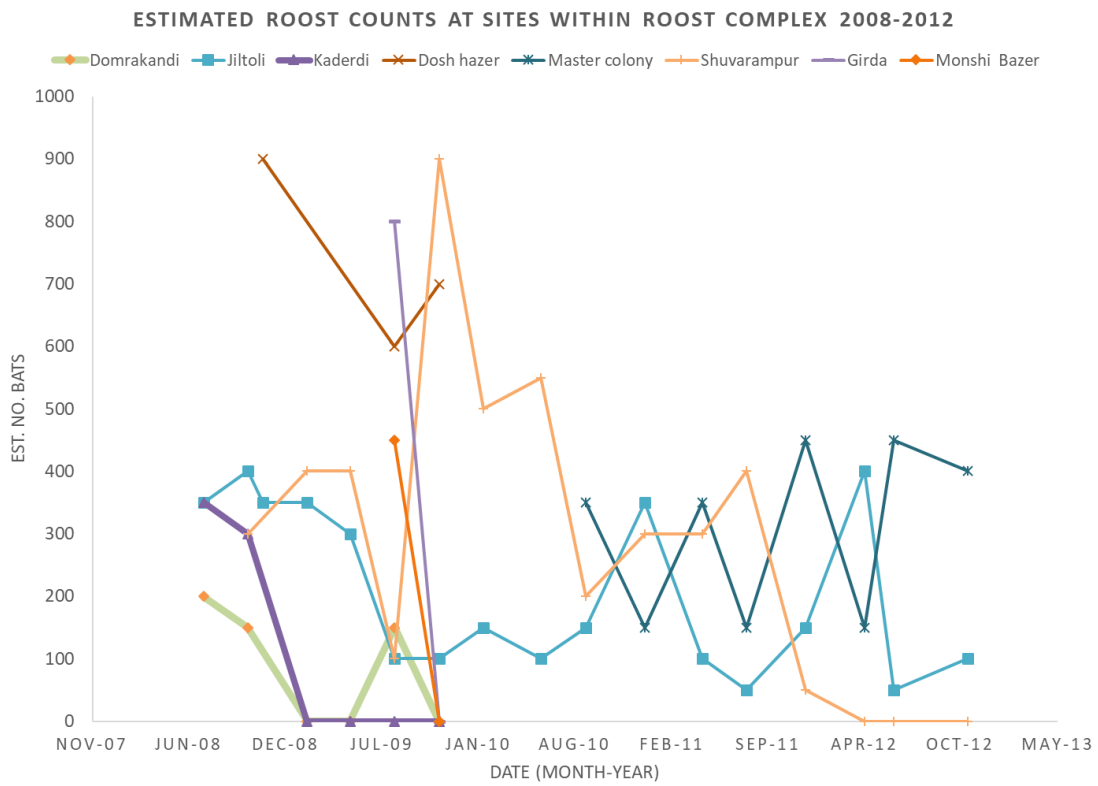

Figure S1. *Pteropus medius* counts from selected roosting sites within the Faridpur Roost Complex: 2008-2012. Sites were included if repeated counts were conducted. Domrakandi and Kaderdi were the two primary roost sites sampled for the longitudinal study and counts were used for the model parameter.

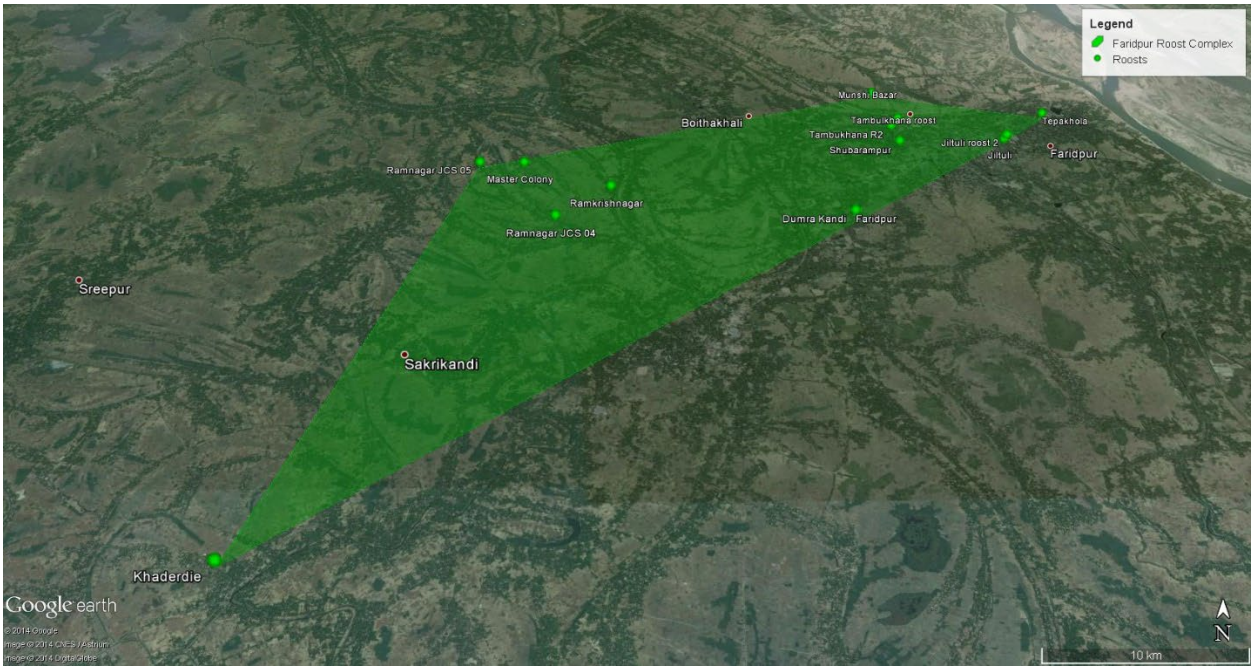

Figure S2. Faridpur Roost Complex. 51 Individual bats were recaptured during the longitudinal study at various locations. 33 bats were recaptured at a different site from where they were originally sampled. 15 unique roosts within an 80km^2 area were identified.

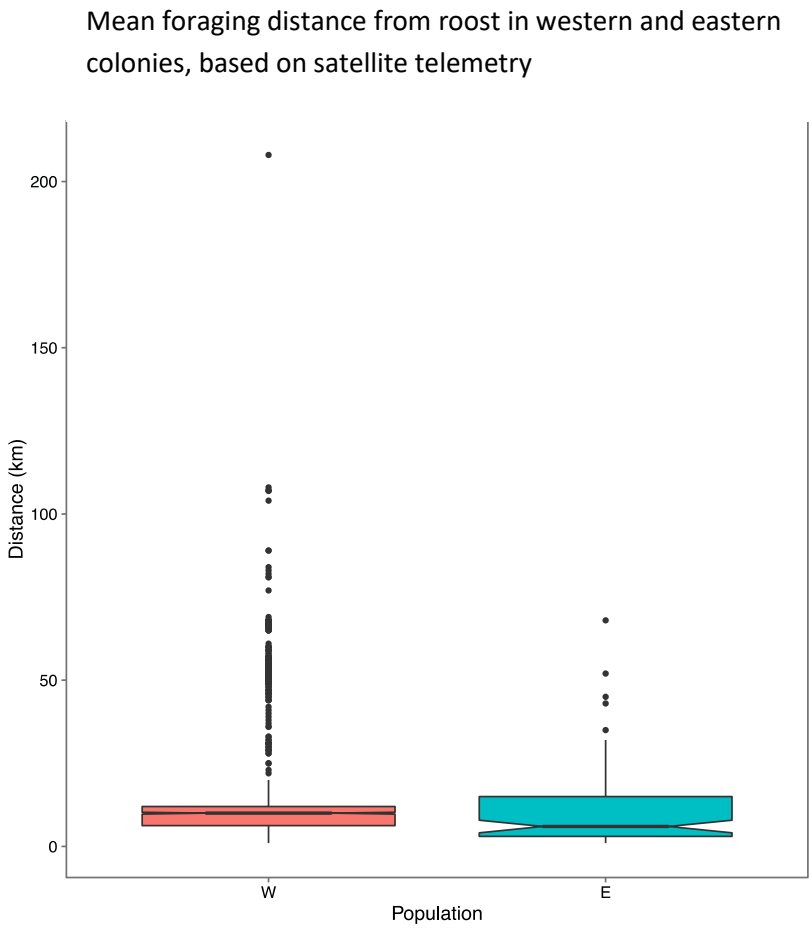

Figure S3. Mean foraging distance of western (W) and eastern (E) bat populations, based on satellite telemetry locations obtained between 1800h and 0600h, when *P. medius* typically forages.

Home range of *Pteropus medius* in wet and dry seasons.

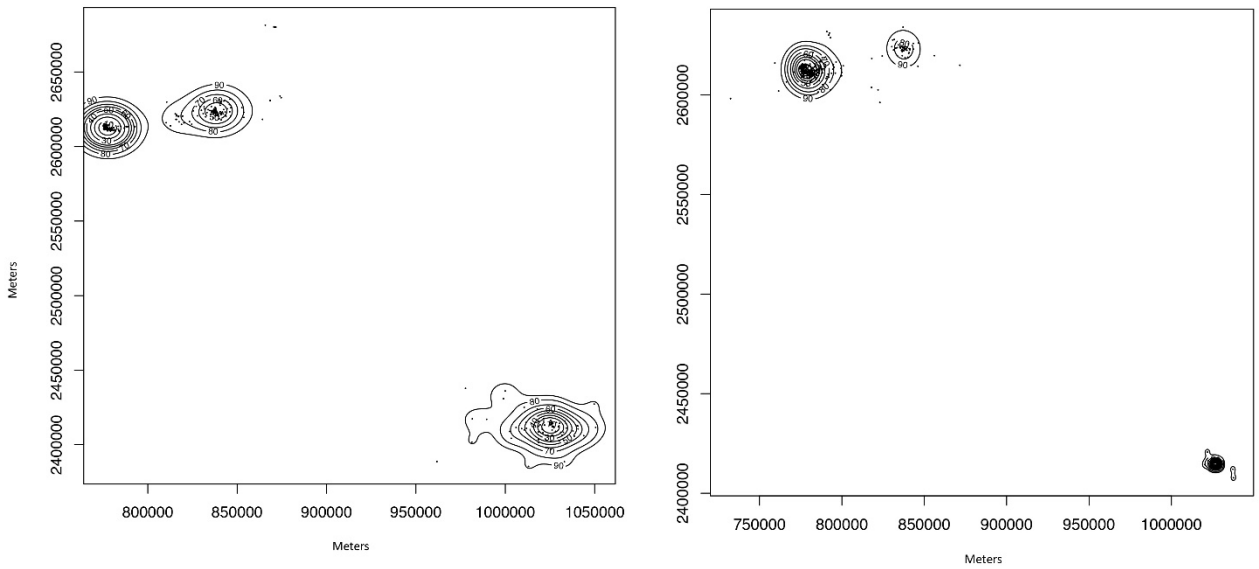

Figure S4. a) Homerange of *Pteropus medius* during the wet season (left) and dry season (right). Maps are projected in UTM (Universal Transverse Mercator) Zone 45 where units are represented in meters. The mean wet season homerange size was 2,746 km^2. Homerange size in the dry season is contracted and represents less than a quarter (618 km^2) of the homerange in the wet season.

Nipah virus phylogenetic tree, based on near complete N gene sequences

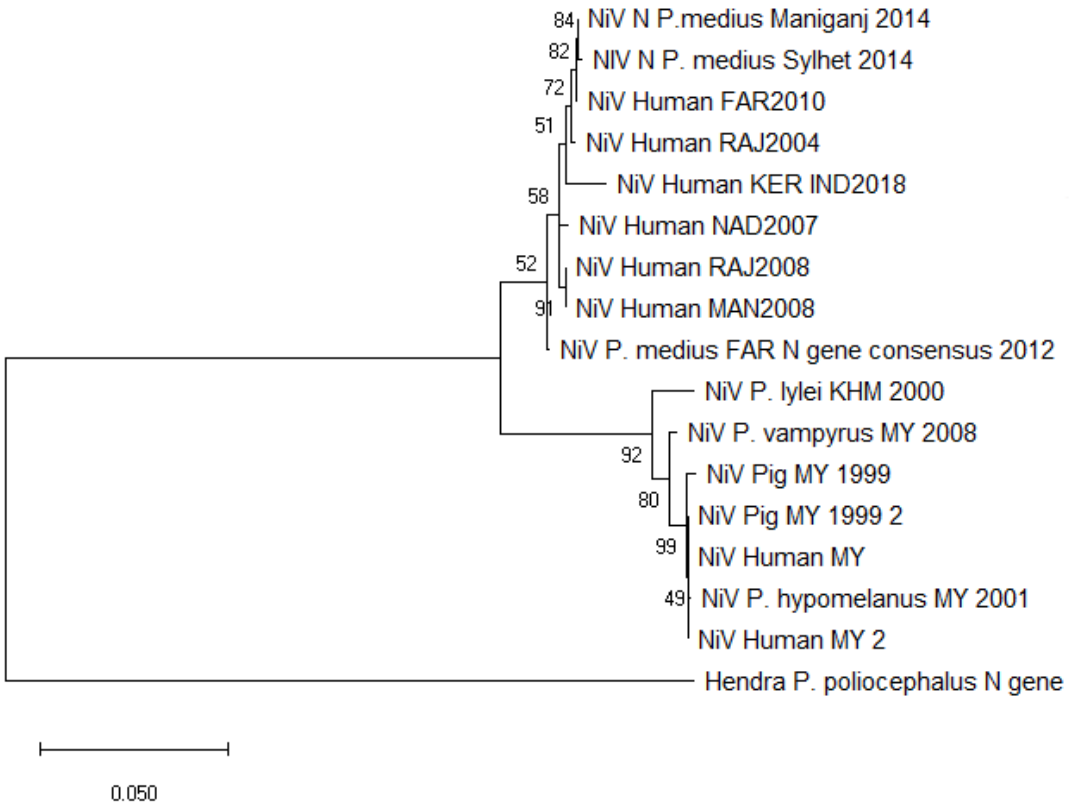

Figure S5. Nipah Virus phylogenetic tree, N gene: Clustal W alignment using nearly whole N gene consensus sequence from *P. medius* (1,592 nt) using Geneious Prime 2019 (*1*). The evolutionary history was inferred by using the Maximum Likelihood method and Hasegawa-Kishino-Yano model (*2*). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X (*3*). Genbank accession numbers for sequences (from top to bottom): *P. medius* Maniganj & Sylhet pending (63); JN808864, AY988601, MH396625, FJ513078, JN808863, JN808857, AY858110, FN869553, AJ627196, AJ564623, AY029767, AF376747, AY029768, JN255803.

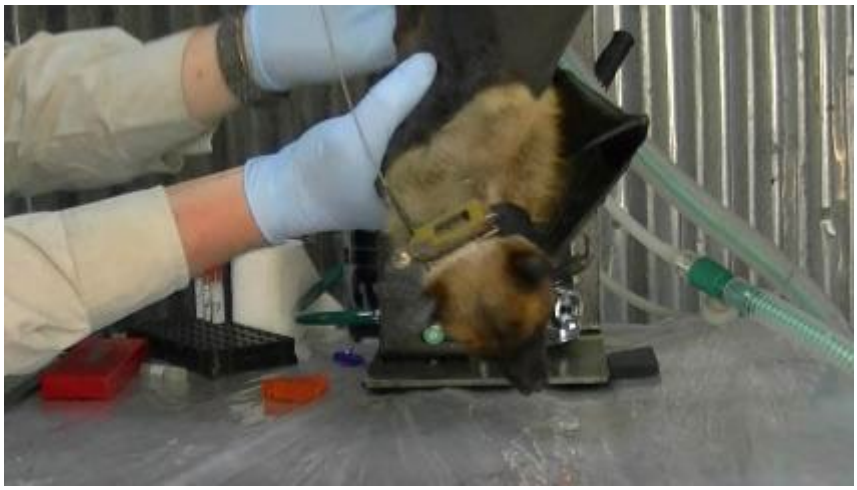

Figure S6. Platform terminal transmitter (PTT) and collar attachment on an anesthetized adult *Pteropus medius*, Bangladesh.

Table S1.

PCR positive bats and their serostatus.

Bat sample ID	Date	Loc	sex	Age	Serology Test result		
					ELISA	Luminex (MFI)	
29	Jan-06	Ramnagar	M	J	Neg	-	
59	Jan-06	Ramnagar	PF	A	Neg		
76	Jan-06	Ramnagar	PF	A	Neg		
21	Feb-09	Faridpur	PF	A	-	25817	Pos
58	May-09	Faridpur	F	A		159	Neg
87	May-09	Faridpur	M	A	-	113	Neg
55	Nov-09	Faridpur	M	A		25955	Pos
26	Jun-10	Faridpur	M	A		30	Neg
32	Jun-10	Faridpur	M	J		758	Pos
28	Jun-10	Faridpur	M	J		377	Neg

Table S2. Maximum likelihood estimates of fitted parameter values and (95% CI). All rates are on a weekly timestep unless otherwise indicated.

Parameter	Name	Maximum likelihood estimate	Lower 95% CI	Upper 95% CI
B_{jj}	Transmision rate, juveniles→juveniles	0.012584	0.00958	0.013021
B_{ja}	Transmision rate, juveniles→adults	0.030008	0.023707	0.03444
B_{aj}	Transmision rate, adults→juveniles	0.002417	0.00195	0.002937
B_{aa}	Transmision rate, adults→adults	0.000465	0	0.004092
R_A/N_A (t=0)	Initial adult seroprevalence	0.018752	0	0.067691
Δ	Recrudescence	2.3E-07	1.41E-08	7.1E-07

$(1-\mu)^{52}$	Adult annual survival	0.754971	0.718554	0.798346
λ	Rate of maternal antibody loss	0.05688	0.040029	0.072817
τ	Rate of adult antibody loss	0.003438	0.002099	0.004082

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Param	MLE	2.50%	97.50%
Bjj	0.012584	0.00958	0.013021
Bja	0.030008	0.023707	0.03444
Baj	0.002417	0.00195	0.002937
Baa	0.000465	0	0.004092
Ias	0.018752	0	0.067691
r	2.3E-07	1.41E-08	7.1E-07
SA_A	0.754971	0.718554	0.798346
MAlr	0.05688	0.040029	0.072817
LAA	0.003438	0.002099	0.004082

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Table S2. Recaptured bats and NiV IgG sero-status from the Faridpur population

Bat Chip ID	Age1	Sex	Capture Date	Location 1	Serostatus1	Age2	Capture Date 2	Serostatus2	Location 2	Status Change	Age3	Capture Date 3	Location 3	Serostatus 3	Status Change 2
17044540	A	M	24/07/08	JH	0	A	19/09/10	1	SH	C					
26774096	A	M	05/10/09	JH	1	A	13/11/09	1	SH	N					
26783883	J	F	15/05/09	SH	1	A	14/02/10	0	SH	R					
26789012	A	M	15/05/09	SH	0	A	02/10/10	1	SH	C					
26791784	A	F	14/05/09	SH	1	A	30/04/12	0	JH	R					
26816627	A	F	11/05/09	SH	0	A	26/08/09	0	DM	N					
26824582	J	M	09/05/09	JH	0	A	20/09/10	0	TP	N	A	05/05/11	TP	0	N
27099360	A	M	24/09/10	RM_MC	0	A	18/08/11	1	TB	C	A	18/11/12	JH	0	R
27102063	A	M	16/11/09	SH	1	A	16/02/10	1	SH	N	A	19/12/11	TP	1	N
27103623	J	M	21/09/10	TP	1	A	17/12/11	0	TP	R					
27105342	J	M	21/09/10	TP	0	A	19/12/11	1	TP	C					
27105562	P	M	24/04/10	RM_JCS	0	J	20/10/10	0	RM_JCS 05	N					
27110270	A	M	24/07/10	RM_JCS	0	A	04/04/11	0	RM_JCS	N					
27111334	A	M	23/07/10	RM_JCS	0	A	19/10/10	0	RM_JCS	N					
27123779	J	F	21/06/10	RM_JCS2	0	J	28/02/11	0	RK	N					
27123803	J	F	21/08/10	RM_JCS1	0	J	28/02/11	0	RK	N					
27123868	J	M	18/02/10	SH	0	A	26/04/12	1	JH	C					
27126256	A	F	10/02/10	SH	0	A	18/12/11	0	TP	N					
27259351	A	M	20/09/10	TP	0	A	30/04/12	0	JH	N					
27259370	A	M	22/07/10	RM_JCS1	0	A	04/04/11	0	RM_JCS1	N					
27261073	J	F	22/06/10	RM_JCS2	1	J	22/07/10	1	RM_JCS1	N					
27261577	A	M	21/09/10	TP	0	A	17/12/11	1	TP	C					
27266775	A	M	21/08/10	RM_JCS1	1	A	18/11/12	0	JH	R					
27291793	A	M	24/09/10	RM_JCS1	0	A	22/01/11	1	RM_JCS1	C					
27296568	A	M	12/11/09	SH	1	A	07/05/11	1	SH	N					
27296851	A	M	22/04/10	RM_JCS1	0	A	25/05/10	0	RM_JCS2	N					
27301580	A	M	17/06/10	SH	1	A	05/05/11	1	TP	N					
27301857	A	M	11/02/10	SH	0	A	22/04/10	1	RM_JCS1	C	A	03/05/11	SH	0	R

Location Codes: Location codes: Jhiltuli (JH); Jiltuli Roost 2 (JH2); Shuvarampur (SH); RamnagarMC (RM); Tepakhola (TP); Ramnagar Roost JCS (RM_JCS); Ramnagar JCS 1 (RM_JCS1); Ramnagar JCS 02 (RM_JCS2); Tambukhana R2 (TB2); Domrakhandi (DM); Khaderdi (KD); Ramkrishnagar (RK); Tepakhola Master Colony (TPMC). Status Change: C = seroconversion, N = No Change, R = sero-reversion.

Table S2 (cont…). Recaptured bats and NiV IgG sero-status from the Faridpur population

Bat Chip ID	Age1	Sex	Capture Date	Location 1	Serostatus1	Age2	Capture Date 2	Serostatus2	Location 2	Status Change	Age3	Capture Date 3	Location 3	Serostatus 3	Status Change 2
27305044	J	M	22/06/10	RM_JCS2	1	A	15/11/12	0	JH	R					
27306794	A	M	15/06/10	SH	1	A	11/07/12	1	TPMC	N					
27306824	A	M	23/07/10	RM_JCS1	1	A	24/09/10	1	RM_JCS1	N	A	18/11/12	JH	1	N
54867532	A	M	23/01/11	JH2	1	A	18/11/12	0	JH	R					
54872600	A	M	19/10/10	RM_JCS2	0	A	30/04/11	0	RM_MC	N					
54877598	J	F	18/01/11	SH	0	A	01/05/12	0	JH	N					
65770323	J	M	04/04/11	RM_JCS1	0	J	01/05/11	0	RM_MC	N					
65780555	A	M	05/05/11	TP	1	A	13/11/12	1	JH	N					
68608827	J	M	14/08/11	TB2	1	J	18/12/11	1	TP	N					
68612032	J	M	15/07/12	TPMC	0		18/11/12	0	JH	N					
80825550	A	M	11/12/07	DM	0	A	13/04/08	1	DM	R					
80855347	A	M	06/12/07	DM	1	A	22/07/08	1	KD	N					
80867630	A	M	11/12/07	DM	0	A	14/05/09	0	SH	N					
80876042	A	M	06/12/07	DM	0	A	14/08/11	0	TB2	N					
80877779	A	M	07/12/07	DM	1	A	21/07/08	1	DM	N					
81030044	A	M	06/02/06	RM_JCS1		A	24/07/10	0	RM_JCS1	NA		04/04/11	RM_JCS1	0	N
81055270	A	F	12/12/07	DM	0	A	20/12/11	1	TP	C					
81095300	A	M	09/12/07	DM	1	A	18/07/08	1	DM	N					
99605347	A	M	15/12/07	DM	0	A	07/04/08	0	DM	N					
99618528	A	M	20/07/08	DM	1	A	16/02/10	1	SH	N					
103821120	A	F	12/04/08	DM	0	A	16/05/09	1	SH	C					
104083112	A	M	03/04/08	DM	1	A	20/07/08	1	DM	N					
65777367	P	M	30/04/11	RM_MC	0	J	14/11/12	0	JH	N					

Location Codes: Location codes: Jhiltuli (JH); Jiltuli Roost 2 (JH2); Shuvarampur (SH); RamnagarMC (RM); Tepakhola (TP); Ramnagar Roost JCS (RM_JCS); Ramnagar JCS 1 (RM_JCS1); Ramnagar JCS 02 (RM_JCS2); Tambukhana R2 (TB2); Domrakhandi (DM); Khaderdi (KD); Ramkrishnagar (RK); Tepakhola Master Colony (TPMC). Status Change: C = seroconversion, N = No Change, R = sero-reversion.

Table S3. Satellite telemetry study: bat characteristics and duration of transmission.

PTT #	Microship ID	Colony location	Date collared	Final location date	Approx.Duration (mo)	Sex	Age	Mass (g)	BCS	Forearm (mm)	Head (mm)	Body (mm)	pregnant	lactating
90831	17035561	F	2/13/2009	5/12/2009	3	F	A	673	G	169.7	74.7	197.5	Y	N
90832	17034004	F	2/13/2009	6/17/2009	4	F	A	663	G	171.4	71.1	195.7	N	N
90833	17019016	F	2/14/2009	4/12/2009	2	M	A	688	G	182.5	78.4	217.6	-	-
90834	080867630*	F	2/14/2009	5/11/2009	3	M	A	665	G	186.4	76.1	201.6	-	-
90835	17027862	F	2/16/2009	7/1/2009	5	M	A	684	G	166.6	68.7	221.6	-	-
90836	17071891	F	2/16/2009	6/3/2009	4	F	A	652	F	181.6	71.8	206.3	Y	Y
101469	54876270	F	1/17/2011	8/13/2011	7	M	A	626	G	175	75	210	-	-
101467	54870019	F	1/17/2011	1/29/2012	12	F	A	603	F	165	70	195	Y	N
101466	54867013	F	2/28/2011	6/8/2011	3	F	A	684	G	164	70	190	Y	N
101468	54883815	F	2/28/2011	4/4/2012	13	M	A	772	G	172.3	71.84	195	-	-
90839	54867601	F	3/1/2011	4/28/2013	25	M	A	771	G	175.53	74	211.14	-	-
101470	65623841	F	3/1/2011	12/8/2011	9	F	A	731	G	172.68	71.6	205.68	Y	N
101471	65628805	C	3/29/2011	5/9/2011	1	M	A	717	G	176	78	220	-	-
90840	65635619	C	3/29/2011	8/8/2011	4	M	A	698	G	169	81	202	-	-
90838	65628094	C	3/30/2011	6/26/2011	3	F	J	446	F	161	73	190	N	N
90837	65775297	C	3/30/2011	6/17/2011	2	M	A	620	F	178	71	190	-	-

PTT = Platform Terminal Transmitter; Colony Location: F=Faridpur, C-Chittagong; BCS = Body Condition Score: G=Good, F=Fair, P=Poor;

1. Geneious Prime 2019. https://www.geneious.com.
2. M. Hasegawa, Kishino, H., Yano, T.-a. J. J. o. m. e., Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. **22**, 160-174 (1985).
3. S. Kumar, Stecher, G., Li, M. *et al.*, MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. **35**, 1547-1549 (2018).

From:	Wang Linfa on behalf of Wang Linfa <linfa.wang@duke-nus.edu.sg>
To:	Jon Epstein; Anthony, Simon J.; Ariful Islam; marm@biology.ucsc.edu; Shahneaz Ali Khan; Noam Ross; ina.smith@csiro.au; Carlos M. Zambrana-Torrelio MSc; Yun Tao; Ausraful Islam; Kevin Olival, PhD; Salah Uddin Khan; Emily Gurley; Dr. Jahangir Hossain; Hume Field; Fielder, Mark; Thomas Briese; Mahmud Rahman; Christopher Broder; Gary Crameri; Stephen Luby; Ian Lipkin; Peter Daszak
Subject:	RE: Nipah dynamics in P medius draft for PNAS
Date:	Friday, January 10, 2020 9:55:11 PM

Well done and good luck to all!

Linfa (Lin-Fa) WANG, PhD FTSE
Professor & Director
Programme in Emerging Infectious Disease
Duke-NUS Medical School,
8 College Road, Singapore 169857
Tel: +65 6516 8397

From: Jon Epstein <epstein@ecohealthalliance.org>
Sent: Saturday, 11 January 2020 1:36 AM
To: Anthony, Simon J. <sja2127@cumc.columbia.edu>; Ariful Islam <arif@ecohealthalliance.org>; marm@biology.ucsc.edu; Shahneaz Ali Khan <shahneazbat@gmail.com>; Noam Ross <ross@ecohealthalliance.org>; ina.smith@csiro.au; Carlos M. Zambrana-Torrelio MSc <zambrana@ecohealthalliance.org>; Yun Tao <yun.tao.86@gmail.com>; Ausraful Islam <islam_ausraf@icddrb.org>; Kevin Olival, PhD <olival@ecohealthalliance.org>; Salah Uddin Khan <sukhanbd@gmail.com>; Emily Gurley <egurley1@jhu.edu>; Dr. Jahangir Hossain <jhossaincsd99@gmail.com>; Hume Field <hume.field@ecohealthalliance.org>; Fielder, Mark <m.fielder@kingston.ac.uk>; Thomas Briese <tb2047@cumc.columbia.edu>; Mahmud Rahman <mahmudur57@gmail.com>; Christopher Broder <christopher.broder@usuhs.edu>; Gary Crameri <garycrameri1@gmail.com>; Wang Linfa <linfa.wang@duke-nus.edu.sg>; Stephen Luby <sluby@stanford.edu>; Ian Lipkin <wil2001@columbia.edu>; Peter Daszak <daszak@ecohealthalliance.org>
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> I have an editor at PNAS willing to send this for review, so please send me any input you may have by December 28th so that I can submit by the 31st. Unless I hear otherwise, I will assume you still consent to co-authorship.
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> Thank you for your patience, and happy holidays!
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absence of ongoing transmission. Additionally, this does not take into account drivers of recrudescence - if this is stress related, then transmission at the population level will also be affected by these broader drivers. Given all the uncertainty around this topic, I think that the wording here needs to be precise. e.g the first sentence should first state the assumption "Assuming that seroconversion results only from new infections, then ...". This could be followed up with a sentence along the lines of "If however, seroconversion can result from recrudescence in the absence of transmission, then broader drivers of recrudescence would also need to be assessed". More clarity here would also help to assess the claims being made in the paragraph beginning 315.

Lines 240 onwards - consider restructuring this paragraph to more clearly step through each stage of the viral dynamics that you are proposing and your assumptions and evidence along the way. For example, you assume infection results in seroconversion, and that antibodies then wane after ~4 years. Are you assuming that all individuals are persistently infected, and it is only when Ab wane that recrudescence can occur? And following that, the individual seroconverts again?

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Lines 245-247: "via recrudescence FROM bats that have previously been infected"? It's also not clear how this links to lines 238-240

Lines 257-259: Consider also cite Peel et al (2018). Scientific Reports, 8(1), e0004796. This study examines waning maternal immunity and waning acquired immunity against henipaviruses in African fruit bats, finding a similar duration of immunity for both

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Lines 295-296 - this links with the SILI hypotheses in Plowright et al (2016) PLoS NTD (see Figure 3)

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Table S1 - there is extra text below Table S1 that looks like it's not supposed to be there
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Dear editor, I have reviewed the manuscript entitled “Nipah virus dynamics in bats and implications for zoonotic spillover to humans”. The manuscript describes a longitudinal surveillance in Pteropid spp. Bats from 2006 until 2012. Biometric data was collected, sera and swabs/urine were analyzed, and inferences were made largely based on serological data. The manuscript is a compendium of relatively loosely compiled data, ranging from seroprevalence in a variety of different sampling sites, but the majority of the samples stem from Faridpur. The problem is that most of the claims by the authors within the paper are not directly supported by the data. The direct problem of the data is the limited amount of detected virus shedding, out of 2789 animals sampled only 11 were found to be shedding the virus. From this only 8 were from the larger study cohort from Faridpur. This directly hampers some of the conclusion of potential spillover dynamics as this cannot be directly inferred from serological data alone. In addition, it does not provide any answers on the occurrence of Nipah spillover in the Nipah belt vs the other regions. Moreover, the significant spillover events in Kerala, India from the last two years, are not discussed. Most emphasis has been put on analyzing the serological results from the Pteropus medius bats. The authors show variation in the seroprevalence within the population based on timing and age status of the animal. The results have been reported before in other natural reservoir-pathogen systems like avian influenza, however even within these systems inferences on spillover can rarely be made. It interesting that the authors did not correlate the positive individual bats in Faridpur and Rajbari with their respective serostatus? Is this data not available? It is unclear to me why the authors have not put more effort in trying to perform full genome analyses on the positive samples obtained throughout this study. Currently there are only 27 full genomes available from Nipah virus and relatively limited amount are from the natural reservoir. Preforming phylogenetic analyses on a 224 nucleotide fragment of a 19kb virus is really not up to standard. Where it might be suitable for identification of the lineage no additional data can be inferred from this. Interestingly, there appears to be full genome sequencing performed but only N is shown in the supplemental data.

Although I do understand the logistics involved with this kind of work, unfortunately some of the claims, especially regarding spillover, need to be supported by more additional data rather than just serology.
Minor points:
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Subject:	RE: Nipah dynamics in P medius draft for PNAS
Date:	Friday, January 10, 2020 7:04:35 PM

Well done – finger's crossed…

Cheers,

Peter

Peter Daszak
President

EcoHealth Alliance
460 West 34th Street – 17th Floor
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Tel. +1 212-380-4474
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From: Fielder, Mark D on behalf of Fielder, Mark D <M.Fielder@kingston.ac.uk>
To: Jon Epstein; Anthony, Simon J.; Ariful Islam; marm@biology.ucsc.edu; Shahneaz Ali Khan; Noam Ross; ina.smith@csiro.au; Carlos M. Zambrana-Torrelio MSc; Yun Tao; Ausraful Islam; Kevin Olival, PhD; Salah Uddin Khan; Emily Gurley; Dr. Jahangir Hossain; Hume Field; Thomas Briese; Mahmud Rahman; Christopher Broder; Gary Crameri; Linfa Wang; Stephen Luby; Ian Lipkin; Peter Daszak
Subject: Re: Nipah dynamics in P medius draft for PNAS
Date: Friday, January 10, 2020 1:53:54 PM

Thanknyou so much for the hard work Jon really appreciated mate
Mark

Science is Vital- http://scienceisvital.org.uk/

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President of the Society for Applied Microbiology
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Webpage http://www.kingston.ac.uk/staff/profile/professor-mark-fielder-336/

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From: Jon Epstein <epstein@ecohealthalliance.org>
Sent: 10 January 2020 17:35:43
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<wil2001@columbia.edu>; Peter Daszak <daszak@ecohealthalliance.org>
Subject: Re: Nipah dynamics in P medius draft for PNAS

Dear co-authors,
Our paper has been submitted to PNAS! Thank you all for your thoughtful and helpful comments.
I think you've each received a link to the submission from PNAS, but if not, here's the version that was submitted.

I'll be in touch when I hear anything.

Cheers,
Jon

On Wed, Dec 18, 2019 at 3:00 PM Jon Epstein <epstein@ecohealthalliance.org> wrote:

> All,
> I hope this email finds you well. Attached is a draft of our manuscript entitled Nipah virus dynamics in bats and implications for spillover to humans. After multiple protracted periods of review in Science Advances, and ultimately a rejection, I've prepared this draft for submission to PNAS. I've revised the manuscript and updated figures and supplemental data in response to the last round of review (comments below, if you're interested).
>
> I have an editor at PNAS willing to send this for review, so please send me any input you may have by December 28th so that I can submit by the 31st. Unless I hear otherwise, I will assume you still consent to co-authorship.
>
> Thank you for your patience, and happy holidays!
>
> Cheers,
> Jon
>
> Reviewer: 1
> Epstein et al aim to better understand the distribution and drivers of Nipah virus infection dynamics in Pteropus medius in Bangladesh by analysing a large set of serological, virological and movement data over a commendable spatial and temporal scale. Overall, this is a highly exciting study with valuable results that are well-deserving of publication. I have a number of minor comments regarding the addition of detail for clarity (and to ensure transparency in the interpretation of results). I think that the conclusions are mostly justified, however, my major criticism is that the integration and interpretation of the results (particularly those presented in the first paragraph of the discussion) requires a little further thought and explanation. The assumptions surrounding the serological implications of within-host persistence and recrudescence needs to be clearly stated. This is likely to become a 'classic' paper, and there are so few studies in this area supported by data that it is important to ensure that the results are not over-interpreted.
>
> Specific comments:
> Abstract:
> Line 32- 33: The wording here is too strong regarding recrudescence. Suggest inserting "model results indicated that" prior to "local transmission dynamics.
> Line 33-34: Similarly - this is too strong. Suggest "likely due to "
> Results:
> Figure 1 - presumably the first three bats in the Comilla represent 2008 and the second three represent 2011, but this is not clear. This should be annotated on the plot
> Figure 1 - "Adult bats had equal or greater seroprevalence than juveniles in each location." - except Tangail?
> Line 129: "detected NiV RNA in 11 individuals, 3 pooled" - insert 'from' before 3
> Line 131: - describe "pooled samples" - pooled under-roost urine samples?
> Line 132 and 137: This 'figure 2' seems to be missing? Figure 2 refers to serological analyses

Line 145: I suggest that the reference to Figure 2 in line 146 should go in the sentence ending on line 145
Line 149: the significant negative association with body condition warrants mentioning in these results.
Line 154-155 and Figure 3: Additional clarity here regarding juvenile vs. yearling terms. Suggest saying "Juveniles in their first year of life (yearlings)" at first mentioning in the text, as well in the figure legend.
Line 159 - incomplete sentence
Lines 160-164: Fascinating results!
Lines 170-171: This information would be helpful to include earlier, with the serological results
Figure 3 and Figure S1 - It would be helpful to have a "total population" size from this roost complex included on Figures 3 and 4. Understanding more about the bat ecology and the size and stability of these populations over time would be a tremendous help to aid interpretation of these results by researchers working in other systems.
Lines 175-176: "Serodynamics in juveniles were strongly driven by inheritance and loss of maternal antibodies". Supported by what result? This should be explained further, and/or it would be helpful to include the serology (fig 3), model output (fig 4) and population size (Figure s1) together as a series of vertical panels in the one figure to aid interpretation
Lines 178-180: I can't see this information in the supplementary information
Line 182 - this paragraph (and ideally also table S2) should provide information on the duration of tracking for each bat. This would be very helpful in assessing the home range information
Line 195 - should the 'of' be 'if?
Figure 6 - the utility of this figure would be greatly improved with some annotation and colouring to help identify the new sequences from this study, and their source location, and the 'groups' that are referred to in the text
Lines 238 - 240 - So, your data has found cyclical serodynamics, but no clear links between those dynamics and detection of NiV in bats or in people. Based on experimental studies, it is still a bit unclear exactly what seropositivy in flying foxes represents. If there is within-host persistence of infection, a cycle of infection > seroconversion (in the absence of clearance) > seroreversion > then recrudescence with serconversion may occur in the absence of ongoing transmission. Additionally, this does not take into account drivers of recrudescence - if this is stress related, then transmission at the population level will also be affected by these broader drivers. Given all the uncertainty around this topic, I think that the wording here needs to be precise. e.g the first sentence should first state the assumption "Assuming that seroconversion results only from new infections, then ...". This could be followed up with a sentence along the lines of "If however, seroconversion can result from recrudescence in the absence of transmission, then broader drivers of recrudescence would also need to be assessed". More clarity here would also help to assess the claims being made in the paragraph beginning 315.
Lines 240 onwards - consider restructuring this paragraph to more clearly step through each stage of the viral dynamics that you are proposing and your assumptions and evidence along the way. For example, you assume infection results in seroconversion, and that antibodies then wane after ~4 years. Are you assuming that all individuals are persistently infected, and it is only when Ab wane that recrudescence can occur? And following that, the individual seroconverts again?
Lines 243 - 245: Not correct - See Brooks et al 2019 JAE Figure S5. That study also involved modelling and has implications for this study more broadly
Lines 245-247: "via recrudescence FROM bats that have previously been infected"? It's also not clear how this links to lines 238-240
Lines 257-259: Consider also cite Peel et al (2018). Scientific Reports, 8(1), e0004796. This study examines waning maternal immunity and waning acquired immunity against henipaviruses in African fruit bats, finding a similar duration of immunity for both
Line 267 - insert "in our study area" after 'Pteropus medius' as you cannot infer whether these patterns hold true for the species across its range. It may be highly dependent on available food resources - as you go on to discuss

Line 277 - replace 'be' with 'result in'
Lines 295-296 - this links with the SILI hypotheses in Plowright et al (2016) PLoS NTD (see Figure 3)
Line 321- suggest "three periods of transmission (significant at the population level) occurred"
Line 315 See my comments re: paragraph beginning Line 238. The sporadic nature of detections is challenging to draw conclusions and much of the paragraph here (starting line 315) seems over-stated. However, that may become clearer if the comments for the earlier paragraph are addressed.
Methods:
Line 364: I can't see reference to the Faripur colony in the list of colonies in lines 358-359. What are its characteristics? Also, I presume it should be "Faridpur'? Also, it would be helpful if the "Faridpur roost complex:" could be described in more detail in the methods as to the structure of this population and what this term means.
Line 366: Delete the first 'between'
Lines 388-390 - check the placement of parentheses here
Line 417: what cutoff was used for the Luminex assay and how was it determined?
Line 425 - fix formatting of second Ct
Line 493- 504: More information on the underlying population dynamic model is required e.g. Were births seasonal or continuous in your model? I can't see where this is stated. If not seasonal, then the effect of this on model output should be addressed. How was the death rate modelled? Was the total population size kept stable inter annually? The latter, in particular, may have implications for interpretation of the density-dependent vs frequency dependent results. The population size is touched on in the discussion in lines 288-291 but never really explained.
Table S1 - there is extra text below Table S1 that looks like it's not supposed to be there
Table S2 - Include what serostatus 0/1 refers to in the table caption

Reviewer: 2
Dear editor, I have reviewed the manuscript entitled “Nipah virus dynamics in bats and implications for zoonotic spillover to humans”. The manuscript describes a longitudinal surveillance in Pteropid spp. Bats from 2006 until 2012. Biometric data was collected, sera and swabs/urine were analyzed, and inferences were made largely based on serological data. The manuscript is a compendium of relatively loosely compiled data, ranging from seroprevalence in a variety of different sampling sites, but the majority of the samples stem from Faridpur. The problem is that most of the claims by the authors within the paper are not directly supported by the data. The direct problem of the data is the limited amount of detected virus shedding, out of 2789 animals sampled only 11 were found to be shedding the virus. From this only 8 were from the larger study cohort from Faridpur. This directly hampers some of the conclusion of potential spillover dynamics as this cannot be directly inferred from serological data alone. In addition, it does not provide any answers on the occurrence of Nipah spillover in the Nipah belt vs the other regions. Moreover, the significant spillover events in Kerala, India from the last two years, are not discussed. Most emphasis has been put on analyzing the serological results from the Pteropus medius bats. The authors show variation in the seroprevalence within the population based on timing and age status of the animal. The results have been reported before in other natural reservoir-pathogen systems like avian influenza, however even within these systems inferences on spillover can rarely be made. It interesting that the authors did not correlate the positive individual bats in Faridpur and Rajbari with their respective serostatus? Is this data not available? It is unclear to me why the authors have not put more effort in trying to perform full genome analyses on the positive samples obtained throughout this study. Currently there are only 27 full genomes available from Nipah virus and relatively limited amount are from the natural reservoir. Preforming phylogenetic analyses on a 224 nucleotide fragment of a 19kb virus is really not up to standard. Where it might be suitable for identification of the lineage no additional data can be inferred from this. Interestingly, there appears to be full genome sequencing performed but only N is shown in the supplemental data.

Although I do understand the logistics involved with this kind of work, unfortunately some of the claims, especially regarding spillover, need to be supported by more additional data rather than just serology.
Minor points:
Line 65: pandemic potential, given the limited amount of h-to-h transmission the pandemic potential of this particular virus appears to relatively limited.
Line 143: n=844 or n=883? Why do the numbers not match-up?
Line 410, include level of biosecurity involved in sample analyses. In addition, heatinactivation is typically to inactivate complement and not inactivation of the pathogen.
>From the current wording it is unclear what the authors mean by this? Complement inactivation or pathogen inactivation?
Line 428, is this data missing? Where is the NSG data? Why not data on the full genomes? I only was able to find the full N gene data in the supplemental figues.
Line 509, given that actual recrudescence in the context of virus shedding in the natural reservoir has never been shown it would be good to treat this a little bit more carefully.

--

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EcoHealth Alliance leads cutting-edge scientific research into the critical connections between human and wildlife health and delicate ecosystems. With this science, we develop solutions that prevent pandemics and promote conservation.

Nipah virus dynamics in bats and implications for spillover to humans

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Classification: Biological Sciences; population biology
Keywords: bats, henipavirus, Nipah virus, *Pteropus medius, Pteropus giganteus,* satellite telemetry, viral phylogeny, disease dynamics, modeling

Author Contributions: Study design: JHE, AMK,EG, MJH, HEF, TB, MR, SPL,WIL, and PD. Data collection/generation and analysis: JHE, SJA, AI, AMK, SAK, MS, NR, IS, CZT, YT, AuI, PLQ, KJO, MSK, TB, CCB, GC, LFW, WIL, and PD. Manuscript writing: JHE, SJA, AI, AMK, CZT, YT, KJO, EG, HEF, MDF, TB, GC, LFW, SPL, WIL, and PD.

This PDF includes: Main text, Figures 1-6; Table 1; Supporting Information Figures S1-S6; Tables S1-S2.

Abstract

Nipah virus (NiV) is an emerging bat-borne zoonotic virus with pandemic potential that causes near-annual outbreaks of fatal encephalitis in South Asia – one of the most populous regions on Earth. In Bangladesh, infection occurs when people drink date palm sap contaminated with bat excreta. Outbreaks are sporadic and the influence of viral dynamics in bats on their temporal and spatial distribution is poorly understood. We analyzed data on host ecology, molecular epidemiology, serological dynamics, and viral genetics to characterize spatio-temporal patterns of NiV dynamics in its wildlife reservoir, *Pteropus medius* bats, in Bangladesh. We found that NiV transmission occurred throughout the country and throughout the year. Model results indicated that local transmission dynamics were modulated by density-dependent transmission, acquired immunity that is lost over time, and recrudescence. Increased transmission followed multi-year periods of declining seroprevalence due to bat population turnover and individual loss of humoral immunity. Individual bats had smaller host ranges than other *Pteropus* spp., although movement data and the discovery of a Malaysia-clade NiV strain in eastern Bangladesh suggest connectivity with bats east of Bangladesh. These data suggest that discrete multi-annual local epidemics in bat populations contribute to the sporadic nature of Nipah virus outbreaks in South Asia. At the same time, the broad spatial and temporal extent of NiV transmission, including the recent outbreak in Kerala, India, highlights the continued risk of spillover to humans wherever they may interact with pteropid bats, and the importance of improving Nipah virus surveillance throughout *Pteropus*'s range.

Significance

Nipah virus (NiV) is a zoonotic virus and WHO priority pathogen that causes near-annual outbreaks in Bangladesh and India associated with >75% mortality. This work advances our understanding of the circulation of NiV in its natural bat reservoir by analyzing data from a 6-year multidisciplinary study of serology, viral phylogenetics, bat ecology and immunology. We show for the first time that outbreaks in *Pteropus* bats are driven by population density, loss of immunity over time and viral recrudescence, resulting in multi-year inter-epidemic periods. Incidence is low, but bats carry NiV across Bangladesh and can shed virus at any time of year, highlighting the importance of routes of transmission to the timing and location of human Nipah virus outbreaks.

Introduction.

Outbreaks of zoonotic diseases are often sporadic, rare events that are difficult to predict, but can have devastating consequences (1). Emerging viral zoonoses of wildlife that have become pandemic include HIV/AIDS, SARS coronavirus, and the 1918 H1N1 influenza virus (2-4). Bats are important hosts for many zoonotic viruses including ebola virus, SARS-CoV, and Nipah virus; the ecological drivers and transmission dynamics of these viruses in their reservoir hosts are poorly understood (5-10). A better understanding of the transmission dynamics of zoonotic pathogens in their natural reservoirs may help anticipate and prevent outbreaks (9, 11).

Nipah virus (NiV) is an emerging zoonotic paramyxovirus (genus *Henipavirus*) that has repeatedly spilled over from bats to cause outbreaks in people and livestock with high case fatality rates across a broad geographic range. To date, human Nipah virus infections have been identified in India, Bangladesh, Malaysia, Singapore, and the Philippines (12-16). It has caused repeated outbreaks in Bangladesh and India, with a mean case fatality rate greater than 70% (12, 17, 18). A single genus of frugivorous bats (*Pteropus*) appears to be the main reservoir for henipaviruses throughout Asia and Australia (19-23), including *Pteropus medius* (formerly *Pteropus giganteus* (24)) in Bangladesh and India (14, 25-27). Nipah virus has several characteristics that make it a significant threat to human and animal health: 1) its bat reservoir hosts are widely distributed throughout Asia, overlapping dense human and livestock populations, providing broad opportunity to cause outbreaks; 2) it can be transmitted directly to humans by bats or via domestic animals; 3) it can be transmitted from person to person; 4) spillover has repeatedly occurred in highly populous and internationally connected regions, giving it pandemic potential; 5) it is associated with high mortality rates in people; and 6) there are currently no commercially available vaccines to prevent infection or drugs to mitigate disease (28-30). As a result, the World Health Organization has listed Nipah virus among the ten most significant threats to global health (31). In May 2018, an outbreak of Nipah virus encephalitis associated with a 91% mortality rate occurred in a new location - Kerala, India - more than 1,200 km southwest of previous Indian and Bangladeshi outbreaks (32). A single case was subsequently reported in Kerala in 2019, and while local *P. medius* populations have been implicated as the local source of infection, the route of spillover in both instances remains unknown (32, 33).

In Malaysia and Bangladesh, consumption of cultivated food resources contaminated with bat excreta such as mangoes in Malaysia and date palm sap in Bangladesh and northeastern India have been identified as the predominant cause of spillover to pigs and people, respectively (34). Human outbreaks occur almost annually in Bangladesh and the seasonal timing (November-April) and spatial distribution of outbreaks coincide with patterns of raw date palm sap consumption in a region termed the “Nipah belt” (35). However, there is variability in the geographic locations and number of spillover events, as well as the number and magnitude of human outbreaks that occur each year (36). Spillover has also occurred outside the predominant season and region of date-palm sap consumption (37). Whereas no human outbreaks have been reported in eastern Bangladesh despite date palm sap harvesting and consumption, human outbreaks have been reported in Kerala, India where date palm sap is not cultivated (35). These observations suggest an alternate route of spillover in certain locations, and a critical need to understand the mechanisms of underlying viral infection dynamics in bats and the extent

of genetic diversity within the virus – each of which may influence the timing, location and epidemiology of human outbreaks (35).

Previous research on the transmission dynamics of Nipah and Hendra viruses in *Pteropus* spp. bats has produced mixed and sometimes contradictory findings. Nipah virus, like Ebola, Marburg, Hendra and some bat coronaviruses, are associated with seasonal spikes in infection that coincide with annual or semi-annual synchronous birth pulses (19, 38-44). Seasonal periods of Nipah virus shedding were observed in *P. lylei* in Thailand and seasonal spikes in NiV (or a related henipavirus) seroprevalence coinciding with pregnancy periods were observed in *Eidolon dupreanum* in Madagascar (45, 46), but not in *P. vampyrus* or *P. hypomelanus* in Peninsular Malaysia (23). Hendra virus prevalence in Australian pteropid bats has shown multi-year inter-epidemic periods during which little virus can be detected, followed by periods of markedly increased viral shedding (47-49). It has been hypothesized that multi-year periodicity in the incidence of henipavirus infections could arise from a build-up and waning of herd immunity in the reservoir host, with re-introduction of virus via immigration or recrudescence or viral persistence (10, 50-52). Some pteropid bat species are migratory and interconnected colonies form a metapopulation which could allow for viral re-introductions (9, 23, 53, 54). In addition, NiV recrudescence has been observed in wild-caught *P. vampyrus* and possibly also in *Eidolon helvum* (55-57). Either of these phenomena could allow it to persist regionally during periods of high local immunity. However, no study has yet shown evidence in open, free-ranging bat populations that favors one or the other hypothesis in driving NiV transmission dynamics.

Here we examine the distribution, dynamics, genetic diversity, and underlying drivers of NiV infection in *Pteropus medius* in Bangladesh to improve our understanding of human outbreak patterns. Specifically, we analyze the spatial, temporal and demographic variation in serological dynamics and viral shedding in bats over a six-year period to determine the spatio-temporal drivers and dynamics of virus transmission. We also analyze the movement patterns of individual bats and analyze NiV phlyogenetics to understand patterns of spatial mixing and virus strain diversity.

Results

Comparative Nipah virus prevalence study in bats inside and outside the Nipah Belt and concurrent longitudinal bat study inside the Nipah Belt.

In a cross-sectional spatial study conducted between January 2006 and July 2012, we caught and tested 883 *P. medius* (~100 per district) from eight colonies in different districts across Bangladesh. We detected anti-Nipah IgG antibodies in all colonies (**Figure 1**). Seroprevalence varied by location (χ^2 = 55.61, p<.001). In all locations except Tangail, adult seroprevalence exceeded juvenile seroprevalence. Viral detection in individuals was rare; overall, we detected NiV RNA in 11/2088 individuals as well as three pooled oropharyngeal samples (representing five bats, but which could not be resolved to an individual) (**Table 1**). We detected viral RNA in individual bats in Faridpur and Rajbari and from pooled samples from Thakurgaon and roost urine samples from Comilla. Of the 11 PCR positive individuals, three had IgG antibodies (**Table S1**). We also detected virus in pooled urine collected from tarps placed below bats at roosts associated with human outbreaks in Bhanga and Joypurhat. The viral prevalence in Rajbari in January 2006 was 3.8% (95% CI: 0% -11%; n=78). In Faridpur, where we also conducted an intensive longitudinal study of (see below), viral prevalence estimates ranged from 0% to 3% (95% CI:

0%-10%; n=100 at each of 18 sampling times) (**Table 1**). Nipah virus RNA was detected in individual bats from inside (Rajbari, Thakurgaon, and Faridpur) and outside (Comilla) the Nipah Belt. There was no significant difference between NiV detection rates from individual bats by the two main sample types: urine/urogenital swabs, 0.37% (n=2,126) and oropharyngeal swabs, 0.15% (n=1973) (χ^2= 1.92 p=0.17). The estimated detection rate from pooled urine samples, collected from tarps placed underneath roosts) across the entire study was 2.5% (n=829), which was significantly higher than either sample type collected from individual bats (χ^2 = 55.6, p < 0.001).

Factors associated with NiV IgG *serostatus in* P. medius

There was no statistical difference between seroprevalence in bats inside the Nipah Belt and outside (odds ratio 90% high density interval of 0.55-2.5)**.** Adults had higher seropositivity than juveniles (OR 2.4, 1.7-3.4 HDI), and males greater than females (OR 1.6, 1.1-2.2 HDI) (**Figure 2**). Among females, odds ratios were weakly higher in pup-carrying (4 times, HDI 0.7-21) and pregnant (1.5 times, HDI 0.89-2.5) individuals. Weight, forearm length nor the W:FA ratio (a proxy for age) did not consistently correlate with seropositivity, however, body condition (an assessment of pectoral muscle mass by palpation) was significantly negatively correlated (Poor/Fair body condition OR = 0.69, HDI 0.52-0.90) with serostatus. Finally, serostatus was strongly correlated in mother-pup pairs, with 71/80 pairs (89%) having matching status.

Longitudinal NiV serodynamics in P. medius*, Faridpur district (2006-2012)*

We sampled bats quarterly from the same population in the Faridpur district, collecting biological samples, as described above, and microchipping a total of 2,345 bats between 2007 and 2012. We used generalized additive models (GAMs) to characterize changes in NiV seroprevalence over time. There were significant fluctuations in adult (>24 mo.) and juvenile (6 – 24 mo.) seroprevalence over the six-year study period (**Figure 3A**). Juvenile seroprevalence ranged from 0% to 44% (95%CI 37%-51%), and decreased over the first year of life for bats born in each year ("yearlings"), consistent with loss of maternal antibodies in juveniles. A more pronounced decrease occurred from mid-October to mid-December. However, the GAM indicating this had only marginal better fit (ΔAIC < 1), than one with a linear decrease over the whole year (**Figure 3B**). The effect of introducing annual cohorts of pups via synchronized birthing pulses was significant on overall seroprevalence.

Adult seroprevalence ranged from 31% (95% CI: 20%-46%) to 82% (95% CI: 77%-87%) with three cycles of clear variability over the course of the study (**Figure 3A**). We found no evidence of regular seasonal fluctuations; a GAM with annual cyclic terms fit worse than one without (ΔAIC > 10). Viral RNA was detected during periods of increasing, decreasing, and stable seroprevalence.

We fitted a series of age-stratified mechanistic models to examine different biological processes influencing serodynamics, including density- vs. frequency-dependent transmission, recrudescence vs. immigration of infected individuals, and seroreversion (loss of antibodies) in both juveniles and adults (see Methods and (**Figure 4**)). Density-dependent models were a far better fit to the data than frequency-dependent models (difference in log-likelihood 10.0; ΔAIC = 20.0), suggesting that movements of bats and fluctuations in colony size alter spatio-temporal variation in the risk of NiV infection in bats. In Faridpur (see "Domrakhandi/Khaderdi" in **Figure S1**) during the period of sampling,

the roost population declined from approximately 300 bats to 185, which decreased transmission potential in the fitted model: R_0 in adult bats was estimated to decrease from 3.5 to 2.1 as the number of bats in the colony decreased. As a result, over the six-year study period, the fitted model predicted that the number of infected bats increased when the seroprevalence of adults fell below 72% (when bat counts were highest - in 2006) and 52% (when bat counts were lowest).

The fitted model suggests that serodynamics in juveniles were strongly driven by inheritance and loss of maternal antibodies. The rate of loss of maternal antibodies was 17.6 weeks (95% CI: 13.7-25.0), which was much quicker than the loss of antibodies in adults (290.8 weeks, 95% CI: 245.0-476.4) (**Table S2**). Finally, models with recrudescence fit the data better than models without recrudescence (**Table S2;** difference in log-likelihood 32.6; ΔAIC = 65.1), and models with recrudescence fit the data better than models with immigration (ΔAIC = 3.76).

Mark-recapture and seroconversion/seroreversion

There were 56 recapture events over the study period (**Table S3**). Thirty-one bats were recaptured at a roost other than the original capture location. This network of roosts or "roost complex" formed a polygon covering approximately 80 Km^2 and included 15 roosts sampled during the longitudinal study (**Figure S2A and S2B**). Ten instances of seroconversion (change from IgG negative to IgG positive) and nine instances of seroreversion (positive to negative) were observed (**Table S3**). The mean time between positive and negative tests in *adults* (excluding juveniles with maternal antibodies) was 588 days (n=6) (range: 124-1,082 days).

Home range and inter-colony connectivity analysis

Home range analysis of satellite telemetry data from 14 bats (mean duration of collar data transmission = 6.25mo; range = 1-25mo; **Table S4**) showed that the majority of bats roosted within 10 km of where the bats were originally collared, in the Faridpur (Nipah belt) colony, and within 7 km from where the bats in the Cox's Bazaar colony were originally collared (315km east of Faridpur). The average foraging radius was 18.7 km (s.d. 21.5 km) for the Faridpur bats and 10.8 km (s.d. 11.9 km) for the Cox's Bazaar bats (**Figure S2**). Home range analysis suggests that bats in Faridpur and Cox's Bazar would have a <5% probability of intermingling (**Figure 5**). Home-range size was larger during the wet season than the dry season (2,746 km^2 vs. 618 km^2) (**Figures S3 & S4**).

NiV phylogenetic analysis.

Phylogenetic analysis of NiV sequences from a 224nt section of the N gene (nt position 1290-1509 [position ref gb|FJ513078.1| India]) suggests that strains from both India and Malaysia clades are present in bats in Bangladesh (**Figure 6**). This finding is supported by an additional analysis of near-whole N gene sequences (~1720 nt) from bats, pigs, and humans, including those from a subset of *P. medius* from this and a more recent study by our group (**Figure S5**) (58). Eleven 224nt N gene sequences obtained from bats between 2006 and 2012 (all from the Faridpur population) were identical. Overall, the N gene sequences identified from the Faridpur, Rajbari and Bhanga colonies between 2006 and 2011 had 98.21%-100% shared nucleotide identity. Sequences from Rajbari district obtained five years apart (January 2006 and January 2011) had only a single nucleotide difference resulting in a synonymous substitution (G to A) at position 1304, which was found in four other bat NiV sequences from this study,

as well as in the NiV isolate from *P. vampyrus* in Malaysia. Five human NiV N gene sequences from various locations within the Nipah belt over the same time period as our bat study show more nucleotide diversity than those from the Faridpur *P. medius* population. Human sequences throughout Bangladesh and from Kerala, India, all nested within the diversity found in *P. medius* (**Figure 6**). By contrast, the sequences found in *P. medius* from Comilla, a location 150Km to the east of Faridpur, showed 80.8%-82.59% shared nucleotide identity with sequences from *P. medius* in Faridpur and clustered within the Malaysia group of NiV sequences. The two Comilla sequences were identical to each other, and had up to 87.95% shared nucleotide identity to sequences from *P. lylei* in Thailand.

Discussion

Our study provides new insights into Nipah virus transmission dynamics, genetics and host ecology. Previous studies from Bangladesh suggested that human NiV outbreaks occur primarily (though not exclusively) within a defined region in western Bangladesh, termed the "Nipah belt," during a defined season (Nov-Apr)(37, 59). These observations raised the question of whether human infections were due solely to the frequency of date palm sap consumption, or whether ecological factors such as the distribution and timing of bat viral infection also influenced the timing and location of human cases (17, 35, 60). We undertook an extensive survey of *Pteropus medius* in Bangladesh to understand Nipah virus infection patterns in its putative reservoir, which is common in Bangladesh and throughout the Indian subcontinent (14, 25, 61).

Overall, our findings suggest viral circulation in bats is not limited to the Nipah belt, but that NiV transmission occurs in bat populations throughout the country. We observed that virus can be shed by bats at any time of year, and that viral dynamics are cyclical but not annual or seasonal. Our models fit to serological data suggest that these cycles may be driven by demographic and immunological factors; the waning of herd immunity through turnover or individual waning in bat populations allows heightened viral transmission when seroprevalence passes below a critical threshold.

A number of mechanisms have been proposed for the maintenance of acute viral infections in bat meta-populations, including synchronous birthing and subsequent loss of maternal antibodies (10, 39, 41), lowered immunity within pregnant females due to stress, nutritional stress and other factors (43) immigration of infected individuals from other colonies (53, 62, 63), and recrudescence within previously-infected individuals (10, 55, 64). Our modeling indicates that NiV is primarily driven by density-dependent transmission dynamics among adult bats, with cycles of higher seroprevalence that would dampen intra-colony transmission followed by waning of antibody titers within individuals and at a population level. Waning humoral immunity against Nipah virus is a consistent feature of henipavirus studies in African pteropodid bats (52, 65). Our recapture data provided the first reported evidence of the loss of detectable NiV IgG antibodies in recaptured individual free-ranging bats, which supports our observation of population level waning immunity. The consistently lower and decreasing seroprevalence that we observed in juveniles suggests that they lose maternal antibodies over their first year, and likely in the first 6-7 months, consistent with other studies of maternal antibodies against henipaviruses in pteropodid bats (43, 52, 56, 66). However, our data and analysis do not support the hypothesis that seasonal pulses of these new seronegative individuals are the primary driver of new outbreaks in adults (41).

Our model outputs suggest that spikes in viral transmission occur within colonies after immunity has waned. Nipah virus reintroduction within a colony may occur from a persistently infected (e.g. recrudescence) or the introduction of an infected individual via immigration. Recrudescence of henipavirus infection has been observed for NiV in captive *P. vampyrus* (55), for henipavirus in captive *E. helvum* (52, 57), and has also been observed in humans infected by NiV (67) and Hendra virus (68). It is difficult to know from serology alone whether wild-caught seronegative bats had been previously infected. Experimental infection of naïve and previously infected *Pteropus medius* that have sero-reverted would provide a better understanding of how humoral immunity influences individual susceptibility to infection, and inform dynamics models attempting to explain viral maintenance within bat populations (65).

Our longitudinal study is limited in that it may not reflect temporal infection dynamics in all bat populations across Bangladesh. Our roost count data and recapture data from microchipped bats showed how roost sizes can fluctuate, and local roost shifting can occur. The observation of individual bats using multiple roost sites suggests that changes in roost count, which our models suggest impacts transmission dynamics, could reflect local shifts in densities rather than fluctuations in regional populations.

Understanding how bat populations connect across landscapes is important for understanding viral maintenance, and studying local and migratory bat movements can provide important ecological information related to viral transmission, including how bats move between different colonies (53, 69). Our satellite telemetry data suggest that *P. medius* exists as a metapopulation, like other pteropid species (10, 63). However, *P. medius* appear to travel shorter distances and remain within a smaller home range (321.46km^2 and 2,865.27km^2 for two groups) compared to *P. vampyrus* in Malaysia (64,000 km^2 and 128,000 km^2) and *Acerodon jubatus* in the Philippines which are similarly sized fruit bats (53, 70). Pteropodid bat migration is primarily driven by seasonal food resource availability (54, 71-73). In Bangladesh, *P. medius* prefer to roost in human-dominated environments in highly fragmented forests (74). The anthropogenic colonization and conversion of land over recent human history has likely led to increased food availability for *P. medius* and reduced the impetus for long-distance migration (34). This may reflect a similar adaptation to anthropogenic food resources as observed over the last few decades in Australian *Pteropus* species (63). Genetic analysis of *P. medius* across Bangladesh has shown that the population is panmictic – that historically, there has been extensive gene flow and intermixing among populations across Bangladesh(69). However, if bat dispersal is currently more localized than population genetic data suggest, as shown in satellite-tracked individuals, then less connectivity among populations may influence Nipah transmission by creating longer inter-epidemic periods and larger amplitude fluctuations in population level immunity compared to more migratory species (63).

Bat movement and population connectivity may also influence the genetic diversity of Nipah virus found in different locations, and genotypic variation has been associated with different clinical outcomes in people. While the overall strain diversity among Nipah virus has not been well characterized due to a dearth of isolates, two distinct NiV clades have been described: A Bangladesh clade, that includes sequences identified in India and Bangladesh; and a Malaysian clade, that comprises sequences from Malaysia, Cambodia, The Philippines and Thailand (16, 58, 75). Strains of NiV from these two clades are associated with differences in pathogenesis, epidemiological and clinical profiles in

humans and animal models and observed shedding patterns in bats (45, 76-80). Phenotypic variation in Nipah virus could influence observed human outbreak patterns by altering transmission to, or pathogenesis in, humans, and the likelihood of smaller outbreaks being identified or reported (81). Human-to-human NiV transmission via contact with respiratory and other secretions has been regularly observed in Bangladesh and India, including the recent 2018 outbreak in Kerala (12, 59, 82), whereas transmission among people was not a common feature of the Malaysia outbreak, despite close contact between cases and health care providers (83, 84). Nipah virus cases in Bangladesh have shown more strain diversity than in the Malaysia outbreak (85).,

Until now, there have been very few Nipah sequences obtained from *P. medius.* We found that Nipah N-gene sequence from bats from the Faridpur population were nearly identical over time, compared to variation in N-gene sequences from bats and humans from other locations observed over the same time period (2006-2010). This suggests that there may be locally prevalent and stable NiV genotypes that persist within bat colonies. Human NiV genotype diversity is likely a reflection of the relative diversity of the NiV strains in the local bats that seed outbreaks (9). This is also supported by viral sequences obtained from human and bats associated with the 2018 NiV outbreak in Kerala, India, where human NiV sequences were most closely related to local *P. medius* sequences *(86)*. We found a significantly divergent NiV strain in Comilla, which clustered within the Malaysia NiV clade, suggesting that strains from both clades are circulating in bats in Bangladesh.

Connectivity of pteropid bats in Bangladesh with those in Southeast Asia could explain the observed strain diversity in our study. Historical interbreeding of *P. medius* with pteropid species found in Myanmar, Thailand, and Malaysia and our telemetry findings showing bats are capable of flying hundreds of kilometers, could explain our discovery of a Malaysia clade NiV sequence in bats from Comilla (69). NiV Bangladesh strains have also been found in *P. lylei* in Thailand (87). The N gene of the Comilla NiV strain differs from those reported from bats in the Nipah belt by 20%, whereas NiV Malaysia and NiV Bangladesh differ by only 6-9% and are associated with different clinical profiles. Whole genome sequence (which could not be obtained) would have allowed for better characterization of the Comilla strain, but the N gene is generally conserved relative to other genes, and suggests the rest of the genome may also be highly divergent. It is therefore plausible that the clinical profile of a 20% divergent NiV strain differs significantly from known strains. Sequence information from an isolated human NiV case in Comilla has not been reported, so comparison to sequence we found in bats was not possible (37). Studies linking viral genotype to clinical phenotype in people would provide insight into the implications of strain diversity in bats for human outbreaks.

Finally, our study sheds light on the sporadic nature of NiV outbreaks with multi-year inter-epidemic periods in South Asia. PCR results show that overall NiV incidence in *P. medius* is low, consistent with previous studies of Hendra and Nipah virus (43, 48, 88, 89). Our modeling suggests that PCR-positive samples are more likely to be identified during viral transmission spikes after periodic reintroduction into populations that have become susceptible through waning immunity (10). Viral detection in bats has also coincided with human outbreaks (58, 86). This is likely a rare or at least sporadic event. In the current study, observed seroprevalence patterns and the fitted model suggest that three periods of transmission occurred over the 6 years of sampling, each of which followed periods of low adult seroprevalence, though not all measurements of low seroprevalence were followed by

outbreaks. We detected NiV RNA during periods of both increasing and decreasing seroprevalence, supporting the fitted model that suggested that shedding can occur even in periods without sustained transmission. This could explain variation in the number of human outbreaks (e.g. spillover events) from year-to year in Bangladesh. Thus, the timing of multiple factors involved in driving transmission dynamics needs to align for intra-colony NiV transmission events and further align with human behavior and availability of a route of spillover for human outbreaks to occur, as previously hypothesized (90). Thus, bat NiV dynamics combined with the seasonality and specific geography of date palm sap consumption in Bangladesh likely explains the sporadic nature of human outbreaks in the region (35).

These findings suggest that Nipah virus outbreaks in other regions of Bangladesh where *Pteropus* spp. bats occur, are also likely to be sporadic and rare, leading to under-reporting or a lack or reporting, particularly given that human neurologic symptoms are similar to other common infections, such as Japanese encephalitis, malaria, and measles (91). Understanding whether some NiV strains are capable of causing mild or asymptomatic cases will provide important insights about why outbreaks may not have been detected in areas such as eastern Bangladesh or other parts of Asia, where host, virus, and potential routes of spillover exist. Mild or asymptomatic cases would be unlikely to be detected by current surveillance systems and it's possible that cryptic spillovers have occurred in Bangladesh, where about half of all outbreaks between 2007 and 2014 were unreported (92). Our work and other reports suggest that Nipah virus transmission is possible wherever *Pteropus* spp. bats and humans live in close association and at any time of year, provided there is an available route of transmission. The 2018 and 2019 spillover events in Kerala, India, which were linked to local *P. medius* colonies and which occurred in an area that does not cultivate date palm sap, further emphasize this point.

Identifying areas where high risk interfaces exist between pteropid bats and people, throughout their range, will be important for monitoring Nipah spillover events and quickly responding to outbreaks, as well as establishing interventions to prevent spillover. Raising awareness of the potential for contaminated food to be a route of Nipah virus transmission and in protecting food resources to limit human or livestock exposure, may be effective in reducing the risk of a more transmissible strain of Nipah virus from emerging and causing an epidemic with significant human and animal mortality.

Methods

The study period was between January 2006 and November 2012. The study was conducted under Tufts University IACUC protocol #G929-07 and icddr,b AEEC protocol 2006-012 with permission from the Forest Department, Government of Bangladesh. Locations were selected based on whether the district had any previously recorded human NiV encephalitis clusters at the time of this study and was therefore inside the Nipah Belt (e.g. Rajbari, Tangail, Thakurgaon, and Kushtia) or whether they had not and were outside the Nipah Belt (e.g. Comilla, Khulna, Sylhet, and Chittagong). The Thakurgaon study was conducted as part of an NiV outbreak investigation, and coincided with ongoing human transmission (93). Between 2006-2012, three different studies of *Pteropus medius*, with similar bat sampling protocols were performed: 1) a cross-sectional spatial study with a single sampling event in each of the eight locations listed above; 2) a longitudinal study of a Faripur bat colony with repeated sampling approximately every 3 months from July 2007 to November 2012; and 3) a longitudinal study of the Rajbari colony with repeated sampling at a monthly interval between 12 month period between April 2010 and May 2011. Opportunistic sampling of *Pteropus medius* was also performed during this time

period during NiV outbreak investigations [Bangha, Faridpur (Feb 2010), Joypurhat (Jan 2012), Rajbari (Dec 2009), West Algi, Faridpur (Jan 2010)]. Bats were captured using mist nets at locations within eight different districts across Bangladesh between January 2006 and December 2012 (**Figure 1**).

Capture and sample collection

For the country-wide cross-sectional and Faridpur longitudinal study, approximately 100 bats were sampled at each sampling event, which lasted 7-10 days. This sample size allowed us to detect at least one exposed bat (IgG antibody-positive) given a seroprevalence of 10% with 95% confidence. Bats were captured using a custom-made mist net of approximately 10 m x 15 m suspended between bamboo poles which were mounted atop trees close to the target bat roost. Catching occurred between 11 pm and 5 am as bats returned from foraging. To minimize bat stress and chance of injury, nets were continuously monitored and each bat was extracted from the net immediately after entanglement. Personal protective equipment was worn during capture and sampling, which included dedicated long-sleeve outerwear or Tyvek suits, P100 respirators (3M, USA), safety glasses, nitrile gloves, and leather welding gloves for bat restraint. Bats were placed into cotton pillowcases and held for a maximum of 6 hours before being released at the site of capture. Bats were sampled at the site of capture using a field lab setup. Bats were anesthetized using isoflurane gas (94) and blood, urine, oropharyngeal swabs, and wing membrane biopsies (for genetic studies) were collected. For some sampling periods, rectal swabs were collected but due to resource constraints, these samples were deemed to likely be lower yield than saliva and urine for NiV, and were discontinued during the study. For each bat sampled we recorded age, weight, sex, physiologic and reproductive status, and morphometric measurements as described previously (25). Bats were classified as either juveniles (approximately four to six months - the age by which most pups are weaned) to two years old (the age when most *Pteropus* species reach sexual maturity) or adults (sexually mature) based on body size and the presence of secondary sexual characteristics, pregnancy, or lactation - indicating reproductive maturity (25, 95).

Up to 3.0 mL of blood were collected from the brachial vein and placed into serum tubes with serum clot activator (Vaccutainer, USA). Blood tubes were stored vertically on ice packs in a cold box and serum was allowed to separate overnight. Serum was drawn from the tube after 24 hours and placed in a screw-top cryovial (Corning, USA) and stored in a liquid nitrogen dewar (Princeton Cryogenics, NJ, USA). Sterile pediatric swabs with polyester tips and aluminum shafts were used to collect urogenital and rectal samples, and larger polyester swabs with plastic shafts (Fisher, USA) were used to collect oropharyngeal samples. All swabs were collected in duplicate, with one set being placed individually in cryotubes containing lysis buffer (either tri-reagent or NucliSENS Lysis buffer, BIOMERIEUX, France) and the second set in viral transport medium. All tubes were stored in liquid nitrogen in the field then transferred to a -80C freezer.

During each sampling event, pooled urine samples were collected beneath bat roosts using polyethylene sheets (2' x 3') distributed evenly under the colony between 3 am and 6 am. Urine was collected from each sheet either using a sterile swab to soak up droplets, or a sterile disposable pipette. Swabs or syringed urine from a single sheet were combined to represent a pooled sample. Each urine sample was divided in half and aliquoted into lysis buffer or VTM at an approximate ratio of one part sample to two parts preservative.

Serological and molecular assays

Sera from the cross-sectional survey were heat inactivated at 56°C for 30 minutes, as described (96) prior to shipment to the Center for Infection and Immunity at Columbia University (New York, USA) for analysis. Samples were screened for anti-NiV IgG antibodies using an enzyme linked immunosorbent assay (ELISA) as described in (25). Sera from the longitudinal studies were sent to the Australian Animal Health Laboratory and were gamma irradiated upon receipt. Because of the large sample size and development of a high throughput multiplex assay of comparable specificity and sensitivity, for these samples we used a Luminex®-based microsphere binding assay to detect anti-Nipah G IgG antibodies reactive to a purified NiV soluble G protein reagent, as described previously (97, 98). Samples resulting in a Median Fluorescent Intensity (MFI) value of over 1000 were considered positive for NiV antibodies in this study. An MFI of 250 and below is considered negative for other bat species and previous studies have reported using a threshold of at least three times the mean MFI of negative sera to determine the cutoff (43, 99-101). This approach is considered appropriate for research purposes for bats, and was used to determine the cutoff in this study.

Total nucleic acids from swabs and urine samples were extracted and cDNA synthesized using Superscript III (Invitrogen) according to manufacturer's instructions. A nested RT-PCR and a real-time assay targeting the N gene were used to detect NiV RNA in samples (102). A RT-qPCR designed to detect the nucleocapsid gene of all known NiV isolates was also utilized (103). Oligonucleotide primers and probe were as described (103). Assays were performed using AgPath-ID One-StepRT-PCR Reagents (Thermofisher) with 250 nM probe, 50 nM forward and 900 nM reverse primers. Thermal cycling was 45◦C for 10 min, followed by 45 cycles of 95◦C for 15 s and 60◦C for 45 s. Cut-off values were cycle threshold (C_T) ≤40 for positive and CT ≥45 for negative. Results with C_T values between 40 and 45 were deemed indeterminate, i.e. not conclusively positive or negative. Samples with NiV RNA detected by real time PCR were confirmed by gel electrophoresis and product sequencing.

A subset of NiV-positive samples was processed by high-throughput sequencing (HTS) on the Ion Torrent PGM platform in order to obtain additional NiV genomic sequence. Libraries were prepared according to the manufacturer's instructions, and 1 million reads allocated per sample. HTS reads were aligned against host reference databases to remove host background using bowtie2 mapper, and host-subtracted reads primer trimmed and filtered based on quality, GC content and sequence complexity. The remaining reads were de novo assembled using Newbler (v2.6) and mapped to the full length NiV genome. Contigs and unique singletons were also subjected to homology search using MegaBlast against the GenBank nucleotide database, in case variance in parts of the genome precluded efficient mapping. From these data, N gene consensus sequences were constructed using Geneious v 7.1, and used for phylogenetic analyses.

Phylogenetic analysis

All *P. medius* NiV sequences have been submitted to Genbank and accession numbers are included in **Figure 6**. Sequence alignments were constructed using ClustalW in Geneious Prime software (104). Phylogenetic trees of NiV N-gene sequences were constructed using Neighbor-Joining algorithms and figures constructed in FigTree 1.4.2.

Satellite telemetry and home range analysis

We developed a collar system to attach 12g solar-powered Platform Terminal Transmitters (PTT) (Microwave Telemetry, Colombia, MD, USA) to adult bats using commercial nylon feline collars with the buckle removed and 0-gauge nylon suture to attach the PTT to the collar and to fasten the collar around the bat's neck. Collars were fitted to the bat such that there was enough space to allow for normal neck movement and swallowing, but so that the collar would not slip over the head of the animal (**Figure S6**). PTTs were programmed with a duty cycle of 10 hours on and 48 hours off. Data was accessed via the Argos online data service (argos-system.org; France). A total of 14 collars were deployed as follows: Feb 2009: 3 males, 3 females from a colony in Shuvarampur, Faridpur district; Feb 2011: 3 males, 2 females from the same colony; and April 2011 Cox's Bazaar, 3 bats from a colony in Cox's Bazaar, Chittagong district. Bats were selected based on size such that the total weight of the collar (~21g) was less than 3% of the bat's body mass (Table S3).

The individual telemetry dataset was combined for each region and its aggregate utilization distributions (UD) computed in R using package 'adehabitatHR' (105). Population-specific home range is represented by the *95% area enclosure of its UD's volume. The volume of intersection between the colonies quantifies the extent of home range overlap. To evaluate the potential for contact with the Sylhet colony, we calculated the most likely distance moved ('mldm') for each sampled bat at Faridpur where the population was more intensively monitored. Movement distance was measured in kilometers with respect to a centroid location (ω) shared by the whole colony; assuming random spatial distribution in tracking rate, we estimated its kernel density function per individual and defined mldm as the mode.

Statistical approach – cross-sectional study

We fit a Bayesian generalized linear model with a logit link and a Bernoulli outcome to identify potential predictors which influenced a bat's serostatus. We included age, sex, age- and sex-normalized mass and forearm length, mass:forearm ratio, body condition, and whether the bat was pregnant, lactating, or carrying a pup, using weak zero-centered normal priors for all (normalized) coefficients. We included location of sampling as a group effect (similar to a random effect in a frequentist context) nested within Nipah Belt or non-Nipah Belt regions. We fit the models and performed posterior predictive checks in R 3.4.3, using the **rstanarm** and **rstan** packages.

Statistical approach – longitudinal study

We fit binomial general additive models (GAMs) (106) to the time series of adult and juvenile seroprevalence in the longitudinal study. We included annual, synchronous birthing that occured between March and April. We assumed that pups weaned from their dams at 3 months, and became independent flyers, and that maternal antibodies waned after 6 months at which point pups transitioned into the "juvenile" class (61, 66). We assumed that juveniles became sexually mature at 24 mo., and entered the "adult" class based on other pteropid species (43, 61, 107). For juveniles, we modeled the birth cohort of bats as separate random effects in a pooled model of juveniles' dynamics starting from June of their birth year, June being the earliest month we sampled free-flying juveniles in any cohort. We determined the cohort year of juveniles by using cluster analysis to group individuals by weight, assuming those in the smallest group were born in the current year and those in the larger

group were born the previous year. 92% of juveniles captured were yearlings. For adults, we modeled dynamics of adults as a single pool over the entire course of the study. We tested models with and without annual cyclic effects.

Where time series had significant temporal autocorrelation (adults only), we aggregated data by week. We determined periods of significant increase in decrease as those where the 95% confidence interval of the GAM prediction's derivative did not overlap zero. We fit the models and performed checks in R 3.4.3, using the **mgcv** package.

To examine the importance of different biological mechanisms in transmission, we fit an age-structured (adult and juvenile) maternally immune (M)-susceptible (S)-infected (I)-recovered (R) model with recrudescence (R to I) and loss of immunity (R to S) to the seroprevalence data on a weekly timescale:

$$\frac{dS_J}{dt} = -S_J(\beta_{JJ}I_J + \beta_{AJ}I_A) - \mu_J S_J + bN_A(t-5)\frac{S_A}{N_A} - bN_A(t-52)\frac{S_J}{N_J}(1-\mu_J)^{52} + \lambda M_J$$

$$\frac{dI_J}{dt} = S_J(\beta_{JJ}I_J + \beta_{AJ}I_A) - \gamma I_J - bN_A(t-52)\frac{I_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dR_J}{dt} = \gamma I_J - \mu_J S_J - \mu_J R_J - bN_A(t-52)\frac{R_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dM_J}{dt} = bA(t-5)\frac{R_A}{N_A} - \lambda M_J - \mu_J M_J - bN_A(t-52)\frac{R_A}{N_A}(1-\mu_J)^{52}\lambda^{52}$$

$$\frac{dS_A}{dt} = -S_A(\beta_{AA}I_A + \beta_{JA}I_J) - \mu_A S_A + \tau R_A + bN_A(t-52)\frac{S_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dI_A}{dt} = S_A(\beta_{AA}I_A + \beta_{JA}I_J) - \gamma I_J - \mu_A I_A + bN_A(t-52)\frac{I_J}{N_J}(1-\mu_J)^{52} + \Delta R_A$$

$$\frac{dR_A}{dt} = \gamma I_A - \mu_A R_A + bN_A(t-52)\frac{R_J}{N_J}(1-\mu_J)^{52} - \tau R_A - \Delta R_A$$

We included a class M for the density of juvenile bats with maternal antibodies to allow for the biological possibility that maternal antibodies are lost at a much higher rate than antibodies acquired following infection. The subscripts J refer to juveniles and A to adults, β is the transmission rate, γ is the recovery rate, μ is the mortality rate, τ is the rate of loss of adult immunity, λ is the rate of loss of maternal antibodies(66), Δ is the adult recrudescence rate (R to I), b is the birth rate (pups join the juvenile population after 5 weeks). Juveniles transition to adults after 52 weeks. We included terms for loss of antibody in adults (τ, S to R), and viral recrudescence (Δ, R to I) based on previous studies on captive bats that demonstrated the existence of these processes without providing enough data to characterize them precisely (55, 56). We fit this deterministic model to the seroprevalence data by maximum likelihood, which assumes that deviations from the model are due to observation error. We estimated the confidence intervals around maximum likelihood parameter estimates using likelihood profiles using the *profile* function in package *bbmle* in R v3.2.2.

We used model fitting and model comparison to examine the need for several of the biological processes in the model above that could influence NiV dynamics. First, we examined both density and frequency-dependent transmission by comparing the fit of the model above to one with transmission

terms that have population size (N_A or N_J) in the denominator. Second, we examined the confidence intervals of the parameters describing viral recrudescence, loss of antibodies in adult bats, and loss of antibodies in juvenile bats. If the confidence bounds for these parameters included zero, then these biological processes are not needed to explain the serological dynamics. Finally, we examined the confidence bounds for parameters describing the loss of maternal and non-maternal antibodies (τ and λ), to determine if the rate of loss of these two types of immunity were different. We note that this model structure has similarities to a susceptible-infected-latently infected(L)-infected (SILI) model (if latently infected individuals are seropositive), but the model above differs in allowing for the possibility of individuals to transition from the R class back to the S class.

Code availability

SIR model code written in R is available upon request.

Data availability

All molecular sequences are available via Genbank. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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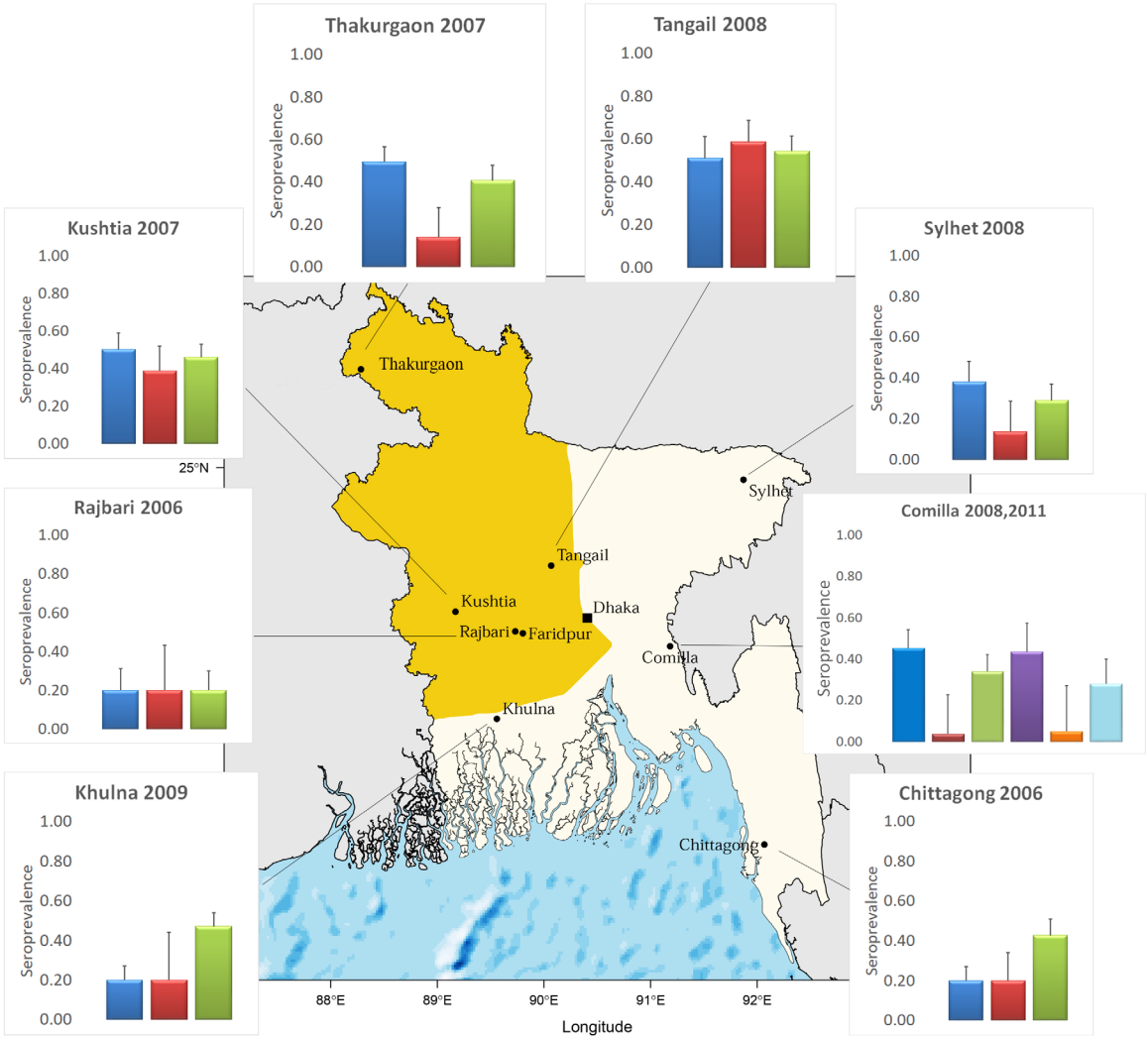


585 **Figure 1.** Map showing age-stratified seroprevalence in *Pteropus medius* colonies, Bangladesh. Bats from eight
586 colonies were sampled and tested for anti-NiV IgG antibodies: four within the "Nipah Belt" (orange shaded) and
587 four outside. Seroprevalence of adults (blue, purple (2011)), juveniles (red, orange (2011)) and total
588 seroprevalence (green, light blue (2011)) are shown. Number (n) of Adult, Juvenile, and Total bats sampled
589 (clockwise): Tangail [53,41,94], Sylhet [63, 37, 100], Comilla 2008 [73,27,100], Comilla 2011 [30,20,50], Chittagong
590 [72,24,96], Khulna[85,15,100], Rajbari [65,15, 80], Kushtia [64,36,100], Thakurgaon [89,29,118]. The shaded region
591 represents the "Nipah Belt" where previous human NiV outbreaks have been reported.

592

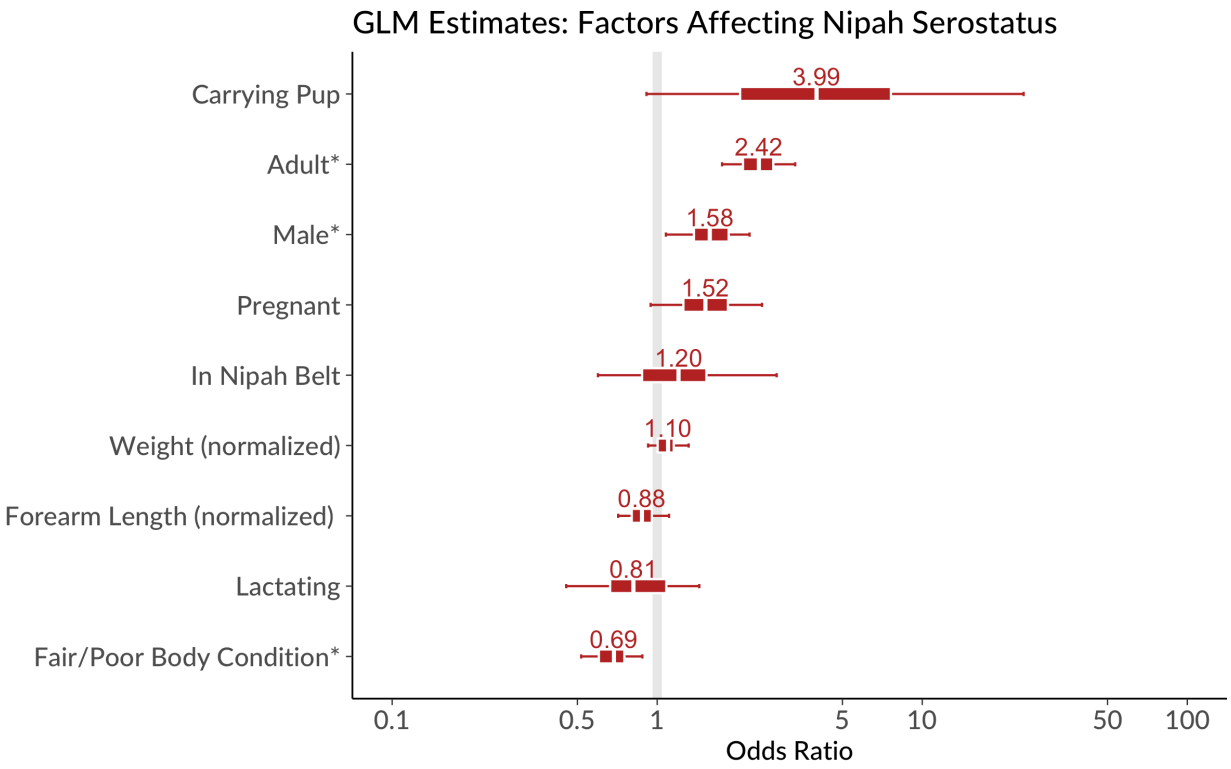


Figure 2. Results of Bayesian GLM of factors affecting Nipah serostatus in bats in cross-sectional study. Bars indicate odds ratios and 50% (inner) and 95% (outer) credible intervals for model parameters. Factors with asterisks (*) have 95% CIs that do not overlap 1. Model intercept (predicted probability of seropositivity for a juvenile, female, bat outside the Nipah belt of mean size and good body condition) was 0.26, (95% CI 0.12-0.56). Random effect of location (not shown) had an estimated SD of 0.63 (95% CI 0.29-1.27)

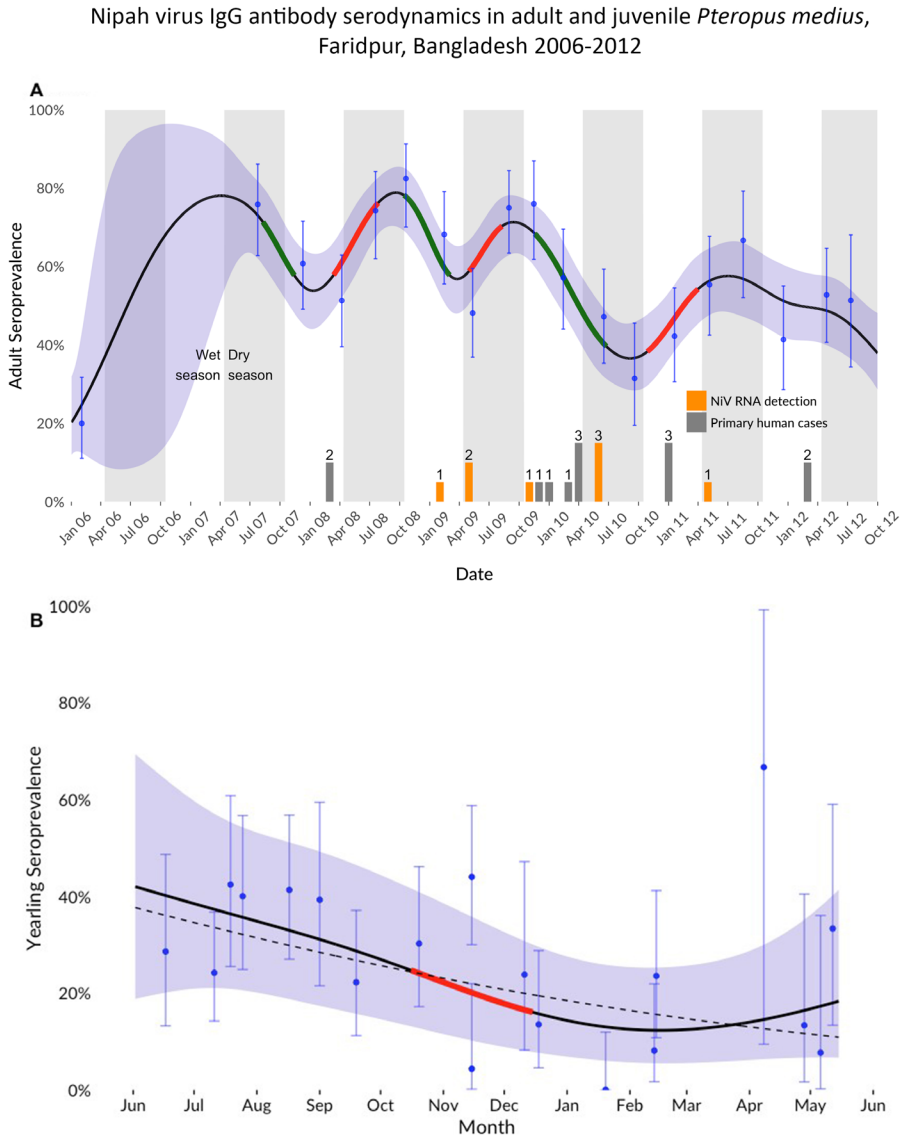


Figure 3 A & B. Serodynamics of the Faridpur bat population. (A) Adult serodynamics, with measured values and 95% CI in blue, and mean GAM prediction and 95% shown with line and surrounding shaded areas. Periods of significant increase (red) and decrease (green) shown where the GAM derivative's 95% CI does not overlap zero. Counts of primary human cases from local district (dark gray, and bat viral detections of approx. 100 sampled (orange, see Table 1), shown on bottom. (B) Juvenile serodynamics during the first year of life ("yearlings"), with all years' measurements overlain to show cohort-level dynamics across all study years. Measured values and 95% CI in blue, and mean and 95% CI for the GAM model pooled across cohorts shown with line and surrounded shaded areas. The period of significant decline in the GAM is shown in red. Also shown is the mean prediction of a model with only a linear mean term, with similar fit (ΔAIC < 1) as the GAM (dotted line).

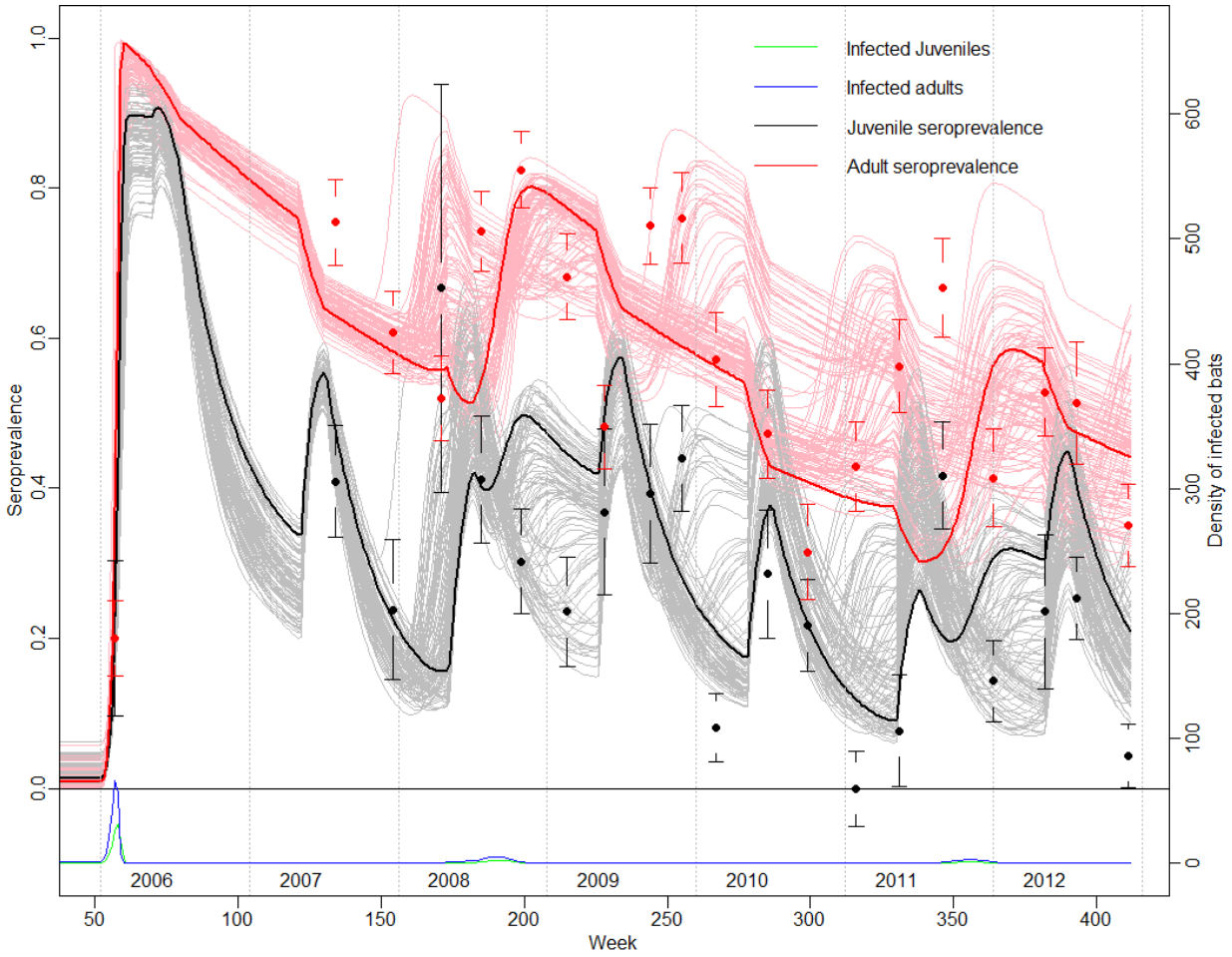
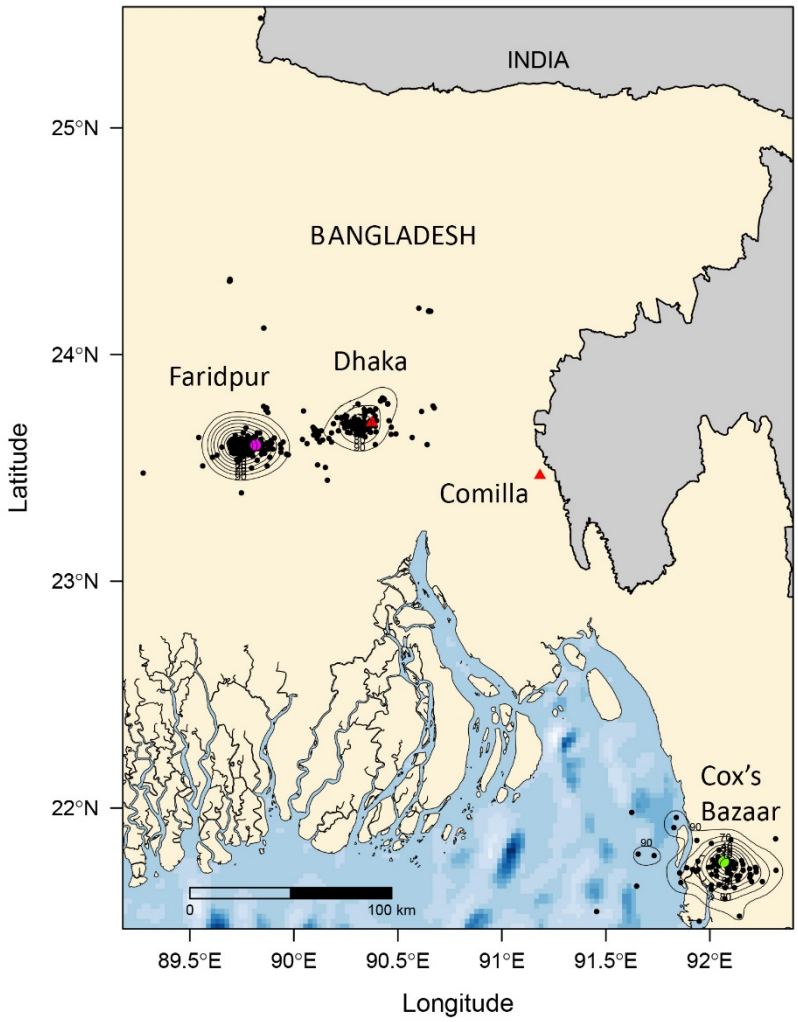


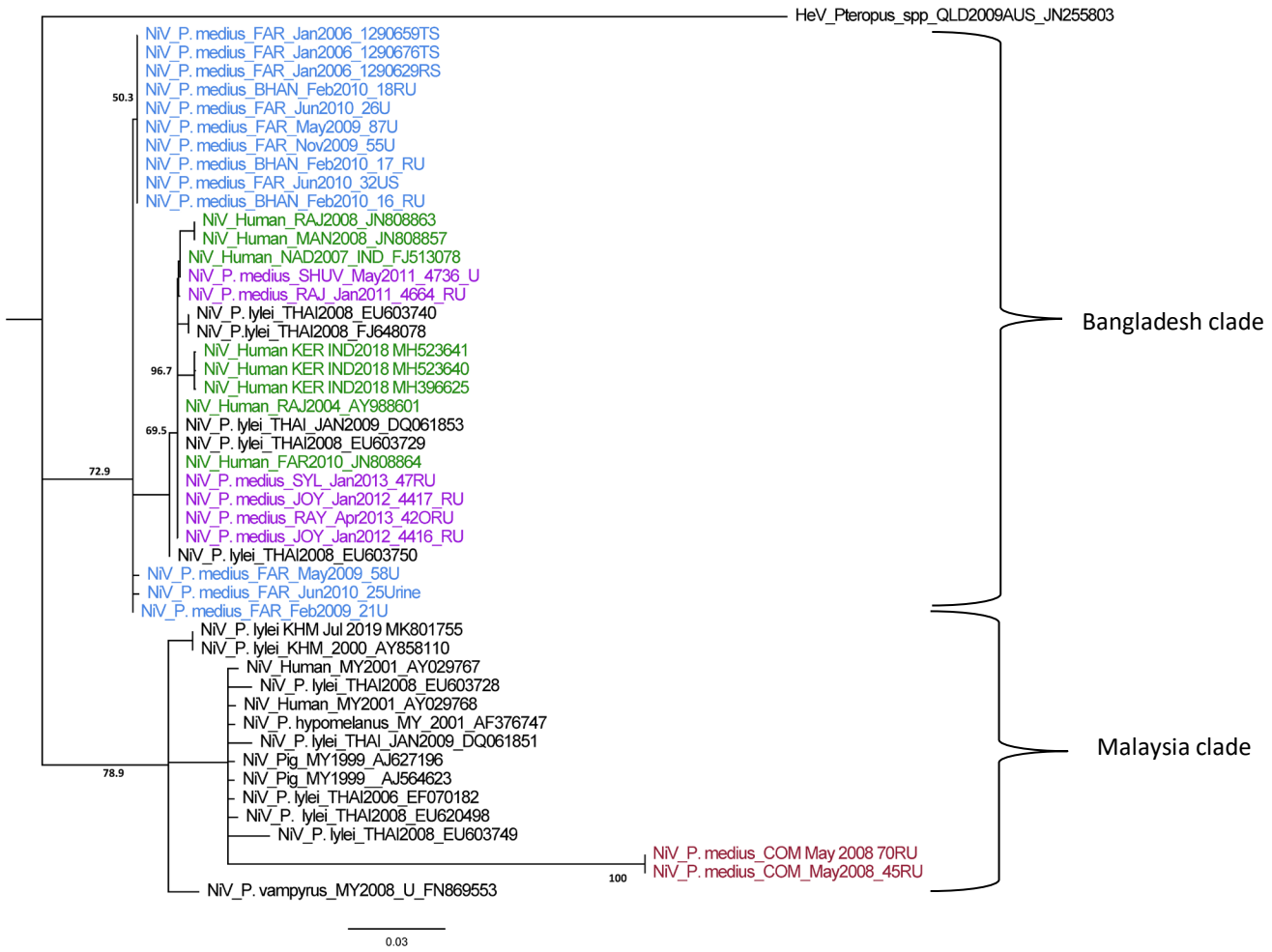
Figure 4. **NiV serological dynamics in adult and juvenile bats.** The observed data (red and black points ±1 SE) and model fit (solid lines; thick lines show the trajectory for the model with maximum likelihood parameter estimates; thin lines show realizations for parameter estimates drawn from the estimated distributions) for the fraction of adults and juveniles seropositive for NiV (left axis), and the model estimated density of infected adult and juvenile bats (bottom panel and right axis). See Methods for details of model structure.

620



621

622 **Figure 5.** Satellite telemetry and home range analysis. Location data from satellite collars (n=14) placed on 11 bats
623 from Faridpur and 3 bats from Cox's Bazaar, Chittagong collected between 2009 and 2011, were used to calculate
624 local and long-range movement patterns and home range for these two groups.



625 **Figure 6**. **Nipah Virus partial N gene phylogeny(224nt).** Phylogenetic Neighbor-joining tree created in Geneious
626 Prime 2019 using a Tamura-Nei model with 1,000 bootstrap replicates, and Hendra virus as an outgroup (108).
627 Branch lengths shown as the number of substitutions per site. Sample collection date, location and Genbank
628 accession numbers are included in the label for each sequence except *P. medius* sequences we collected: Genbank
629 accession numbers MK995284 – MK995302. Blue labels indicate bat sequences from Faridpur and Bhanga (an
630 outbreak response in Faridpur). Purple sequences are from *P. medius* from other roosts sampled during the
631 longitudinal study. Red sequences are from *P. medius* in Comilla. Green sequences are human NiV sequences from
632 Bangladesh and India.
633

Table 1. PCR detection of NiV RNA in *Pteropus medius* 2006-2012.

Location	Date	Bats Sampled	Throat Swabs Tested	Throat Swabs Positive	Urine Tested	Urine Positive	Rectal Swabs Tested	Rectal Swabs Positive	Paired Samples	Bats Positive	Bats w >1 Positive Sample	Prevalence	+/- 95% CI	Roost Urine	Roost Urine Positive
Spatial Study															
Rajbari	Jan-06	99	79	**3**	78	0	79	**1**	78	3	**1**	**0.04**	**0.11**	-	-
Thakurgaon	Mar-07	118	115	**3***	72	0	-	-	70	unk.	0	0.00	-	-	-
Kushtia	Aug-07	101	100	0	99	0	-	-	98	0	0	0.00	-	-	-
Tangail	Jun-08	100	61	0	77	0	-	-	60	0	0	0.00	-	81	0
Chittagong	Aug-06	115	19	0	-	-	-	-	-	0	-	-	-	-	0
Comilla	May-08	100	0	0	50	0	-	-	0	0	-	-	-	100	**2**
Sylhet	Sep-08	100	100	0	49	0	-	-	48	0	0	0.00	-	100	0
Khulna	Jan-09	100	50	0	80	0	-	-	32	0	0	0.00	-	50	0
Comilla	Mar-11	50	50	0	50	0	-	-	0	0	0	0.00	-	-	-
Outbreak Investigation															
Bangha	Feb-10													19	3
Joypurhat	Jan-12													19	**16**$^{\alpha}$
Rajbari	Dec-09													35	0
West Algi	Jan-10													31	0
Longitudinal Study															
Faridpur	Jul-07	102	64	0	50	0	-	-	22	0	0	0.00			
Faridpur	Dec-07	101	N/A	N/A	N/A	-	-	-		0					
Faridpur	Apr-08	100	64	0	88	0	-	-	54	0	0	0.00		51	0
Faridpur	Jul-08	100	58	0	74	0	-	-	54	0	0	0.00			
Faridpur	Oct-08	100	98	0	99	0	-	-	98	0	0	0.00			
Faridpur	Feb-09	100	50	0	100	**1**	-	-	49	**1**	0	**0.01**	**0.10**	50	0
Faripdur	May-09	101	100	0	99	**2**	-	-	99	**2**	**0**	**0.02**	**0.10**	9	0
Faridpur	Aug-09	100	100	0	99	0	-	-	95	0	0	0.00		3	0
Faridpur	Nov-09	100	100	0	82	**1**	-	-	82	**1**	**0**	**0.01**	**0.11**	50	0
Faridpur	Feb-10	100	100	0	100	0	-	-	100	0	0	0.00		45	0
Faridpur	Jun-10	100	100	0	100	**3**	-	-	100	**3**	**0**	**0.03**	**0.10**	25	0
Faridpur	Sep-10	100	100	0	100	0	-	-	-	0	-	-		20	0
Faridpur	Jan-11	100	100	0	100	0	-	-	0	0	0	0.00		15	0
Faridpur	May-11	102	102	0	102	**1**	-	-	0	**1**	0	**0.01**	**0.10**	20	0
Faridpur	Aug-11	100	100	0	100	0	-	-	-	0	-	-		10	0
Faridpur	Dec-11	100	100	0	100	0	-	-	-	0	-	-		16	0
Faridpur	Apr-12	100	78	0	78	0	-	-	-	0	-	-		50	0
Faridpur	Jul-12	100	100	0	100	0	-	-	-	0	-	-		30	0
Faridpur	Nov-12	100	100	0	100	0	-	-	-	0	0			34	0
Total		**2789**	**2088**	**6**	**2126**	**8**	**79**	**1**		**11**	**1**	**0.005**	**0.02**	**863**	**21**

*NiV RNA was detected in three pooled oropharyngeal samples, confirmed by sequencing, although confirmation from individual samples could not be made. These data re not used in prevalence estimates. $^{\alpha}$ Detection by qPCR, Ct ranges 20-38.

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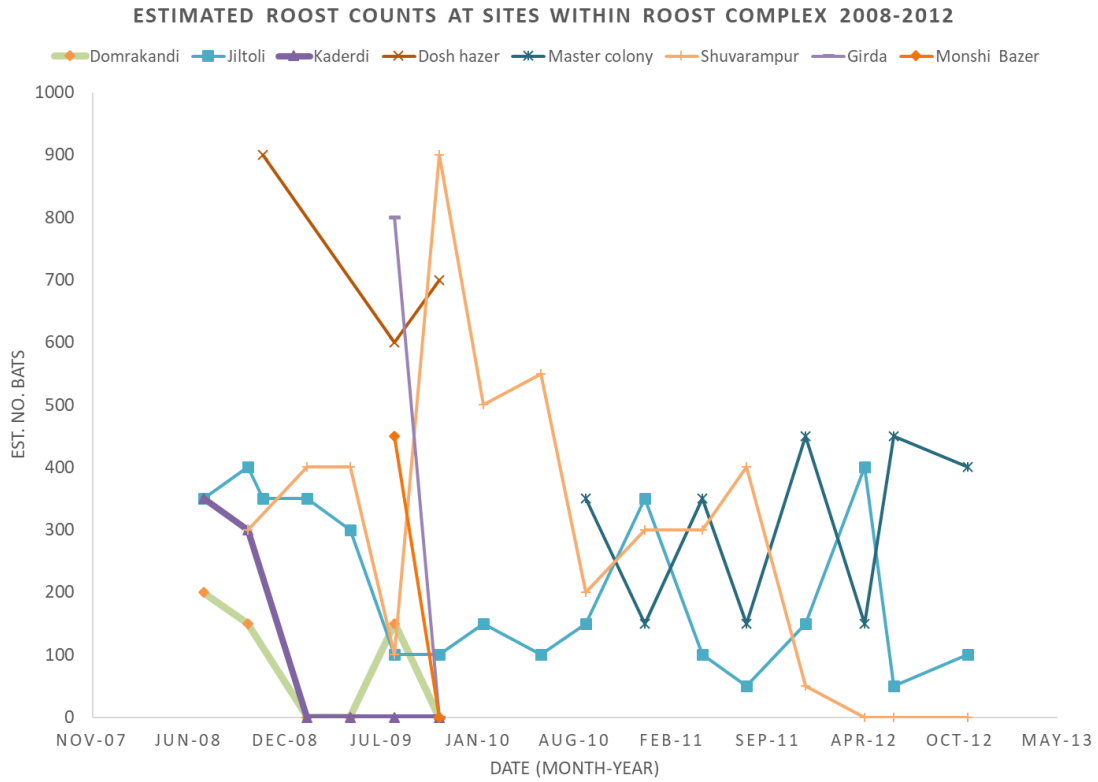
877 Supporting Information



878

879 **Figure S1**. *Pteropus medius* counts from selected roosting sites within the Faridpur Roost Complex: 2008-2012.

880 Sites were included if repeated counts were conducted. Domrakandi and Kaderdi were the two primary roost sites

881 sampled for the longitudinal study and counts were used for the model parameter.

882

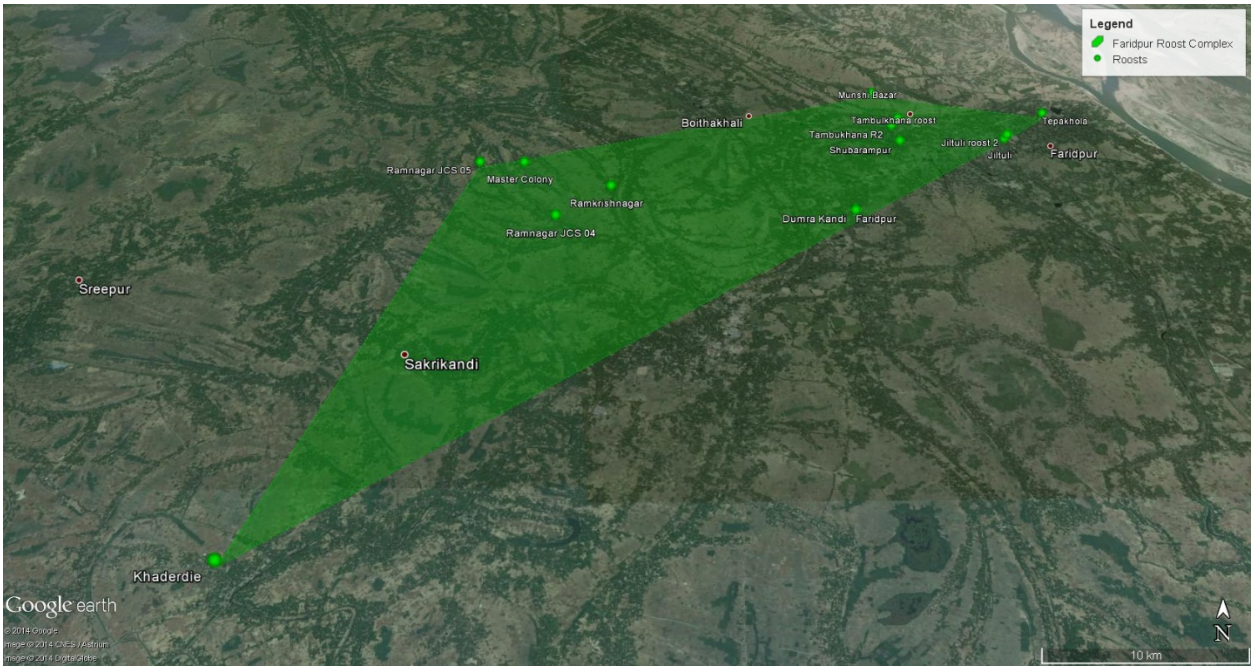


Figure S2. Faridpur Roost Complex. 51 Individual bats were recaptured during the longitudinal study at various locations. 33 bats were recaptured at a different site from where they were originally sampled. 15 unique roosts within an 80km^2 area were identified.

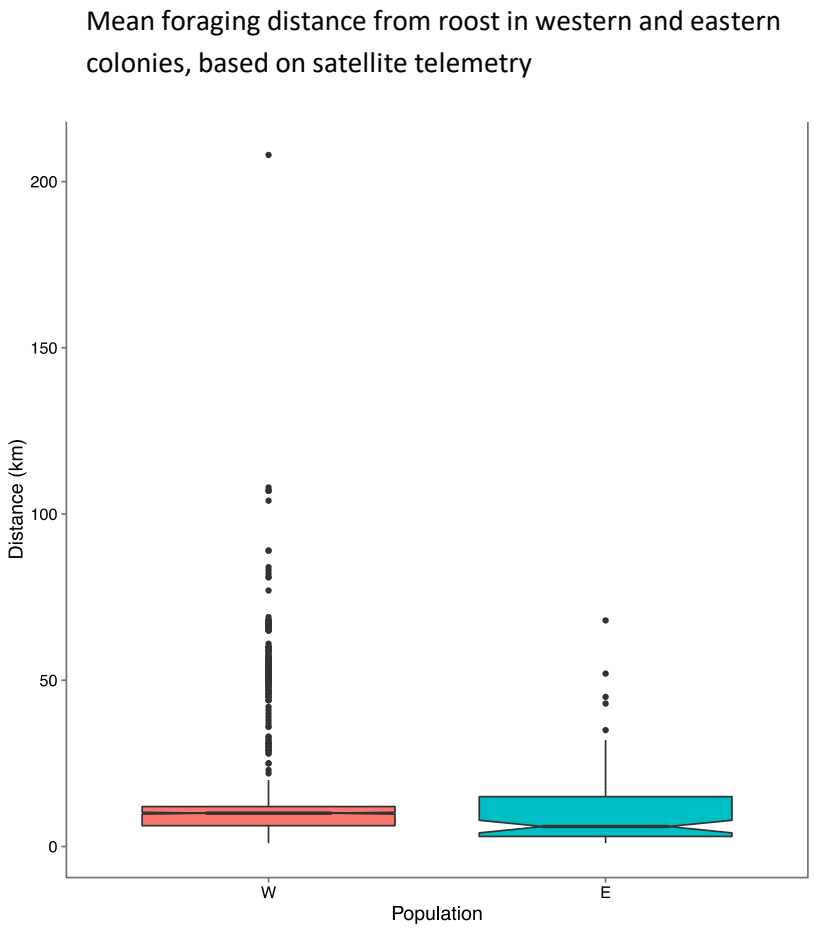


Figure S3. Mean foraging distance of western (W) and eastern (E) bat populations, based on satellite telemetry locations obtained between 1800h and 0600h, when *P. medius* typically forages.

Home range of *Pteropus medius* in wet and dry seasons.

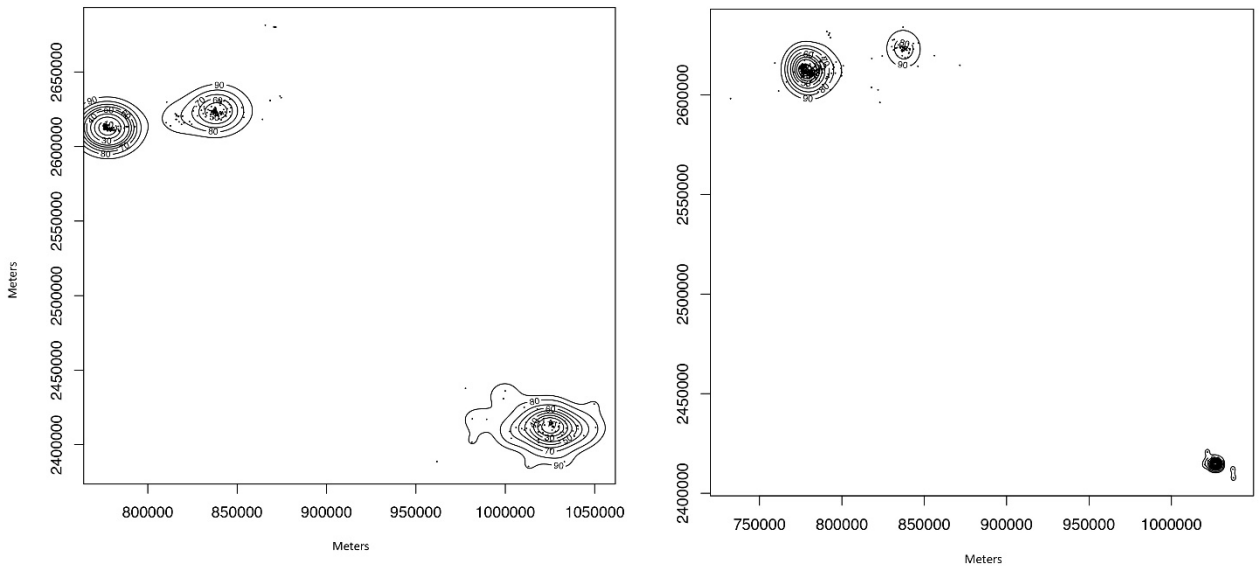


Figure S4. a) Homerange of *Pteropus medius* during the wet season (left) and dry season (right). Maps are projected in UTM (Universal Transverse Mercator) Zone 45 where units are represented in meters. The mean wet season homerange size was 2,746 km^2. Homerange size in the dry season is contracted and represents less than a quarter (618 km^2) of the homerange in the wet season.

Nipah virus phylogenetic tree, based on near complete N gene sequences

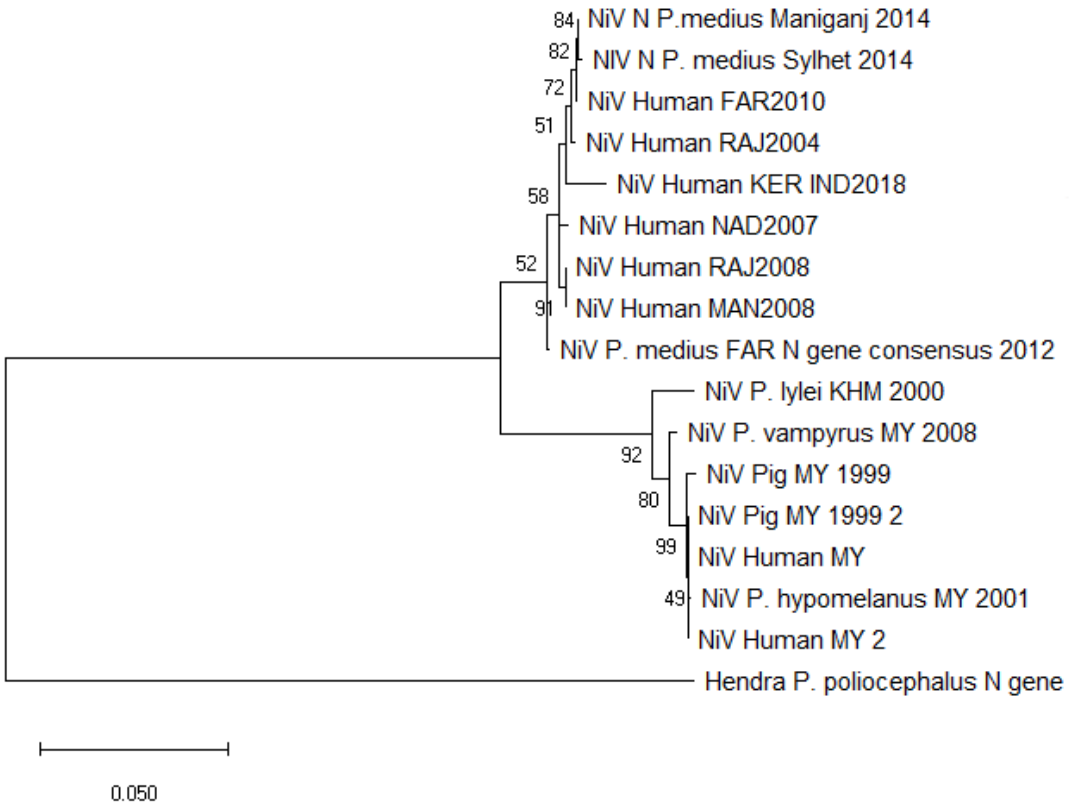


Figure S5. Nipah Virus phylogenetic tree, N gene: Clustal W alignment using nearly whole N gene consensus sequence from *P. medius* (1,592 nt) using Geneious Prime 2019 (108). The evolutionary history was inferred by using the Maximum Likelihood method and Hasegawa-Kishino-Yano model (109). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X (110). Genbank accession numbers for sequences (from top to bottom): *P. medius* Maniganj & Sylhet pending (63); JN808864, AY988601, MH396625, FJ513078, JN808863, JN808857, AY858110, FN869553, AJ627196, AJ564623, AY029767, AF376747, AY029768, JN255803.

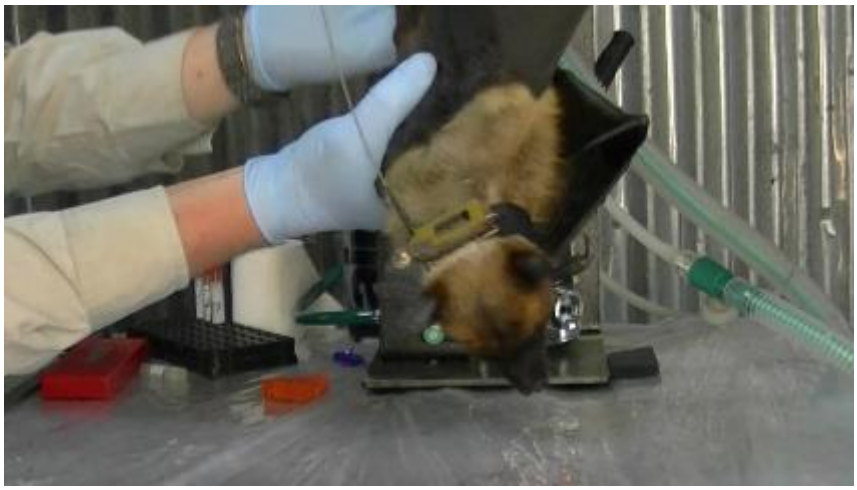


Figure S6. Platform terminal transmitter (PTT) and collar attachment on an anesthetized adult *Pteropus medius*, Bangladesh.

Table S1.

PCR positive bats and their serostatus.

Bat sample ID	Date	Loc	sex	Age	Serology Test result		
					ELISA	Luminex (MFI)	
29	Jan-06	Ramnagar	M	J	Neg	-	
59	Jan-06	Ramnagar	PF	A	Neg		
76	Jan-06	Ramnagar	PF	A	Neg		
21	Feb-09	Faridpur	PF	A	-	25817	Pos
58	May-09	Faridpur	F	A		159	Neg
87	May-09	Faridpur	M	A	-	113	Neg
55	Nov-09	Faridpur	M	A		25955	Pos
26	Jun-10	Faridpur	M	A		30	Neg
32	Jun-10	Faridpur	M	J		758	Pos
28	Jun-10	Faridpur	M	J		377	Neg

Table S2. Maximum likelihood estimates of fitted parameter values and (95% CI). All rates are on a weekly timestep unless otherwise indicated.

Parameter	Name	Maximum likelihood estimate	Lower 95% CI	Upper 95% CI
B_{jj}	Transmision rate, juveniles→juveniles	0.012584	0.00958	0.013021
B_{ja}	Transmision rate, juveniles→adults	0.030008	0.023707	0.03444
B_{aj}	Transmision rate, adults→juveniles	0.002417	0.00195	0.002937
B_{aa}	Transmision rate, adults→adults	0.000465	0	0.004092
R_A/N_A (t=0)	Initial adult seroprevalence	0.018752	0	0.067691
Δ	Recrudescence	2.3E-07	1.41E-08	7.1E-07
$(1-\mu)^{52}$	Adult annual survival	0.754971	0.718554	0.798346
λ	Rate of maternal antibody loss	0.05688	0.040029	0.072817

τ	Rate of adult antibody loss	0.003438	0.002099	0.004082

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Param	MLE	2.50%	97.50%
Bjj	0.012584	0.00958	0.013021
Bja	0.030008	0.023707	0.03444
Baj	0.002417	0.00195	0.002937
Baa	0.000465	0	0.004092
Ias	0.018752	0	0.067691
r	2.3E-07	1.41E-08	7.1E-07
SA_A	0.754971	0.718554	0.798346
MAlr	0.05688	0.040029	0.072817
LAA	0.003438	0.002099	0.004082

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Table S2. Recaptured bats and NiV IgG sero-status from the Faridpur population

Bat Chip ID	Age1	Sex	Capture Date	Location 1	Serostatus1	Age2	Capture Date 2	Serostatus2	Location 2	Status Change	Age3	Capture Date 3	Location 3	Serostatus 3	Status Change 2
17044540	A	M	24/07/08	JH	0	A	19/09/10	1	SH	C					
26774096	A	M	05/10/09	JH	1	A	13/11/09	1	SH	N					
26783883	J	F	15/05/09	SH	1	A	14/02/10	0	SH	R					
26789012	A	M	15/05/09	SH	0	A	02/10/10	1	SH	C					
26791784	A	F	14/05/09	SH	1	A	30/04/12	0	JH	R					
26816627	A	F	11/05/09	SH	0	A	26/08/09	0	DM	N					
26824582	J	M	09/05/09	JH	0	A	20/09/10	0	TP	N	A	05/05/11	TP	0	N
27099360	A	M	24/09/10	RM_MC	0	A	18/08/11	1	TB	C	A	18/11/12	JH	0	R
27102063	A	M	16/11/09	SH	1	A	16/02/10	1	SH	N	A	19/12/11	TP	1	N
27103623	J	M	21/09/10	TP	1	A	17/12/11	0	TP	R					
27105342	J	M	21/09/10	TP	0	A	19/12/11	1	TP	C					
27105562	P	M	24/04/10	RM_JCS	0	J	20/10/10	0	RM_JCS 05	N					
27110270	A	M	24/07/10	RM_JCS	0	A	04/04/11	0	RM_JCS	N					
27111334	A	M	23/07/10	RM_JCS	0	A	19/10/10	0	RM_JCS	N					
27123779	J	F	21/06/10	RM_JCS2	0	J	28/02/11	0	RK	N					
27123803	J	F	21/08/10	RM_JCS1	0	J	28/02/11	0	RK	N					
27123868	J	M	18/02/10	SH	0	A	26/04/12	1	JH	C					
27126256	A	F	10/02/10	SH	0	A	18/12/11	0	TP	N					
27259351	A	M	20/09/10	TP	0	A	30/04/12	0	JH	N					
27259370	A	M	22/07/10	RM_JCS1	0	A	04/04/11	0	RM_JCS1	N					
27261073	J	F	22/06/10	RM_JCS2	1	J	22/07/10	1	RM_JCS1	N					
27261577	A	M	21/09/10	TP	0	A	17/12/11	1	TP	C					
27266775	A	M	21/08/10	RM_JCS1	1	A	18/11/12	0	JH	R					
27291793	A	M	24/09/10	RM_JCS1	0	A	22/01/11	1	RM_JCS1	C					
27296568	A	M	12/11/09	SH	1	A	07/05/11	1	SH	N					
27296851	A	M	22/04/10	RM_JCS1	0	A	25/05/10	0	RM_JCS2	N					
27301580	A	M	17/06/10	SH	1	A	05/05/11	1	TP	N					
27301857	A	M	11/02/10	SH	0	A	22/04/10	1	RM_JCS1	C	A	03/05/11	SH	0	R

Location Codes: Location codes: Jhiltuli (JH); Jiltuli Roost 2 (JH2); Shuvarampur (SH); RamnagarMC (RM); Tepakhola (TP); Ramnagar Roost JCS (RM_JCS); Ramnagar JCS 1 (RM_JCS1); Ramnagar JCS 02 (RM_JCS2); Tambukhana R2 (TB2); Domrakhandi (DM); Khaderdi (KD); Ramkrishnagar (RK); Tepakhola Master Colony (TPMC). Status Change: C = seroconversion, N = No Change, R = sero-reversion.

Table S2 (cont…). Recaptured bats and NiV IgG sero-status from the Faridpur population

Bat Chip ID	Age1	Sex	Capture Date	Location 1	Serostatus1	Age2	Capture Date 2	Serostatus2	Location 2	Status Change	Age3	Capture Date 3	Location 3	Serostatus 3	Status Change 2
27305044	J	M	22/06/10	RM_JCS2	1	A	15/11/12	0	JH	R					
27306794	A	M	15/06/10	SH	1	A	11/07/12	1	TPMC	N					
27306824	A	M	23/07/10	RM_JCS1	1	A	24/09/10	1	RM_JCS1	N	A	18/11/12	JH	1	N
54867532	A	M	23/01/11	JH2	1	A	18/11/12	0	JH	R					
54872600	A	M	19/10/10	RM_JCS2	0	A	30/04/11	0	RM_MC	N					
54877598	J	F	18/01/11	SH	0	A	01/05/12	0	JH	N					
65770323	J	M	04/04/11	RM_JCS1	0	J	01/05/11	0	RM_MC	N					
65780555	A	M	05/05/11	TP	1	A	13/11/12	1	JH	N					
68608827	J	M	14/08/11	TB2	1	J	18/12/11	1	TP	N					
68612032	J	M	15/07/12	TPMC	0		18/11/12	0	JH	N					
80825550	A	M	11/12/07	DM	0	A	13/04/08	1	DM	R					
80855347	A	M	06/12/07	DM	1	A	22/07/08	1	KD	N					
80867630	A	M	11/12/07	DM	0	A	14/05/09	0	SH	N					
80876042	A	M	06/12/07	DM	0	A	14/08/11	0	TB2	N					
80877779	A	M	07/12/07	DM	1	A	21/07/08	1	DM	N					
81030044	A	M	06/02/06	RM_JCS1		A	24/07/10	0	RM_JCS1	NA		04/04/11	RM_JCS1	0	N
81055270	A	F	12/12/07	DM	0	A	20/12/11	1	TP	C					
81095300	A	M	09/12/07	DM	1	A	18/07/08	1	DM	N					
99605347	A	M	15/12/07	DM	0	A	07/04/08	0	DM	N					
99618528	A	M	20/07/08	DM	1	A	16/02/10	1	SH	N					
103821120	A	F	12/04/08	DM	0	A	16/05/09	1	SH	C					
104083112	A	M	03/04/08	DM	1	A	20/07/08	1	DM	N					
65777367	P	M	30/04/11	RM_MC	0	J	14/11/12	0	JH	N					

Location Codes: Location codes: Jhiltuli (JH); Jiltuli Roost 2 (JH2); Shuvarampur (SH); RamnagarMC (RM); Tepakhola (TP); Ramnagar Roost JCS (RM_JCS); Ramnagar JCS 1 (RM_JCS1); Ramnagar JCS 02 (RM_JCS2); Tambukhana R2 (TB2); Domrakhandi (DM); Khaderdi (KD); Ramkrishnagar (RK); Tepakhola Master Colony (TPMC). Status Change: C = seroconversion, N = No Change, R = sero-reversion.

Table S3. Satellite telemetry study: bat characteristics and duration of transmission.

PTT #	Microship ID	Colony location	Date collared	Final location date	Approx.Duration (mo)	Sex	Age	Mass (g)	BCS	Forearm (mm)	Head (mm)	Body (mm)	pregnant	lactating
90831	17035561	F	2/13/2009	5/12/2009	3	F	A	673	G	169.7	74.7	197.5	Y	N
90832	17034004	F	2/13/2009	6/17/2009	4	F	A	663	G	171.4	71.1	195.7	N	N
90833	17019016	F	2/14/2009	4/12/2009	2	M	A	688	G	182.5	78.4	217.6	-	-
90834	080867630*	F	2/14/2009	5/11/2009	3	M	A	665	G	186.4	76.1	201.6	-	-
90835	17027862	F	2/16/2009	7/1/2009	5	M	A	684	G	166.6	68.7	221.6	-	-
90836	17071891	F	2/16/2009	6/3/2009	4	F	A	652	F	181.6	71.8	206.3	Y	Y
101469	54876270	F	1/17/2011	8/13/2011	7	M	A	626	G	175	75	210	-	-
101467	54870019	F	1/17/2011	1/29/2012	12	F	A	603	F	165	70	195	Y	N
101466	54867013	F	2/28/2011	6/8/2011	3	F	A	684	G	164	70	190	Y	N
101468	54883815	F	2/28/2011	4/4/2012	13	M	A	772	G	172.3	71.84	195	-	-
90839	54867601	F	3/1/2011	4/28/2013	25	M	A	771	G	175.53	74	211.14	-	-
101470	65623841	F	3/1/2011	12/8/2011	9	F	A	731	G	172.68	71.6	205.68	Y	N
101471	65628805	C	3/29/2011	5/9/2011	1	M	A	717	G	176	78	220	-	-
90840	65635619	C	3/29/2011	8/8/2011	4	M	A	698	G	169	81	202	-	-
90838	65628094	C	3/30/2011	6/26/2011	3	F	J	446	F	161	73	190	N	N
90837	65775297	C	3/30/2011	6/17/2011	2	M	A	620	F	178	71	190	-	-

PTT = Platform Terminal Transmitter; Colony Location: F=Faridpur, C-Chittagong; BCS = Body Condition Score: G=Good, F=Fair, P=Poor;

From:	Jon Epstein on behalf of Jon Epstein <epstein@ecohealthalliance.org>
To:	Anthony, Simon J.; Ariful Islam; marm@biology.ucsc.edu; Shahneaz Ali Khan; Noam Ross; ina.smith@csiro.au; Carlos M. Zambrana-Torrelio MSc; Yun Tao; Ausraful Islam; Kevin Olival, PhD; Salah Uddin Khan; Emily Gurley; Dr. Jahangir Hossain; Hume Field; Fielder, Mark; Thomas Briese; Mahmud Rahman; Christopher Broder; Gary Crameri; Linfa Wang; Stephen Luby; Ian Lipkin; Peter Daszak
Subject:	Re: Nipah dynamics in P medius draft for PNAS
Date:	Friday, January 10, 2020 12:37:01 PM
Attachments:	Nipah dynamics in bats_Epstein et al 2020_complete.pdf

Dear co-authors,
Our paper has been submitted to PNAS! Thank you all for your thoughtful and helpful comments.
I think you've each received a link to the submission from PNAS, but if not, here's the version that was submitted.

I'll be in touch when I hear anything.

Cheers,
Jon

On Wed, Dec 18, 2019 at 3:00 PM Jon Epstein <epstein@ecohealthalliance.org> wrote:

> All,
> I hope this email finds you well. Attached is a draft of our manuscript entitled Nipah virus dynamics in bats and implications for spillover to humans. After multiple protracted periods of review in Science Advances, and ultimately a rejection, I've prepared this draft for submission to PNAS. I've revised the manuscript and updated figures and supplemental data in response to the last round of review (comments below, if you're interested).
>
> I have an editor at PNAS willing to send this for review, so please send me any input you may have by December 28th so that I can submit by the 31st. Unless I hear otherwise, I will assume you still consent to co-authorship.
>
> Thank you for your patience, and happy holidays!
>
> Cheers,
> Jon
>
> Reviewer: 1
> Epstein et al aim to better understand the distribution and drivers of Nipah virus infection dynamics in Pteropus medius in Bangladesh by analysing a large set of serological, virological and movement data over a commendable spatial and temporal scale. Overall, this is a highly exciting study with valuable results that are well-deserving of publication. I have a number of minor comments regarding the addition of detail for clarity (and to ensure transparency in the interpretation of results). I think that the conclusions are mostly justified, however, my major criticism is that the integration and interpretation of the results (particularly those presented in the first paragraph of the discussion) requires a little further thought and explanation. The assumptions surrounding the serological implications of within-host persistence and recrudescence needs to be clearly stated. This is likely to become a 'classic' paper, and there are so few studies in this area supported by data that it is important to ensure that the results are not over-interpreted.
>
> Specific comments:
> Abstract:
> Line 32- 33: The wording here is too strong regarding recrudescence. Suggest inserting "model results indicated that" prior to "local transmission dynamics.
> Line 33-34: Similarly - this is too strong. Suggest "likely due to "
> Results:
> Figure 1 - presumably the first three bats in the Comilla represent 2008 and the second three represent 2011, but this is not clear. This should be annotated on the plot
> Figure 1 - "Adult bats had equal or greater seroprevalence than juveniles in each location."

- except Tangail?
Line 129: "detected NiV RNA in 11 individuals, 3 pooled" - insert 'from' before 3
Line 131: - describe "pooled samples" - pooled under-roost urine samples?
Line 132 and 137: This 'figure 2' seems to be missing? Figure 2 refers to serological analyses
Line 145: I suggest that the reference to Figure 2 in line 146 should go in the sentence ending on line 145
Line 149: the significant negative association with body condition warrants mentioning in these results.
Line 154-155 and Figure 3: Additional clarity here regarding juvenile vs. yearling terms. Suggest saying "Juveniles in their first year of life (yearlings)" at first mentioning in the text, as well in the figure legend.
Line 159 - incomplete sentence
Lines 160-164: Fascinating results!
Lines 170-171: This information would be helpful to include earlier, with the serological results
Figure 3 and Figure S1 - It would be helpful to have a "total population" size from this roost complex included on Figures 3 and 4. Understanding more about the bat ecology and the size and stability of these populations over time would be a tremendous help to aid interpretation of these results by researchers working in other systems.
Lines 175-176: "Serodynamics in juveniles were strongly driven by inheritance and loss of maternal antibodies". Supported by what result? This should be explained further, and/or it would be helpful to include the serology (fig 3), model output (fig 4) and population size (Figure s1) together as a series of vertical panels in the one figure to aid interpretation
Lines 178-180: I can't see this information in the supplementary information
Line 182 - this paragraph (and ideally also table S2) should provide information on the duration of tracking for each bat. This would be very helpful in assessing the home range information
Line 195 - should the 'of' be 'if?
Figure 6 - the utility of this figure would be greatly improved with some annotation and colouring to help identify the new sequences from this study, and their source location, and the 'groups' that are referred to in the text
Lines 238 - 240 - So, your data has found cyclical serodynamics, but no clear links between those dynamics and detection of NiV in bats or in people. Based on experimental studies, it is still a bit unclear exactly what seropositivy in flying foxes represents. If there is within-host persistence of infection, a cycle of infection > seroconversion (in the absence of clearance) > seroreversion > then recrudescence with serconversion may occur in the absence of ongoing transmission. Additionally, this does not take into account drivers of recrudescence - if this is stress related, then transmission at the population level will also be affected by these broader drivers. Given all the uncertainty around this topic, I think that the wording here needs to be precise. e.g the first sentence should first state the assumption "Assuming that seroconversion results only from new infections, then ...". This could be followed up with a sentence along the lines of "If however, seroconversion can result from recrudescence in the absence of transmission, then broader drivers of recrudescence would also need to be assessed". More clarity here would also help to assess the claims being made in the paragraph beginning 315.
Lines 240 onwards - consider restructuring this paragraph to more clearly step through each stage of the viral dynamics that you are proposing and your assumptions and evidence along the way. For example, you assume infection results in seroconversion, and that antibodies then wane after ~4 years. Are you assuming that all individuals are persistently infected, and it is only when Ab wane that recrudescence can occur? And following that, the individual seroconverts again?
Lines 243 - 245: Not correct - See Brooks et al 2019 JAE Figure S5. That study also involved modelling and has implications for this study more broadly
Lines 245-247: "via recrudescence FROM bats that have previously been infected"? It's also not clear how this links to lines 238-240
Lines 257-259: Consider also cite Peel et al (2018). Scientific Reports, 8(1), e0004796.

This study examines waning maternal immunity and waning acquired immunity against henipaviruses in African fruit bats, finding a similar duration of immunity for both
Line 267 - insert "in our study area" after 'Pteropus medius' as you cannot infer whether these patterns hold true for the species across its range. It may be highly dependent on available food resources - as you go on to discuss
Line 277 - replace 'be' with 'result in'
Lines 295-296 - this links with the SILI hypotheses in Plowright et al (2016) PLoS NTD (see Figure 3)
Line 321- suggest "three periods of transmission (significant at the population level) occurred"
Line 315 See my comments re: paragraph beginning Line 238. The sporadic nature of detections is challenging to draw conclusions and much of the paragraph here (starting line 315) seems over-stated. However, that may become clearer if the comments for the earlier paragraph are addressed.
Methods:
Line 364: I can't see reference to the Faripur colony in the list of colonies in lines 358-359. What are its characteristics? Also, I presume it should be "Faridpur'? Also, it would be helpful if the "Faridpur roost complex:" could be described in more detail in the methods as to the structure of this population and what this term means.
Line 366: Delete the first 'between'
Lines 388-390 - check the placement of parentheses here
Line 417: what cutoff was used for the Luminex assay and how was it determined?
Line 425 - fix formatting of second Ct
Line 493- 504: More information on the underlying population dynamic model is required e.g. Were births seasonal or continuous in your model? I can't see where this is stated. If not seasonal, then the effect of this on model output should be addressed. How was the death rate modelled? Was the total population size kept stable inter annually? The latter, in particular, may have implications for interpretation of the density-dependent vs frequency dependent results. The population size is touched on in the discussion in lines 288-291 but never really explained.
Table S1 - there is extra text below Table S1 that looks like it's not supposed to be there
Table S2 - Include what serostatus 0/1 refers to in the table caption

Reviewer: 2
Dear editor, I have reviewed the manuscript entitled “Nipah virus dynamics in bats and implications for zoonotic spillover to humans”. The manuscript describes a longitudinal surveillance in Pteropid spp. Bats from 2006 until 2012. Biometric data was collected, sera and swabs/urine were analyzed, and inferences were made largely based on serological data. The manuscript is a compendium of relatively loosely compiled data, ranging from seroprevalence in a variety of different sampling sites, but the majority of the samples stem from Faridpur. The problem is that most of the claims by the authors within the paper are not directly supported by the data. The direct problem of the data is the limited amount of detected virus shedding, out of 2789 animals sampled only 11 were found to be shedding the virus. From this only 8 were from the larger study cohort from Faridpur. This directly hampers some of the conclusion of potential spillover dynamics as this cannot be directly inferred from serological data alone. In addition, it does not provide any answers on the occurrence of Nipah spillover in the Nipah belt vs the other regions. Moreover, the significant spillover events in Kerala, India from the last two years, are not discussed. Most emphasis has been put on analyzing the serological results from the Pteropus medius bats. The authors show variation in the seroprevalence within the population based on timing and age status of the animal. The results have been reported before in other natural reservoir-pathogen systems like avian influenza, however even within these systems inferences on spillover can rarely be made. It interesting that the authors did not correlate the positive individual bats in Faridpur and Rajbari with their respective serostatus? Is this data not available? It is unclear to me why the authors have not put more effort in trying to perform full genome analyses on the positive samples obtained throughout this study. Currently there are only 27 full genomes available from Nipah virus

and relatively limited amount are from the natural reservoir. Preforming phylogenetic analyses on a 224 nucleotide fragment of a 19kb virus is really not up to standard. Where it might be suitable for identification of the lineage no additional data can be inferred from this. Interestingly, there appears to be full genome sequencing performed but only N is shown in the supplemental data.
Although I do understand the logistics involved with this kind of work, unfortunately some of the claims, especially regarding spillover, need to be supported by more additional data rather than just serology.
Minor points:
Line 65: pandemic potential, given the limited amount of h-to-h transmission the pandemic potential of this particular virus appears to relatively limited.
Line 143: n=844 or n=883? Why do the numbers not match-up?
Line 410, include level of biosecurity involved in sample analyses. In addition, heatinactivation is typically to inactivate complement and not inactivation of the pathogen.
>From the current wording it is unclear what the authors mean by this? Complement inactivation or pathogen inactivation?
Line 428, is this data missing? Where is the NSG data? Why not data on the full genomes? I only was able to find the full N gene data in the supplemental figues.
Line 509, given that actual recrudescence in the context of virus shedding in the natural reservoir has never been shown it would be good to treat this a little bit more carefully.

--

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EcoHealth Alliance leads cutting-edge scientific research into the critical connections between human and wildlife health and delicate ecosystems. With this science, we develop solutions that prevent pandemics and promote conservation.

Nipah virus dynamics in bats and implications for spillover to humans

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Summary of Comments on Email 32 - Attachment 1 - Nipah dynamics in bats_Epstein et al 2019_amk sl (002).pdf

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Although obviously a good hook, the data we present are only weakly useful for this since we find no correlation b/w bat dynamics and human cases. If the editor will already send it out for review I'd cut this. Overselling what the paper is about is why the paper was rejected at Science Advances.

Abstract

Nipah virus (NiV) is an emerging bat-borne zoonotic virus with pandemic potential that causes near-annual outbreaks of fatal encephalitis in South Asia – one of the most populous regions on Earth. In Bangladesh, infection occurs when people drink date palm sap contaminated with bat excreta, but a recent outbreak in India involved a different, but yet unknown, route of spillover. Outbreaks are sporadic and the influence of viral dynamics in bats on their temporal and spatial distribution is poorly understood. We analyzed data on host ecology, molecular epidemiology, serological dynamics, and viral genetics to characterize spatio-temporal patterns of NiV dynamics in its wildlife reservoir, *Pteropus medius* bats, in Bangladesh. We found that NiV transmission occurred throughout the country and throughout the year. Model results indicated that local transmission dynamics were driven by density-dependent transmission, acquired immunity which is lost over time, and recrudescence. Increased transmission followed multi-year periods of declining seroprevalence due to bat population turnover and individual loss of humoral immunity. Individual bats had smaller host ranges than other *Pteropus* spp., although movement data and the discovery of a Malaysia-clade NiV strain in eastern Bangladesh suggest connectivity with bats east of Bangladesh. These data suggest that discrete multi-annual local epidemics in bat populations contribute to the sporadic nature of Nipah virus outbreaks in South Asia. At the same time, the broad spatial and temporal extent of NiV transmission, including the recent outbreak in Kerala, India, highlights the continued risk of spillover to humans wherever they may interact with pteropid bats, and the importance of improving Nipah virus surveillance throughout *Pteropus*'s range.

Keywords: bats, henipavirus, Nipah virus, *Pteropus medius, Pteropus giganteus,* satellite telemetry, viral phylogeny, disease dynamics, modeling

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This is interesting but isn't relevant to this paper and we have no data to address it so I'd remove this, especially in the abstract. The mention of it below doesn't require this text. Mentioning it here makes the reader expect us to address it and we can't.

Introduction.

Outbreaks of zoonotic diseases are often sporadic, rare events that are inherently difficult to predict, but can have devastating consequences (*1*). Several emerging viral zoonoses with wildlife reservoirs have become pandemics, including HIV/AIDS, SARS coronavirus, and 1918 Pandemic Influenza H1N1 (*2-4*). Bats are important hosts for many zoonotic viruses (*5*) including Ebola virus, SARS-CoV, and Nipah virus, but the ecological drivers and transmission dynamics of these viruses in their reservoir hosts are poorly understood (*6-10*). A better understanding of the transmission dynamics of zoonotic pathogens in their natural reservoirs may help anticipate and prevent outbreaks (*9, 11*).

Nipah virus (NiV) is an emerging zoonotic paramyxovirus (genus *Henipavirus*) that has repeatedly spilled over from bats to cause outbreaks in people and livestock with high case fatality rates across a broad geographic range, making it a significant threat to global health. It has caused repeated outbreaks in Bangladesh and India, with a mean case fatality rate greater than 70% (*12-14*). A single genus of frugivorous bats (*Pteropus*) appears to be the main reservoir for henipaviruses throughout Asia and Australia (*15-19*), including *Pteropus medius* (formerly *Pteropus giganteus* (*20*)) in Bangladesh and India (*21-24*). Nipah virus has several characteristics that make it a significant threat to human and animal health (*25-27*): 1) its bat reservoir hosts are widely distributed throughout Asia, overlapping dense human and livestock populations, providing broad opportunity to cause outbreaks; 2) it can be transmitted directly to humans by bats or via domestic animals; 3) it can be transmitted from person to person; 4) spillover has repeatedly occurred in highly populous and internationally connected regions, giving it pandemic potential; 5) it is associated with high mortality rates in people; and 6) there are currently no commercially available vaccines or therapeutics. As a result, the World Health Organization has listed Nipah virus among the ten most significant threats to global health (*28*). To date, human Nipah virus infections have been identified in India, Bangladesh, Malaysia, Singapore, and the Philippines (*12, 22, 29-31*). In May 2018, an outbreak of Nipah virus encephalitis associated with a 91% mortality rate occurred in a new location - Kerala, India - more than 1,200 km southwest of previous Indian and Bangladeshi outbreaks (*32*). A single case was subsequently reported in Kerala in 2019, and while local *P. medius* populations have been implicated as the local source of infection, the route of spillover in both instances remains unknown (*32, 33*).

In Malaysia and Bangladesh, consumption of cultivated food resources contaminated with bat excreta such as mangoes in Malaysia and date palm sap in Bangladesh and northeastern India have been identified as the predominant cause of spillover to pigs and people respectively (*34*). Human outbreaks

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Error D7. Since you repeat this with further qualification in the next sentence. Best to drop it here.

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I'd put the refs after the statements (even if you cite the same ref multiple times), because otherwise it's hard to know which paper to find the info.

78 occur almost annually in Bangladesh and the seasonal timing (November-April) and spatial distribution
79 of outbreaks coincide with patterns of raw date palm sap consumption in a region termed the "Nipah
80 belt" (*35*). However, there is variability in the number of spillover events and magnitude of the
81 outbreaks that occur each year (*36*), and spillover has occasionally occurred outside the predominant
82 season and region of date-palm sap consumption (*37*). Further, date palm sap harvesting and
83 consumption also occurs in eastern Bangladesh, yet no human outbreaks have been reported, while
84 date palm sap is not cultivated in Kerala, India at all, suggesting an alternate route of spillover (*35*).
85 While the full range of mechanisms for zoonotic transmission remain unknown, so too are the
86 underlying viral infection dynamics in bats and the extent of genetic diversity within the virus – each of
87 which may influence the timing, location and epidemiology of human outbreaks (*35*).

88 Previous research on the transmission dynamics of Nipah and Hendra viruses in *Pteropus* spp.
89 bats have produced mixed and sometimes contradictory findings. Nipah virus, like Ebola, Marburg,
90 Hendra and some bat coronaviruses, has been hypothesized to have seasonal spikes in infection that
91 coincide with annual or semi-annual synchronous birth pulses (*15, 38-44*). Seasonal periods of Nipah
92 virus shedding were observed in *P. lylei* in Thailand and seasonal spikes in NiV (or a related henipavirus)
93 seroprevalence coinciding with pregnancy periods were observed in *Eidolon dupreanum* in Madagascar
94 (*45, 46*), but not in *P. vampyrus* or *P. hypomelanus* in Peninsular Malaysia (*19*). Hendra virus prevalence
95 in Australian pteropid bats has shown multi-year inter-epidemic periods where very little virus can be
96 detected, followed by periods of increased viral shedding, suggesting that viral dynamics are not annual
97 (*47-49*). It has been hypothesized that multi-year periodicity in henipavirus infection dynamics could
98 arise from a build-up and waning of herd immunity in the reservoir host, with re-introduction of virus via
99 immigration or recrudescence or viral persistence (*10, 50-52*). Some pteropid bat species are migratory
100 and interconnected colonies form a metapopulation which could allow for viral re-introductions (*9, 19,*
101 *53, 54*). In addition, NiV recrudescence has been observed in wild-caught *P. vampyrus* and *Eidolon*
102 *helvum* and either of these phenomena could allow it to persist regionally during periods of high local
103 immunity (*55*). However, no study has yet shown evidence in open, free-ranging bat populations that
104 favors one or the other hypothesis in driving NiV transmission dynamics.

105 The goal of the current study was to determine the distribution and drivers of NiV infection
106 dynamics and NiV diversity in *Pteropus medius* in Bangladesh to try to understand patterns of human
107 outbreaks. We examined spatial, temporal and demographic variation in serological dynamics and viral
108 shedding in bats over a six-year period to determine the spatio-temporal drivers and dynamics of virus

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Isn't there also variation in exactly where there are cases even if there are cases in most years? In short, I think we want to make the pattern of cases in a given place (I'm not sure what scale, but the approximate scale of a bat colony would be useful) as clear as possible. I think the strongest thing we found from the model-fitting analysis is that large epidemics in bats don't occur every year, and thus we wouldn't expect many spill over events in the same communities in consecutive years (although we don't predict zero either, because of recrudescence)). If the human spillover data are consistent with that pattern, it would be suggestive that the bat data and fitted model are telling us something useful.

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This reference does not seem appropriate for this point or maybe its because there are 2 sets of references, and I am referring to the "wrong" one.

transmission. We also studied movement patterns of individual bats and analyzed NiV phlyogenetics to understand patterns of spatial mixing and virus strain diversity.

Results

Comparative Nipah virus prevalence study in bats inside and outside the Nipah Belt and concurrent longitudinal bat study inside the Nipah Belt (2006-2012)

We caught and tested 883 *P. medius* (~100 per district) from eight different districts across Bangladesh. We detected anti-Nipah IgG antibodies in all colonies (**Figure 1**). Seroprevalence varied by location (χ^2 = 55.61, p<.001), but there was no statistical difference between seroprevalence in bats inside the Nipah Belt and outside**.** In all locations except Tangail, adult seroprevalence exceeded juvenile seroprevalence. Viral detection in individuals was rare; overall, we detected NiV RNA in 11/XXX individuals as well as 3 pooled oropharyngeal samples (representing 5 bats, but which could not be resolved to an individual) and 21 or XXX pooled urine samples ("roost urine") collected from tarps underneath the roost (**Table 1**). We detected viral RNA in individual bats in Faridpur and Rajbari and from pooled samples from Thakurgaon and roost urine samples from Comilla. Of the 10 PCR positive individuals, three had detectable IgG antibodies (**Table S1**). We also detected virus in pooled urine collected from tarps placed below bats at roosts associated with human outbreaks in Bhanga and Joypurhat. The viral prevalence in Rajbari in January 2006 was 3.8% (95% CI: 0% -11%; n=78). In Faridpur, where we also conducted an intensive longitudinal study (see below), viral prevalence estimates ranged from 0% to 3% (95% CI: 0%-10%; n=100 at each of 18 sampling times) (**Table 1**). Nipah virus RNA was detected in individual bats from inside (Rajbari, Thakurgaon, and Faridpur) and outside (Comilla) the Nipah Belt. We detected viral RNA in bats both with and without detectable IgG antibodies (**Table S1**). Urine samples provided the highest NiV detection rate. Detection rates in individual bats by sample type were: urine/urogenital swab = 4% (n=2,126); oropharyngeal swab 3% (n=2,088); and rectal swab = 1.3% (n=79). The estimated detection rate from pooled urine samples across the entire study was 2.7% (+/- 1.6%; n=829).

Factors associated with NiV IgG *serostatus in* P. medius

Among adult and juvenile bats sampled in the aforementioned cross-sectional study from which we got blood samples (844 of 883), seropositivity was 2.4 times more likely among adults than juveniles, and 1.6 times more likely among males than females (**Figure 2**). Among females, seropositivity was higher in pup-carrying (4 times) and pregnant (1.5 times) individuals. Weight or forearm length did not

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This is a key result and we need statistics to support this claim. I did these stats a while back and they are probably in a previous version of the paper.

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Out of how many? We should give a denominator and the percent.

Number: 3 Author: Author Date: Indeterminate
Same comment.

Number: 4 Author: Author Date: Indeterminate
It says 11 above.

Number: 5 Author: Author Date: Indeterminate
This is already clear from the sentence above. I'd delete this.

Number: 6 Author: Author Date: Indeterminate
This needs a statistical test to support it. I think I did the stats for this a while back.

Number: 7 Author: Author Date: Indeterminate
Odds ratios don't actually mean the prob of seropositivity (unless the prob of seropositivity is very small in which case they are approximately similar). See this page:
https://en.wikipedia.org/wiki/Odds_ratio
You can predict the actual seroprevalence values using the fitted models and then report these actual ratios of seropositivity, but the odds ratios can't be described this way.

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Same for all of these.

consistently correlate with seropositivity, however, body condition (an assessment of pectoral muscle mass by palpation) was significantly negatively correlated (Poor/Fair body condition OR = 0.69) with serostatus. Finally, serostatus was strongly correlated in mother-pup pairs, with 71/80 pairs (89%) having matching status.

NiV serodynamics over time in a population of P. medius*, Faridpur district (2006-2012)*

We conducted an intensive longitudinal study of NiV serology in a population of bats in the Faridpur district and used flexible generalized additive models (GAMs) to characterize changes over time. There were significant fluctuations in adult (>24 mo.) and juvenile (6 – 24 mo.) seroprevalence over the six-year study period (**Figure 3A**). Juvenile seroprevalence ranged from 0% to 44% (95%CI 37%-51%), and decreased over the first year of life for bats born in each year ("yearlings"), consistent with loss of maternal antibodies in juveniles. A more pronounced decrease occurred from mid-October to mid-December. However, the GAM indicating this had only marginal better fit (ΔAIC < 1), than one with a linear decrease over the whole year (**Figure 3B**). The effect of birth cohort was significant on overall seroprevalence.

Adult seroprevalence ranged from 31% (95% CI: 20%-46%) to 82% (95% CI: 77%-87%) and went through three periods of significant decrease then increase over the course of the study (**Figure 3A**). We found no evidence of regular seasonal fluctuations; a GAM with annual cyclic terms fit worse than one without (ΔAIC > 10). Viral RNA detections occurred in periods of increasing, decreasing, and stable seroprevalence.

We fitted a series of age-stratified mechanistic models to examine different biological processes influencing serodynamics, including density- vs. frequency-dependent transmission, recrudescence vs. immigration of infected individuals, and seroreversion (loss of antibodies) in both juveniles and adults (**Figure 4**). We included annual, synchronous birthing, which occured between March and April. We assumed that pups weaned from their dams at 3 months, and became independent flyers, and that maternal antibodies waned after 6 months at which point we had pups transition into the "juvenile" class (*56, 57*). We assumed that juveniles became sexually mature at 24 mo., and entered the "adult" class based on other pteropid species(*43, 56, 58*). Density-dependent models were a far better fit to the data than frequency-dependent models (difference in log-likelihood 10.0; ΔAIC = 20.0), suggesting that movements of bats and fluctuations in colony size alter spatio-temporal variation in the risk of NiV epidemic spillover to humans. In this colony (Lomrakhandi/Khaderdi) during the period of sampling, the roost population declined from approximately 300 bats to 185, which decreased transmission potential

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What does this mean?

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Given that you already show a nice figure with these values, I'd just report the factors that are positively and negatively correlated with seropositivity and their relative strength but you don't need to give all the individual values.

in the fitted model: R_0 in adult bats was estimated to decrease from 3.5 to 2.1 as the number of bats in the colony decreased. As a result, over the six-year study period, the fitted model predicted that the number of infected bats increased when the seroprevalence of adults fell below 72% (when bat counts were highest - in 2006) and 52% (when bat counts were lowest). The fitted model suggested that serodynamics in juveniles were strongly driven by inheritance and loss of maternal antibodies. The rate of loss of maternal antibodies was 17.6 weeks (95% CI: 13.7-25.0), which was much quicker than the loss of antibodies in adults (290.8 weeks, 95% CI: 245.0-476.4) (**Table S2**). Finally, models with recrudescence fit the data better than models without recrudescence (**Table S2;** difference in log-likelihood 32.6; ΔAIC = 65.1), and models with recrudescence fit the data better than models with immigration (ΔAIC = 3.76).

Mark-recapture and seroconversion/seroreversion

A total of 2,345 bats from the Faridpur/Rajbari region were sampled and microchipped between 2007 and 2012. There were 56 recapture events (**Table S3**). Thirty-one bats were recaptured at a roost other than the original capture location. This network of roosts or "roost complex" formed a polygon covering approximately 80 Km^2 and included 15 roosts sampled during the longitudinal study (**Figure S2A and S2B**). Ten instances of seroconversion (change from IgG negative to IgG positive) and nine instances of seroreversion (positive to negative) were observed (**Table S3**). The mean time between positive and negative tests in *adults* (excluding juveniles with maternal antibodies) was 588 days (n=6) (range: 124-1,082 days).

Home range and inter-colony connectivity analysis

Home range analysis of satellite telemetry data from 14 bats (**Table S4**) showed that the majority of these bats roosted within 10 km of where the bats were originally collared, in the Faridpur (Nipah belt) colony, and within 7 km from where the bats in the Cox's Bazaar colony were originally collared (315km east of Faridpur). The average foraging radius was 18.7 km (s.d. 21.5 km) for the Faridpur bats and 10.8 km (s.d. 11.9 km) for the Cox's Bazaar bats (**Figure S2**). Homerange analysis suggests that bats in Faridpur and Cox's Bazar would have a <5% probability of intermingling (**Figure 5**). Homerange size was significantly larger during the wet season than the dry season (2,746 km^2 vs. 618 km^2) (**Figures S3 & S4**).

NiV phylogenetic analysis.

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I think this is 2 words. If so, I'd change it throughout.

Phylogenetic analysis of NiV sequences from a 224nt section of the N gene (nt position 1290-1509 [position ref gb|FJ513078.1| India]) suggests that strains from both India and Malaysia clades are present in Bangladesh bats (**Figure 6**). This finding is supported by an analysis of near-whole N gene sequences (~1720 nt) from bats, pigs, and humans, including those from a subset of *P. medius* from this and a more recent study by our group (**Figure S5**) (*59*). Eleven 224nt N gene sequences obtained from bats between 2006 and 2012 (all from the Faridpur population) were identical. Overall, the N gene sequences identified from the Faridpur, Rajbari and Bhanga colonies between 2006 and 2011 had 98.21%-100% shared nucleotide identity. Sequences from Rajbari district obtained five years apart (January 2006 and January 2011) had only a single nucleotide difference resulting in a synonymous substitution (G to A) at position 1304, which was found in four other bat NiV sequences from this study, as well as in the NiV isolate from *P. vampyrus* in Malaysia. Five Human NiV N gene sequences from various locations within the Nipah belt over the same time period as our bat study show more nucleotide diversity than those from the Faridpur *P. medius* population. Human sequences throughout Bangladesh and from Kerala, India, all nested within the diversity found in *P. medius* (**Figure 6**). By contrast, the sequences found in *P. medius* from Comilla, a location 150Km to the east of Faridpur, showed 80.8%-82.59% shared nucleotide identity with sequences from *P. medius* in Faridpur and clustered within the Malaysia group of NiV sequences. The two Comilla sequences were identical to each other, and had up to 87.95% shared nucleotide identity to sequences from *P. lylei* in Thailand. *Pteropus lylei* bats in Thailand were also found to carry NiV strains from both Malaysia and Bangladesh groups.

Discussion

Our study provides new insights into Nipah virus transmission dynamics, genetics and host ecology. Previous studies from Bangladesh suggested that human NiV outbreaks occur only within a defined region in western Bangladesh, termed the “Nipah belt,” and during a defined season (Nov-Apr), which raised the question of whether that observation was entirely due to date palm sap consumption, or whether ecological factors such as the distribution and timing of bat viral infection also influenced the timing and location of human cases (*13, 35, 60*). We undertook the most geographically extensive survey of *Pteropus medius* in Bangladesh to date to understand Nipah virus infection patterns in its putative reservoir, *Pteropus medius*, which is common in Bangladesh and throughout the Indian subcontinent (*21, 22, 56*).

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I believe we have previously reported the Comilla case.

Overall, our findings suggest viral circulation is not limited to the Nipah belt, but that NiV transmission occurs in bat populations throughout the country. We observed that virus can be shed at any time of year, and that viral dynamics are not annual or seasonal, but driven by demographic and immunological factors. Analysis of serological data from our longitudinal study suggests that the underlying mechanism driving the timing of NiV transmission in bats is the waning of herd immunity in bat populations allowing heightened viral transmission, but recrudescence can result in sporadic shedding at any time.

A number of mechanisms have been proposed for the maintenance of acute viral infections in bat metapopulations, including synchronous birthing and subsequent loss of maternal antibodies (*10, 39, 41*), lowered immunity within pregnant females due to stress, nutritional stress and other factors (*43*) immigration of infected individuals from other colonies (*53, 61, 62*), and recrudescence within previously-infected individuals (*10, 55, 63*). Our modeling indicates that NiV is primarily driven by density-dependent transmission dynamics among adult bats, with cycles of higher seroprevalence that would dampen intra-colony transmission followed by waning of antibody titers within individuals and at a population level. Waning humoral immunity against Nipah virus is a consistent feature of henipavirus studies in African pteropodid bats (*52, 64*). Our recapture data provided the first reported evidence of the loss of detectable NiV IgG antibodies in recaptured individual free-ranging bats, which supports our observation of population level waning immunity. The consistently lower and decreasing seroprevalence that we observed in juveniles suggests that they lose maternal antibodies over their first year, and likely in the first 6-7 months, consistent with other studies of maternal antibodies against henipaviruses in pteropodid bats (*43, 52, 57, 65*). However, our analysis does not support the hypothesis that seasonal pulses of these new seronegative individuals are the primary driver of new outbreaks in adults (*41*).

Our model outputs suggest that spikes in viral transmission occur after virus is reintroduced into colonies after immunity has waned. The model fitting suggests that serodynamics are more consistent with recrudescence, but immigration of infected individuals could also reintroduce the virus. Recrudescence would presumably occur in individuals with antibody titers that have waned below a neutralizing titer, if loss of humoral immunity following a primary infection is sufficient to allow a second infection. Recrudescence of henipavirus infection has been observed for NiV in captive *P. vampyrus* (*55*), for henipavirus in captive *E. helvum* (*52, 66*), and has also been observed in humans infected by NiV (*67*) and Hendra virus (*68*). It is difficult to know from serology alone whether wild-caught seronegative bats had been previously infected. Experimental infection of naïve and previously infected *Pteropus medius* that have sero-reverted would provide a better understanding of how humoral immunity influences

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Does not seem the optimal conjunction.

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This is a bit less aggressive. If the paper goes to one of the authors of ref 41 it'd be better to be more gentle.

individual susceptibility to infection, and inform dynamics models attempting to explain viral maintenance within bat populations (*64*).

Our longitudinal study is limited in that it may not necessarily reflect temporal infection dynamics in all bat populations across Bangladesh. Our roost count data and recapture data from microchipped bats showed how roost sizes can fluctuate, and local roost shifting can occur. The observation of individual bats using multiple roost sites suggests that changes in roost count, which our models suggest impacts transmission dynamics, could reflect local shifts in densities rather than fluctuations in regional populations.

Understanding how bat populations connect across landscapes is important for understanding viral maintenance, and studying local and migratory bat movements can provide important ecological information related to viral transmission, including how bats move between different colonies (*53*). Our satellite telemetry data suggest that *P. medius* exists as a metapopulation, like other pteropid species (*10, 62*). However, *Pteropus medius* in Bangladesh appear to travel shorter distances and remain within a smaller home range (321.46km^2 and 2,865.27km^2 for two groups) compared to *P. vampyrus* in Malaysia (64,000 km^2 and 128,000 km^2) and *Acerodon jubatus* in the Philippines which are similarly sized fruit bats (*53, 69*). Pteropodid bat migration is primarily driven by seasonal food resource availability (*54, 70-72*). In Bangladesh, *P. medius* prefer to roost in human-dominated environments in highly fragmented forests (*73*). The anthropogenic colonization and conversion of land over recent human history has likely led to increased food availability for *P. medius* and reduced necessity for long-distance migration (*34*). This may reflect a similar adaptation to anthropogenic food resources as observed over the last few decades in Australian *Pteropus* species (*62*). Genetic analysis of *P. medius* across Bangladesh has shown that the population is panmictic – that historically, there has been interbreeding among populations across Bangladesh(*74*). If movements are generally more localized, as suggested by telemetry, then less connectivity among flying fox populations may influence Nipah transmission by creating longer inter-epidemic periods and larger amplitude fluctuations in population level immunity compared to more migratory species (*62*).

Bat movement and population connectivity may also influence the genetic diversity of Nipah virus found in different locations, and genotypic variation has been associated with different clinical outcomes in people. While the overall strain diversity among Nipah virus has not been well characterized due to a dearth of isolates, two distinct NiV clades have been described: A Bangladesh clade, which includes sequences identified in India and Bangladesh; and a Malaysian clade, which comprises sequences from Malaysia, Cambodia, The Philippines and Thailand (*31, 59, 75*). Strains of NiV

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It's not a limitation in model parameterization – it was a limitation in the data. If we had seroprevalence data in many individual roosts we could examine the importance of local vs regional population processes. But that would be a ton of work!

from these two clades are associated with differences in pathogenesis, epidemiological and clinical profiles in humans and animal models and observed shedding patterns in bats (*45, 76-80*). Phenotypic variation in Nipah virus could influence observed human outbreak patterns by altering transmission to, or pathogenesis in, humans, and the likelihood of smaller outbreaks being identified or reported (*81*). Human-to-human NiV transmission via contact with respiratory and other secretions has been regularly observed in Bangladesh and India, including the recent 2018 outbreak in Kerala (*12, 82, 83*), whereas transmission among people was not a common feature of the Malaysia outbreak, despite close contact between cases and health care providers (*84, 85*). Nipah virus cases in Bangladesh have shown more strain diversity than in the Malaysia outbreak (*86*).,

Until now, there have been very few Nipah sequences obtained from *P. medius.* We found that Nipah N-gene sequence from bats from the Faridpur population were nearly identical over time, compared to variation in N-gene sequences from bats and humans from other locations observed over the same time period (2006-2010). This suggests that there may be locally prevalent and stable NiV genotypes that persist within bat colonies. Human NiV genotype diversity is likely a reflection of the relative diversity of the NiV strains in the local bats that seed outbreaks (*9*). This is also supported by viral sequences obtained from human and bats associated with the 2018 NiV outbreak in Kerala, India, where human NiV sequences were most closely related to local *P. medius* sequences *(87)*. We found a significantly divergent NiV strain in Comilla, which clustered within the Malaysia NiV clade, suggesting that strains from both clades are circulating in bats in Bangladesh.

Connectivity of pteropid bats in Bangladesh with those in Southeast Asia could explain the observed strain diversity in our study. Historical interbreeding of *P. medius* with pteropid species found in Myanmar, Thailand, and Malaysia and our telemetry findings showing bats are capable of flying hundreds of kilometers, could explain our discovery of a Malaysia clade NiV sequence in bats from Comilla (*74*). NiV Bangladesh strains have also been found in *P. lylei* in Thailand (*88*). The N gene of the Comilla NiV strain differs from those reported from bats in the Nipah belt by 20%, whereas NiV Malaysia and NiV Bangladesh differ by only 6-9% and are associated with different clinical profiles. Whole genome sequence (which could not be obtained) would have allowed for better characterization of the Comilla strain, but the N gene is generally conserved relative to other genes, and suggests the rest of the genome may also be highly divergent. It is therefore plausible that the clinical profile of a 20% divergent NiV strain differs significantly from known strains. Further studies linking viral genotype to clinical phenotype would provide insight into the implications of strain diversity in bats for human outbreaks.

Finally, our study sheds light on the sporadic nature of NiV outbreaks with multi-year inter-epidemic periods in South Asia. First, PCR results show that overall NiV incidence in *P. medius* is low, consistent with previous studies of Hendra and Nipah virus (*43, 48, 89, 90*). The data and our modeling suggests that PCR-positive samples are more likely to be identified during viral transmission spikes after periodic reintroduction into populations that have become susceptible through waning population-level immunity (*10*). Viral detection in bats has also coincided with human outbreaks (*59, 87*). This is likely a rare or at least sporadic event. In the current study, observed seroprevalence patterns and the fitted model suggest that three periods of transmission occurred over the 6 years of sampling, each of which followed periods of low adult seroprevalence, though not all measurements of low seroprevalence were followed by outbreaks. We detected NiV RNA during periods of both increasing and decreasing seroprevalence, supporting the fitted model which suggested that shedding can occur through recrudescence at low levels in bats even in periods without sustained transmission. Our observation that not every instance of rising seroprevalence resulted in detectable viral shedding suggests that not all episodes of viral circulation in bats are of equal magnitude, and that other factors (e.g. variation in human-bat contact and exposure) may affect likelihood of spillover. Together, this evidence suggests that outbreaks can occur in bats when the population falls below a protective threshold of immunity in any season, but variability in how many bats become infected may impact the likelihood of spillover to humans, assuming a route of transmission is available. This could explain variation in the number of human outbreaks (e.g. spillover events) from year-to year in Bangladesh. Thus, the timing of multiple factors involved in driving transmission dynamics needs to align for intra-colony NiV transmission events and further align with human behavior and availability of a route of spillover for human outbreaks to occur, as previously hypothesized (*91*). This, and the seasonality and specific geography of date palm sap consumption in Bangladesh likely explains the somewhat sporadic nature of human outbreaks in the region, albeit that when spillover occurs, it is within the well-defined date palm sap collection season and geographic zone (*35*).

These findings suggest that Nipah virus outbreaks in other regions of Bangladesh where *Pteropus* spp. bats occur, are also likely to be sporadic and rare, leading to under-reporting or a lack or reporting, particularly given that human neurologic symptoms are similar to other common infections, such as Japanese encephalitis, malaria, and measles (*92*). Understanding whether some NiV strains are capable of causing mild or asymptomatic cases will provide important insights about why outbreaks may not have been detected in areas such as eastern Bangladesh or other parts of Asia, where host, virus, and potential routes of spillover exist. Mild or asymptomatic cases would be unlikely to be detected by

current surveillance systems and it's possible that cryptic spillovers have occurred in Bangladesh, where about analysis of health care seeking behavior suggests that half of all outbreaks between 2007 and 2014 were unreported (*93*). Our work and other reports suggest that Nipah virus transmission is possible wherever *Pteropus* spp. bats and humans live in close association and at any time of year, provided there is an available pathway of transmission. The 2018 and 2019 spillover events in Kerala, India, which were linked to local *P. medius* colonies and which occurred in an area that does not cultivate date palm sap, further emphasize this point.

Identifying areas where high risk interfaces exist between pteropid bats and people, throughout their range, will be important for monitoring Nipah spillover events and quickly responding to outbreaks, as well as establishing interventions to prevent spillover. Raising awareness of the potential for contaminated food to be a route of Nipah virus transmission and in protecting food resources to limit human or livestock exposure, may be effective in reducing the risk of a more transmissible strain of Nipah virus from emerging and causing an epidemic with significant human and animal mortality.

Methods

The study period was between January 2006 and November 2012. The study was conducted under Tufts University IACUC protocol #G929-07. Locations were selected based on whether the district had any previously recorded human NiV encephalitis clusters at the time of this study and was therefore inside the Nipah Belt (e.g. Rajbari, Tangail, Thakurgaon, and Kushtia) or whether they had not and were outside the Nipah Belt (e.g. Comilla, Khulna, Sylhet, and Chittagong). The Thakurgaon study was conducted as part of an NiV outbreak investigation, and coincided with ongoing human transmission (*94*). Between 2006-2012, three different studies of *Pteropus medius*, with similar bat sampling protocols were performed: 1) a cross-sectional spatial study with a single sampling event in each of the eight locations listed above; 2) a longitudinal study of a Faripur bat colony with repeated sampling approximately every 3 months from July 2007 to November 2012; and 3) a longitudinal study of the Rajbari colony with repeated sampling at a monthly interval between 12 month period between April 2010 and May 2011. Opportunistic sampling of *Pteropus medius* was also performed during this time period during NiV outbreak investigations [Bangha, Faridpur (Feb 2010), Joypurhat (Jan 2012), Rajbari (Dec 2009), West Algi, Faridpur (Jan 2010)]. Bats were captured using mist nets at locations within eight different districts across Bangladesh between January 2006 and December 2012 (**Figure 1**).

Capture and sample collection

For the country-wide cross-sectional and Faridpur longitudinal study, on average, 100 bats were sampled at each sampling event, which lasted 7-10 days. This sample size allowed us to detect at least one exposed bat (IgG antibody-positive) given a seroprevalence of 10% with 95% confidence. Bats were captured using a custom-made mist net of approximately 10 m x 15 m suspended between bamboo poles which were mounted atop trees close to the target bat roost. Catching occurred between 11 pm and 5 am as bats returned from foraging. To minimize bat stress and chance of injury, nets were continuously monitored and each bat was extracted from the net immediately after entanglement. Personal protective equipment was worn during capture and sampling, which included dedicated long-sleeve outerwear or Tyvek suits, P100 respirators (3M, USA), safety glasses, nitrile gloves, and leather welding gloves for bat restraint. Bats were placed into cotton pillowcases and held for a maximum of 6 hours before being released at the site of capture. Bats were sampled at the site of capture using a field lab setup. Bats were anesthetized using isoflurane gas (*95*) and blood, urine, oropharyngeal swabs, and wing membrane biopsies (for genetic studies) were collected. For some sampling periods, rectal swabs were collected but due to resource constraints, these samples were deemed to likely be lower yield than

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Recommend including local institutional approval.

saliva and urine for NiV, and were discontinued during the study. For each bat sampled we recorded age, weight, sex, physiologic and reproductive status, and morphometric measurements as described previously (*21*). Bats were classified as either juveniles (approximately four to six months - the age by which most pups are weaned) to two years old (the age when most *Pteropus* species reach sexual maturity) or adults (sexually mature) based on body size and the presence of secondary sexual characteristics, pregnancy, or lactation - indicating reproductive maturity (*21, 96*).

Up to 3.0 mL of blood were collected from the brachial vein and placed into serum tubes with serum clot activator (Vaccutainer, USA). Blood tubes were stored vertically on ice packs in a cold box and serum was allowed to separate overnight. Serum was drawn from the tube after 24 hours and placed in a screw-top cryovial (Corning, USA) and stored in a liquid nitrogen dewar (Princeton Cryogenics, NJ, USA). Sterile pediatric swabs with polyester tips and aluminum shafts were used to collect urogenital and rectal samples, and larger polyester swabs with plastic shafts (Fisher, USA) were used to collect oropharyngeal samples. All swabs were collected in duplicate, with one set being placed individually in cryotubes containing lysis buffer (either tri-reagent or NucliSENS Lysis buffer, BIOMERIEUX, France) and the second set in viral transport medium. All tubes were stored in liquid nitrogen in the field then transferred to a -80C freezer.

During each sampling event, pooled urine samples were collected beneath bat roosts using polyethylene sheets (2' x 3') distributed evenly under the colony between 3 am and 6 am. Urine was collected from each sheet either using a sterile swab to soak up droplets, or a sterile disposable pipette. Swabs or syringed urine from a single sheet were combined to represent a pooled sample. Each urine sample was divided in half and aliquoted into lysis buffer or VTM at an approximate ratio of one part sample to two parts preservative.

Serological and molecular assays

Sera from the cross-sectional survey were heat inactivated at 56^{o}C for 30 minutes, as described (*97*) prior to shipment to the Center for Infection and Immunity at Columbia University (New York, USA) for analysis. Samples were screened for anti-NiV IgG antibodies using an enzyme linked immunosorbent assay (ELISA) as described in (*21*). Sera from the longitudinal studies were sent to the Australian Animal Health Laboratory and were gamma irradiated upon receipt. Because of the large sample size and development of a high throughput multiplex assay of comparable specificity and sensitivity, for these samples we used a Luminex-based assay to detect anti-Nipah G IgG antibodies reactive to a purified NiV soluble G protein reagent (*98, 99*).

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Jen Barr – please insert language about MFI cutoff that was used for each protein. We used the NiVsG value to decide positive or negative status, but should we discuss NiVsF status, or just focus on G?

Total nucleic acids from swabs and urine samples were extracted and cDNA synthesized using Superscript III (Invitrogen) according to manufacturer's instructions. A nested RT-PCR and a real-time assay targeting the N gene were used to detect NiV RNA in samples (*100*). A RT-qPCR designed to detect the nucleocapsid gene of all known NiV isolates was also utilized (*101*). Oligonucleotide primers and probe were as described (*101*). Assays were performed using AgPath-ID One-StepRT-PCR Reagents (Thermofisher) with 250 nM probe, 50 nM forward and 900 nM reverse primers. Thermal cycling was 45◦C for 10 min, followed by 45 cycles of 95◦C for 15 s and 60◦C for 45 s. Cut-off values were cycle threshold (C_T) ≤40 for positive and CT ≥45 for negative. Results with C_T values between 40 and 45 were deemed indeterminate, i.e. not conclusively positive or negative. Samples with NiV RNA detected by real time PCR were confirmed by gel electrophoresis and product sequencing.

A subset of NiV-positive samples was processed by high-throughput sequencing (HTS) on the Ion Torrent PGM platform in order to obtain additional NiV genomic sequence. Libraries were prepared according to the manufacturer's instructions, and 1 million reads allocated per sample. HTS reads were aligned against host reference databases to remove host background using bowtie2 mapper, and host-subtracted reads primer trimmed and filtered based on quality, GC content and sequence complexity. The remaining reads were de novo assembled using Newbler (v2.6) and mapped to the full length NiV genome. Contigs and unique singletons were also subjected to homology search using MegaBlast against the GenBank nucleotide database, in case variance in parts of the genome precluded efficient mapping. From these data, N gene consensus sequences were constructed using Geneious v 7.1, and used for phylogenetic analyses.

Phylogenetic analysis

All *P. medius* NiV sequences have been submitted to Genbank and accession numbers are included in **Figure 6**. Sequence alignments were constructed using ClustalW in Geneious Prime software (*102*). Phylogenetic trees of NiV N-gene sequences were constructed using Neighbor-Joining, Maximum-Likelihood algorithms and figures constructed in FigTree 1.4.2.

Satellite telemetry and homerange analysis

We developed a collar system to attach 12g solar-powered Platform Terminal Transmitters (PTT) (Microwave Telemetry, Colombia, MD, USA) to adult bats using commercial nylon feline collars with the buckle removed and 0-gauge nylon suture to attach the PTT to the collar and to fasten the collar around the bat's neck. Collars were fitted to the bat such that there was enough space to allow for normal neck

movement and swallowing, but so that the collar would not slip over the head of the animal (**Figure S6**). PTTs were programmed with a duty cycle of 10 hours on and 48 hours off. Data was accessed via the Argos online data service (argos-system.org; France). A total of 14 collars were deployed as follows: Feb 2009: 3 males, 3 females from a colony in Shuvarampur, Faridpur district; Feb 2011: 3 males, 2 females from the same colony; and April 2011 Cox's Bazaar, 3 bats from a colony in Cox's Bazaar, Chittagong district. Bats were selected based on size such that the total weight of the collar (~21g) was less than 3% of the bat's body mass (Table S3).

The individual telemetry dataset was combined for each region and its aggregate utilization distributions (UD) computed in R using package 'adehabitatHR' (*103*). Population-specific home range is represented by the *95% area enclosure of its UD's volume. The volume of intersection between the colonies quantifies the extent of home range overlap. To evaluate the potential for contact with the Sylhet colony, we calculated the most likely distance moved ('mldm') for each sampled bat at Faridpur where the population was more intensively monitored. Movement distance was measured in kilometers with respect to a centroid location (ω) shared by the whole colony; assuming random spatial distribution in tracking rate, we estimated its kernel density function per individual and defined mldm as the mode.

Statistical approach – cross-sectional study

We fit a Bayesian generalized linear model with a logit link and a Bernoulli distribution to identify potential predictors which influenced a bat's serostatus. We included age, sex, age- and sex-normalized mass and forearm length, mass:forearm ratio, body condition, and whether the bat was pregnant, lactating, or carrying a pup. We included location of sampling as a group effect (similar to a random effect in a frequentist context) nested within Nipah Belt or non-Nipah Belt regions. We fit the models and performed posterior predictive checks in R 3.4.3, using the **brms** and **rstan** packages.

Statistical approach – longitudinal study

We fit binomial general additive models (GAMs) (*104*) to the time series of adult and juvenile seroprevalence in the longitudinal study. For juveniles, we modeled the birth cohort of bats as separate random effects in a pooled model of juveniles' dynamics starting from June of their birth year, June being the earliest month we sampled free-flying juveniles in any cohort. We determined the cohort year of juveniles by using cluster analysis to group individuals by weight, assuming those in the smallest group were born in the current year and those in the larger group were born the previous year. 92% of

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If you used a Bayesian approach you need to specify what priors you used for each coefficient and whether the predictors were standardized, since this determines how informative those priors are.

juveniles captured were yearlings. For adults, we modeled dynamics of adults as a single pool over the entire course of the study. We tested models with and without annual cyclic effects.

Where time series had significant temporal autocorrelation (adults only), we aggregated data by week. We determined periods of significant increase in decrease as those where the 95% confidence interval of the GAM prediction's derivative did not overlap zero. We fit the models and performed checks in R 3.4.3, using the **mgcv** package.

To examine the importance of different biological mechanisms in transmission, we fit an age-structured (adult and juvenile) maternally immune (M)-susceptible (S)-infected (I)-recovered (R) model with recrudescence (R to I) and loss of immunity (R to S) to the seroprevalence data on a weekly timescale:

$$\frac{dS_J}{dt} = -S_J(\beta_{JJ}I_J + \beta_{AJ}I_A) - \mu_J S_J + bN_A(t-5)\frac{S_A}{N_A} - bN_A(t-52)\frac{S_J}{N_J}(1-\mu_J)^{52} + \lambda M_J$$

$$\frac{dI_J}{dt} = S_J(\beta_{JJ}I_J + \beta_{AJ}I_A) - \gamma I_J - bN_A(t-52)\frac{I_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dR_J}{dt} = \gamma I_J - \mu_J S_J - \mu_J R_J - bN_A(t-52)\frac{R_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dM_J}{dt} = bA(t-5)\frac{R_A}{N_A} - \lambda M_J - \mu_J M_J - bN_A(t-52)\frac{R_A}{N_A}(1-\mu_J)^{52}\lambda^{52}$$

$$\frac{dS_A}{dt} = -S_A(\beta_{AA}I_A + \beta_{JA}I_J) - \mu_A S_A + \tau R_A + bN_A(t-52)\frac{S_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dI_A}{dt} = S_A(\beta_{AA}I_A + \beta_{JA}I_J) - \gamma I_J - \mu_A I_A + bN_A(t-52)\frac{I_J}{N_J}(1-\mu_J)^{52} + \Delta R_A$$

$$\frac{dR_A}{dt} = \gamma I_A - \mu_A R_A + bN_A(t-52)\frac{R_J}{N_J}(1-\mu_J)^{52} - \tau R_A - \Delta R_A$$

We included a class M for the density of juvenile bats with maternal antibodies to allow for the biological possibility that maternal antibodies are lost at a much higher rate than antibodies acquired following infection. The subscripts J refer to juveniles and A to adults, β is the transmission rate, γ is the recovery rate, μ is the mortality rate, τ is the rate of loss of adult immunity, λ is the rate of loss of maternal antibodies(*57*), Δ is the adult recrudescence rate (R to I), b is the birth rate (pups join the juvenile population after 5 weeks). Juveniles transition to adults after 52 weeks. We included terms for loss of antibody in adults (τ, S to R), and viral recrudescence (Δ, R to I) based on previous studies on captive bats that demonstrated the existence of these processes without providing enough data to characterize them precisely (*55, 65*). We fit this deterministic model to the seroprevalence data by maximum likelihood,

which assumes that deviations from the model are due to observation error. We estimated the confidence intervals around maximum likelihood parameter estimates using likelihood profiles using the *profile* function in package *bbmle* in R v3.2.2.

We used model fitting and model comparison to examine the need for several of the biological processes in the model above that could influence NiV dynamics. First, we examined both density and frequency-dependent transmission by comparing the fit of the model above to one with transmission terms that have population size (N_A or N_J) in the denominator. Second, we examined the confidence intervals of the parameters describing viral recrudescence, loss of antibodies in adult bats, and loss of antibodies in juvenile bats. If the confidence bounds for these parameters included zero, then these biological processes are not needed to explain the serological dynamics. Finally, we examined the confidence bounds for parameters describing the loss of maternal and non-maternal antibodies (τ and λ), to determine if the rate of loss of these two types of immunity were different. We note that this model structure has similarities to a susceptible-infected-latently infected(L)-infected (SILI) model (if latently infected individuals are seropositive), but the model above differs in allowing for the possibility of individuals to transition from the R class back to the S class.

Code availability

SIR model code written in R is available upon request.

Data availability

All molecular sequences are available via Genbank. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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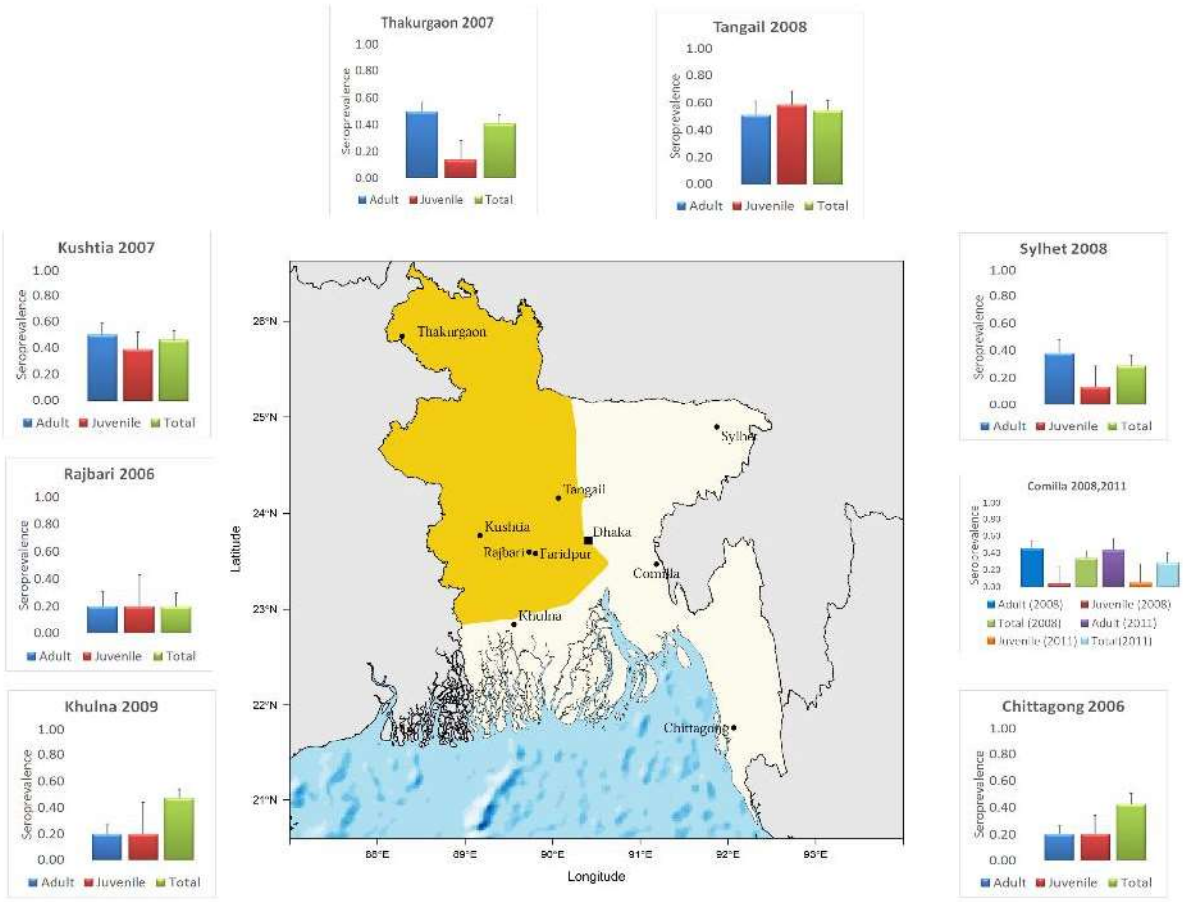

784

785 **Figure 1.** Map showing age-stratified seroprevalence in *Pteropus medius* colonies, Bangladesh. Bats from eight
786 colonies were sampled and tested for anti-NiV IgG antibodies: four within the "Nipah Belt" (orange shaded) and
787 four outside. Seroprevalence of adults (blue, purple), juveniles (red, orange) and total seroprevalence (green, light
788 blue) are shown. Number (n) of Adult, Juvenile, and Total bats sampled (clockwise): Tangail [53,41,94], Sylhet [63,
789 37, 100], Comilla 2008 [73,27,100], Comilla 2011 [30,20,50], Chittagong [72,24,96], Khulna[85,15,100], Rajbari
790 [65,15, 80], Kushtia [64,36,100], Thakurgaon [89,29,118]. The shaded region represents the "Nipah Belt" where
791 previous human NiV outbreaks have been reported.

792

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You only need one legend since it is the same for all panels. This would all you to make the panels a little bigger. I would try to reduce the white-space and zoom in on the map a little and make the graphs bigger. Finally I'd add lines b/w the locations on the map and the graphs.

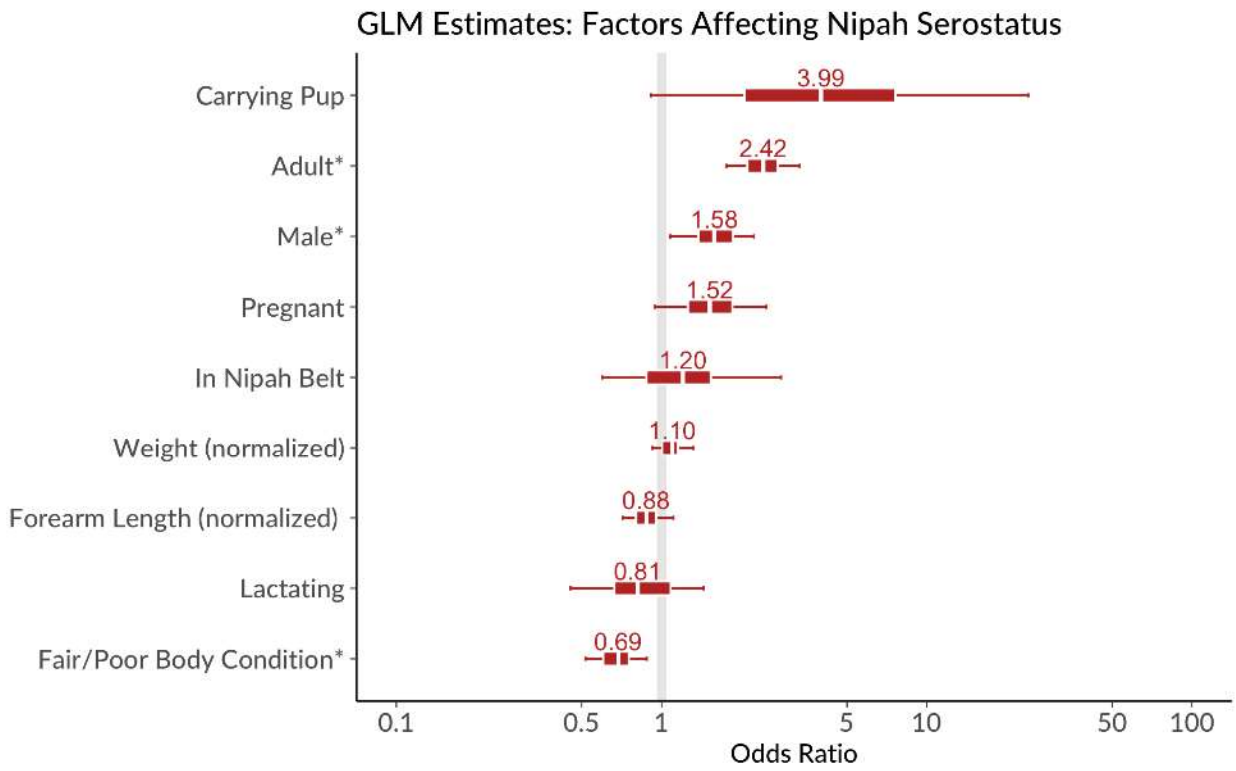

Figure 2. Results of Bayesian GLM of factors affecting Nipah serostatus in bats in cross-sectional study. Bars indicate odds ratios and 50% (inner) and 95% (outer) credible intervals for model parameters. Factors with asterisks (*) have 95% CIs that do not overlap 1. Model intercept (predicted probability of seropositivity for a juvenile, female, bat outside the Nipah belt of mean size and good body condition) was 0.26, (95% CI 0.12-0.56). Random effect of location (not shown) had an estimated SD of 0.63 (95% CI 0.29-1.27)

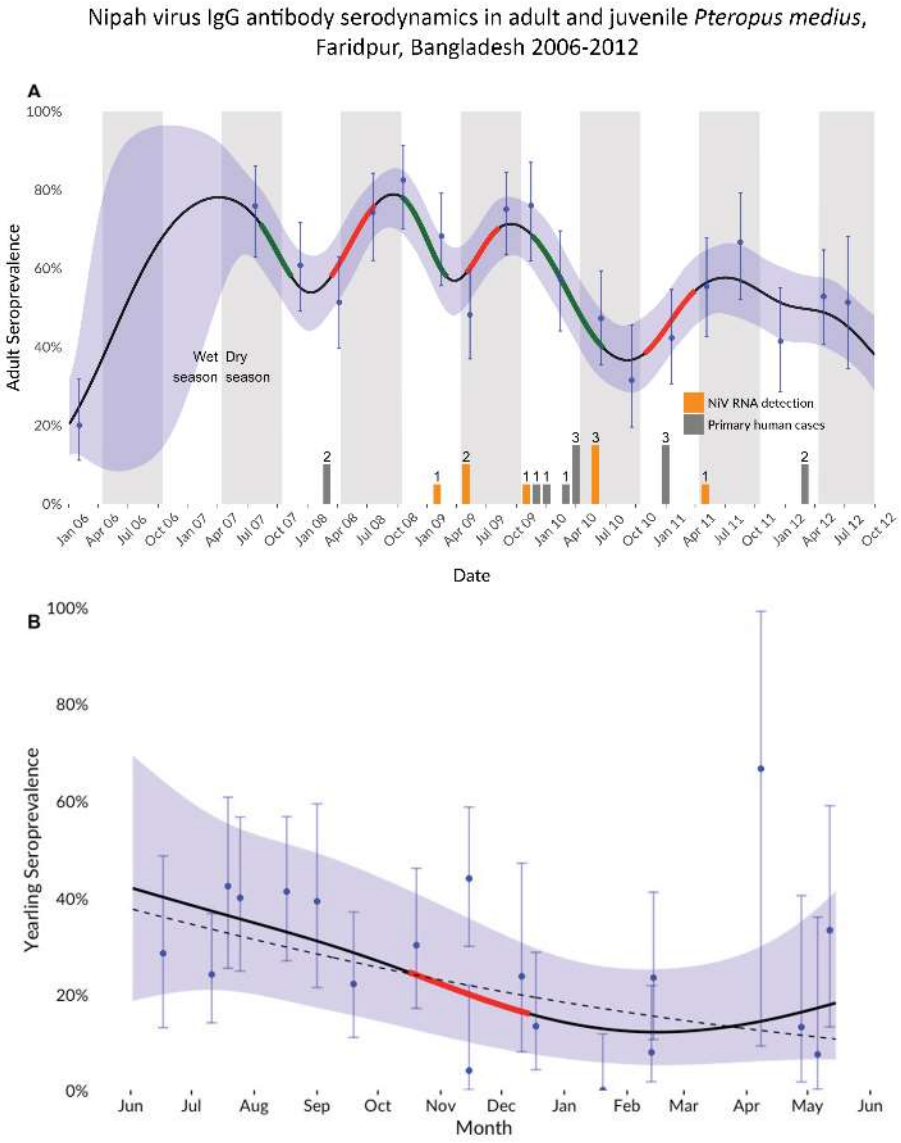

Figure 3 A & B. Serodynamics of the Faridpur bat population. (A) Adult serodynamics, with measured values and 95% CI in blue, and mean GAM prediction and 95% shown with line and surrounding shaded areas. Periods of significant increase (red) and decrease (green) shown where the GAM derivative's 95% CI does not overlap zero. Counts of primary human cases from local district (orange, and bat viral detections (dark grey, see Table 1), shown on bottom. (B) Juvenile serodynamics during the first year of life ("yearlings"), with all years' measurements overlain to show cohort-level dynamics across all study years. Measured values and 95% CI in blue, and mean and 95% CI for the GAM model pooled across cohorts shown with line and surrounded shaded areas. The period of significant decline in the GAM is shown in red. Also shown is the mean prediction of a model with only a linear mean term, with similar fit (ΔAIC < 1) as the GAM (dotted line).

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I'd show these as a % of samples collected, not the raw #. Otherwise you are confounding prevalence with sampling effort, unless the # of samples was the same for every sample.

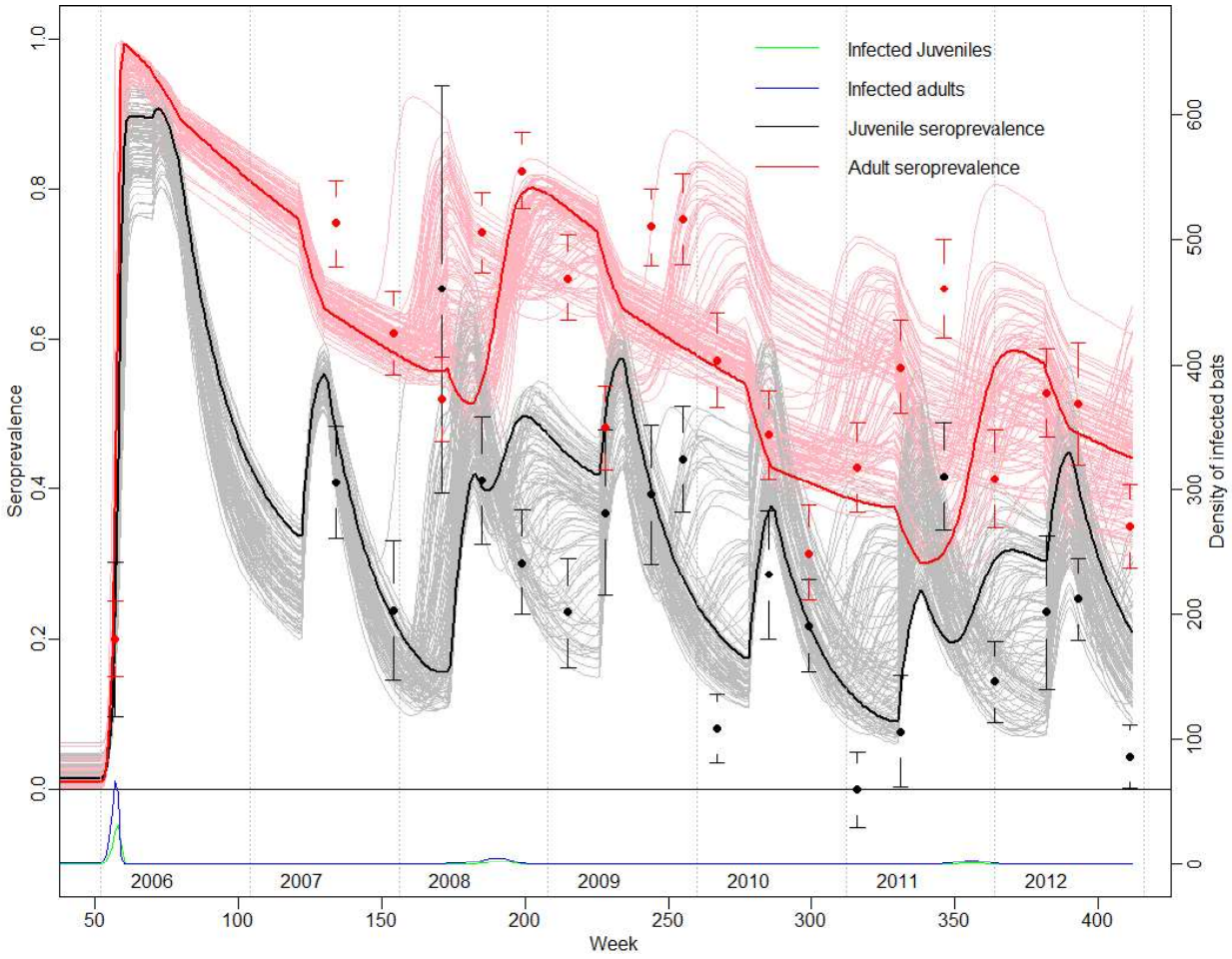

Figure 4. **NiV serological dynamics in adult and juvenile bats.** The observed data (red and black points ±1 SE) and model fit (solid lines; thick lines show the trajectory for the model with maximum likelihood parameter estimates; thin lines show realizations for parameter estimates drawn from the estimated distributions) for the fraction of adults and juveniles seropositive for NiV (left axis), and the model estimated density of infected adult and juvenile bats (bottom panel and right axis). See Methods for details of model structure.

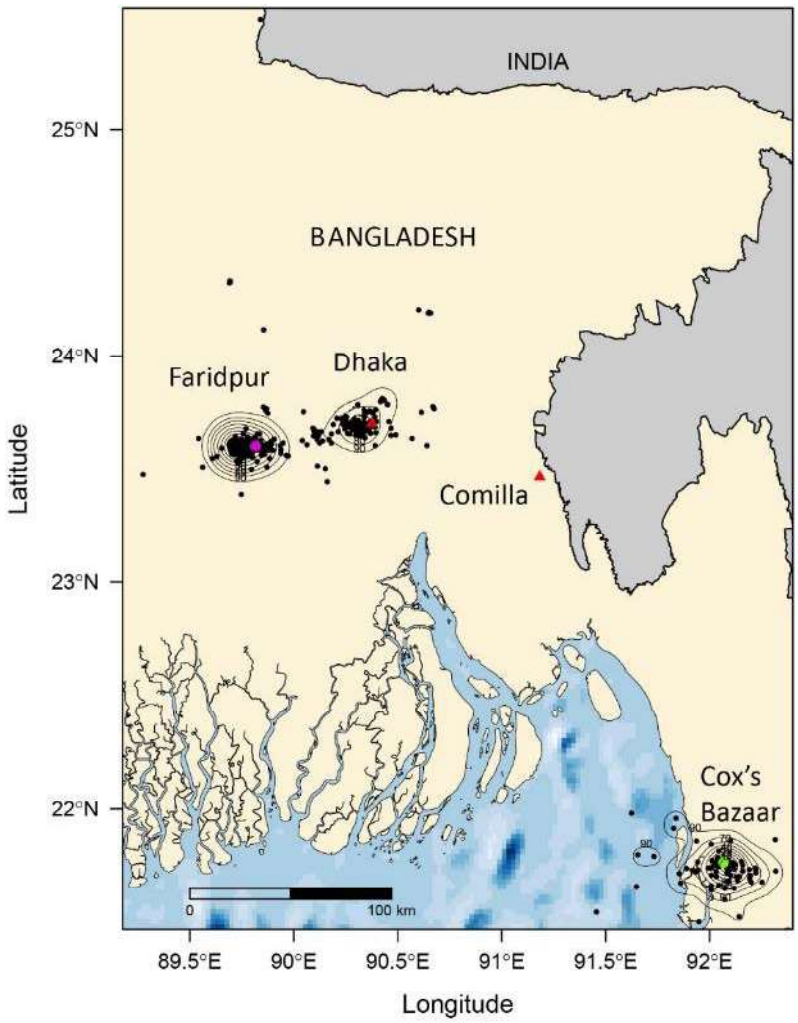

Figure 5. Satellite telemetry and homerange analysis. Location data from satellite collars (n=14) placed on 11 bats from Faridpur and 3 bats from Cox's Bazaar, Chittagong collected between 2009 and 2011, were used to calculate local and long-range movement patterns and home range for these two groups.

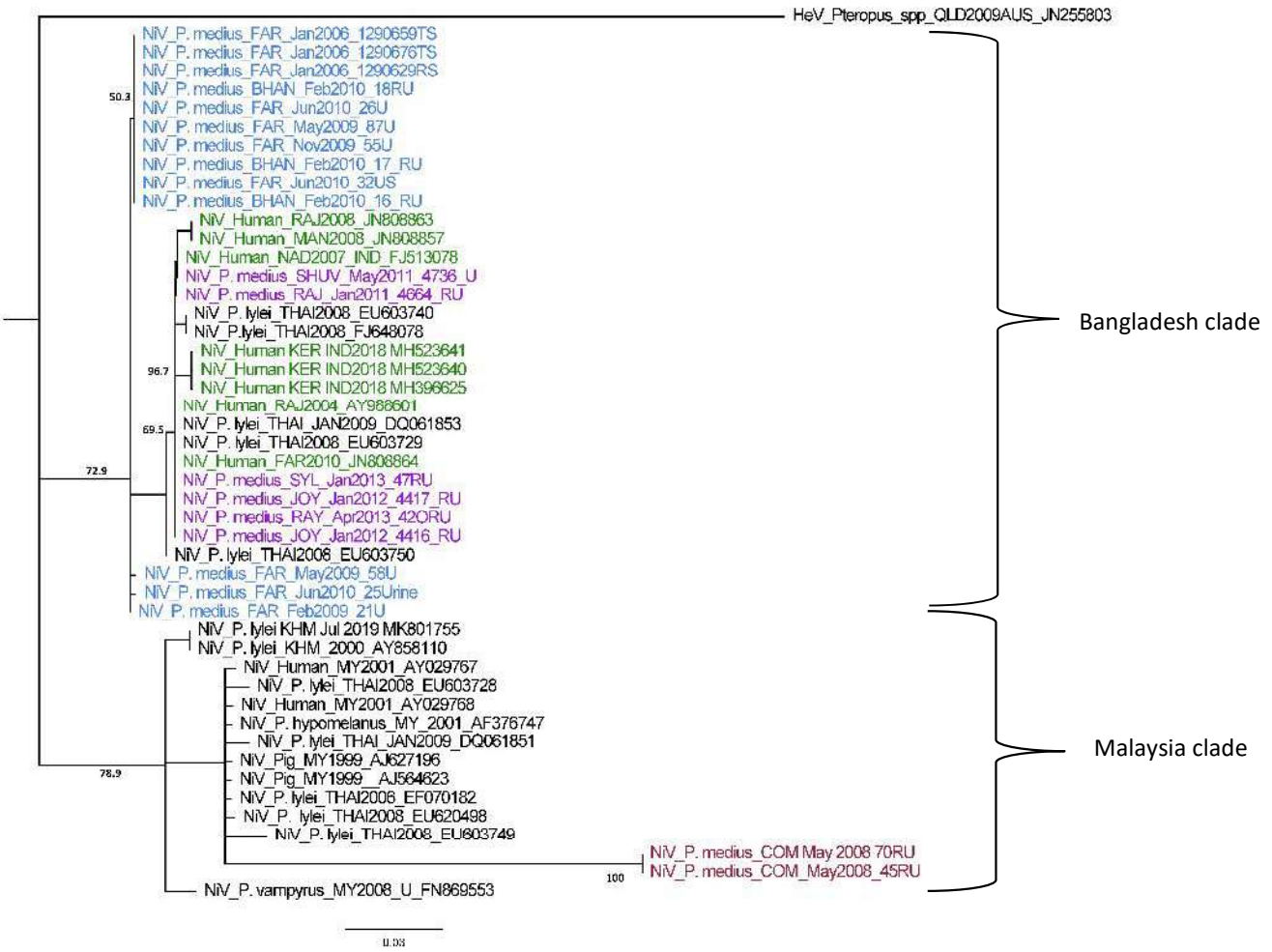

Figure 6. **Nipah Virus N gene phylogenetic tree (224nt):** Tree created in Geneious Prime 2019 using a Neighbor-joining Tamura-Nei model with 1,000 replicates (*105*). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Hendra virus was used as an outgroup. Sample collection date, location and Genbank accession numbers are included in the label for each sequence except *P. medius* sequences which are accession Nos MK995284 – MK995302. Blue labels indicate bat sequences from Faridpur and Bhanga (an outbreak response in Faridpur). Purple sequences are from *P. medius* from other roosts sampled during the longitudinal study. Red sequences are from *P. medius* in Comilla. Green sequences are human NiV sequences from Bangladesh and India.

Table 1. PCR detection of NiV RNA in *Pteropus medius* 2006-2012.

Location	date	Bats Sampled	Throat Tested	Throat Pos	Urine Tested	Urine Pos	Rectal Tested	Rectal Pos	Paired samples	Pos. Bats	Bats w multi pos samples	prev.	95% CI	Roost Urine	Roost Urine pos.
Spatial Study															
Rajbari	Jan-06	99	79	**3**	78	0	79	**1**	78	3	**1**	**0.04**	**0.11**	-	-
Thakurgaon	Mar-07	118	115	**3***	72	0	-	-	70	unk.	0	0.00	-	-	-
Kushtia	Aug-07	101	100	0	99	0	-	-	98	0	0	0.00	-	-	-
Tangail	Jun-08	100	61	0	77	0	-	-	60	0	0	0.00	-	81	0
Chittagong	Aug-06	115	19	0	-	-	-	-	-	0	-	-	-	-	0
Comilla	May-08	100	0	0	50	0	-	-	0	0	-	-	-	100	**2**
Sylhet	Sep-08	100	100	0	49	0	-	-	48	0	0	0.00	-	100	0
Khulna	Jan-09	100	50	0	80	0	-	-	32	0	0	0.00	-	50	0
Comilla	Mar-11	50	50	0	50	0	-	-	0	0	0	0.00	-	-	-
Outbreak Investigation															
Bangha	Feb-10													19	3
Joypurhat	Jan-12													19	**16**[α]
Rajbari	Dec-09													35	0
West Algi	Jan-10													31	0
Longitudinal Study															
Faridpur	Jul-07	102	64	0	50	0	-	-	22	0	0	0.00			
Faridpur	Dec-07	101	N/A	N/A	N/A	-	-	-		0					
Faridpur	Apr-08	100	64	0	88	0	-	-	54	0	0	0.00		51	0
Faridpur	Jul-08	100	58	0	74	0	-	-	54	0	0	0.00			
Faridpur	Oct-08	100	98	0	99	0	-	-	98	0	0	0.00			
Faridpur	Feb-09	100	50	0	100	**1**	-	-	49	**1**	0	**0.01**	**0.10**	50	0
Faripdur	May-09	101	100	0	99	**2**	-	-	99	**2**	**0**	**0.02**	**0.10**	9	0
Faridpur	Aug-09	100	100	0	99	0	-	-	95	0	0	0.00		3	0
Faridpur	Nov-09	100	100	0	82	**1**	-	-	82	**1**	**0**	**0.01**	**0.11**	50	0
Faridpur	Feb-10	100	100	0	100	0	-	-	100	0	0	0.00		45	0
Faridpur	Jun-10	100	100	0	100	**3**	-	-	100	**3**	**0**	**0.03**	**0.10**	25	0
Faridpur	Sep-10	100	100	0	100	0	-	-	-	0	-	-		20	0
Faridpur	Jan-11	100	100	0	100	0	-	-	0	0	0	0.00		15	0
Faridpur	May-11	102	102	0	102	**1**	-	-	0	**1**	0	**0.01**	**0.10**	20	0
Faridpur	Aug-11	100	100	0	100	0	-	-	-	0	-	-		10	0
Faridpur	Dec-11	100	100	0	100	0	-	-	-	0	-	-		16	0
Faridpur	Apr-12	100	78	0	78	0	-	-	-	0	-	-		50	0
Faridpur	Jul-12	100	100	0	100	0	-	-	-	0	-	-		30	0
Faridpur	Nov-12	100	100	0	100	0	-	-	-	0	0			34	0
Total		**2789**	**2088**	**6**	**2126**	**8**	**79**	**1**		**11**	**1**	**0.005**	**0.02**	**829**	

*NiV RNA was detected in three pooled oropharyngeal samples, confirmed by sequencing, although confirmation from individual samples could not be made. These data re not used in prevalence estimates. [α] Detection by qPCR, Ct ranges 20-38.

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From:	Steve Luby on behalf of Steve Luby <sluby@stanford.edu>
To:	Jon Epstein
Cc:	Anthony, Simon J.; Ariful Islam; Shahneaz Ali Khan; Noam Ross; ina.smith@csiro.au; Carlos M. Zambrana-Torrelio MSc; Yun Tao; Ausraful Islam; Kevin Olival, PhD; Salah Uddin Khan; Emily Gurley; Dr. Jahangir Hossain; Hume Field; Fielder, Mark; Thomas Briese; Mahmud Rahman; Christopher Broder; Gary Crameri; Linfa Wang; Ian Lipkin; Peter Daszak; A. Marm Kilpatrick
Subject:	Re: Nipah dynamics in P medius draft for PNAS
Date:	Saturday, December 21, 2019 4:08:43 PM
Attachments:	Nipah dynamics in bats_Epstein et al 2019_amk sl.docx

Nice work Jon.

Attached are a few minor comments.

Good luck with submission.

Steve

A. Marm Kilpatrick Friday, December 20, 2019 3:09 AM

Hi Jon,

It's reading pretty well! See my suggested revisions and comments on the main doc and supplemental material. Let me know if you have questions!

marm

On 12/18/2019 10:00 AM, Jon Epstein wrote:

--
A. Marm Kilpatrick
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Jon Epstein Wednesday, December 18, 2019 2:00 PM

All,

I hope this email finds you well. Attached is a draft of our manuscript entitled Nipah virus dynamics in bats and implications for spillover to humans. After multiple protracted periods of review in Science Advances, and ultimately a rejection, I've prepared this draft for submission to PNAS. I've revised the manuscript and updated figures and supplemental data in response to the last round of review (comments below, if you're interested).

I have an editor at PNAS willing to send this for review, so please send me any input you may have by December 28th so that I can submit by the 31st. Unless I hear otherwise, I will assume you still consent to co-authorship.

Thank you for your patience, and happy holidays!

Cheers,
Jon

Reviewer: 1
Epstein et al aim to better understand the distribution and drivers of Nipah virus infection dynamics in Pteropus medius in Bangladesh by analysing a large set of serological, virological and movement data over a commendable spatial and temporal scale. Overall, this is a highly exciting study with valuable results that are well-deserving of publication. I have a number of minor comments regarding the addition of detail for clarity (and to ensure transparency in the interpretation of results). I think that the conclusions are mostly justified, however, my major criticism is that the integration and interpretation of the results (particularly those presented in the first paragraph of the discussion) requires a little further thought and explanation. The assumptions surrounding the serological implications of within-host persistence and recrudescence needs to be clearly stated. This is likely to become a 'classic' paper, and there are so few studies in this area supported by data that it is important to ensure that the results are not over-interpreted.

Specific comments:
Abstract:
Line 32- 33: The wording here is too strong regarding recrudescence. Suggest inserting "model results indicated that" prior to "local transmission dynamics.
Line 33-34: Similarly - this is too strong. Suggest "likely due to "
Results:
Figure 1 - presumably the first three bats in the Comilla represent 2008 and the second three represent 2011, but this is not clear. This should be annotated on the plot
Figure 1 - "Adult bats had equal or greater seroprevalence than juveniles in each location." - except Tangail?
Line 129: "detected NiV RNA in 11 individuals, 3 pooled" - insert 'from' before 3
Line 131: - describe "pooled samples" - pooled under-roost urine samples?
Line 132 and 137: This 'figure 2' seems to be missing? Figure 2 refers to serological analyses
Line 145: I suggest that the reference to Figure 2 in line 146 should go in the sentence ending on line 145
Line 149: the significant negative association with body condition warrants mentioning in these results.
Line 154-155 and Figure 3: Additional clarity here regarding juvenile vs. yearling terms. Suggest saying "Juveniles in their first year of life (yearlings)" at first mentioning in the text,
as well in the figure legend.
Line 159 - incomplete sentence
Lines 160-164: Fascinating results!
Lines 170-171: This information would be helpful to include earlier, with the serological results
Figure 3 and Figure S1 - It would be helpful to have a "total population" size from this roost

complex included on Figures 3 and 4. Understanding more about the bat ecology and the size and stability of these populations over time would be a tremendous help to aid interpretation of these results by researchers working in other systems.
Lines 175-176: "Serodynamics in juveniles were strongly driven by inheritance and loss of maternal antibodies". Supported by what result? This should be explained further, and/or it would be helpful to include the serology (fig 3), model output (fig 4) and population size (Figure s1) together as a series of vertical panels in the one figure to aid interpretation
Lines 178-180: I can't see this information in the supplementary information
Line 182 - this paragraph (and ideally also table S2) should provide information on the duration of tracking for each bat. This would be very helpful in assessing the home range information
Line 195 - should the 'of' be 'if?
Figure 6 - the utility of this figure would be greatly improved with some annotation and colouring to help identify the new sequences from this study, and their source location, and the 'groups' that are referred to in the text
Lines 238 - 240 - So, your data has found cyclical serodynamics, but no clear links between those dynamics and detection of NiV in bats or in people. Based on experimental studies, it is still a bit unclear exactly what seropositivy in flying foxes represents. If there is within-host persistence of infection, a cycle of infection > seroconversion (in the absence of clearance) > seroreversion > then recrudescence with serconversion may occur in the absence of ongoing transmission. Additionally, this does not take into account drivers of recrudescence - if this is stress related, then transmission at the population level will also be affected by these broader drivers. Given all the uncertainty around this topic, I think that the wording here needs to be precise. e.g the first sentence should first state the assumption "Assuming that seroconversion results only from new infections, then ...". This could be followed up with a sentence along the lines of "If however, seroconversion can result from recrudescence in the absence of transmission, then broader drivers of recrudescence would also need to be assessed". More clarity here would also help to assess the claims being made in the paragraph beginning 315.
Lines 240 onwards - consider restructuring this paragraph to more clearly step through each stage of the viral dynamics that you are proposing and your assumptions and evidence along the way. For example, you assume infection results in seroconversion, and that antibodies then wane after ~4 years. Are you assuming that all individuals are persistently infected, and it is only when Ab wane that recrudescence can occur? And following that, the individual seroconverts again?
Lines 243 - 245: Not correct - See Brooks et al 2019 JAE Figure S5. That study also involved modelling and has implications for this study more broadly
Lines 245-247: "via recrudescence FROM bats that have previously been infected"? It's also not clear how this links to lines 238-240
Lines 257-259: Consider also cite Peel et al (2018). Scientific Reports, 8(1), e0004796. This study examines waning maternal immunity and waning acquired immunity against henipaviruses in African fruit bats, finding a similar duration of immunity for both
Line 267 - insert "in our study area" after 'Pteropus medius' as you cannot infer whether these patterns hold true for the species across its range. It may be highly dependent on available food resources - as you go on to discuss
Line 277 - replace 'be' with 'result in'
Lines 295-296 - this links with the SILI hypotheses in Plowright et al (2016) PLoS NTD (see Figure 3)
Line 321- suggest "three periods of transmission (significant at the population level) occurred"
Line 315 See my comments re: paragraph beginning Line 238. The sporadic nature of detections is challenging to draw conclusions and much of the paragraph here (starting line 315) seems over-stated. However, that may become clearer if the comments for the earlier paragraph are addressed.
Methods:
Line 364: I can't see reference to the Faripur colony in the list of colonies in lines 358-359. What are its characteristics? Also, I presume it should be "Faridpur"? Also, it would be

helpful if the "Faridpur roost complex:" could be described in more detail in the methods as to the structure of this population and what this term means.
Line 366: Delete the first 'between'
Lines 388-390 - check the placement of parentheses here
Line 417: what cutoff was used for the Luminex assay and how was it determined?
Line 425 - fix formatting of second Ct
Line 493- 504: More information on the underlying population dynamic model is required e.g. Were births seasonal or continuous in your model? I can't see where this is stated. If not seasonal, then the effect of this on model output should be addressed. How was the death rate modelled? Was the total population size kept stable inter annually? The latter, in particular, may have implications for interpretation of the density-dependent vs frequency dependent results. The population size is touched on in the discussion in lines 288-291 but never really explained.
Table S1 - there is extra text below Table S1 that looks like it's not supposed to be there
Table S2 - Include what serostatus 0/1 refers to in the table caption

Reviewer: 2
Dear editor, I have reviewed the manuscript entitled “Nipah virus dynamics in bats and implications for zoonotic spillover to humans”. The manuscript describes a longitudinal surveillance in Pteropid spp. Bats from 2006 until 2012. Biometric data was collected, sera and swabs/urine were analyzed, and inferences were made largely based on serological data. The manuscript is a compendium of relatively loosely compiled data, ranging from seroprevalence in a variety of different sampling sites, but the majority of the samples stem from Faridpur. The problem is that most of the claims by the authors within the paper are not directly supported by the data. The direct problem of the data is the limited amount of detected virus shedding, out of 2789 animals sampled only 11 were found to be shedding the virus. >>From this only 8 were from the larger study cohort from Faridpur. This
directly hampers some of the conclusion of potential spillover dynamics as this cannot be directly inferred from serological data alone. In addition, it does not provide any answers on the occurrence of Nipah spillover in the Nipah belt vs the other regions. Moreover, the significant spillover events in Kerala, India from the last two years, are not discussed.
Most emphasis has been put on analyzing the serological results from the Pteropus medius bats. The authors show variation in the seroprevalence within the population based on timing and age status of the animal. The results have been reported before in other natural reservoir-pathogen systems like avian influenza, however even within these systems inferences on spillover can rarely be made. It interesting that the authors did not correlate the positive individual bats in Faridpur and Rajbari with their respective serostatus? Is this data not available? It is unclear to me why the authors have not put more effort in trying to perform full genome analyses on the positive samples obtained throughout this study. Currently there are only 27 full genomes available from Nipah virus and relatively limited amount are from the natural reservoir. Preforming phylogenetic analyses on a 224 nucleotide fragment of a 19kb virus is really not up to standard. Where it might be suitable for identification of the lineage no additional data can be inferred from this. Interestingly, there appears to be full genome sequencing performed but only N is shown in the supplemental data.
Although I do understand the logistics involved with this kind of work, unfortunately some of the claims, especially regarding spillover, need to be supported by more additional data rather than just serology.
Minor points:
Line 65: pandemic potential, given the limited amount of h-to-h transmission the pandemic potential of this particular virus appears to relatively limited.
Line 143: n=844 or n=883? Why do the numbers not match-up?
Line 410, include level of biosecurity involved in sample analyses. In addition, heatinactivation
is typically to inactivate complement and not inactivation of the pathogen.
From the current wording it is unclear what the authors mean by this? Complement

inactivation or pathogen inactivation?
Line 428, is this data missing? Where is the NSG data? Why not data on the full genomes? I only was able to find the full N gene data in the supplemental figues.
Line 509, given that actual recrudescence in the context of virus shedding in the natural reservoir has never been shown it would be good to treat this a little bit more carefully.

--

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Supplemental Data

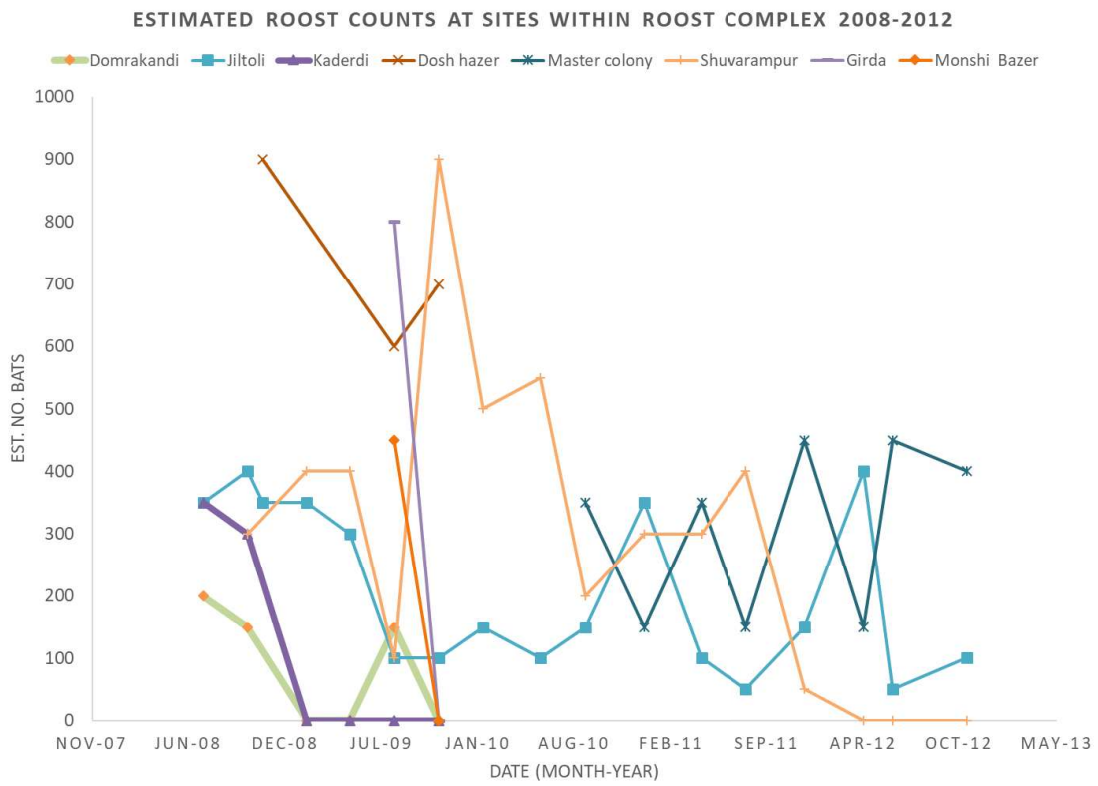

Figure S1. *Pteropus medius* counts from selected roosting sites within the Faridpur Roost Complex: 2008-2012. Sites were included if repeated counts were conducted. Domrakandi and Kaderdi were the two primary roost sites sampled for the longitudinal study and counts were used for the model parameter.

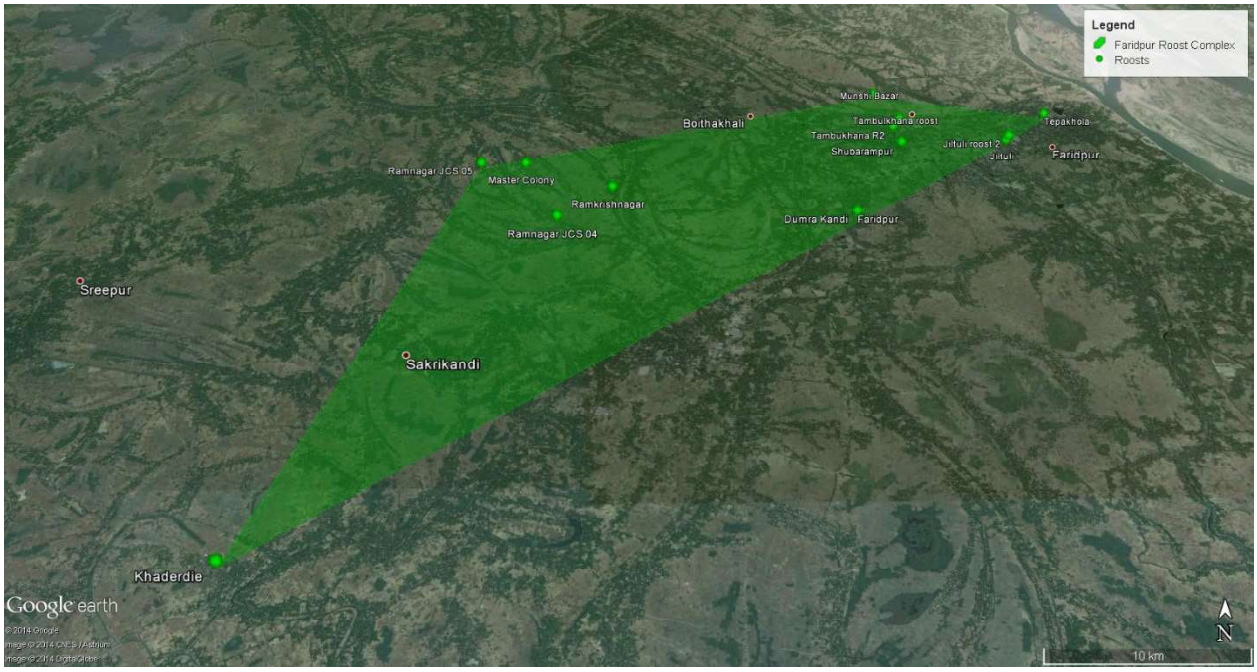

Figure S2. Faridpur Roost Complex. 51 Individual bats were recaptured during the longitudinal study at various locations. 33 bats were recaptured at a different site from where they were originally sampled. 15 unique roosts within an 80km^2 area were identified.

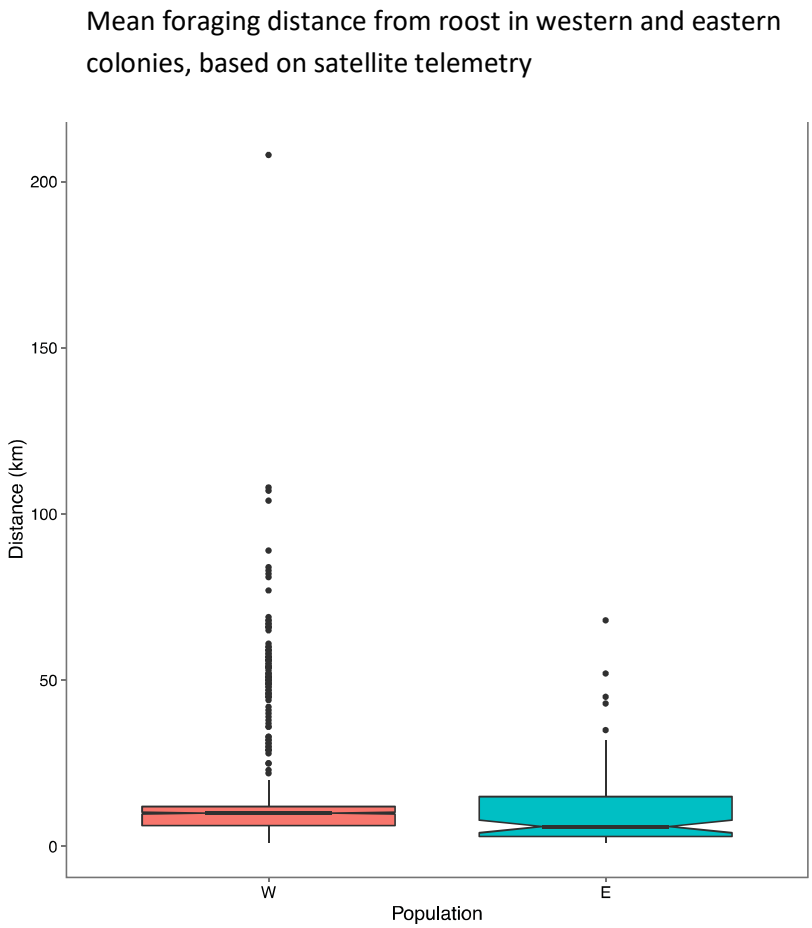

Figure S3. Mean foraging distance of western (W) and eastern (E) bat populations, based on satellite telemetry locations obtained between 1800h and 0600h, when *P. medius* typically forages.

Home range of *Pteropus medius* in wet and dry seasons.

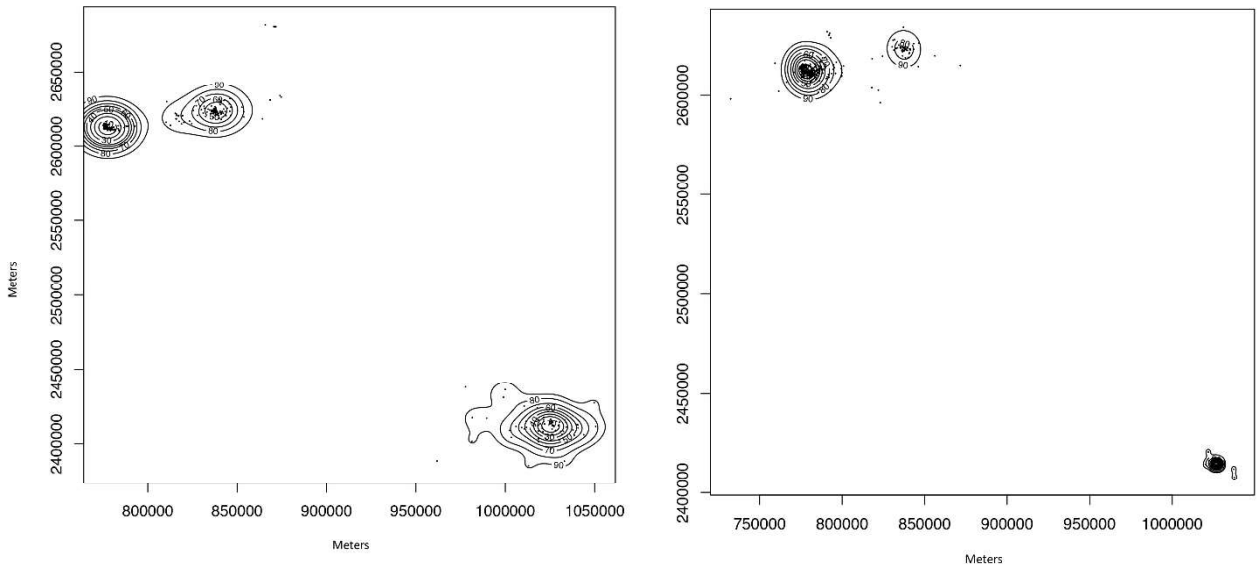

Figure S4. a) Homerange of *Pteropus medius* during the wet season (left) and dry season (right). Maps are projected in UTM (Universal Transverse Mercator) Zone 45 where units are represented in meters. The mean wet season homerange size was 2,746 km^2. Homerange size in the dry season is contracted and represents less than a quarter (618 km^2) of the homerange in the wet season.

Nipah virus phylogenetic tree, based on near complete N gene sequences

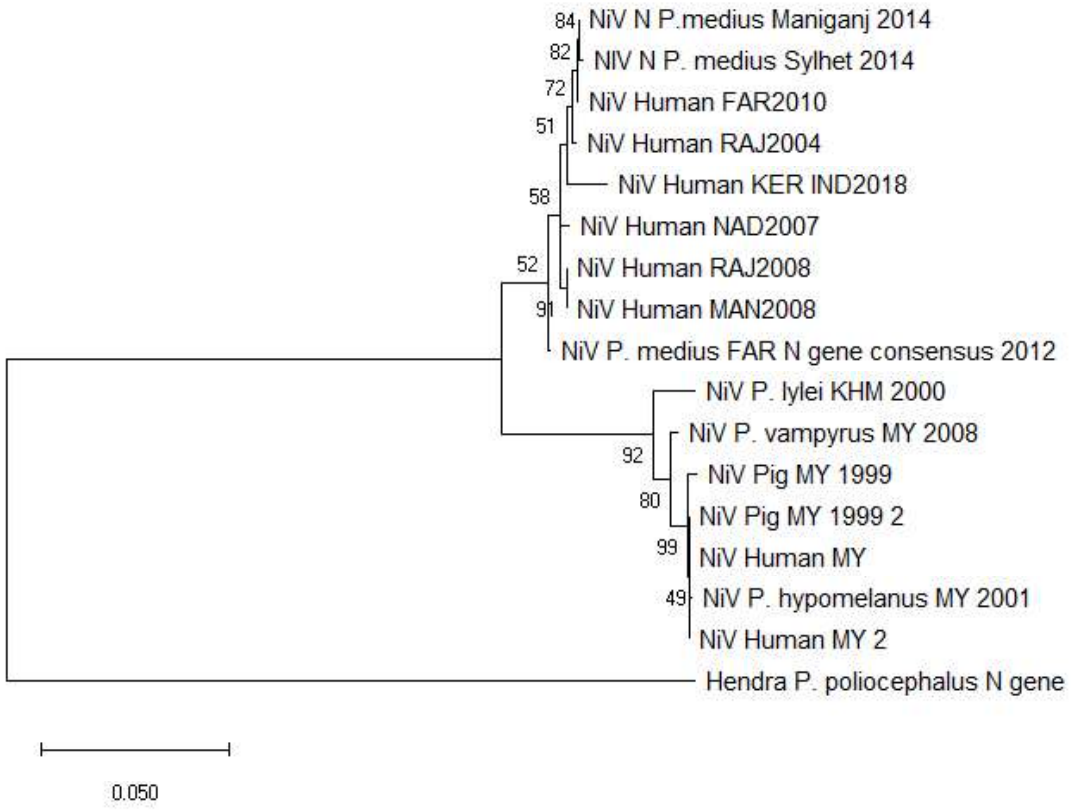

Figure S5. Nipah Virus phylogenetic tree, N gene: Clustal W alignment using nearly whole N gene consensus sequence from *P. medius* (1,592 nt) using Geneious Prime 2019 (*1*). The evolutionary history was inferred by using the Maximum Likelihood method and Hasegawa-Kishino-Yano model (*2*). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X (*3*). Genbank accession numbers for sequences (from top to bottom): *P. medius* Maniganj & Sylhet pending (63); JN808864, AY988601, MH396625, FJ513078, JN808863, JN808857, AY858110, FN869553, AJ627196, AJ564623, AY029767, AF376747, AY029768, JN255803.

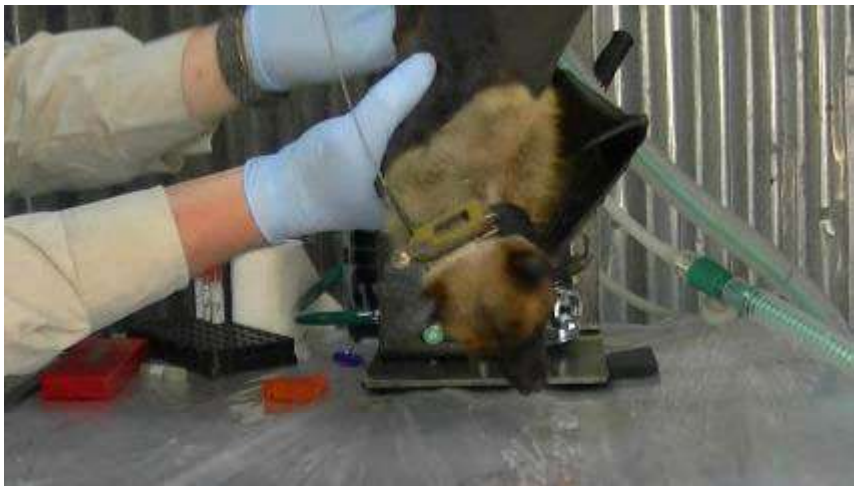

Figure S6. Platform terminal transmitter (PTT) and collar attachment on an anesthetized adult *Pteropus medius*, Bangladesh.

Table S1.

PCR positive bats and their serostatus.

Bat sample ID	Date	Loc	sex	Age	Serology Test result		
					ELISA	Luminex (MFI)	
29	Jan-06	Ramnagar	M	J	Neg	-	
59	Jan-06	Ramnagar	PF	A	Neg		
76	Jan-06	Ramnagar	PF	A	Neg		
21	Feb-09	Faridpur	PF	A	-	25817	Pos
58	May-09	Faridpur	F	A		159	Neg
87	May-09	Faridpur	M	A	-	113	Neg
55	Nov-09	Faridpur	M	A		25955	Pos
26	Jun-10	Faridpur	M	A		30	Neg
32	Jun-10	Faridpur	M	J		758	Pos
28	Jun-10	Faridpur	M	J		377	Neg

Table S2. Maximum likelihood estimates of fitted parameter values and (95% CI). All rates are on a weekly timestep unless otherwise indicated.

Parameter	Name	Maximum likelihood estimate	Lower 95% CI	Upper 95% CI
B_{jj}	Transmission rate, juveniles→ juveniles	0.013	0.0096	0.013
B_{ja}	Transmission rate, juveniles→ adults	0.030	0.024	0.034
B_{aj}	Transmission rate, adults→ juveniles	0.0024	0.00195	0.0029
B_{aa}	Transmission rate, adults→ adults	0.00047	0	0.0041
R_A/N_A (t=0)	Initial adult sero-prevalence	0.019	0	0.068

Summary of Comments on Email 33 - Attachment 1 - Supplemental Data_PNAS 2019_amk (002).pdf

Page: 7

Number: 1 Author: Auston Marmaduke Kilpatrick Date: 12/19/2019 11:05:00 PM -05'00'

Somehow I mis-spelled “transmission” and left way too many significant digits. I also had left the small table with the coefs in the word doc. I’ve corrected these three things.

Δ	Recrudescence	2.30E-07	1.41E-08	7.10E-07
$(1-\mu)^{52}$	Adult annual survival	0.75	0.718554	0.80
λ	Rate of maternal antibody loss	0.057	0.040	0.073
τ	Rate of adult antibody loss	0.0034	0.0021	0.0041

106

108

Table S2. Recaptured bats and NiV IgG sero-status from the Faridpur population

Bat Chip ID	Age1	Sex	Capture Date	Location 1	Serostatus1	Age2	Capture Date 2	Serostatus2	Location 2	Status Change	Age3	Capture Date 3	Location 3	Serostatus 3	Status Change 2
17044540	A	M	24/07/08	JH	0	A	19/09/10	1	SH	C					
26774096	A	M	05/10/09	JH	1	A	13/11/09	1	SH	N					
26783883	J	F	15/05/09	SH	1	A	14/02/10	0	SH	R					
26789012	A	M	15/05/09	SH	0	A	02/10/10	1	SH	C					
26791784	A	F	14/05/09	SH	1	A	30/04/12	0	JH	R					
26816627	A	F	11/05/09	SH	0	A	26/08/09	0	DM	N					
26824582	J	M	09/05/09	JH	0	A	20/09/10	0	TP	N	A	05/05/11	TP	0	N
27099360	A	M	24/09/10	RM_MC	0	A	18/08/11	1	TB	C	A	18/11/12	JH	0	R
27102063	A	M	16/11/09	SH	1	A	16/02/10	1	SH	N	A	19/12/11	TP	1	N
27103623	J	M	21/09/10	TP	1	A	17/12/11	0	TP	R					
27105342	J	M	21/09/10	TP	0	A	19/12/11	1	TP	C					
27105562	P	M	24/04/10	RM_JCS	0	J	20/10/10	0	RM_JCS 05	N					
27110270	A	M	24/07/10	RM_JCS	0	A	04/04/11	0	RM_JCS	N					
27111334	A	M	23/07/10	RM_JCS	0	A	19/10/10	0	RM_JCS	N					
27123779	J	F	21/06/10	RM_JCS2	0	J	28/02/11	0	RK	N					
27123803	J	F	21/08/10	RM_JCS1	0	J	28/02/11	0	RK	N					
27123868	J	M	18/02/10	SH	0	A	26/04/12	1	JH	C					
27126256	A	F	10/02/10	SH	0	A	18/12/11	0	TP	N					
27259351	A	M	20/09/10	TP	0	A	30/04/12	0	JH	N					
27259370	A	M	22/07/10	RM_JCS1	0	A	04/04/11	0	RM_JCS1	N					
27261073	J	F	22/06/10	RM_JCS2	1	J	22/07/10	1	RM_JCS1	N					
27261577	A	M	21/09/10	TP	0	A	17/12/11	1	TP	C					
27266775	A	M	21/08/10	RM_JCS1	1	A	18/11/12	0	JH	R					
27291793	A	M	24/09/10	RM_JCS1	0	A	22/01/11	1	RM_JCS1	C					
27296568	A	M	12/11/09	SH	1	A	07/05/11	1	SH	N					
27296851	A	M	22/04/10	RM_JCS1	0	A	25/05/10	0	RM_JCS2	N					
27301580	A	M	17/06/10	SH	1	A	05/05/11	1	TP	N					
27301857	A	M	11/02/10	SH	0	A	22/04/10	1	RM_JCS1	C	A	03/05/11	SH	0	R

Location Codes: Location codes: Jhiltuli (JH); Jiltuli Roost 2 (JH2); Shuvarampur (SH); RamnagarMC (RM); Tepakhola (TP); Ramnagar Roost JCS (RM_JCS); Ramnagar JCS 1 (RM_JCS1); Ramnagar JCS 02 (RM_JCS2); Tambukhana R2 (TB2); Domrakhandi (DM); Khaderdi (KD); Ramkrishnagar (RK); Tepakhola Master Colony (TPMC). Status Change: C = seroconversion, N = No Change, R = sero-reversion.

Table S2 (cont…). Recaptured bats and NiV IgG sero-status from the Faridpur population

Bat Chip ID	Age1	Sex	Capture Date	Location 1	Serostatus1	Age2	Capture Date 2	Serostatus2	Location 2	Status Change	Age3	Capture Date 3	Location 3	Serostatus 3	Status Change 2
27305044	J	M	22/06/10	RM_JCS2	1	A	15/11/12	0	JH	R					
27306794	A	M	15/06/10	SH	1	A	11/07/12	1	TPMC	N					
27306824	A	M	23/07/10	RM_JCS1	1	A	24/09/10	1	RM_JCS1	N	A	18/11/12	JH	1	N
54867532	A	M	23/01/11	JH2	1	A	18/11/12	0	JH	R					
54872600	A	M	19/10/10	RM_JCS2	0	A	30/04/11	0	RM_MC	N					
54877598	J	F	18/01/11	SH	0	A	01/05/12	0	JH	N					
65770323	J	M	04/04/11	RM_JCS1	0	J	01/05/11	0	RM_MC	N					
65780555	A	M	05/05/11	TP	1	A	13/11/12	1	JH	N					
68608827	J	M	14/08/11	TB2	1	J	18/12/11	1	TP	N					
68612032	J	M	15/07/12	TPMC	0		18/11/12	0	JH	N					
80825550	A	M	11/12/07	DM	0	A	13/04/08	1	DM	R					
80855347	A	M	06/12/07	DM	1	A	22/07/08	1	KD	N					
80867630	A	M	11/12/07	DM	0	A	14/05/09	0	SH	N					
80876042	A	M	06/12/07	DM	0	A	14/08/11	0	TB2	N					
80877779	A	M	07/12/07	DM	1	A	21/07/08	1	DM	N					
81030044	A	M	06/02/06	RM_JCS1		A	24/07/10	0	RM_JCS1	NA		04/04/11	RM_JCS1	0	N
81055270	A	F	12/12/07	DM	0	A	20/12/11	1	TP	C					
81095300	A	M	09/12/07	DM	1	A	18/07/08	1	DM	N					
99605347	A	M	15/12/07	DM	0	A	07/04/08	0	DM	N					
99618528	A	M	20/07/08	DM	1	A	16/02/10	1	SH	N					
103821120	A	F	12/04/08	DM	0	A	16/05/09	1	SH	C					
104083112	A	M	03/04/08	DM	1	A	20/07/08	1	DM	N					
65777367	P	M	30/04/11	RM_MC	0	J	14/11/12	0	JH	N					

Location Codes: Location codes: Jhiltuli (JH); Jiltuli Roost 2 (JH2); Shuvarampur (SH); RamnagarMC (RM); Tepakhola (TP); Ramnagar Roost JCS (RM_JCS); Ramnagar JCS 1 (RM_JCS1); Ramnagar JCS 02 (RM_JCS2); Tambukhana R2 (TB2); Domrakhandi (DM); Khaderdi (KD); Ramkrishnagar (RK); Tepakhola Master Colony (TPMC). Status Change: C = seroconversion, N = No Change, R = sero-reversion.

Table S3. Satellite telemetry study: bat characteristics and duration of transmission.

PTT #	Microship ID	Colony location	Date collared	Final location date	Approx.Duration (mo)	Sex	Age	Mass (g)	BCS	Forearm (mm)	Head (mm)	Body (mm)	pregnant	lactating
90831	17035561	F	2/13/2009	5/12/2009	3	F	A	673	G	169.7	74.7	197.5	Y	N
90832	17034004	F	2/13/2009	6/17/2009	4	F	A	663	G	171.4	71.1	195.7	N	N
90833	17019016	F	2/14/2009	4/12/2009	2	M	A	688	G	182.5	78.4	217.6	-	-
90834	080867630*	F	2/14/2009	5/11/2009	3	M	A	665	G	186.4	76.1	201.6	-	-
90835	17027862	F	2/16/2009	7/1/2009	5	M	A	684	G	166.6	68.7	221.6	-	-
90836	17071891	F	2/16/2009	6/3/2009	4	F	A	652	F	181.6	71.8	206.3	Y	Y
101469	54876270	F	1/17/2011	8/13/2011	7	M	A	626	G	175	75	210	-	-
101467	54870019	F	1/17/2011	1/29/2012	12	F	A	603	F	165	70	195	Y	N
101466	54867013	F	2/28/2011	6/8/2011	3	F	A	684	G	164	70	190	Y	N
101468	54883815	F	2/28/2011	4/4/2012	13	M	A	772	G	172.3	71.84	195	-	-
90839	54867601	F	3/1/2011	4/28/2013	25	M	A	771	G	175.53	74	211.14	-	-
101470	65623841	F	3/1/2011	12/8/2011	9	F	A	731	G	172.68	71.6	205.68	Y	N
101471	65628805	C	3/29/2011	5/9/2011	1	M	A	717	G	176	78	220	-	-
90840	65635619	C	3/29/2011	8/8/2011	4	M	A	698	G	169	81	202	-	-
90838	65628094	C	3/30/2011	6/26/2011	3	F	J	446	F	161	73	190	N	N
90837	65775297	C	3/30/2011	6/17/2011	2	M	A	620	F	178	71	190	-	-

PTT = Platform Terminal Transmitter; Colony Location: F=Faridpur, C-Chittagong; BCS = Body Condition Score: G=Good, F=Fair, P=Poor;

1. Geneious Prime 2019. https://www.geneious.com.
2. M. Hasegawa, Kishino, H., Yano, T.-a. J. J. o. m. e., Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. **22**, 160-174 (1985).
3. S. Kumar, Stecher, G., Li, M. *et al.*, MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. **35**, 1547-1549 (2018).

Nipah virus dynamics in bats and implications for spillover to humans

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Summary of Comments on Email 33 - Attachment 2 - Nipah dynamics in bats_Epstein et al 2019_amk (002).pdf

Page: 1

Number: 1 Author: Author Date: Indeterminate

Although obviously a good hook, the data we present are only weakly useful for this since we find no correlation b/w bat dynamics and human cases. If the editor will already send it out for review I'd cut this. Overselling what the paper is about is why the paper was rejected at Science Advances.

Abstract

Nipah virus (NiV) is an emerging bat-borne zoonotic virus with pandemic potential that causes near-annual outbreaks of fatal encephalitis in South Asia – one of the most populous regions on Earth. In Bangladesh, infection occurs when people drink date palm sap contaminated with bat excreta, but a recent outbreak in India involved a different, but yet unknown, route of spillover. Outbreaks are sporadic and the influence of viral dynamics in bats on their temporal and spatial distribution is poorly understood. We analyzed data on host ecology, molecular epidemiology, serological dynamics, and viral genetics to characterize spatio-temporal patterns of NiV dynamics in its wildlife reservoir, *Pteropus medius* bats, in Bangladesh. We found that NiV transmission occurred throughout the country and throughout the year. Model results indicated that local transmission dynamics were driven by density-dependent transmission, acquired immunity which is lost over time, and recrudescence. Increased transmission followed multi-year periods of declining seroprevalence due to bat population turnover and individual loss of humoral immunity. Individual bats had smaller host ranges than other *Pteropus* spp., although movement data and the discovery of a Malaysia-clade NiV strain in eastern Bangladesh suggest connectivity with bats east of Bangladesh. These data suggest that discrete multi-annual local epidemics in bat populations contribute to the sporadic nature of Nipah virus outbreaks in South Asia. At the same time, the broad spatial and temporal extent of NiV transmission, including the recent outbreak in Kerala, India, highlights the continued risk of spillover to humans wherever they may interact with pteropid bats, and the importance of improving Nipah virus surveillance throughout *Pteropus*'s range.

Keywords: bats, henipavirus, Nipah virus, *Pteropus medius, Pteropus giganteus,* satellite telemetry, viral phylogeny, disease dynamics, modeling

Page: 2

Number: 1 Author: Author Date: Indeterminate

This is interesting but isn't relevant to this paper and we have no data to address it so I'd remove this, especially in the abstract. The mention of it below doesn't require this text. Mentioning it here makes the reader expect us to address it and we can't.

Introduction.

Outbreaks of zoonotic diseases are often sporadic, rare events that are inherently difficult to predict, but can have devastating consequences (*1*). Several emerging viral zoonoses with wildlife reservoirs have become pandemics, including HIV/AIDS, SARS coronavirus, and 1918 Pandemic Influenza H1N1 (*2-4*). Bats are important hosts for many zoonotic viruses (*5*) including Ebola virus, SARS-CoV, and Nipah virus, but the ecological drivers and transmission dynamics of these viruses in their reservoir hosts are poorly understood (*6-10*). A better understanding of the transmission dynamics of zoonotic pathogens in their natural reservoirs may help anticipate and prevent outbreaks (*9, 11*).

Nipah virus (NiV) is an emerging zoonotic paramyxovirus (genus *Henipavirus*) that has repeatedly spilled over from bats to cause outbreaks in people and livestock with high case fatality rates across a broad geographic range, making it a significant threat to global health. It has caused repeated outbreaks in Bangladesh and India, with a mean case fatality rate greater than 70% (*12-14*). A single genus of frugivorous bats (*Pteropus*) appears to be the main reservoir for henipaviruses throughout Asia and Australia (*15-19*), including *Pteropus medius* (formerly *Pteropus giganteus* (*20*)) in Bangladesh and India (*21-24*). Nipah virus has several characteristics that make it a significant threat to human and animal health (*25-27*): 1) its bat reservoir hosts are widely distributed throughout Asia, overlapping dense human and livestock populations, providing broad opportunity to cause outbreaks; 2) it can be transmitted directly to humans by bats or via domestic animals; 3) it can be transmitted from person to person; 4) spillover has repeatedly occurred in highly populous and internationally connected regions, giving it pandemic potential; 5) it is associated with high mortality rates in people; and 6) there are currently no commercially available vaccines or therapeutics. As a result, the World Health Organization has listed Nipah virus among the ten most significant threats to global health (*28*). To date, human Nipah virus infections have been identified in India, Bangladesh, Malaysia, Singapore, and the Philippines (*12, 22, 29-31*). In May 2018, an outbreak of Nipah virus encephalitis associated with a 91% mortality rate occurred in a new location - Kerala, India - more than 1,200 km southwest of previous Indian and Bangladeshi outbreaks (*32*). A single case was subsequently reported in Kerala in 2019, and while local *P. medius* populations have been implicated as the local source of infection, the route of spillover in both instances remains unknown (*32, 33*).

In Malaysia and Bangladesh, consumption of cultivated food resources contaminated with bat excreta such as mangoes in Malaysia and date palm sap in Bangladesh and northeastern India have been identified as the predominant cause of spillover to pigs and people respectively (*34*). Human outbreaks

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occur almost annually in Bangladesh and the seasonal timing (November-April) and spatial distribution of outbreaks coincide with patterns of raw date palm sap consumption in a region termed the "Nipah belt" (*35*). However, there is variability in the number of spillover events and magnitude of the outbreaks that occur each year (*36*), and spillover has occasionally occurred outside the predominant season and region of date-palm sap consumption (*37*). Further, date palm sap harvesting and consumption also occurs in eastern Bangladesh, yet no human outbreaks have been reported, while date palm sap is not cultivated in Kerala, India at all, suggesting an alternate route of spillover (*35*). While the full range of mechanisms for zoonotic transmission remain unknown, so too are the underlying viral infection dynamics in bats and the extent of genetic diversity within the virus – each of which may influence the timing, location and epidemiology of human outbreaks (*35*).

Previous research on the transmission dynamics of Nipah and Hendra viruses in *Pteropus* spp. bats have produced mixed and sometimes contradictory findings. Nipah virus, like Ebola, Marburg, Hendra and some bat coronaviruses, has been hypothesized to have seasonal spikes in infection that coincide with annual or semi-annual synchronous birth pulses (*15, 38-44*). Seasonal periods of Nipah virus shedding were observed in *P. lylei* in Thailand and seasonal spikes in NiV (or a related henipavirus) seroprevalence coinciding with pregnancy periods were observed in *Eidolon dupreanum* in Madagascar (*45, 46*), but not in *P. vampyrus* or *P. hypomelanus* in Peninsular Malaysia (*19*). Hendra virus prevalence in Australian pteropid bats has shown multi-year inter-epidemic periods where very little virus can be detected, followed by periods of increased viral shedding, suggesting that viral dynamics are not annual (*47-49*). It has been hypothesized that multi-year periodicity in henipavirus infection dynamics could arise from a build-up and waning of herd immunity in the reservoir host, with re-introduction of virus via immigration or recrudescence or viral persistence (*10, 50-52*). Some pteropid bat species are migratory and interconnected colonies form a metapopulation which could allow for viral re-introductions (*9, 19, 53, 54*). In addition, NiV recrudescence has been observed in wild-caught *P. vampyrus* and *Eidolon helvum* and either of these phenomena could allow it to persist regionally during periods of high local immunity (*55*). However, no study has yet shown evidence in open, free-ranging bat populations that favors one or the other hypothesis in driving NiV transmission dynamics.

The goal of the current study was to determine the distribution and drivers of NiV infection dynamics and NiV diversity in *Pteropus medius* in Bangladesh to try to understand patterns of human outbreaks. We examined spatial, temporal and demographic variation in serological dynamics and viral shedding in bats over a six-year period to determine the spatio-temporal drivers and dynamics of virus

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transmission. We also studied movement patterns of individual bats and analyzed NiV phlyogenetics to understand patterns of spatial mixing and virus strain diversity.

Results

Comparative Nipah virus prevalence study in bats inside and outside the Nipah Belt and concurrent longitudinal bat study inside the Nipah Belt (2006-2012)

We caught and tested 883 *P. medius* (~100 per district) from eight different districts across Bangladesh. We detected anti-Nipah IgG antibodies in all colonies (**Figure 1**). Seroprevalence varied by location (χ^2 = 55.61, p<.001), but there was no statistical difference between seroprevalence in bats inside the Nipah Belt and outside**.** In all locations except Tangail, adult seroprevalence exceeded juvenile seroprevalence. Viral detection in individuals was rare; overall, we detected NiV RNA in 11/XXX individuals as well as 3 pooled oropharyngeal samples (representing 5 bats, but which could not be resolved to an individual) and 21 or XXX pooled urine samples ("roost urine") collected from tarps underneath the roost (**Table 1**). We detected viral RNA in individual bats in Faridpur and Rajbari and from pooled samples from Thakurgaon and roost urine samples from Comilla. Of the 10 PCR positive individuals, three had detectable IgG antibodies (**Table S1**). We also detected virus in pooled urine collected from tarps placed below bats at roosts associated with human outbreaks in Bhanga and Joypurhat. The viral prevalence in Rajbari in January 2006 was 3.8% (95% CI: 0% -11%; n=78). In Faridpur, where we also conducted an intensive longitudinal study (see below), viral prevalence estimates ranged from 0% to 3% (95% CI: 0%-10%; n=100 at each of 18 sampling times) (**Table 1**). Nipah virus RNA was detected in individual bats from inside (Rajbari, Thakurgaon, and Faridpur) and outside (Comilla) the Nipah Belt. We detected viral RNA in bats both with and without detectable IgG antibodies (**Table S1**). Urine samples provided the highest NiV detection rate. Detection rates in individual bats by sample type were: urine/urogenital swab = 4% (n=2,126); oropharyngeal swab 3% (n=2,088); and rectal swab = 1.3% (n=79). The estimated detection rate from pooled urine samples across the entire study was 2.7% (+/- 1.6%; n=829).

Factors associated with NiV IgG *serostatus in* P. medius

Among adult and juvenile bats sampled in the aforementioned cross-sectional study from which we got blood samples (844 of 883), seropositivity was 2.4 times more likely among adults than juveniles, and 1.6 times more likely among males than females (**Figure 2**). Among females, seropositivity was higher in pup-carrying (4 times) and pregnant (1.5 times) individuals. Weight or forearm length did not

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https://en.wikipedia.org/wiki/Odds_ratio
You can predict the actual seroprevalence values using the fitted models and then report these actual ratios of seropositivity, but the odds ratios can't be described this way.

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consistently correlate with seropositivity, however, body condition (an assessment of pectoral muscle mass by palpation) was significantly negatively correlated (Poor/Fair body condition OR = 0.69) with serostatus. Finally, serostatus was strongly correlated in mother-pup pairs, with 71/80 pairs (89%) having matching status.

NiV serodynamics over time in a population of P. medius*, Faridpur district (2006-2012)*

We conducted an intensive longitudinal study of NiV serology in a population of bats in the Faridpur district and used flexible generalized additive models (GAMs) to characterize changes over time. There were significant fluctuations in adult (>24 mo.) and juvenile (6 – 24 mo.) seroprevalence over the six-year study period (**Figure 3A**). Juvenile seroprevalence ranged from 0% to 44% (95%CI 37%-51%), and decreased over the first year of life for bats born in each year ("yearlings"), consistent with loss of maternal antibodies in juveniles. A more pronounced decrease occurred from mid-October to mid-December. However, the GAM indicating this had only marginal better fit (ΔAIC < 1), than one with a linear decrease over the whole year (**Figure 3B**). The effect of birth cohort was significant on overall seroprevalence.

Adult seroprevalence ranged from 31% (95% CI: 20%-46%) to 82% (95% CI: 77%-87%) and went through three periods of significant decrease then increase over the course of the study (**Figure 3A**). We found no evidence of regular seasonal fluctuations; a GAM with annual cyclic terms fit worse than one without (ΔAIC > 10). Viral RNA detections occurred in periods of increasing, decreasing, and stable seroprevalence.

We fitted a series of age-stratified mechanistic models to examine different biological processes influencing serodynamics, including density- vs. frequency-dependent transmission, recrudescence vs. immigration of infected individuals, and seroreversion (loss of antibodies) in both juveniles and adults (**Figure 4**). We included annual, synchronous birthing, which occured between March and April. We assumed that pups weaned from their dams at 3 months, and became independent flyers, and that maternal antibodies waned after 6 months at which point we had pups transition into the "juvenile" class (*56, 57*). We assumed that juveniles became sexually mature at 24 mo., and entered the "adult" class based on other pteropid species(*43, 56, 58*). Density-dependent models were a far better fit to the data than frequency-dependent models (difference in log-likelihood 10.0; ΔAIC = 20.0), suggesting that movements of bats and fluctuations in colony size alter spatio-temporal variation in the risk of NiV epidemic spillover to humans. In this colony (Domrakhandi/Khaderdi) during the period of sampling, the roost population declined from approximately 300 bats to 185, which decreased transmission potential

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in the fitted model: R_0 in adult bats was estimated to decrease from 3.5 to 2.1 as the number of bats in the colony decreased. As a result, over the six-year study period, the fitted model predicted that the number of infected bats increased when the seroprevalence of adults fell below 72% (when bat counts were highest - in 2006) and 52% (when bat counts were lowest). The fitted model suggested that serodynamics in juveniles were strongly driven by inheritance and loss of maternal antibodies. The rate of loss of maternal antibodies was 17.6 weeks (95% CI: 13.7-25.0), which was much quicker than the loss of antibodies in adults (290.8 weeks, 95% CI: 245.0-476.4) (**Table S2**). Finally, models with recrudescence fit the data better than models without recrudescence (**Table S2;** difference in log-likelihood 32.6; ΔAIC = 65.1), and models with recrudescence fit the data better than models with immigration (ΔAIC = 3.76).

Mark-recapture and seroconversion/seroreversion

A total of 2,345 bats from the Faridpur/Rajbari region were sampled and microchipped between 2007 and 2012. There were 56 recapture events (**Table S3**). Thirty-one bats were recaptured at a roost other than the original capture location. This network of roosts or "roost complex" formed a polygon covering approximately 80 Km^2 and included 15 roosts sampled during the longitudinal study (**Figure S2A and S2B**). Ten instances of seroconversion (change from IgG negative to IgG positive) and nine instances of seroreversion (positive to negative) were observed (**Table S3**). The mean time between positive and negative tests in *adults* (excluding juveniles with maternal antibodies) was 588 days (n=6) (range: 124-1,082 days).

Home range and inter-colony connectivity analysis

Home range analysis of satellite telemetry data from 14 bats (**Table S4**) showed that the majority of bats roosted within 10 km of where the bats were originally collared, in the Faridpur (Nipah belt) colony, and within 7 km from where the bats in the Cox's Bazaar colony were originally collared (315km east of Faridpur). The average foraging radius was 18.7 km (s.d. 21.5 km) for the Faridpur bats and 10.8 km (s.d. 11.9 km) for the Cox's Bazaar bats (**Figure S2**). Homerange analysis suggests that bats in Faridpur and Cox's Bazar would have a <5% probability of intermingling (**Figure 5**). Homerange size was significantly larger during the wet season than the dry season (2,746 km^2 vs. 618 km^2) (**Figures S3 & S4**).

NiV phylogenetic analysis.

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Phylogenetic analysis of NiV sequences from a 224nt section of the N gene (nt position 1290-1509 [position ref gb|FJ513078.1| India]) suggests that strains from both India and Malaysia clades are present in Bangladesh bats (**Figure 6**). This finding is supported by an analysis of near-whole N gene sequences (~1720 nt) from bats, pigs, and humans, including those from a subset of *P. medius* from this and a more recent study by our group (**Figure S5**) (*59*). Eleven 224nt N gene sequences obtained from bats between 2006 and 2012 (all from the Faridpur population) were identical. Overall, the N gene sequences identified from the Faridpur, Rajbari and Bhanga colonies between 2006 and 2011 had 98.21%-100% shared nucleotide identity. Sequences from Rajbari district obtained five years apart (January 2006 and January 2011) had only a single nucleotide difference resulting in a synonymous substitution (G to A) at position 1304, which was found in four other bat NiV sequences from this study, as well as in the NiV isolate from *P. vampyrus* in Malaysia. Five Human NiV N gene sequences from various locations within the Nipah belt over the same time period as our bat study show more nucleotide diversity than those from the Faridpur *P. medius* population. Human sequences throughout Bangladesh and from Kerala, India, all nested within the diversity found in *P. medius* (**Figure 6**). By contrast, the sequences found in *P. medius* from Comilla, a location 150Km to the east of Faridpur, showed 80.8%-82.59% shared nucleotide identity with sequences from *P. medius* in Faridpur and clustered within the Malaysia group of NiV sequences. The two Comilla sequences were identical to each other, and had up to 87.95% shared nucleotide identity to sequences from *P. lylei* in Thailand. *Pteropus lylei* bats in Thailand were also found to carry NiV strains from both Malaysia and Bangladesh groups.

Discussion

Our study provides new insights into Nipah virus transmission dynamics, genetics and host ecology. Previous studies from Bangladesh suggested that human NiV outbreaks occur only within a defined region in western Bangladesh, termed the “Nipah belt,” and during a defined season (Nov-Apr), which raised the question of whether that observation was entirely due to date palm sap consumption, or whether ecological factors such as the distribution and timing of bat viral infection also influenced the timing and location of human cases (*13, 35, 60*). We undertook the most geographically extensive survey of *Pteropus medius* in Bangladesh to date to understand Nipah virus infection patterns in its putative reservoir, *Pteropus medius*, which is common in Bangladesh and throughout the Indian subcontinent (*21, 22, 56*).

Overall, our findings suggest viral circulation is not limited to the Nipah belt, but that NiV transmission occurs in bat populations throughout the country. We observed that virus can be shed at any time of year, and that viral dynamics are not annual or seasonal, but driven by demographic and immunological factors. Analysis of serological data from our longitudinal study suggests that the underlying mechanism driving the timing of NiV transmission in bats is the waning of herd immunity in bat populations allowing heightened viral transmission, but recrudescence can result in sporadic shedding at any time.

A number of mechanisms have been proposed for the maintenance of acute viral infections in bat metapopulations, including synchronous birthing and subsequent loss of maternal antibodies (*10, 39, 41*), lowered immunity within pregnant females due to stress, nutritional stress and other factors (*43*) immigration of infected individuals from other colonies (*53, 61, 62*), and recrudescence within previously-infected individuals (*10, 55, 63*). Our modeling indicates that NiV is primarily driven by density-dependent transmission dynamics among adult bats, with cycles of higher seroprevalence that would dampen intra-colony transmission followed by waning of antibody titers within individuals and at a population level. Waning humoral immunity against Nipah virus is a consistent feature of henipavirus studies in African pteropodid bats (*52, 64*). Our recapture data provided the first reported evidence of the loss of detectable NiV IgG antibodies in recaptured individual free-ranging bats, which supports our observation of population level waning immunity. The consistently lower and decreasing seroprevalence that we observed in juveniles suggests that they lose maternal antibodies over their first year, and likely in the first 6-7 months, consistent with other studies of maternal antibodies against henipaviruses in pteropodid bats (*43, 52, 57, 65*). However, our analysis does not support the hypothesis that seasonal pulses of these new seronegative individuals are the primary driver of new outbreaks in adults (*41*).

Our model outputs suggest that spikes in viral transmission occur after virus is reintroduced into colonies after immunity has waned. The model fitting suggests that serodynamics are more consistent with recrudescence, but immigration of infected individuals could also reintroduce the virus. Recrudescence would presumably occur in individuals with antibody titers that have waned below a neutralizing titer, if loss of humoral immunity following a primary infection is sufficient to allow a second infection. Recrudescence of henipavirus infection has been observed for NiV in captive *P. vampyrus* (*55*), for henipavirus in captive *E. helvum* (*52, 66*), and has also been observed in humans infected by NiV (*67*) and Hendra virus (*68*). It is difficult to know from serology alone whether wild-caught seronegative bats had been previously infected. Experimental infection of naïve and previously infected *Pteropus medius* that have sero-reverted would provide a better understanding of how humoral immunity influences

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individual susceptibility to infection, and inform dynamics models attempting to explain viral maintenance within bat populations (*64*).

Our longitudinal study is limited in that it may not necessarily reflect temporal infection dynamics in all bat populations across Bangladesh. Our roost count data and recapture data from microchipped bats showed how roost sizes can fluctuate, and local roost shifting can occur. The observation of individual bats using multiple roost sites suggests that changes in roost count, which our models suggest impacts transmission dynamics, could reflect local shifts in densities rather than fluctuations in regional populations.

Understanding how bat populations connect across landscapes is important for understanding viral maintenance, and studying local and migratory bat movements can provide important ecological information related to viral transmission, including how bats move between different colonies (*53*). Our satellite telemetry data suggest that *P. medius* exists as a metapopulation, like other pteropid species (*10, 62*). However, *Pteropus medius* appear to travel shorter distances and remain within a smaller home range (321.46km^2 and 2,865.27km^2 for two groups) compared to *P. vampyrus* in Malaysia (64,000 km^2 and 128,000 km^2) and *Acerodon jubatus* in the Philippines which are similarly sized fruit bats (*53, 69*). Pteropodid bat migration is primarily driven by seasonal food resource availability (*54, 70-72*). In Bangladesh, *P. medius* prefer to roost in human-dominated environments in highly fragmented forests (*73*). The anthropogenic colonization and conversion of land over recent human history has likely led to increased food availability for *P. medius* and reduced necessity for long-distance migration (*34*). This may reflect a similar adaptation to anthropogenic food resources as observed over the last few decades in Australian *Pteropus* species (*62*). Genetic analysis of *P. medius* across Bangladesh has shown that the population is panmictic – that historically, there has been interbreeding among populations across Bangladesh(*74*). If movements are generally more localized, as suggested by telemetry, then less connectivity among flying fox populations may influence Nipah transmission by creating longer inter-epidemic periods and larger amplitude fluctuations in population level immunity compared to more migratory species (*62*).

Bat movement and population connectivity may also influence the genetic diversity of Nipah virus found in different locations, and genotypic variation has been associated with different clinical outcomes in people. While the overall strain diversity among Nipah virus has not been well characterized due to a dearth of isolates, two distinct NiV clades have been described: A Bangladesh clade, which includes sequences identified in India and Bangladesh; and a Malaysian clade, which comprises sequences from Malaysia, Cambodia, The Philippines and Thailand (*31, 59, 75*). Strains of NiV

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It's not a limitation in model parameterization – it was a limitation in the data. If we had seroprevalence data in many individual roosts we could examine the importance of local vs regional population processes. But that would be a ton of work!

from these two clades are associated with differences in pathogenesis, epidemiological and clinical profiles in humans and animal models and observed shedding patterns in bats (*45, 76-80*). Phenotypic variation in Nipah virus could influence observed human outbreak patterns by altering transmission to, or pathogenesis in, humans, and the likelihood of smaller outbreaks being identified or reported (*81*). Human-to-human NiV transmission via contact with respiratory and other secretions has been regularly observed in Bangladesh and India, including the recent 2018 outbreak in Kerala (*12, 82, 83*), whereas transmission among people was not a common feature of the Malaysia outbreak, despite close contact between cases and health care providers (*84, 85*). Nipah virus cases in Bangladesh have shown more strain diversity than in the Malaysia outbreak (*86*).,

Until now, there have been very few Nipah sequences obtained from *P. medius.* We found that Nipah N-gene sequence from bats from the Faridpur population were nearly identical over time, compared to variation in N-gene sequences from bats and humans from other locations observed over the same time period (2006-2010). This suggests that there may be locally prevalent and stable NiV genotypes that persist within bat colonies. Human NiV genotype diversity is likely a reflection of the relative diversity of the NiV strains in the local bats that seed outbreaks (*9*). This is also supported by viral sequences obtained from human and bats associated with the 2018 NiV outbreak in Kerala, India, where human NiV sequences were most closely related to local *P. medius* sequences *(87)*. We found a significantly divergent NiV strain in Comilla, which clustered within the Malaysia NiV clade, suggesting that strains from both clades are circulating in bats in Bangladesh.

Connectivity of pteropid bats in Bangladesh with those in Southeast Asia could explain the observed strain diversity in our study. Historical interbreeding of *P. medius* with pteropid species found in Myanmar, Thailand, and Malaysia and our telemetry findings showing bats are capable of flying hundreds of kilometers, could explain our discovery of a Malaysia clade NiV sequence in bats from Comilla (*74*). NiV Bangladesh strains have also been found in *P. lylei* in Thailand (*88*). The N gene of the Comilla NiV strain differs from those reported from bats in the Nipah belt by 20%, whereas NiV Malaysia and NiV Bangladesh differ by only 6-9% and are associated with different clinical profiles. Whole genome sequence (which could not be obtained) would have allowed for better characterization of the Comilla strain, but the N gene is generally conserved relative to other genes, and suggests the rest of the genome may also be highly divergent. It is therefore plausible that the clinical profile of a 20% divergent NiV strain differs significantly from known strains. Further studies linking viral genotype to clinical phenotype would provide insight into the implications of strain diversity in bats for human outbreaks.

Finally, our study sheds light on the sporadic nature of NiV outbreaks with multi-year inter-epidemic periods in South Asia. First, PCR results show that overall NiV incidence in *P. medius* is low, consistent with previous studies of Hendra and Nipah virus (*43, 48, 89, 90*). The data and our modeling suggests that PCR-positive samples are more likely to be identified during viral transmission spikes after periodic reintroduction into populations that have become susceptible through waning population-level immunity (*10*). Viral detection in bats has also coincided with human outbreaks (*59, 87*). This is likely a rare or at least sporadic event. In the current study, observed seroprevalence patterns and the fitted model suggest that three periods of transmission occurred over the 6 years of sampling, each of which followed periods of low adult seroprevalence, though not all measurements of low seroprevalence were followed by outbreaks. We detected NiV RNA during periods of both increasing and decreasing seroprevalence, supporting the fitted model which suggested that shedding can occur through recrudescence at low levels in bats even in periods without sustained transmission. Our observation that not every instance of rising seroprevalence resulted in detectable viral shedding suggests that not all episodes of viral circulation in bats are of equal magnitude, and that other factors (e.g. variation in human-bat contact and exposure) may affect likelihood of spillover. Together, this evidence suggests that outbreaks can occur in bats when the population falls below a protective threshold of immunity in any season, but variability in how many bats become infected may impact the likelihood of spillover to humans, assuming a route of transmission is available. This could explain variation in the number of human outbreaks (e.g. spillover events) from year-to year in Bangladesh. Thus, the timing of multiple factors involved in driving transmission dynamics needs to align for intra-colony NiV transmission events and further align with human behavior and availability of a route of spillover for human outbreaks to occur, as previously hypothesized (*91*). This, and the seasonality and specific geography of date palm sap consumption in Bangladesh likely explains the somewhat sporadic nature of human outbreaks in the region, albeit that when spillover occurs, it is within the well-defined date palm sap collection season and geographic zone (*35*).

These findings suggest that Nipah virus outbreaks in other regions of Bangladesh where *Pteropus* spp. bats occur, are also likely to be sporadic and rare, leading to under-reporting or a lack or reporting, particularly given that human neurologic symptoms are similar to other common infections, such as Japanese encephalitis, malaria, and measles (*92*). Understanding whether some NiV strains are capable of causing mild or asymptomatic cases will provide important insights about why outbreaks may not have been detected in areas such as eastern Bangladesh or other parts of Asia, where host, virus, and potential routes of spillover exist. Mild or asymptomatic cases would be unlikely to be detected by

current surveillance systems and it's possible that cryptic spillovers have occurred in Bangladesh, where about half of all outbreaks between 2007 and 2014 were unreported (*93*). Our work and other reports suggest that Nipah virus transmission is possible wherever *Pteropus* spp. bats and humans live in close association and at any time of year, provided there is an available route of transmission. The 2018 and 2019 spillover events in Kerala, India, which were linked to local *P. medius* colonies and which occurred in an area that does not cultivate date palm sap, further emphasize this point.

Identifying areas where high risk interfaces exist between pteropid bats and people, throughout their range, will be important for monitoring Nipah spillover events and quickly responding to outbreaks, as well as establishing interventions to prevent spillover. Raising awareness of the potential for contaminated food to be a route of Nipah virus transmission and in protecting food resources to limit human or livestock exposure, may be effective in reducing the risk of a more transmissible strain of Nipah virus from emerging and causing an epidemic with significant human and animal mortality.

Methods

The study period was between January 2006 and November 2012. The study was conducted under Tufts University IACUC protocol #G929-07. Locations were selected based on whether the district had any previously recorded human NiV encephalitis clusters at the time of this study and was therefore inside the Nipah Belt (e.g. Rajbari, Tangail, Thakurgaon, and Kushtia) or whether they had not and were outside the Nipah Belt (e.g. Comilla, Khulna, Sylhet, and Chittagong). The Thakurgaon study was conducted as part of an NiV outbreak investigation, and coincided with ongoing human transmission (*94*). Between 2006-2012, three different studies of *Pteropus medius*, with similar bat sampling protocols were performed: 1) a cross-sectional spatial study with a single sampling event in each of the eight locations listed above; 2) a longitudinal study of a Faripur bat colony with repeated sampling approximately every 3 months from July 2007 to November 2012; and 3) a longitudinal study of the Rajbari colony with repeated sampling at a monthly interval between 12 month period between April 2010 and May 2011. Opportunistic sampling of *Pteropus medius* was also performed during this time period during NiV outbreak investigations [Bangha, Faridpur (Feb 2010), Joypurhat (Jan 2012), Rajbari (Dec 2009), West Algi, Faridpur (Jan 2010)]. Bats were captured using mist nets at locations within eight different districts across Bangladesh between January 2006 and December 2012 (**Figure 1**).

Capture and sample collection

For the country-wide cross-sectional and Faridpur longitudinal study, on average, 100 bats were sampled at each sampling event, which lasted 7-10 days. This sample size allowed us to detect at least one exposed bat (IgG antibody-positive) given a seroprevalence of 10% with 95% confidence. Bats were captured using a custom-made mist net of approximately 10 m x 15 m suspended between bamboo poles which were mounted atop trees close to the target bat roost. Catching occurred between 11 pm and 5 am as bats returned from foraging. To minimize bat stress and chance of injury, nets were continuously monitored and each bat was extracted from the net immediately after entanglement. Personal protective equipment was worn during capture and sampling, which included dedicated long-sleeve outerwear or Tyvek suits, P100 respirators (3M, USA), safety glasses, nitrile gloves, and leather welding gloves for bat restraint. Bats were placed into cotton pillowcases and held for a maximum of 6 hours before being released at the site of capture. Bats were sampled at the site of capture using a field lab setup. Bats were anesthetized using isoflurane gas (*95*) and blood, urine, oropharyngeal swabs, and wing membrane biopsies (for genetic studies) were collected. For some sampling periods, rectal swabs were collected but due to resource constraints, these samples were deemed to likely be lower yield than

saliva and urine for NiV, and were discontinued during the study. For each bat sampled we recorded age, weight, sex, physiologic and reproductive status, and morphometric measurements as described previously (*21*). Bats were classified as either juveniles (approximately four to six months - the age by which most pups are weaned) to two years old (the age when most *Pteropus* species reach sexual maturity) or adults (sexually mature) based on body size and the presence of secondary sexual characteristics, pregnancy, or lactation - indicating reproductive maturity (*21, 96*).

Up to 3.0 mL of blood were collected from the brachial vein and placed into serum tubes with serum clot activator (Vaccutainer, USA). Blood tubes were stored vertically on ice packs in a cold box and serum was allowed to separate overnight. Serum was drawn from the tube after 24 hours and placed in a screw-top cryovial (Corning, USA) and stored in a liquid nitrogen dewar (Princeton Cryogenics, NJ, USA). Sterile pediatric swabs with polyester tips and aluminum shafts were used to collect urogenital and rectal samples, and larger polyester swabs with plastic shafts (Fisher, USA) were used to collect oropharyngeal samples. All swabs were collected in duplicate, with one set being placed individually in cryotubes containing lysis buffer (either tri-reagent or NucliSENS Lysis buffer, BIOMERIEUX, France) and the second set in viral transport medium. All tubes were stored in liquid nitrogen in the field then transferred to a -80C freezer.

During each sampling event, pooled urine samples were collected beneath bat roosts using polyethylene sheets (2' x 3') distributed evenly under the colony between 3 am and 6 am. Urine was collected from each sheet either using a sterile swab to soak up droplets, or a sterile disposable pipette. Swabs or syringed urine from a single sheet were combined to represent a pooled sample. Each urine sample was divided in half and aliquoted into lysis buffer or VTM at an approximate ratio of one part sample to two parts preservative.

Serological and molecular assays

Sera from the cross-sectional survey were heat inactivated at 56^{o}C for 30 minutes, as described (*97*) prior to shipment to the Center for Infection and Immunity at Columbia University (New York, USA) for analysis. Samples were screened for anti-NiV IgG antibodies using an enzyme linked immunosorbent assay (ELISA) as described in (*21*). Sera from the longitudinal studies were sent to the Australian Animal Health Laboratory and were gamma irradiated upon receipt. Because of the large sample size and development of a high throughput multiplex assay of comparable specificity and sensitivity, for these samples we used a Luminex-based assay to detect anti-Nipah G IgG antibodies reactive to a purified NiV soluble G protein reagent (*98, 99*).

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Jen Barr – please insert language about MFI cutoff that was used for each protein. We used the NiVsG value to decide positive or negative status, but should we discuss NiVsF status, or just focus on G?

Total nucleic acids from swabs and urine samples were extracted and cDNA synthesized using Superscript III (Invitrogen) according to manufacturer's instructions. A nested RT-PCR and a real-time assay targeting the N gene were used to detect NiV RNA in samples (*100*). A RT-qPCR designed to detect the nucleocapsid gene of all known NiV isolates was also utilized (*101*). Oligonucleotide primers and probe were as described (*101*). Assays were performed using AgPath-ID One-StepRT-PCR Reagents (Thermofisher) with 250 nM probe, 50 nM forward and 900 nM reverse primers. Thermal cycling was 45◦C for 10 min, followed by 45 cycles of 95◦C for 15 s and 60◦C for 45 s. Cut-off values were cycle threshold (C_T) ≤40 for positive and CT ≥45 for negative. Results with C_T values between 40 and 45 were deemed indeterminate, i.e. not conclusively positive or negative. Samples with NiV RNA detected by real time PCR were confirmed by gel electrophoresis and product sequencing.

A subset of NiV-positive samples was processed by high-throughput sequencing (HTS) on the Ion Torrent PGM platform in order to obtain additional NiV genomic sequence. Libraries were prepared according to the manufacturer's instructions, and 1 million reads allocated per sample. HTS reads were aligned against host reference databases to remove host background using bowtie2 mapper, and host-subtracted reads primer trimmed and filtered based on quality, GC content and sequence complexity. The remaining reads were de novo assembled using Newbler (v2.6) and mapped to the full length NiV genome. Contigs and unique singletons were also subjected to homology search using MegaBlast against the GenBank nucleotide database, in case variance in parts of the genome precluded efficient mapping. From these data, N gene consensus sequences were constructed using Geneious v 7.1, and used for phylogenetic analyses.

Phylogenetic analysis

All *P. medius* NiV sequences have been submitted to Genbank and accession numbers are included in **Figure 6**. Sequence alignments were constructed using ClustalW in Geneious Prime software (*102*). Phylogenetic trees of NiV N-gene sequences were constructed using Neighbor-Joining, Maximum-Likelihood algorithms and figures constructed in FigTree 1.4.2.

Satellite telemetry and homerange analysis

We developed a collar system to attach 12g solar-powered Platform Terminal Transmitters (PTT) (Microwave Telemetry, Colombia, MD, USA) to adult bats using commercial nylon feline collars with the buckle removed and 0-gauge nylon suture to attach the PTT to the collar and to fasten the collar around the bat's neck. Collars were fitted to the bat such that there was enough space to allow for normal neck

movement and swallowing, but so that the collar would not slip over the head of the animal (**Figure S6**). PTTs were programmed with a duty cycle of 10 hours on and 48 hours off. Data was accessed via the Argos online data service (argos-system.org; France). A total of 14 collars were deployed as follows: Feb 2009: 3 males, 3 females from a colony in Shuvarampur, Faridpur district; Feb 2011: 3 males, 2 females from the same colony; and April 2011 Cox's Bazaar, 3 bats from a colony in Cox's Bazaar, Chittagong district. Bats were selected based on size such that the total weight of the collar (~21g) was less than 3% of the bat's body mass (Table S3).

The individual telemetry dataset was combined for each region and its aggregate utilization distributions (UD) computed in R using package 'adehabitatHR' (*103*). Population-specific home range is represented by the *95% area enclosure of its UD's volume. The volume of intersection between the colonies quantifies the extent of home range overlap. To evaluate the potential for contact with the Sylhet colony, we calculated the most likely distance moved ('mldm') for each sampled bat at Faridpur where the population was more intensively monitored. Movement distance was measured in kilometers with respect to a centroid location (ω) shared by the whole colony; assuming random spatial distribution in tracking rate, we estimated its kernel density function per individual and defined mldm as the mode.

Statistical approach – cross-sectional study

We fit a Bayesian generalized linear model with a logit link and a Bernoulli distribution to identify potential predictors which influenced a bat's serostatus. We included age, sex, age- and sex-normalized mass and forearm length, mass:forearm ratio, body condition, and whether the bat was pregnant, lactating, or carrying a pup. We included location of sampling as a group effect (similar to a random effect in a frequentist context) nested within Nipah Belt or non-Nipah Belt regions. We fit the models and performed posterior predictive checks in R 3.4.3, using the **brms** and **rstan** packages.

Statistical approach – longitudinal study

We fit binomial general additive models (GAMs) (*104*) to the time series of adult and juvenile seroprevalence in the longitudinal study. For juveniles, we modeled the birth cohort of bats as separate random effects in a pooled model of juveniles' dynamics starting from June of their birth year, June being the earliest month we sampled free-flying juveniles in any cohort. We determined the cohort year of juveniles by using cluster analysis to group individuals by weight, assuming those in the smallest group were born in the current year and those in the larger group were born the previous year. 92% of

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If you used a Bayesian approach you need to specify what priors you used for each coefficient and whether the predictors were standardized, since this determines how informative those priors are.

juveniles captured were yearlings. For adults, we modeled dynamics of adults as a single pool over the entire course of the study. We tested models with and without annual cyclic effects.

Where time series had significant temporal autocorrelation (adults only), we aggregated data by week. We determined periods of significant increase in decrease as those where the 95% confidence interval of the GAM prediction's derivative did not overlap zero. We fit the models and performed checks in R 3.4.3, using the **mgcv** package.

To examine the importance of different biological mechanisms in transmission, we fit an age-structured (adult and juvenile) maternally immune (M)-susceptible (S)-infected (I)-recovered (R) model with recrudescence (R to I) and loss of immunity (R to S) to the seroprevalence data on a weekly timescale:

$$\frac{dS_J}{dt} = -S_J(\beta_{JJ}I_J + \beta_{AJ}I_A) - \mu_J S_J + bN_A(t-5)\frac{S_A}{N_A} - bN_A(t-52)\frac{S_J}{N_J}(1-\mu_J)^{52} + \lambda M_J$$

$$\frac{dI_J}{dt} = S_J(\beta_{JJ}I_J + \beta_{AJ}I_A) - \gamma I_J - bN_A(t-52)\frac{I_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dR_J}{dt} = \gamma I_J - \mu_J S_J - \mu_J R_J - bN_A(t-52)\frac{R_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dM_J}{dt} = bA(t-5)\frac{R_A}{N_A} - \lambda M_J - \mu_J M_J - bN_A(t-52)\frac{R_A}{N_A}(1-\mu_J)^{52}\lambda^{52}$$

$$\frac{dS_A}{dt} = -S_A(\beta_{AA}I_A + \beta_{JA}I_J) - \mu_A S_A + \tau R_A + bN_A(t-52)\frac{S_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dI_A}{dt} = S_A(\beta_{AA}I_A + \beta_{JA}I_J) - \gamma I_J - \mu_A I_A + bN_A(t-52)\frac{I_J}{N_J}(1-\mu_J)^{52} + \Delta R_A$$

$$\frac{dR_A}{dt} = \gamma I_A - \mu_A R_A + bN_A(t-52)\frac{R_J}{N_J}(1-\mu_J)^{52} - \tau R_A - \Delta R_A$$

We included a class M for the density of juvenile bats with maternal antibodies to allow for the biological possibility that maternal antibodies are lost at a much higher rate than antibodies acquired following infection. The subscripts J refer to juveniles and A to adults, β is the transmission rate, γ is the recovery rate, μ is the mortality rate, τ is the rate of loss of adult immunity, λ is the rate of loss of maternal antibodies(*57*), Δ is the adult recrudescence rate (R to I), b is the birth rate (pups join the juvenile population after 5 weeks). Juveniles transition to adults after 52 weeks. We included terms for loss of antibody in adults (τ, S to R), and viral recrudescence (Δ, R to I) based on previous studies on captive bats that demonstrated the existence of these processes without providing enough data to characterize them precisely (*55, 65*). We fit this deterministic model to the seroprevalence data by maximum likelihood,

which assumes that deviations from the model are due to observation error. We estimated the confidence intervals around maximum likelihood parameter estimates using likelihood profiles using the *profile* function in package *bbmle* in R v3.2.2.

We used model fitting and model comparison to examine the need for several of the biological processes in the model above that could influence NiV dynamics. First, we examined both density and frequency-dependent transmission by comparing the fit of the model above to one with transmission terms that have population size (N_A or N_J) in the denominator. Second, we examined the confidence intervals of the parameters describing viral recrudescence, loss of antibodies in adult bats, and loss of antibodies in juvenile bats. If the confidence bounds for these parameters included zero, then these biological processes are not needed to explain the serological dynamics. Finally, we examined the confidence bounds for parameters describing the loss of maternal and non-maternal antibodies (τ and λ), to determine if the rate of loss of these two types of immunity were different. We note that this model structure has similarities to a susceptible-infected-latently infected(L)-infected (SILI) model (if latently infected individuals are seropositive), but the model above differs in allowing for the possibility of individuals to transition from the R class back to the S class.

Code availability

SIR model code written in R is available upon request.

Data availability

All molecular sequences are available via Genbank. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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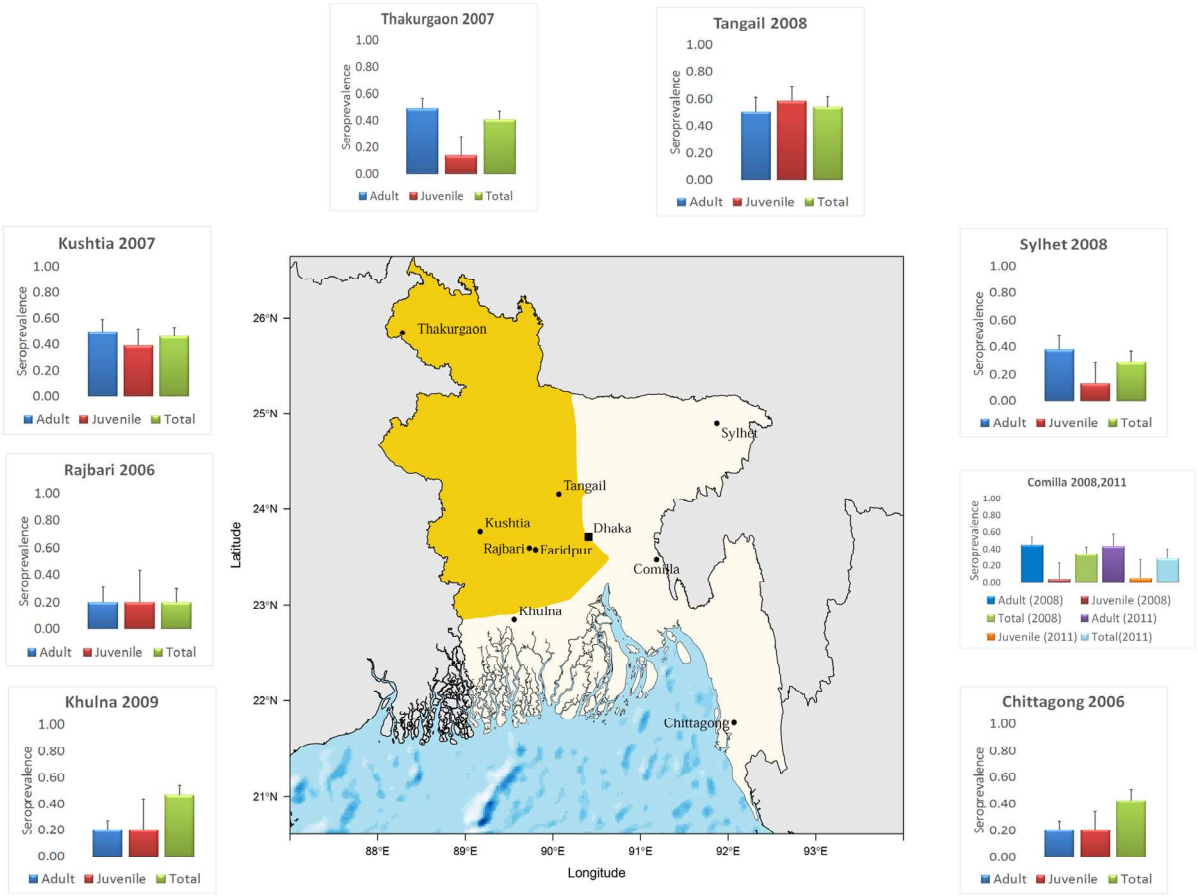

Figure 1. Map showing age-stratified seroprevalence in *Pteropus medius* colonies, Bangladesh. Bats from eight colonies were sampled and tested for anti-NiV IgG antibodies: four within the "Nipah Belt" (orange shaded) and four outside. Seroprevalence of adults (blue, purple), juveniles (red, orange) and total seroprevalence (green, light blue) are shown. Number (n) of Adult, Juvenile, and Total bats sampled (clockwise): Tangail [53,41,94], Sylhet [63, 37, 100], Comilla 2008 [73,27,100], Comilla 2011 [30,20,50], Chittagong [72,24,96], Khulna[85,15,100], Rajbari [65,15, 80], Kushtia [64,36,100], Thakurgaon [89,29,118]. The shaded region represents the "Nipah Belt" where previous human NiV outbreaks have been reported.

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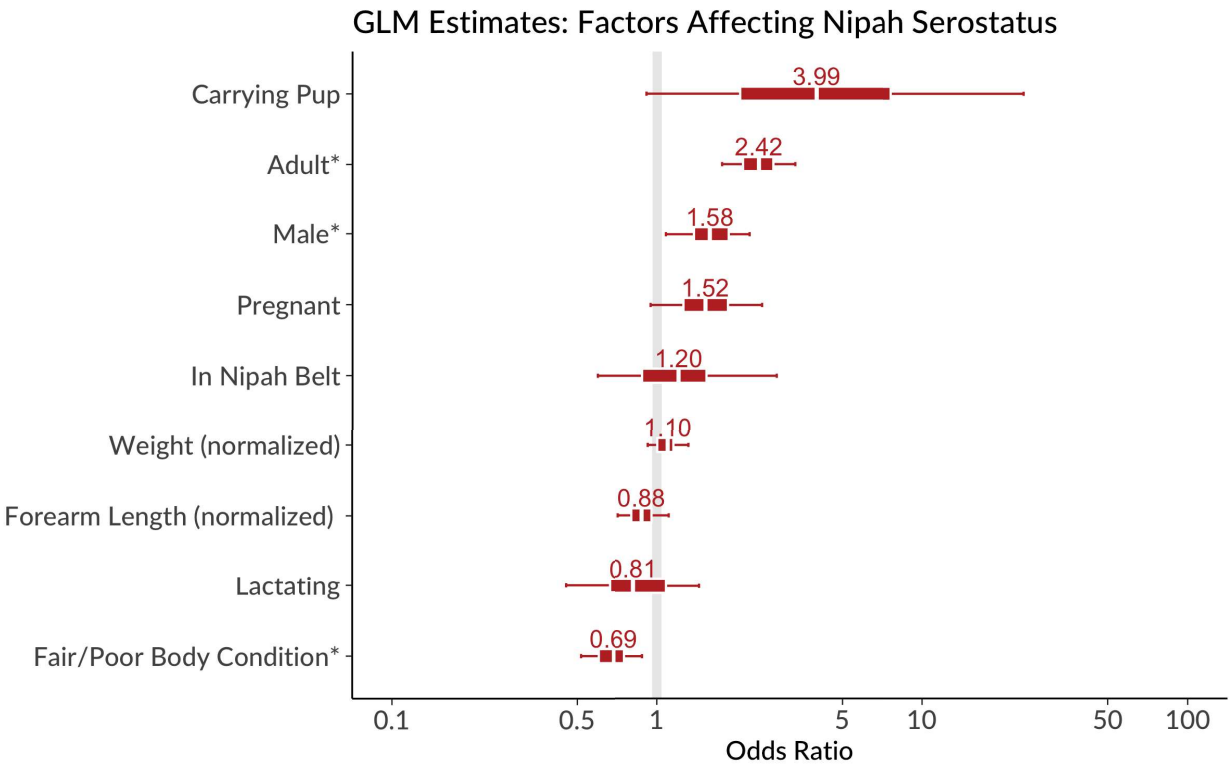
You only need one legend since it is the same for all panels. This would all you to make the panels a little bigger. I would try to reduce the white-space and zoom in on the map a little and make the graphs bigger. Finally I'd add lines b/w the locations on the map and the graphs.



792

793 **Figure 2.** Results of Bayesian GLM of factors affecting Nipah serostatus in bats in cross-sectional study. Bars
794 indicate odds ratios and 50% (inner) and 95% (outer) credible intervals for model parameters. Factors with
795 asterisks (*) have 95% CIs that do not overlap 1. Model intercept (predicted probability of seropositivity for a
796 juvenile, female, bat outside the Nipah belt of mean size and good body condition) was 0.26, (95% CI 0.12-0.56).
797 Random effect of location (not shown) had an estimated SD of 0.63 (95% CI 0.29-1.27)

798

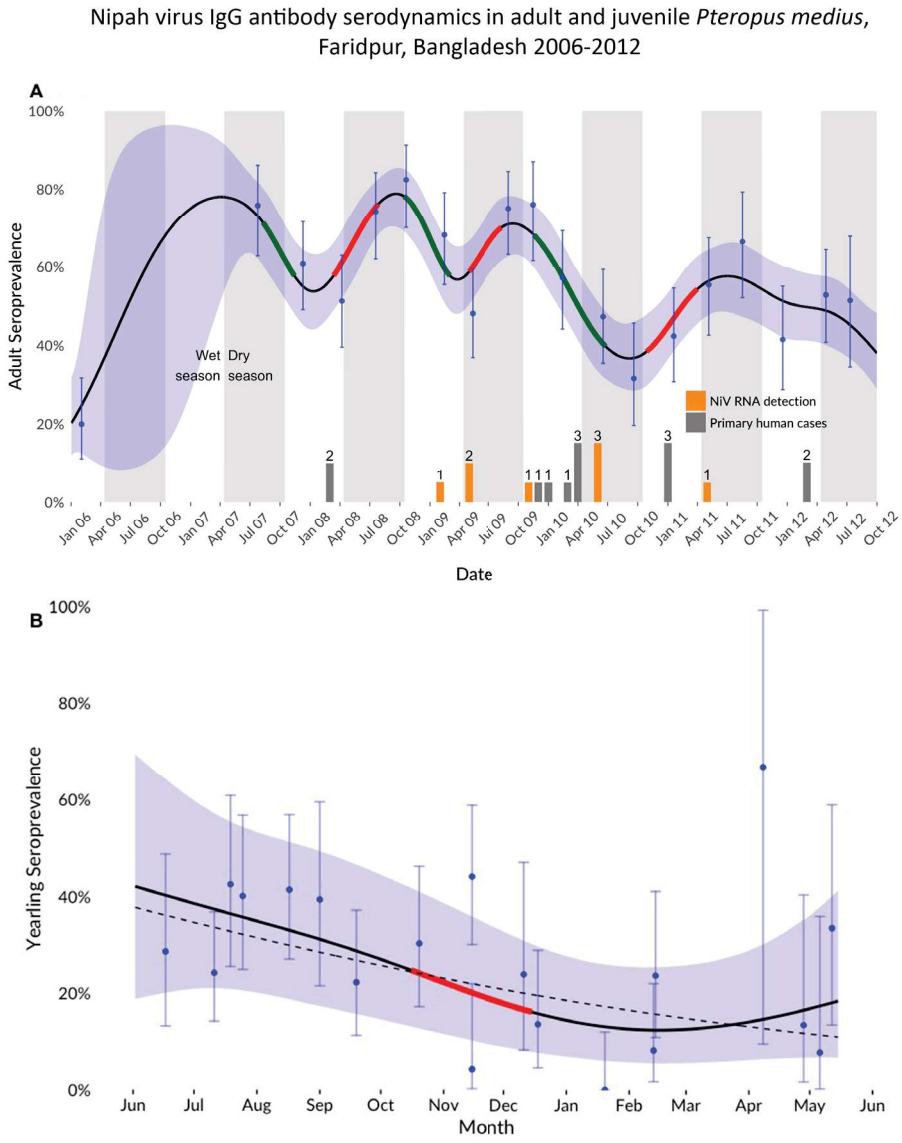


Figure 3 A & B. Serodynamics of the Faridpur bat population. (A) Adult serodynamics, with measured values and 95% CI in blue, and mean GAM prediction and 95% shown with line and surrounding shaded areas. Periods of significant increase (red) and decrease (green) shown where the GAM derivative's 95% CI does not overlap zero. Counts of primary human cases from local district (orange, and bat viral detections (dark grey, see Table 1), shown on bottom. (B) Juvenile serodynamics during the first year of life ("yearlings"), with all years' measurements overlain to show cohort-level dynamics across all study years. Measured values and 95% CI in blue, and mean and 95% CI for the GAM model pooled across cohorts shown with line and surrounded shaded areas. The period of significant decline in the GAM is shown in red. Also shown is the mean prediction of a model with only a linear mean term, with similar fit (ΔAIC < 1) as the GAM (dotted line).

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I'd show these as a % of samples collected, not the raw #. Otherwise you are confounding prevalence with sampling effort, unless the # of samples was the same for every sample.

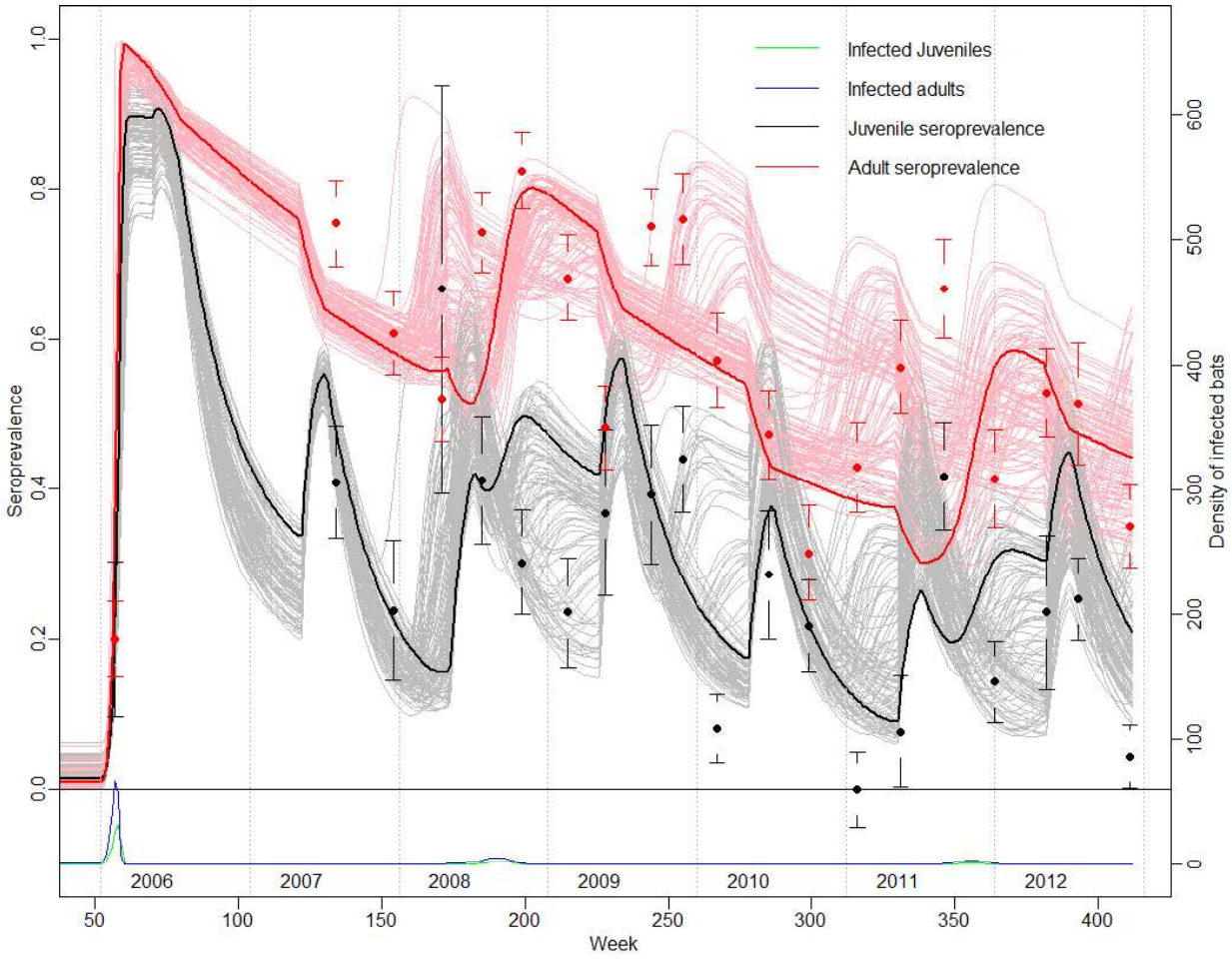

Figure 4. **NiV serological dynamics in adult and juvenile bats.** The observed data (red and black points ±1 SE) and model fit (solid lines; thick lines show the trajectory for the model with maximum likelihood parameter estimates; thin lines show realizations for parameter estimates drawn from the estimated distributions) for the fraction of adults and juveniles seropositive for NiV (left axis), and the model estimated density of infected adult and juvenile bats (bottom panel and right axis). See Methods for details of model structure.

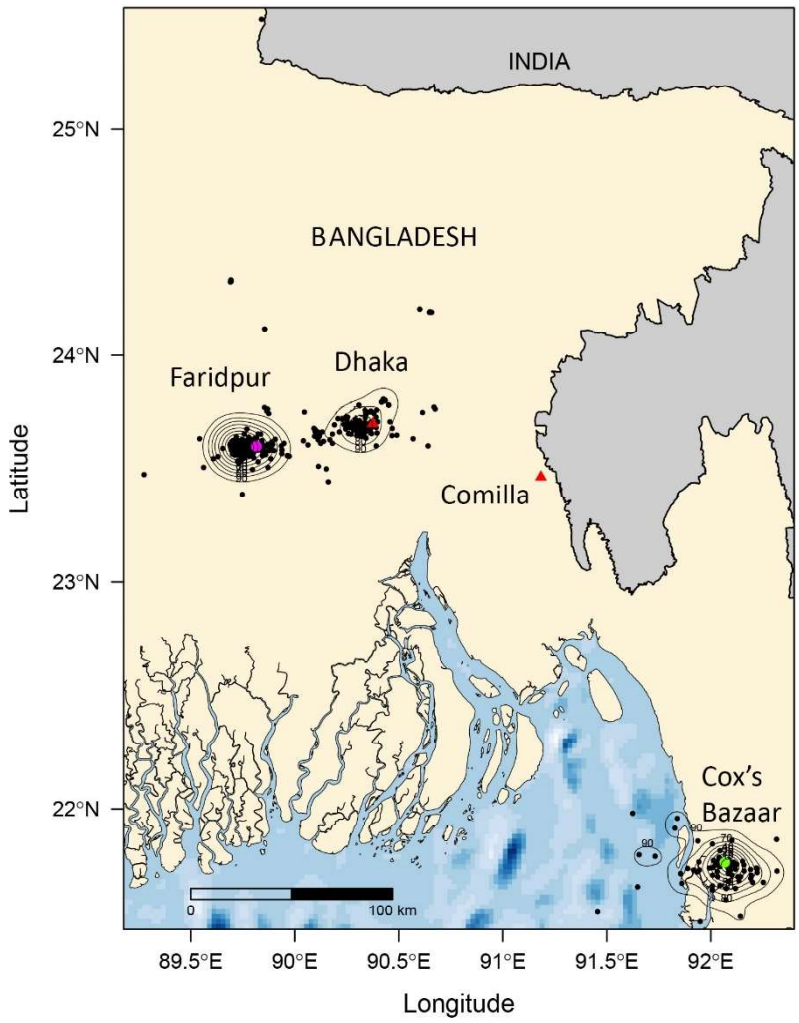

Figure 5. Satellite telemetry and homerange analysis. Location data from satellite collars (n=14) placed on 11 bats from Faridpur and 3 bats from Cox's Bazaar, Chittagong collected between 2009 and 2011, were used to calculate local and long-range movement patterns and home range for these two groups.

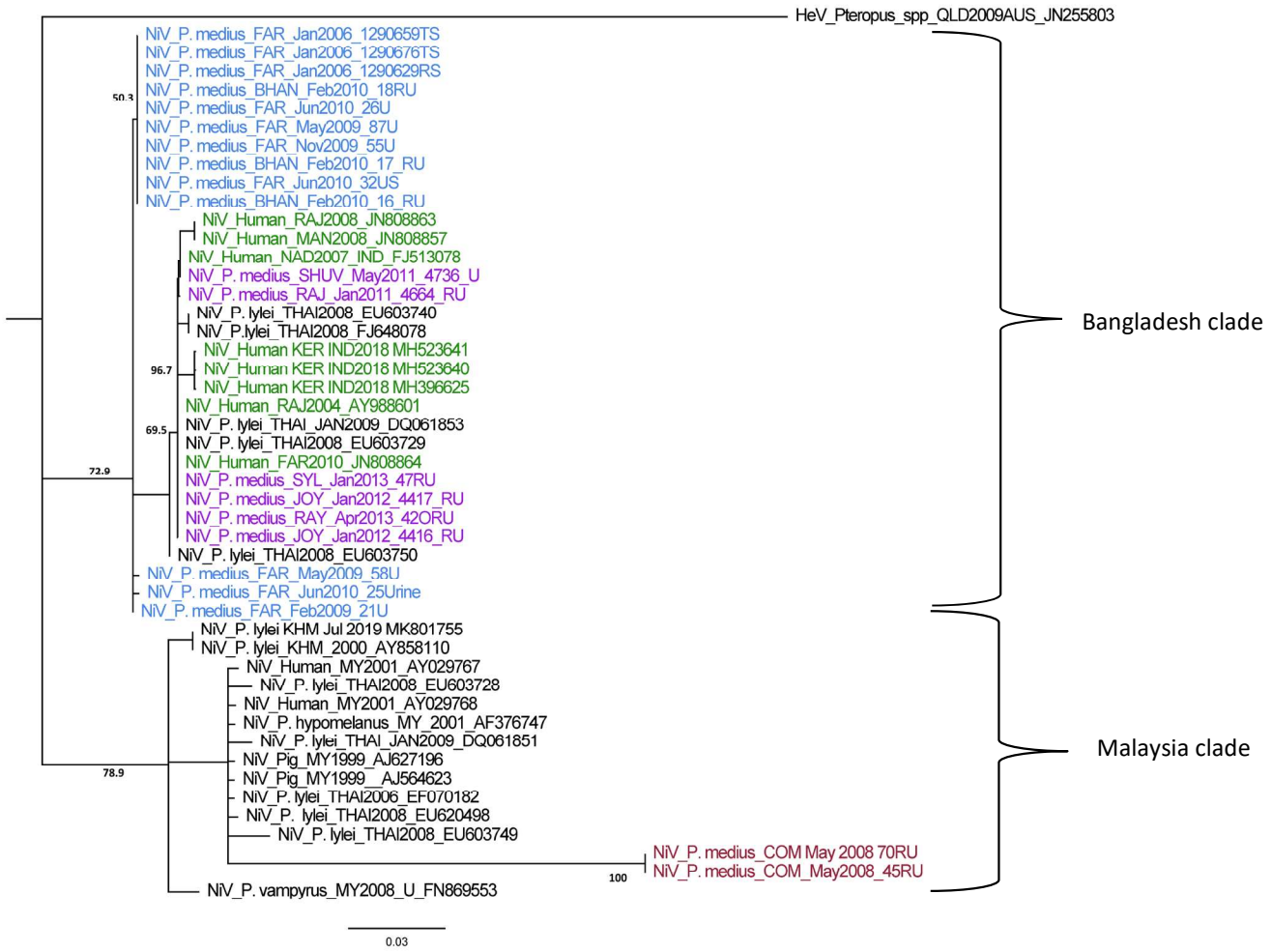

Figure 6. **Nipah Virus N gene phylogenetic tree (224nt):** Tree created in Geneious Prime 2019 using a Neighbor-joining Tamura-Nei model with 1,000 replicates (*105*). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Hendra virus was used as an outgroup. Sample collection date, location and Genbank accession numbers are included in the label for each sequence except *P. medius* sequences which are accession Nos MK995284 – MK995302. Blue labels indicate bat sequences from Faridpur and Bhanga (an outbreak response in Faridpur). Purple sequences are from *P. medius* from other roosts sampled during the longitudinal study. Red sequences are from *P. medius* in Comilla. Green sequences are human NiV sequences from Bangladesh and India.

Table 1. PCR detection of NiV RNA in *Pteropus medius* 2006-2012.

Location	date	Bats Sampled	Throat Tested	Throat Pos	Urine Tested	Urine Pos	Rectal Tested	Rectal Pos	Paired samples	Pos. Bats	Bats w multi pos samples	prev.	95% CI	Roost Urine	Roost Urine pos.
Spatial Study															
Rajbari	Jan-06	99	79	**3**	78	0	79	**1**	78	3	**1**	**0.04**	**0.11**	-	-
Thakurgaon	Mar-07	118	115	**3***	72	0	-	-	70	unk.	0	0.00	-	-	-
Kushtia	Aug-07	101	100	0	99	0	-	-	98	0	0	0.00	-	-	-
Tangail	Jun-08	100	61	0	77	0	-	-	60	0	0	0.00	-	81	0
Chittagong	Aug-06	115	19	0	-	-	-	-	-	0	-	-	-	-	0
Comilla	May-08	100	0	0	50	0	-	-	0	0	-	-	-	100	**2**
Sylhet	Sep-08	100	100	0	49	0	-	-	48	0	0	0.00	-	100	0
Khulna	Jan-09	100	50	0	80	0	-	-	32	0	0	0.00	-	50	0
Comilla	Mar-11	50	50	0	50	0	-	-	0	0	0	0.00	-	-	-
Outbreak Investigation															
Bangha	Feb-10													19	3
Joypurhat	Jan-12													19	**16**[α]
Rajbari	Dec-09													35	0
West Algi	Jan-10													31	0
Longitudinal Study															
Faridpur	Jul-07	102	64	0	50	0	-	-	22	0	0	0.00			
Faridpur	Dec-07	101	N/A	N/A	N/A	-	-	-		0					
Faridpur	Apr-08	100	64	0	88	0	-	-	54	0	0	0.00		51	0
Faridpur	Jul-08	100	58	0	74	0	-	-	54	0	0	0.00			
Faridpur	Oct-08	100	98	0	99	0	-	-	98	0	0	0.00			
Faridpur	Feb-09	100	50	0	100	**1**	-	-	49	**1**	0	**0.01**	**0.10**	50	0
Faripdur	May-09	101	100	0	99	**2**	-	-	99	**2**	**0**	**0.02**	**0.10**	9	0
Faridpur	Aug-09	100	100	0	99	0	-	-	95	0	0	0.00		3	0
Faridpur	Nov-09	100	100	0	82	**1**	-	-	82	**1**	**0**	**0.01**	**0.11**	50	0
Faridpur	Feb-10	100	100	0	100	0	-	-	100	0	0	0.00		45	0
Faridpur	Jun-10	100	100	0	100	**3**	-	-	100	**3**	**0**	**0.03**	**0.10**	25	0
Faridpur	Sep-10	100	100	0	100	0	-	-	-	0	-	-		20	0
Faridpur	Jan-11	100	100	0	100	0	-	-	0	0	0	0.00		15	0
Faridpur	May-11	102	102	0	102	**1**	-	-	0	**1**	0	**0.01**	**0.10**	20	0
Faridpur	Aug-11	100	100	0	100	0	-	-	-	0	-	-		10	0
Faridpur	Dec-11	100	100	0	100	0	-	-	-	0	-	-		16	0
Faridpur	Apr-12	100	78	0	78	0	-	-	-	0	-	-		50	0
Faridpur	Jul-12	100	100	0	100	0	-	-	-	0	-	-		30	0
Faridpur	Nov-12	100	100	0	100	0	-	-	-	0	0			34	0
Total		**2789**	**2088**	**6**	**2126**	**8**	**79**	**1**		**11**	**1**	**0.005**	**0.02**	**829**	

*NiV RNA was detected in three pooled oropharyngeal samples, confirmed by sequencing, although confirmation from individual samples could not be made. These data re not used in prevalence estimates. [α] Detection by qPCR, Ct ranges 20-38.

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From: A. Marm Kilpatrick on behalf of A. Marm Kilpatrick <akilpatr@ucsc.edu>
To: Jon Epstein; Anthony, Simon J.; Ariful Islam; Shahneaz Ali Khan; Noam Ross; ina.smith@csiro.au; Carlos M. Zambrana-Torrelio MSc; Yun Tao; Ausraful Islam; Kevin Olival, PhD; Salah Uddin Khan; Emily Gurley; Dr. Jahangir Hossain; Hume Field; Fielder, Mark; Thomas Briese; Mahmud Rahman; Christopher Broder; Gary Crameri; Linfa Wang; Stephen Luby; Ian Lipkin; Peter Daszak
Subject: Re: Nipah dynamics in P medius draft for PNAS
Date: Friday, December 20, 2019 4:12:47 AM
Attachments: Supplemental Data_PNAS 2019_amk.docx
Nipah dynamics in bats_Epstein et al 2019_amk.docx

Hi Jon,

It's reading pretty well! See my suggested revisions and comments on the main doc and supplemental material. Let me know if you have questions!

marm

On 12/18/2019 10:00 AM, Jon Epstein wrote:

> All,
> I hope this email finds you well. Attached is a draft of our manuscript entitled Nipah virus dynamics in bats and implications for spillover to humans. After multiple protracted periods of review in Science Advances, and ultimately a rejection, I've prepared this draft for submission to PNAS. I've revised the manuscript and updated figures and supplemental data in response to the last round of review (comments below, if you're interested).
>
> I have an editor at PNAS willing to send this for review, so please send me any input you may have by December 28th so that I can submit by the 31st. Unless I hear otherwise, I will assume you still consent to co-authorship.
>
> Thank you for your patience, and happy holidays!
>
> Cheers,
> Jon
>
> Reviewer: 1
> Epstein et al aim to better understand the distribution and drivers of Nipah virus infection dynamics in Pteropus medius in Bangladesh by analysing a large set of serological, virological and movement data over a commendable spatial and temporal scale. Overall, this is a highly exciting study with valuable results that are well-deserving of publication. I have a number of minor comments regarding the addition of detail for clarity (and to ensure transparency in the interpretation of results). I think that the conclusions are mostly justified, however, my major criticism is that the integration and interpretation of the results (particularly those presented in the first paragraph of the discussion) requires a little further thought and explanation. The assumptions surrounding the serological implications of within-host persistence and recrudescence needs to be clearly stated. This is likely to become a 'classic' paper, and there are so few studies in this area supported by data that it is important to ensure that the results are not over-interpreted.
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> Specific comments:
> Abstract:
> Line 32- 33: The wording here is too strong regarding recrudescence. Suggest inserting "model results indicated that" prior to "local transmission dynamics.

Line 33-34: Similarly - this is too strong. Suggest "likely due to "
Results:
Figure 1 - presumably the first three bats in the Comilla represent 2008 and the second three represent 2011, but this is not clear. This should be annotated on the plot
Figure 1 - "Adult bats had equal or greater seroprevalence than juveniles in each location." - except Tangail?
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Line 131: - describe "pooled samples" - pooled under-roost urine samples?
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Line 159 - incomplete sentence
Lines 160-164: Fascinating results!
Lines 170-171: This information would be helpful to include earlier, with the serological results
Figure 3 and Figure S1 - It would be helpful to have a "total population" size from this roost complex included on Figures 3 and 4. Understanding more about the bat ecology and the size and stability of these populations over time would be a tremendous help to aid interpretation of these results by researchers working in other systems.
Lines 175-176: "Serodynamics in juveniles were strongly driven by inheritance and loss of maternal antibodies". Supported by what result? This should be explained further, and/or it would be helpful to include the serology (fig 3), model output (fig 4) and population size (Figure s1) together as a series of vertical panels in the one figure to aid interpretation
Lines 178-180: I can't see this information in the supplementary information
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Line 195 - should the 'of' be 'if?
Figure 6 - the utility of this figure would be greatly improved with some annotation and colouring to help identify the new sequences from this study, and their source location, and the 'groups' that are referred to in the text
Lines 238 - 240 - So, your data has found cyclical serodynamics, but no clear links between those dynamics and detection of NiV in bats or in people. Based on experimental studies, it is still a bit unclear exactly what seropositivy in flying foxes represents. If there is within-host persistence of infection, a cycle of infection > seroconversion (in the absence of clearance) > seroreversion > then recrudescence with serconversion may occur in the absence of ongoing transmission. Additionally, this does not take into account drivers of recrudescence - if this is stress related, then transmission at the population level will also be affected by these broader drivers. Given all the uncertainty around this topic, I think that the wording here needs to be precise. e.g the first sentence should first state the assumption "Assuming that seroconversion results only from new infections, then ...". This could be followed up with a sentence along the lines of "If however, seroconversion can result from recrudescence in the absence of transmission, then broader drivers of recrudescence would also need to be assessed". More clarity here would also help to assess the claims being made in the paragraph beginning 315.
Lines 240 onwards - consider restructuring this paragraph to more clearly step through each stage of the viral dynamics that you are proposing and your assumptions and evidence along the way. For example, you assume infection results in seroconversion, and that antibodies then wane after ~4 years. Are you assuming that all individuals are persistently infected, and it is only when Ab wane that recrudescence can occur? And following that, the individual seroconverts again?

Lines 243 - 245: Not correct - See Brooks et al 2019 JAE Figure S5. That study also involved modelling and has implications for this study more broadly
Lines 245-247: "via recrudescence FROM bats that have previously been infected"? It's also not clear how this links to lines 238-240
Lines 257-259: Consider also cite Peel et al (2018). Scientific Reports, 8(1), e0004796. This study examines waning maternal immunity and waning acquired immunity against henipaviruses in African fruit bats, finding a similar duration of immunity for both
Line 267 - insert "in our study area" after 'Pteropus medius' as you cannot infer whether these patterns hold true for the species across its range. It may be highly dependent on available food resources - as you go on to discuss
Line 277 - replace 'be' with 'result in'
Lines 295-296 - this links with the SILI hypotheses in Plowright et al (2016) PLoS NTD (see Figure 3)
Line 321- suggest "three periods of transmission (significant at the population level) occurred"
Line 315 See my comments re: paragraph beginning Line 238. The sporadic nature of detections is challenging to draw conclusions and much of the paragraph here (starting line 315) seems over-stated. However, that may become clearer if the comments for the earlier paragraph are addressed.
Methods:
Line 364: I can't see reference to the Faripur colony in the list of colonies in lines 358-359. What are its characteristics? Also, I presume it should be "Faridpur"? Also, it would be helpful if the "Faridpur roost complex:" could be described in more detail in the methods as to the structure of this population and what this term means.
Line 366: Delete the first 'between'
Lines 388-390 - check the placement of parentheses here
Line 417: what cutoff was used for the Luminex assay and how was it determined?
Line 425 - fix formatting of second Ct
Line 493- 504: More information on the underlying population dynamic model is required e.g. Were births seasonal or continuous in your model? I can't see where this is stated. If not seasonal, then the effect of this on model output should be addressed. How was the death rate modelled? Was the total population size kept stable inter annually? The latter, in particular, may have implications for interpretation of the density-dependent vs frequency dependent results. The population size is touched on in the discussion in lines 288-291 but never really explained.
Table S1 - there is extra text below Table S1 that looks like it's not supposed to be there
Table S2 - Include what serostatus 0/1 refers to in the table caption

Reviewer: 2
Dear editor, I have reviewed the manuscript entitled “Nipah virus dynamics in bats and implications for zoonotic spillover to humans”. The manuscript describes a longitudinal surveillance in Pteropid spp. Bats from 2006 until 2012. Biometric data was collected, sera and swabs/urine were analyzed, and inferences were made largely based on serological data. The manuscript is a compendium of relatively loosely compiled data, ranging from seroprevalence in a variety of different sampling sites, but the majority of the samples stem from Faridpur. The problem is that most of the claims by the authors within the paper are not directly supported by the data. The direct problem of the data is the limited amount of detected virus shedding, out of 2789 animals sampled only 11 were found to be shedding the virus. From this only 8 were from the larger study cohort from Faridpur. This directly hampers some of the conclusion of potential spillover dynamics as this cannot be directly inferred from serological data alone. In addition, it does not provide any answers on the occurrence of Nipah spillover in the Nipah belt vs the other regions. Moreover, the significant spillover events in Kerala, India from the last two years, are not discussed. Most emphasis has been put on analyzing the serological results from the Pteropus medius bats. The authors show variation in the seroprevalence within the population based on timing and age status of the animal. The results have been reported before in other natural reservoir-pathogen systems like avian influenza, however even within

these systems inferences on spillover can rarely be made. It interesting that the authors did not correlate the positive individual bats in Faridpur and Rajbari with their respective serostatus? Is this data not available? It is unclear to me why the authors have not put more effort in trying to perform full genome analyses on the positive samples obtained throughout this study. Currently there are only 27 full genomes available from Nipah virus and relatively limited amount are from the natural reservoir. Preforming phylogenetic analyses on a 224 nucleotide fragment of a 19kb virus is really not up to standard. Where it might be suitable for identification of the lineage no additional data can be inferred from this. Interestingly, there appears to be full genome sequencing performed but only N is shown in the supplemental data.
Although I do understand the logistics involved with this kind of work, unfortunately some of the claims, especially regarding spillover, need to be supported by more additional data rather than just serology.
Minor points:
Line 65: pandemic potential, given the limited amount of h-to-h transmission the pandemic potential of this particular virus appears to relatively limited.
Line 143: n=844 or n=883? Why do the numbers not match-up?
Line 410, include level of biosecurity involved in sample analyses. In addition, heatinactivation is typically to inactivate complement and not inactivation of the pathogen.
From the current wording it is unclear what the authors mean by this? Complement inactivation or pathogen inactivation?
Line 428, is this data missing? Where is the NSG data? Why not data on the full genomes? I only was able to find the full N gene data in the supplemental figues.
Line 509, given that actual recrudescence in the context of virus shedding in the natural reservoir has never been shown it would be good to treat this a little bit more carefully.

--

Jonathan H. Epstein DVM, MPH, PhD

Vice President for Science and Outreach

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EcoHealth Alliance leads cutting-edge scientific research into the critical connections between human and wildlife health and delicate ecosystems. With this science, we develop solutions that prevent pandemics and promote conservation.

--
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130 McAllister Way
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http://kilpatrick.eeb.ucsc.edu/

From: Broder, Christopher on behalf of Broder, Christopher <christopher.broder@usuhs.edu>
To: Jon Epstein
Subject: Re: Nipah dynamics in P medius draft for PNAS
Date: Thursday, December 19, 2019 3:03:45 PM

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good luck jon

boy reviewer #2 was harsh....
wonder who that was?

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Line 267 - insert "in our study area" after 'Pteropus medius' as you cannot infer whether these patterns hold true for the species across its range. It may be highly dependent on

available food resources - as you go on to discuss
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shown in the supplemental data.
Although I do understand the logistics involved with this kind of work, unfortunately some of the claims, especially regarding spillover, need to be supported by more additional data rather than just serology.
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--

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Vice President for Science and Outreach

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From: Wang Linfa on behalf of Wang Linfa <linfa.wang@duke-nus.edu.sg>
To: Jon Epstein; Anthony, Simon J.; Ariful Islam; marm@biology.ucsc.edu; Shahneaz Ali Khan; Noam Ross; ina.smith@csiro.au; Carlos M. Zambrana-Torrelio MSc; Yun Tao; Ausraful Islam; Kevin Olival, PhD; Salah Uddin Khan; Emily Gurley; Dr. Jahangir Hossain; Hume Field; Fielder, Mark; Thomas Briese; Mahmud Rahman; Christopher Broder; Gary Crameri; Stephen Luby; Ian Lipkin; Peter Daszak
Subject: RE: Nipah dynamics in P medius draft for PNAS
Date: Wednesday, December 18, 2019 9:47:28 PM

Dear Jon,

I had a quick read and have nothing to add.

Fingers crossed!

Thanks

LF

Linfa (Lin-Fa) Wang, PhD FTSE
Professor & Director
Programme in Emerging Infectious Diseases
Duke-NUS Medical School
8 College Road, Singapore 169875
Tel: +65 65168397

From: Jon Epstein [mailto:epstein@ecohealthalliance.org]
Sent: Thursday, 19 December 2019 4:01 AM
To: Anthony, Simon J. <sja2127@cumc.columbia.edu>; Ariful Islam <arif@ecohealthalliance.org>; marm@biology.ucsc.edu; Shahneaz Ali Khan <shahneazbat@gmail.com>; Noam Ross <ross@ecohealthalliance.org>; ina.smith@csiro.au; Carlos M. Zambrana-Torrelio MSc <zambrana@ecohealthalliance.org>; Yun Tao <yun.tao.86@gmail.com>; Ausraful Islam <islam_ausraf@icddrb.org>; Kevin Olival, PhD <olival@ecohealthalliance.org>; Salah Uddin Khan <sukhanbd@gmail.com>; Emily Gurley <egurley1@jhu.edu>; Dr. Jahangir Hossain <jhossaincsd99@gmail.com>; Hume Field <hume.field@ecohealthalliance.org>; Fielder, Mark <m.fielder@kingston.ac.uk>; Thomas Briese <tb2047@cumc.columbia.edu>; Mahmud Rahman <mahmudur57@gmail.com>; Christopher Broder <christopher.broder@usuhs.edu>; Gary Crameri <garycrameri1@gmail.com>; Wang Linfa <linfa.wang@duke-nus.edu.sg>; Stephen Luby <sluby@stanford.edu>; Ian Lipkin <wil2001@columbia.edu>; Peter Daszak <daszak@ecohealthalliance.org>
Subject: Nipah dynamics in P medius draft for PNAS

All,
I hope this email finds you well. Attached is a draft of our manuscript entitled Nipah virus dynamics in bats and implications for spillover to humans. After multiple protracted periods of

review in Science Advances, and ultimately a rejection, I've prepared this draft for submission to PNAS. I've revised the manuscript and updated figures and supplemental data in response to the last round of review (comments below, if you're interested).

I have an editor at PNAS willing to send this for review, so please send me any input you may have by December 28th so that I can submit by the 31st. Unless I hear otherwise, I will assume you still consent to co-authorship.

Thank you for your patience, and happy holidays!

Cheers,
Jon

Reviewer: 1
Epstein et al aim to better understand the distribution and drivers of Nipah virus infection dynamics in Pteropus medius in Bangladesh by analysing a large set of serological, virological and movement data over a commendable spatial and temporal scale. Overall, this is a highly exciting study with valuable results that are well-deserving of publication. I have a number of minor comments regarding the addition of detail for clarity (and to ensure transparency in the interpretation of results). I think that the conclusions are mostly justified, however, my major criticism is that the integration and interpretation of the results (particularly those presented in the first paragraph of the discussion) requires a little further thought and explanation. The assumptions surrounding the serological implications of within-host persistence and recrudescence needs to be clearly stated. This is likely to become a 'classic' paper, and there are so few studies in this area supported by data that it is important to ensure that the results are not over-interpreted.

Specific comments:
Abstract:
Line 32- 33: The wording here is too strong regarding recrudescence. Suggest inserting "model results indicated that" prior to "local transmission dynamics.
Line 33-34: Similarly - this is too strong. Suggest "likely due to "
Results:
Figure 1 - presumably the first three bats in the Comilla represent 2008 and the second three represent 2011, but this is not clear. This should be annotated on the plot
Figure 1 - "Adult bats had equal or greater seroprevalence than juveniles in each location." - except Tangail?
Line 129: "detected NiV RNA in 11 individuals, 3 pooled" - insert 'from' before 3
Line 131: - describe "pooled samples" - pooled under-roost urine samples?
Line 132 and 137: This 'figure 2' seems to be missing? Figure 2 refers to serological analyses
Line 145: I suggest that the reference to Figure 2 in line 146 should go in the sentence ending on line 145
Line 149: the significant negative association with body condition warrants mentioning in these results.
Line 154-155 and Figure 3: Additional clarity here regarding juvenile vs. yearling terms. Suggest saying "Juveniles in their first year of life (yearlings)" at first mentioning in the text, as well in the figure legend.
Line 159 - incomplete sentence

Lines 160-164: Fascinating results!
Lines 170-171: This information would be helpful to include earlier, with the serological results
Figure 3 and Figure S1 - It would be helpful to have a "total population" size from this roost complex included on Figures 3 and 4. Understanding more about the bat ecology and the size and stability of these populations over time would be a tremendous help to aid interpretation of these results by researchers working in other systems.
Lines 175-176: "Serodynamics in juveniles were strongly driven by inheritance and loss of maternal antibodies". Supported by what result? This should be explained further, and/or it would be helpful to include the serology (fig 3), model output (fig 4) and population size (Figure s1) together as a series of vertical panels in the one figure to aid interpretation
Lines 178-180: I can't see this information in the supplementary information
Line 182 - this paragraph (and ideally also table S2) should provide information on the duration of tracking for each bat. This would be very helpful in assessing the home range information
Line 195 - should the 'of' be 'if?
Figure 6 - the utility of this figure would be greatly improved with some annotation and colouring to help identify the new sequences from this study, and their source location, and the 'groups' that are referred to in the text
Lines 238 - 240 - So, your data has found cyclical serodynamics, but no clear links between those dynamics and detection of NiV in bats or in people. Based on experimental studies, it is still a bit unclear exactly what seropositivy in flying foxes represents. If there is within-host persistence of infection, a cycle of infection > seroconversion (in the absence of clearance) > seroreversion > then recrudescence with serconversion may occur in the absence of ongoing transmission. Additionally, this does not take into account drivers of recrudescence - if this is stress related, then transmission at the population level will also be affected by these broader drivers. Given all the uncertainty around this topic, I think that the wording here needs to be precise. e.g the first sentence should first state the assumption "Assuming that seroconversion results only from new infections, then ...". This could be followed up with a sentence along the lines of "If however, seroconversion can result from recrudescence in the absence of transmission, then broader drivers of recrudescence would also need to be assessed". More clarity here would also help to assess the claims being made in the paragraph beginning 315.
Lines 240 onwards - consider restructuring this paragraph to more clearly step through each stage of the viral dynamics that you are proposing and your assumptions and evidence along the way. For example, you assume infection results in seroconversion, and that antibodies then wane after ~4 years. Are you assuming that all individuals are persistently infected, and it is only when Ab wane that recrudescence can occur? And following that, the individual seroconverts again?
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New York, NY 10001

1.212.380.4467 (direct)
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web: ecohealthalliance.org
Twitter: @epsteinjon

SUMMARY STATEMENT

PROGRAM CONTACT: **(Privileged Communication)** ***Release Date:*** **12/03/2019**
Jean Patterson ***Revised Date:***

Application Number: **1 U01 AI151797-01**

Principal Investigator

DASZAK, PETER

Applicant Organization: ECOHEALTH ALLIANCE, INC.

Review Group: **ZAI1 EC-M (J2)**
National Institute of Allergy and Infectious Diseases Special Emphasis Panel
Emerging Infectious Diseases Research Centers (U01 Clinical Trial Not Allowed)

Meeting Date: **11/04/2019** ***RFA/PA:*** **AI19-028**
Council: **JAN 2020** ***PCC:*** **M32F**
Requested Start: **03/01/2020**

Project Title: **Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia**
SRG Action: **Impact Score:32**
Next Steps: **Visit https://grants.nih.gov/grants/next_steps.htm**
Human Subjects: **30-Human subjects involved - Certified, no SRG concerns**
Animal Subjects: **30-Vertebrate animals involved - no SRG concerns noted**
Gender: **1A-Both genders, scientifically acceptable**
Minority: **5A-Only foreign subjects, scientifically acceptable**
Age: **1A-Children, Adults, Older Adults, scientifically acceptable**

Project Year	Direct Costs Requested	Estimated Total Cost
1	1,050,579	1,514,744
2	1,050,579	1,514,744
3	1,050,579	1,514,744
4	1,050,579	1,514,744
5	1,050,579	1,514,744
TOTAL	5,252,895	7,573,721

ADMINISTRATIVE BUDGET NOTE: The budget shown is the requested budget and has not been adjusted to reflect any recommendations made by reviewers. If an award is planned, the costs will be calculated by Institute grants management staff based on the recommendations outlined below in the COMMITTEE BUDGET RECOMMENDATIONS section.

1U01AI151797-01 Daszak, P

FOREIGN INSTITUTION
SCIENTIFIC REVIEW OFFICER'S ADMINISTRATIVE NOTES
SELECT AGENTS

RESUME AND SUMMARY OF DISCUSSION: This excellent new Research Project Cooperative Agreement application entitled “Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia” was submitted in response to AI19-028: Emerging Infectious Diseases Research Centers (U01) by the Ecohealth Alliance, Inc., New York, New York with Dr. Peter Daszak as Principal Investigator (PI).

The application has many strengths. The study proposed is adequately focused on the identification of new emerging virus in Southeast Asia, which is a hotspot of viral activity with significant threat to human health. The approach is based on the identification of viral spillovers by studying the pathogen in wild animals and performing surveillance of high-risk communities. There is a strong emphasis to identify and characterize viral etiology of ‘cryptic’ outbreaks in clinical cohorts by using surveys to look for causal links between syndromes and the viruses detected. The PI, Dr. Daszak, is a well-recognized expert with strong track record in working with zoonotic diseases and researching spillover events. The team of investigators have significance experience working in the SE and includes accomplished scientists such as Dr. Baric, who is a well-recognized coronavirus expert. They have built a network of local collaborators with access to regional clinics in Thailand, Singapore, and Malaysia. Adequate analytic and laboratory techniques are in place for conducting human and animal surveillance to complete the proposed studies. The feasibility of the strategy proposed is supported by the availability of samples from previous studies and the preliminary data presented. The data management plan and statistical plan are well described. The environment and resources are excellent at the established participating institutions. Data management plan and statistical plan are well described.

The application has some weaknesses. There is an overemphasis in the results obtained in previous work. The virus families targeted are somewhat restricted to those previously identified by the team. The roles and responsibilities of the Executive Committee are not clearly described. Some of the key personnel listed does not appear to have appropriate expertise for the tasks proposed. The sample sites for the wildlife studies are not clearly identified. There will be challenges to find new human pathogens by using serological-based field studies. The team does not include a veterinary pathologist nor an expert in entomology/vectors approach. No emphasis is made of efforts to build in-country infrastructure. There is no planned integration or clear leveraging with NIAID resources. The potential role of the movement of animals, people and products within the region and with neighboring countries such as China is not mentioned. The lack of their own lab facilities by the applicant organization is seen also as a weakness as well as the absence from the team of a US-based clinical scientist.

Based upon the evaluation of scientific and technical merit, this application received an Impact/Priority score of 32.

DESCRIPTION (provided by applicant**):** Southeast Asia is one of the world’s highest-risk EID hotspots, the origin of the SARS pandemic, Nipah virus, and repeated outbreaks of influenza. This is driven by high diversity of wildlife and rapidly expanding demography that brings human and wildlife populations closer. This proposal will launch the Emerging Infectious Diseases - South East Asia Research Collaboration Hub (EID-SEARCH), a collaboration among leaders in emerging disease research in the USA, Thailand, Singapore and the 3 major Malaysian administrative regions. These researchers have

networks that span >50 clinics, laboratories and research institutions across almost all SE Asian countries and will use the EID-SEARCH as an early warning system for outbreaks involving exchange of information, reagents, samples and technology, and a collaborative power- house for fundamental and translational research. The EID-SEARCH will also act as a significant asset to scale-up and deploy resources in the case of an outbreak in the region. This EIDRC will conduct research to: 1) Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife, by analyzing previously-archived wildlife samples, conducting targeted wildlife surveillance, and using serology & PCR assays to identify novel viruses. These will be characterized to assess risk of spillover to people, and a series of in vitro (receptor binding, cell culture) and in vivo (humanized mouse and collaborative cross models) assays used to assess their potential to infect people and cause disease; 2) Collect samples and questionnaire data from human communities that live in EID hotspots and have high cultural and behavioral risk of animal exposure (e.g. wildlife hunting, bat guano collection). These will be tested with serological assays to identify evidence of novel virus spillover and analyzed against metadata to identify key risk pathways for transmission; 3) Identify and characterize viral etiology of ‘cryptic’ outbreaks in clinical cohorts. We will conduct syndromic surveillance at clinics serving the populations in Aim 2, enroll patients with undiagnosed illness and symptoms consistent with emerging viral pathogens, and test samples with molecular and follow-up serological assays to identify causal links between these syndromes and novel viruses. This research will advance our understanding of the risk of novel viral emergence in a uniquely important region. It will also strengthen in-country research capacity by linking local infectious disease scientists with an international collaborative network that has proven capacity to conduct this work and produce significant findings. The large body of high impact collaborative research from this EIDRC leadership team provides proof-of-concept that EID-SEARCH has the background, collaborative network, experience, and skillset to act as a unique early warning system for novel EIDs of any etiology threatening to emerge in this hottest of the EID hotspots.
PUBLIC HEALTH RELEVANCE: This proposed EID Research Center (EID-SEARCH) brings leaders in emerging disease research from the US, Thailand, Singapore and the 3 major Malaysian administrative regions together to build an early warning system to safeguard against pandemic disease threats. This team will identify novel viruses from Southeast Asian wildlife, characterize their capacity to infect and cause illness in people, and use serological assays of samples from people in rural communities with high wildlife contact to identify the background rate of exposure, and risk factors that drive this. They will conduct in-depth surveillance of clinical cohorts at hospitals serving these communities to examine if ‘cryptic’ outbreaks are caused by these novel agents, and to build significant capacity to rapidly detect and respond should there be a major outbreak of a virus in the region.

CRITIQUE: The comments in the CRITIQUE section were prepared by the reviewers assigned to this application and are provided without significant modification or editing by staff. They are included to indicate the range of comments made during the discussion and may not reflect the outcome. The RESUME AND SUMMARY OF DISCUSSION section summarizes the final opinion of the committee after the discussion and is the basis for the assigned Overall Impact/Priority score.

CRITIQUE 1

Significance:	3
Investigator(s):	3
Innovation:	2
Approach:	4
Environment:	2

Overall Impact: The focus of this application is to identify new pathogens from wild animals and monitor their spillover in high-risk communities. The countries of interest include Thailand and Malaysia. Some of the research team members have excellent expertise relevant to the proposed studies. The availability of samples from previous studies is a strength. The PI has the experience to handle this type of application. This application has a few minor weaknesses and the proposed studies are overly ambitious.

1. Significance:

Strengths

- The identification spillovers of new pathogens from wild animals (bats, rodents, and nonhuman primates) to humans will be significant.
- Surveillance of high-risk communities for new pathogens could be useful to prevent potential pandemic event/s.

Weaknesses

- They seem to be constrained in their efforts since they are thinking more about coronaviruses (CoVs), paramyxoviruses (PMVs, e.g., henipaviruses) and filoviruses (FVs).
- Outcomes will be hard to evaluate due to complex experimental design.

2. Investigators:

Strengths

- The PI has the relevant expertise and experience to lead this type of multi-national research initiative.
- The research team includes scientists from EcoHealth Alliance, University of North Carolina, Duke-NUS Medical School, Chulalongkorn University Hospital, Gleneagles Hospital, Hospital University Malaysia Sabah, Queen Elizabeth State Hospital, Lintang Clinic of Kuala Kangsar District Health Office, University Malaysia Sarawak, Bario Clinic of Rural Area Service under Ministry of Health Malaysia, Conservation Medicine Ltd., Uniformed Services University, BU NEIDL, University of North Carolina at Chapel Hill, and Hospital Miri.
- Most research team members have the needed expertise for the proposed studies.

Weaknesses

- It seems that Hongying Li joined a PhD program at Kingston University, UK in 2018, therefore, it is unclear how this person will serve as a China Coordinator.
- Some of the key personnel do not seem to have expertise in the area of proposed research.
- It is unclear who will collect samples and perform various assays at Chulalongkorn Hospital.
- Roles of some of team players could have been explained better.
- A veterinary pathologist is needed in their team.

3. Innovation:

Strengths

- The concept of investigating new pathogen spillovers by studying the pathogen in the wild animals and humans is innovative.

Weaknesses

- None significant.

4. Approach:

Strengths

- Preliminary results provide support to some of the proposed studies. The availability of samples from previous studies is a plus.
- Identify, characterize and evaluate spillover risk of high zoonotic potential viruses from wildlife (bats, rodents, primates).
- Surveillance of high-risk communities will be pursued to find evidence of viral spillovers.
- Efforts will be made to Identify and characterize viral etiology of ‘cryptic’ outbreaks in clinical cohorts.
- Administrative plan seems fine.
- Data management plan and statistical plan are well described.

Weaknesses

- It is unclear what else they anticipate finding in addition to their earlier findings. They are also focusing their efforts on pathogens already identified by their team.
- The application is overly ambitious.
- In vitro and in vivo characterization of new viral pathogens to evaluate their potential for human infections may lead to misleading results.
- They have not yet identified the wildlife sampling sites.
- Antibody cross-reactivity may sometime be misleading.
- They do not plan to collect samples from NHPs.
- Reverse genetic approach to generate new viruses is doable but will distract from their focus.
- It is difficult to find new pathogens by sero-surveillance.

5. Environment:

Strengths

- The research team has all the necessary facilities and resources for the proposed studies.

Weaknesses

- The lead organization, EcoHealth Alliance, has limited wet lab research facilities.
- Limited capacity building at the partner sites.

CRITIQUE 2

Significance:	1
Investigator(s):	1
Innovation:	1
Approach:	2
Environment:	1

Overall Impact: This application is highly focused on the identification of emerging zoonotic viruses. The work will be carried out by a highly experienced and accomplished investigative team that has already had substantial success in this area. The strategies for sampling and for prioritization of testing resources is detailed and well planned. Modeling has been incorporated into the plan in a way that increases the likelihood that surveillance and testing will result in the identification of emergent viruses, which then will be efficiently characterized and followed by detection in high risk human cohorts.

1. Significance:

Strengths

- The application incorporates surveillance data spanning wildlife, human and livestock populations.
- The use of clinical surveys to look for causal links between syndromes and the viruses detected in Aim 1 is an integrated and elegant strategy.
- The work is very focused around the goal of identifying emergent zoonotic viruses. The team has extensively worked in this area with several examples of key discoveries from this group of viruses in the families around which the application is based.

Weaknesses

- Given the reliance on geographic-based prediction and targeting in Aim 1, it could be possible for the Aim 1-identified viruses to be in a region or in a type of animal-human interface that is not relevant to the established cohorts in use in Aim 3. This would require Aim 3 work to focus on a different, non-integrated, set of viruses.

2. Investigators:

Strengths

- The PI and the applicant institution have a long track record in working with zoonotic diseases and researching spillover events.
- The investigator team includes established experts and there is a pre-existing history of collaboration between the partners including a history of joint publications and grant implementation with Dr. Daszak and Dr. Baric.
- The investigator team includes experience with working in SE Asia, conducting human and animal surveillance, and analytic and laboratory techniques necessary to complete the proposed objectives.

Weaknesses

- None noted.

3. Innovation:

Strengths

- The use of combined geographic hotspot and reservoir-specific targeting is innovative and is an improvement on surveillance efforts driven by convenience sampling.
- While not innovative in its individual components, the integration of the components of the project is such that this is a refinement on existing early warning and investigative strategies for zoonotic emergences.

Weaknesses

- None noted.

4. Approach:

Strengths

- The approach is detailed and integrated across each aim, with a logical progression from zoonotic discovery, to basic science work, to clinical surveillance. Preliminary data is presented to support likelihood of success at each stage.
- While the application is primarily focused on a limited number of virus families, the expertise and capacity are available to expand this further into other viruses if needed. The application demonstrates examples of the team's ability to conduct rapid response.
- Targeted, systematic sampling strategies are used for prioritization of viruses.
- The sampling infrastructure needed for Aim 1 appears to already be in place.
- Preliminary testing in one of the proposed sample sets has already been conducted and several novel viruses have already been identified.
- Statistical approaches are listed in detail and are appropriate for the scientific questions (including the use of LASSO).
- This proposal will collect a very large number of specimens, only a subset will be used for the intensive laboratory analysis proposed in Aim 2. Testing in Aim 2 is informed by the findings in Aim 1 following a detailed plan for prioritization of resources.
- For studies in human specimens, the investigators have access to established population surveillance efforts in communities that are likely to be most impacted by the spillover mechanism that is hypothesized in this application. Serosurvey efforts are augmented by the appropriate epidemiologic data needed to integrate Aim 2 with Aim 3.
- Meetings with the community are included in the specimen collection plans.

Weaknesses

- The application mentions processing of PBMCs after serologic testing confirms exposure. Given issues of timing in processing PBMCs, it is uncertain to what degree systematic PBMC collection is feasible.

5. Environment:

Strengths

- Serology testing capabilities are available at individual sites.
- The environment is excellent with strength both in established clinical sites and in superb laboratory infrastructure.

Weaknesses

- There is no planned integration with NIAID resources, however there is integration with USAID programs and the investigator team has extensive experience working with NIAID.

CRITIQUE 3

Significance: 2

Investigator(s):	3
Innovation:	2
Approach:	3
Environment:	2

Overall Impact: The application by Daszak and colleagues plans to build off their collective work under PREDICT and several R01s to conduct research to identify, characterize, assess potential for human infection and risk factors of zoonotic viruses in South East Asia. The team will be led by the EcoHealth Alliance, an NGO based in New York that has experience with similar projects and working with the consortium members. The application contains three aims to identify and characterize zoonotic viruses utilizing a large biorepository of animal and clinical specimens, determining the risk of spill-over into the human and other animal populations in the region linking to their large network in the three countries (Malaysia, Thailand and Singapore, and characterize cryptic outbreaks of viral EIDs in the region. The proposal has many strengths, including the use of the humanized mouse model to determine infectivity of novel viruses, efficient use of banked specimens, multidisciplinary team able to respond to potential and actual outbreaks in the three countries including neighboring regions, local capacity building, access to BSL3+ facilities and bioinformatic and other analytical expertise. I have identified several mild weaknesses in the proposal including: no clear veterinary or entomological expertise, limited clinical expertise amongst the US collaborating institutions which might be important for future diagnostic and treatment protocols, no clear expertise to identify and characterize non-zoonotic viruses, including bacteria, and lack of clear responsibilities for the Executive Committee to manage this complex project. Overall, I believe the project has significant strengths with several mild weaknesses.

1. Significance:

Strengths

- Zoonoses are common in SE Asia and have led to significant human and domestic animal outbreaks, with evidence that viruses are circulating in animals in the region
- Team will use an early warning approach to identify EIDs

Weaknesses

- While the team is taking a regional approach, there is no mention of the movement of animals, people and products within the region, as well as with neighboring countries such as China

2. Investigators:

Strengths

- Dr. Daszak is an internationally recognized leader in emerging viral zoonosis, with the administrative experience to direct this project
- The team is multidisciplinary, albeit with a bias towards ID ecologist, microbiologists, etc.

Weaknesses

- No clear expertise in veterinary medicine nor entomology to address new EIDs
- Little clinical research expertise from US-based investigators which could be required for advancing therapeutic and diagnostic studies

3. Innovation:

Strengths

- Evaluation of infectivity of novel viruses using the humanized mouse model

Weaknesses

- Unclear how team will identify previously uncharacterized EID viruses, and if needed non-viral EIDs in the region as needed during the project time period

4. Approach:

Strengths

- The focus on zoonotic viral EIDs is relevant to SE Asia and the US
- Use of banked specimens will ensure success of Aim 1
- In order to determine the impact to human health, the team will use transgenic mice to test infectivity of humanized cells
- Use of validated instruments to test for associations with risk exposure in human populations
- Use of case-control cross-sectional studies to determine risk of infection

Weaknesses

- The responsibilities of the Executive Committee are not clearly laid out

5. Environment:

Strengths

- The EcoHealth Alliance is an NGO that will serve to coordinate this project in the field and with its US partners. The Alliance has managed related projects including the USAID-PREDICT as a partner
- The Partner institutions have BSL3+ facilities
- Access to many banked specimens for screening of human and animals throughout the region—this will be a very efficient aspect of this project

Weaknesses

- No clear leveraging of existing networks, other than the recently completed PREDICT

THE FOLLOWING RESUME SECTIONS WERE PREPARED BY THE SCIENTIFIC REVIEW OFFICER TO SUMMARIZE THE OUTCOME OF DISCUSSIONS OF THE REVIEW COMMITTEE ON THE FOLLOWING ISSUES:

PROTECTION OF HUMAN SUBJECTS: ACCEPTABLE (CODE 30). This application is a study of human spillover and exposure to animal coronaviruses, henipaviruses, and filoviruses in Southeast Asia, with active sample collection in Thailand and Malaysia and testing of archived samples in Singapore.

Study participants will be recruited for this project within the study sites in Thailand, Malaysia, and Singapore. The protection of Human Subjects is adequately described.

INCLUSION OF WOMEN PLAN: Male and female subjects are anticipated (G1A)
The inclusion plan is appropriate for the scientific goals of the research.

INCLUSION OF MINORITIES PLAN: ACCEPTABLE (Code M5A). The study population will be selected from the hotspot regions of Ratchaburi (Thailand), Chonburi (Thailand), Peninsular Malaysia, Sabah Malaysia, and Sarawak. The inclusion plan is appropriate for the scientific goals of the research.

INCLUSION OF INDIVIDUALS ACROSS THE LIFESPAN: ACCEPTABLE (C1A). All individuals across the lifespan will be enrolled with no exclusion based on age or gender. The inclusion plan is appropriate for the scientific goals of the research.

VERTEBRATE ANIMAL: ACCEPTABLE. (CODE 30). This application involves working with vertebrate animals in Thailand and Malaysia for field sampling and the University of North Carolina, Chapel Hill, and the National Emerging Infectious Diseases Laboratory (NEIDL), Boston, USA for mouse model experimental infections. Wild animals capture includes bats, rodents and non-human primates. The protection of Vertebrate Animal welfare is adequately described.

BIOHAZARD COMMENT: ACCEPTABLE. The plan to prevent risks during handling of biohazard materials or samples is adequate. The proposed research requires higher biosafety level containment (BSL3/4) for the selected select agents that are available at the University of North Carolina at Chapel Hill (UNC) and the National Emerging Infectious Diseases Laboratories (NEIDL), who will conduct select agent research. All biohazards that are shipped or received for the planned projects will meet the standards of the High Hazard Materials Management policy.

SELECT AGENTS: ACCEPTABLE. The pathogen surveillance activities proposed will result in the identification of the following select agents SARS-CoV (coronaviruses); wildtype Nipah, Hendra and related bat viruses (Henipaviruses); and wildtype Ebola viruses (Filovruses).

RESOURCE SHARING PLANS

DATA SHARING PLAN: ACCEPTABLE. Data will be shared in accordance with NIH guidelines. Plan will need to be improved by Data Coordination Center

MODEL ORGANISM SHARING PLAN: ACCEPTABLE.
There is an adequate plan for sharing of model organisms that may be generated in the project.

GENOMIC DATA SHARING PLAN: Applicant will ensure compliance with NIH's Genomic Data Sharing plans for all viral sequence data generated in this project.

FOREIGN INSTITUTION: JUSTIFIED. The involvement of foreign organizations in Southeast Asia in this application is properly justified.

AUTHENTICATION OF KEY BIOLOGICAL AND/OR CHEMICAL RESOURCES: ACCEPTABLE
Adequate plans for authentication of key resources are outlined in the application.

BUDGETARY OVERLAP: NON-APPLICABLE.

SCIENTIFIC REVIEW OFFICER'S ADMINISTRATIVE NOTES:

During the review of this application, reviewers and/or NIH staff noted that one or more biosketches did not comply with the required format (NOT-OD-15-032). NIH has the authority to withdraw such applications from review or consideration for funding.

COMMITTEE BUDGET RECOMMENDATIONS: The budget is recommended as requested in all years.

Footnotes for 1 U01 AI151797-01; PI Name: DASZAK, PETER

NIH has modified its policy regarding the receipt of resubmissions (amended applications). See Guide Notice NOT-OD-14-074 at http://grants.nih.gov/grants/guide/notice-files/NOT-OD-14-074.html. The impact/priority score is calculated after discussion of an application by averaging the overall scores (1-9) given by all voting reviewers on the committee and multiplying by 10. The criterion scores are submitted prior to the meeting by the individual reviewers assigned to an application, and are not discussed specifically at the review meeting or calculated into the overall impact score. Some applications also receive a percentile ranking. For details on the review process, see http://grants.nih.gov/grants/peer_review_process.htm#scoring.

MEETING ROSTER

National Institute of Allergy and Infectious Diseases Special Emphasis Panel
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
Emerging Infectious Diseases Research Centers (U01 Clinical Trial Not Allowed)

ZAI1 EC-M (J2)
11/04/2019 - 11/05/2019

Notice of NIH Policy to All Applicants: Meeting rosters are provided for information purposes only. Applicant investigators and institutional officials must not communicate directly with study section members about an application before or after the review. Failure to observe this policy will create a serious breach of integrity in the peer review process, and may lead to actions outlined in NOT-OD-14-073 at https://grants.nih.gov/grants/guide/notice-files/NOT-OD-14-073.html and NOT-OD-15-106 at https://grants.nih.gov/grants/guide/notice-files/NOT-OD-15-106.html, including removal of the application from immediate review.

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ROCKVILLE, MD 20892

Consultants are required to absent themselves from the room during the review of any application if their presence would constitute or appear to constitute a conflict of interest.

From:	Peter Daszak on behalf of Peter Daszak <daszak@ecohealthalliance.org>
To:	Laing, Eric; Thomas Hughes
Cc:	Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Supaporn Wacharapluesadee; Aleksei Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross; Kevin Olival; Jon Epstein
Subject:	RE: EIDRC Summary Statement from NIAID
Date:	Friday, December 6, 2019 7:08:35 PM
Attachments:	Summary Statement - EIDRC Grant.pdf
Importance:	High

Dear All,

Attached is the summary statement from our EIDRC review. We didn't do badly at all – 3 reviewers, two of whom gave us 2's and 3's, and the second reviewer who loved it (mainly 1's).

The good news is that the summary statement starts with "in this excellent …proposal", which is a good sign, although there were some negatives of course.

In my opinion, we're touch and go on whether we'll get funded on this, so I quickly wanted to check in with who of you will be at the Singapore Nipah meeting over the next few days? I'll be there, as will Jon Epstein (cc'd here so he can help). Linfa and Danielle, obviously. **Supaporn, Chris, Ralph – will you be there in Singapore?**

The reason I'm asking is that Cristina Cassetti, and possibly others from NIH/NIAID/DMID will be there as well, and they're in a key position to decide whether to push this forwards for funding. The council meeting is on January 27th.

Linfa or Danielle – can you email me a list of who's registered for the meeting from NIAID or NIH please? Jean Patterson is the Program Officer and Eun-Chung Park will be part of the process also.

For those of you going – we can't lobby NIH directly, but it will be good for us to be seen as a cohesive team at that meeting, and talk very positively about our work together over the past few years, including on PREDICT and the samples that have been collected that could be also used in this project. Also, if you have chance, please re-read some of the proposal in case we do get chance to talk with them about it (attached in a previous email).

Fingers crossed on this one!

Cheers,

Peter

Peter Daszak
President

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Website: www.ecohealthalliance.org
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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

From: Peter Daszak
Sent: Monday, November 18, 2019 9:24 PM
To: 'Laing, Eric'; Thomas Hughes
Cc: 'Wang Linfa'; 'Danielle E. ANDERSON PhD'; 'Ralph S. Baric'; 'Baric, Toni C'; 'Sims, Amy C'; 'Chris Broder'; 'Supaporn Wacharapluesadee'; Aleksei Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross; Kevin Olival
Subject: EIDRC impact scores from NIH
Importance: High

Dear All,

Just to let you know that we've got the results back from NIH. EHA submitted two EIDRC proposals, and we were linked to a third one as a subcontract. Out of these, the only one to get a score was the Southeast Asia EIDRC (EID-SEARCH) which you are all collaborators on. I also know of another good group that wasn't scored, so just to get a score is actually a good result…

Our 'impact score' was 32, which normally would put us right on the edge of being funded. I've already contacted the NIAID program officer (Jean Patterson) to ask about the likelihood of being funded. She told me that NIH staff are currently working out the structure of the network and a funding plan. She felt our proposal was well-received and scored well enough to be considered, and she asked me to hold off for a week or two and then they'd know more.

My take on this is that they're now deciding what the geography and scope of the network should be, how many EIDRC centers they can afford to fund, and then they'll decide priorities for funding. With this being strongly driven by NIAID's internal concerns about their reach into different geographies, it's really going to be a decision driven by their own considerations of who else is in the mix and what strengths/weaknesses different proposals have. That means we're in with a chance, but we don't really know the factors driving a decision.

I'll contact NIAID again in a few days, but in the meantime, please do the following: 1) try not to share this information with colleagues because we don't want to have a situation where they scored better than us and we got funded, leading to a bad feeling (or worse, the other way round!); 2) if you meet people from NIAID, tell them how keen and enthusiastic you are about the proposal and the work we're conducting (I've attached the final text again as a reminder) – you never know how this might help. Key people at NIAID who will be making this decision are: Tony Fauci (head of NIAID),

Cristina Cassetti (Deputy Head, and in charge of this line of work), Jean Patterson (Program Officer directly in charge of the EIDRCs), Eun-Chung Park (also directly in charge of this work), Pat Repik (I think she's involved).

Finally – I've attached the Review Committee Roster for you to look at who the people are who either liked or disliked our proposal! I know a few of them, and I'm sure you know the others…

Will be in touch as soon as I hear more.

Cheers,

Peter

Peter Daszak
President

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Website: www.ecohealthalliance.org
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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

From: Peter Daszak
Sent: Friday, June 28, 2019 8:59 PM
To: 'Laing, Eric'; Thomas Hughes
Cc: 'Wang Linfa'; 'Danielle E. ANDERSON PhD'; 'Ralph S. Baric'; 'Baric, Toni C'; 'Sims, Amy C'; 'Chris Broder'; 'Supaporn Wacharapluesadee'; Aleksei Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross; Kevin Olival
Subject: EIDRC grant submitted
Importance: High

Dear All,

Just to let you all know that the grant was successfully submitted today with time to spare, and no errors. It's in the system, and it's all down to the reviewers now! Thanks to all of you for your help and support in getting this finalized and completed. Please pass on my personal thanks to the Co-Investigators, Key Personnel and Consultants who you also brought into the team.

I've attached a pdf of the final proposal text and will send the Word version in a few minutes. It's actually a good read, and I'm especially grateful to Hongying who generated all the cool graphics, based largely on Ralph's previous NIH grant proposals.

As you read the text, please remember that the wording is very carefully targeted to a typical US-based NIH reviewer, and to the Program Officers, with the sole purpose of trying to win the grant. If I've exaggerated or made mistakes, or used language that isn't quite right, I apologize, but I did it for the key goal of getting funded.

In the meantime, please don't share this proposal beyond our group on this email chain, so we don't give our competitors an edge!

Good luck, and I'll be keeping my fingers crossed all summer in the hope that we win this…

Cheers,

Peter

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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

From: Peter Daszak
Sent: Wednesday, June 26, 2019 5:19 PM
To: 'Laing, Eric'; Thomas Hughes
Cc: Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Supaporn Wacharapluesadee; Aleksei Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross; Kevin Olival
Subject: EID-SEARCH Comments

Thanks everyone for sending the comments back. Working on v5 now, while others in the office are cranking out sample size calculations, new figures, human and vert. subjects and all the additional information needed after the research plan.

As I go through all your comments, I'll send the occasional email with questions to clarify specific points, so please be on standby for a quick turnaround.

Cheers,

Peter

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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

From: Laing, Eric [mailto:eric.laing@usuhs.edu]
Sent: Wednesday, June 26, 2019 12:59 PM
To: Thomas Hughes
Cc: Peter Daszak; Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Supaporn Wacharapluesadee; Aleksei Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross; Kevin Olival
Subject: Re: EID-SEARCH v4

Hi Peter,

Built on top of Chris' suggestions. Included a prelimin data figure from Hughes et al, in prep. Double check that is ok with Tom?

- Eric

Eric D. Laing, Ph.D.
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On Wed, Jun 26, 2019 at 11:26 AM Tom Hughes <tom.hughes@ecohealthalliance.org> wrote:

> Hi Peter,
>
> I have added my edits and comments to Kevin's.
>
> Please let me know if you ave any questions or need more details.
>
> Thanks.
>
> Tom

From: Kevin Olival <olival@ecohealthalliance.org>
Sent: 26 June 2019 2:51 PM
To: Peter Daszak
Cc: Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Eric Laing; Thomas Hughes; Supaporn Wacharapluesadee; Aleksei Avery Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross
Subject: Re: EID-SEARCH v4

Peter, v4 with my edits, some revised figs, and additional comments.

Cheers,
Kevin

Kevin J. Olival, PhD
Vice President for Research

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On Jun 25, 2019, at 9:26 AM, Peter Daszak <daszak@ecohealthalliance.org> wrote:

Dear All,

Here's version 4. Apologies for the delay. It's in better shape now, but there are still lots of areas that need your attention. Please put in time today, and tomorrow to edit and firm up the text. I've put your names in some of the comment boxes where I need specific help but I also need you to read this critically now and edit and tighten it up. We have 30 pages of space, and our research plan is now 21 pages, so we need to lose maybe 3 or 4 pages. There are also some weak points, specifically on Filoviruses, on the details of where we'll do our clinical work, and on what we'll do with the animal models for henipas and filos.

Please get comments back with me before Wednesday 26th 4pm New York time, so that I have Wendesday evening and all day Thursday to incorporate them. In the meantime, I'll be working with folks here on better figures, and all the extra sections that come after the research plan. I'll need you all to help with those on Thursday while I'm finishing off the draft.

Thanks to all of you in advance and look forward to the next round.

Cheers,

Peter

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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

From: Peter Daszak
Sent: Thursday, June 20, 2019 9:41 PM
To: Wang Linfa; Danielle Anderson (danielle.anderson@duke-nus.edu.sg); Ralph Baric (rbaric@email.unc.edu); Baric, Toni C; Sims, Amy C; Christopher Broder (christopher.broder@usuhs.edu); Eric Laing; 'Tom Hughes'
Cc: Aleksei Chmura (chmura@ecohealthalliance.org); Alison Andre; Luke Hamel (hamel@ecohealthalliance.org); Kevin Olival, PhD (olival@ecohealthalliance.org)
Subject: EIDRC-SEA v.3
Importance: High

Dear all,

Thanks to everyone for comments and text for the earlier draft at the beginning of the week. Kevin's spent the last couple of days pulling all the comments together into one draft and inserting his changes, and I've now attached a v3 of the draft. It's still rough, and now too long, but it's getting there. I wanted you to look at this rough version because I'm stuck in Liberia in meetings Friday, then flying back home Saturday. I'll continue editing this draft on the plane and will be working solely on this grant from home on Sunday, Monday, Tues, Wed, Thurs.

Please work on this rapidly if possible. If you can all push your comments through, using track changes, and get them back to me **by Sunday evening New York Time**, I will turn round a polished draft by Tuesday, giving us time for significant last go-over on Wed, Thurs. It's tight, but unfortunately, it is what it is, so please commit the time if you can!! As well as general edits (using track changes, with references in comment boxes), please do the following…

Linfa/Danielle – We especially need your input in section 1.1, 1.3, 1.4 and 2.5

Ralph/Amy - Please work your magic on this draft, inserting text, editing and reducing text, and making sure the technical details of the characterization are correct and fit the proposed work on CoVs, PMVs and Henipaviruses

Chris/Eric – Eric – congratulations on your (b) (6) and thanks for working on it at this time! Chris – please help and edit/finesse the language in the relevant sections for you

Tom – Your edits crossed over with Kevin's changes, so please insert all the bits that were new in the last version you sent to me, into this version. I think these are mainly the sections and comments from the other Malaysian colleagues, but please check and re-insert all key text. We especially need to be specific about which partner in Malaysia is doing what cohorts, and where, and about who's doing the testing

Thanks again all, and I'm looking forward to non-stop work on this as soon as I get on my flight in 24 hours…

Cheers,

Peter

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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

<EIDRC Southeast Asia v4.docx>

MEETING ROSTER
National Institute of Allergy and Infectious Diseases Special Emphasis Panel
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
Emerging Infectious Diseases Research Centers (U01 Clinical Trial Not Allowed)
ZAI1 EC-M (J2)
11/04/2019 - 11/05/2019

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MEETING ROSTER
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Consultants are required to absent themselves from the room during the review of any application if their presence would constitute or appear to constitute a conflict of interest.

II. Research Strategy:

1. Significance: Southeast Asia is a critical hotspot for emerging diseases due to its high biodiversity of wildlife and their viruses, and the presence of key ecological and socioeconomic drivers of emergence **(Fig. 1)** (*1*). These include dense human populations living in networks of rural and urban communities, with strong cultural and occupational connection to wildlife and livestock farming and trading, and intimate connection to global travel networks (*2*). The region is undergoing rapid environmental and demographic change, both of which increase the risk of disease emergence and spread. Pathogens that emerge in this region often spread and sometimes enter the USA (e.g. prior influenza pandemics, SARS) and threaten global health security.

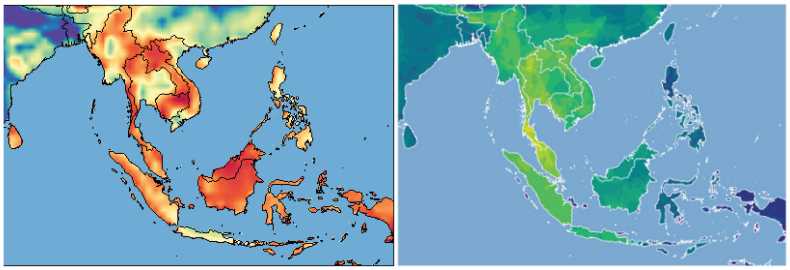


Fig. 1: Southeast Asia, and in particular, parts of Thailand and Malaysia are hotspots of risk for disease emergence **(left)**, and predicted high diversity of 'missing' or as-yet undiscovered viruses, yellow = highest diversity **(left)**. From (*1-3*).

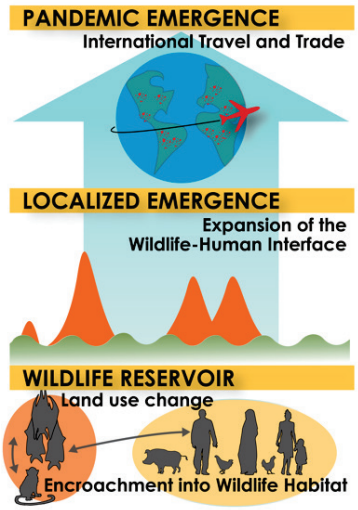
Not surprisingly novel viruses, including near-neighbors of known agents, have emerged in the region leading to often unusual clinical presentations **(Table 1)**.This adds to a significant background burden of repeated Dengue virus outbreaks in the region (*20*), and over 30 known *Flavivirus* species circulating throughout S. and SE Asia (*21*). The events in **Table 1** are dominated by three viral families: coronaviruses (CoVs), paramyxoviruses (PMVs - particularly henipaviruses) and filoviruses (FVs), which are initial examples of our team's research focus and capabilities. These viral groups have led to globally important emerging zoonoses (*4, 5, 22-31*), causing tens of thousands of deaths, and costing billions of dollars (*32-34*). Furthermore, near-neighbors of known pathogens in these families have been reported throughout the region, including by members of our consortium: henipaviruses in bats in Thailand, Cambodia, Philippines, Laos, Indonesia, China, Malaysia, Bangladesh and India (*35-41*); a novel henipavirus, Mòjiāng virus, in wild rats in Yunnan (*14*); serological evidence of FVs in bats in Bangladesh (*42*) and Singapore (*43*); Nipah- and Ebola-like viruses in *Hipposideros*, *Cynopterus* and *Rhinolophus* species in Malaysia (Hughes *et al.*, in prep.); evidence of novel FVs in bats in China (*44-46*), including Měnglà virus that appears capable of infecting human cells (*47*); novel PMVs in bats and rodents consumed as bushmeat in Vietnam (*48*); a lineage C β-CoV in bats in China using the same cell receptor as MERS-CoV to infect human cells *in vitro* (*49*); MERSr-CoVs in bat guano harvested as fertilizer in Thailand (*50*), and directly in bats (Wacharapluesadee *et al.*, in prep.); 172 related viruses.

Viral agent	Site, date	Impact	Novelty of event	Ref.
Nipah virus	Malaysia, Singapore 1998-9	~246 human cases, ~40% fatal	2nd emergence of a zoonotic henipavirus, 1st large outbreak	(*4-6*)
Melaka & Kampar virus	Malaysia 2006	SARI in family group, individual	1st disease due to bat-origin reoviruses, also Singapore, Vietnam etc.	(*7-10*)
Reston filovirus	Philippines 2008	Seropositive people killed pigs,	No prior FVs in pigs	(*11*)
Thrombocytopenia Syndrome virus	E. Asia 2009	100s of deaths in people	Novel tick-borne zoonosis with large caseload	(*12*)
Reston filovirus	Shanghai 2011	PCR positive pigs	Further evidence of pig RESTV infection	(*13*)
Mòjiāng virus	Yunnan 2012	Death of 3 mineworkers	1st evidence of rodent origin henipavirus in people	(*14*)
Nipah-like virus	Philippines 2015	Killed horses	No prior horse infection for NiV, known for HeV	(*15*)
SARSr-CoV & HKU10-CoV	S. China 2015	Seropositive people	1st evidence human infection HKU10 & SARSr-CoV	(*16*)
SADS-CoV	China 2017	>25,000 pig deaths seronegative people	Novel emergence of bat-origin CoV	(*17*)
Nipah virus	Kerala 2018, 2019	Killed 17/19 people 2018, 1 infected 2019	1st outbreak of NiV outside Bangladesh, W. Bengal focus	(*18, 19*)

Table 1: Recent emergence events in SE Asia indicating potential for novel pathways of emergence, or unusual presentations for known or

novel β-CoVs (52 novel SARSr-CoVs) and a new β-CoV clade ("lineage E") in bats in S. China that occur throughout the region (*24, 49, 51, 52*); 9 novel wildlife-origin CoVs and 27 PMVs in Thailand, and 9 novel CoVs in Malaysia reported by us from USAID-PREDICT funding (*51, 53*). These discoveries underscore the clear and present danger of zoonotic events in the region. The wide diversity of potentially pathogenic viral strains also has significant potential use in broadly active vaccine, immunotherapeutic and drug development (*49*).

Most high-impact zoonotic viruses originate in wildlife reservoirs, sometimes spilling over first into livestock 'amplifier' hosts, or directly into localized human populations with high levels of animal contact **(Fig. 2)**. Efforts to prevent emerging zoonoses have targeted these high-risk populations in regions prone to disease emergence (*31, 54*). However, these regions tend to be in the developing world, where nations often lack the resources to investigate novel zoonotic events, or wildlife reservoirs of zoonoses, and prioritize more immediate public health concerns such as Dengue virus or NTDs (*20*). Zoonotic disease surveillance and control is also hampered by inadequate information on basic disease ecology (e.g. range of wildlife reservoirs, risk factors for spillover) and pathogen biology (viral diversity, pathogenesis), and by logistical challenges sharing novel diagnostics, animal models, samples and reagents at sites where outbreaks begin (*55*). Countermeasure and vaccine development is challenged by the small number of isolates available, and the presence of closely-related strains, some with evidence of transmission to people but with unknown pathogenicity. For example, using NIH and other funding, we found serological evidence of exposure to African henipaviruses in 3-4% of a sample of people in Cameroon, with a significant risk factor being butchering of bats; and serological responses to bat SARSr-CoVs in 6/209 (2.87%) and bat HKU10-CoV in 2/412 (0.5%) people living close to a bat cave in Yunnan, all novel viruses with unknown clinical impact (*16, 56*). We discovered two novel bat-borne reoviruses, Melaka and Pulau viruses, and showed that 14/109 (13%) of people living in close proximity to bat roosts on Tioman Island, Malaysia were exposed to these viruses (*7*) as well as 12/856 (1.4%) of a random sample screened in Singapore (*8*). Previous studies in the Philippines found famers and butchers who had contact with sick pigs tested positive for Reston ebolavirus antibodies but had not experienced illness (*11*), and it was our team that discovered Philippine bats as natural reservoir hosts for Reston virus (*57*). In preliminary screening in Malaysia with funding from DTRA we found serological evidence of exposure to henipaviruses (Hendra, Nipah and Cedar) in 14 bats and 6 human samples, and Ebola (Zaire and Sudan)-related viruses in 11 bats, 2 non-human primates (NHP) and 3 human samples (Hughes, in prep.).



Even when spillover leads to outbreaks of illness and mortality, they are often unreported, undiagnosed or misdiagnosed. We initiated targeted syndromic surveillance of encephalitis patients in Bangladesh clinics and showed that Nipah virus (NiV) causes repeated annual outbreaks with an overall mortality rate of ~70% (*58*). NiV has been reported in people in West Bengal, India, which borders Bangladesh (*28*), in bats in Central North India (*59*), and now in people in Kerala, South India in 2018 and 2019, raising the specter of future spillover at other sites in the region (*18, 60*).

Fig. 2: Our Early Warning Approach: Most zoonotic viruses emerge from wildlife hosts, sometimes via livestock **(lower panel)**, to cause small scale outbreaks (green) or larger chains (red) of human-to-human transmission **(middle)**. In some cases, these spread more widely via air travel **(upper)**. Our EIDRC proposal targets each stage: **SA1** examines the diversity and spillover potential of viral threats in targeted SE Asian wildlife; **SA2** seeks evidence of their spillover into focused high-risk human populations; **SA3** identifies their capacity to cause illness and to spread more widely. Figure from (*54*).

Under a current NIAID R01, we have shown that we can use analytical approaches to target surveillance of wildlife and people and identify likely sites of spillover. We used phylogenetic analysis of bat hosts and their CoVs to identify a key cave complex in SW Yunnan Province that harbors all the genetic elements of SARS-CoV (*61*), as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics (*24, 49, 51, 52, 62-64*). We conducted risk factor surveys and biological sampling of human populations living close to this cave and found serological evidence of spillover in people highly exposed to wildlife (*16*). **This work provides proof-of-**

concept for an early warning approach that we will expand in this EIDRC to target other viral groups in one of the world's most high-risk EID hotspots.

The overall premise of this EIDRC proposal is that there is substantial evidence that: 1) diverse CoVs, henipaviruses and FVs related to known human pathogens are circulating in wildlife reservoirs in Southeast Asia; **2)** these viruses likely spillover regularly to people, are often unreported or misdiagnosed, and their clinical manifestations and potential to cause pandemics are unknown and underestimated; and **3)** our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence. To scale-up this approach, we propose to launch **EID-SEARCH** (the **E**merging **I**nfectious **D**iseases - **S**outh **E**ast **A**sia **R**esearch **C**ollaboration **H**ub), to better understand, and respond to, the risk of zoonotic viral emergence in Southeast Asia. We will initially focus on the diversity of known and novel CoVs, henipaviruses, and FVs in wildlife reservoirs. We will use this work to build our center's interactive network throughout the region, bridge barriers, provide early focal point for basic and translational studies, develop sample handling and storage routines, communication networks, cohort management, infrastructure development, and cross-training in surveillance and novel diagnostic platforms. We will develop and transfer technology to test these viruses' capacity to infect human cells and mouse models, develop new specific and sensitive serological and molecular diagnostic tools, conduct surveillance of human communities with high risk of exposure to wildlife, and clinical cohorts to identify evidence of spillover of viruses causing previously 'cryptic' clinical syndromes in people. We will test the capacity of novel viruses to infect human cells and/or mouse models, and thus asses potential for spillover and spread which will feed into our risk models. Our group also has extensive experience in other viruses, including vector-borne flaviviruses, and alphaviruses, and will use this experience to provide robust long-term surveillance, diagnostics, and basic science opportunities. While the current proposal focuses its basic and applied research agendas on this group of viral EID, we note that the EID-SEARCH is poised to rapidly respond to any outbreak. **Thus, FVs, henipaviruses and CoVs are examples of sentinel surveillance systems in place that illustrate EID-SEARCH's capacity to identify and initiate a rapid response targeting any novel microbial pathogen that emerges in this region.**

2. Innovation: Previous work by our consortium has developed strategies to target surveillance in wildlife and people to better anticipate and identify early spillover events and pre-empt outbreaks of emerging viruses. In the EID-SEARCH we scale up this approach in a central research "hub" made up of leading laboratories, clinics and surveillance sites in three critically high-risk EID hotspot countries in SE Asia. EID-SEARCH's reach is expanded through its regional network of collaborators that covers nearly all of the greater SE Asia region. The innovation of the EID-SEARCH is in: **1)** Its multidisciplinary approach that combines modeling to target geographic and taxonomic sampling targets with on-the-ground zoonotic disease surveillance in human and wildlife populations; **2)** the biological characterization approach that identifies how likely viruses are to be able to infect people, and enables evaluation of existing countermeasure technologies; an approach that is grounded in our successful work on SARSr-CoVs; and **3)** the strategic sampling of both high-risk communities with extensive animal contact at the front-lines of viral spillover *and* syndromic clinical cohorts that present with signs of novel viral emergence, to identify risk of viral spillover in an early warning system approach **(Fig. 2)**. **In Aim 1, we will target viruses in archived and newly collected wildlife samples, and characterize their risk of spillover to people**. We will use spatial and phylogeographic analyses to identify the geography and species of wildlife to test samples from, and to further characterize viruses we have recently discovered. We will use *in silico* methods (novel ecological-phylogenetic risk ranking and mapping of receptor binding domains), cell culture, and animal models to assess their potential for spillover into high-risk human populations. **In Aim 2, we will conduct targeted cross-sectional serological surveys of human communities with high levels of animal contact to find evidence of viral spillover, and identify occupational and other risk factors for zoonotic virus transmission.** We will use serological tests targeting viruses identified in Aim 1 to identify the baseline spillover of known or novel CoVs, henipaviruses and FVs in these populations. Serological findings will be analyzed together with subject metadata (including a simple animal-contact survey, location data, viral sequence/isolates etc.) to identify zoonotic risk factors and routes of exposure from specific wildlife or vector species. **In Aim 3, we will use syndromic surveillance of clinical human cohorts to identify evidence of viral etiology for otherwise 'cryptic' outbreaks.** We will enroll and

collect biological samples from patients in local clinics and hospitals who present with syndromes previously linked to known viral agents and live within communities at high risk for disease emergence. We will conduct molecular and follow-up serological diagnostic assays to identify likely viral etiology of syndromes that may represent otherwise cryptic events. Where viruses are identified, we will attempt to isolate or molecularly re-derive them, and use survey data, ecological and phylogenetic analysis to identify likely reservoir hosts and inform intervention programs. Our US partner BSL-4 laboratory, NEIDL, will attempt isolation and characterization of viruses requiring BSL-4 of containment (e.g. novel filoviruses, close relatives of Hev/NiV).

3. Approach: 3.1. Research team: The EID-SEARCH builds on long-term collaboration among world leaders in emerging virus research with proven experience collaborating internationally on field, lab and human surveillance research **(Fig. 3)**. Over the past two decades, our consortium partners have conducted high profile research on EIDs within the region and globally, including identifying the wildlife reservoirs for NiV and Hendra virus (HeV), MERS- and SARS-CoVs (*24, 27, 59, 61, 65-67*), discovering SADS-CoV (*17*), and developing an array of serological, molecular, *in vitro* and *in vivo* approaches to characterizing high-risk CoVs, henipaviruses, and FVs (*62-64, 68-81*). Our team has substantial experience conducting human surveillance in the community and during outbreaks **(Sections 2.2.a., 2.2.b., 2.2.c, 3.2.a, 3.2.b)**, including: USAID-PREDICT, and DTRA-funded viral surveillance of indigenous communities in Peninsular Malaysia and syndromic surveillance of hospital patients in Sabah (Co-I Hughes); collection of >2,000 serum samples from healthy blood donors and sanitation workers, febrile and influenza like illness (ILI), and diarrheal stool samples from children under 5 years of age across Borneo Malaysia (Co-I Kamruddin); collaboration on the MONKEYBAR project with the London School of Hygiene and Tropical Medicine to analyze risk and prevalence of the zoonotic malaria *Plasmodium knowlesi* through case control and cross-sectional studies in Sabah villagers (n=~800, 10,000 resp.) and clinical observational studies to determine the etiology of central nervous system infections and acute undifferentiated febrile illness in Sabah. (Co-Is William, Rajahram, Yeo); and community-based surveillance of hundreds of bat-exposed villagers in Thailand (Co-Is Wacharapluesedee, Hemachudha). As a proof-of-concept of how our team can rapidly scale up resources for diagnostics and surveillance in the face of a new EID, in 2016, we discovered a novel bat-origin HKU-2 CoV (*82, 83*) killing >20,000 pigs in S. China, designed PCR and serological assays, surveyed pig farmers for evidence of exposure, and conducted experimental infections, pathogenesis and molecular studies, **all in the span of 3 months** (*17, 84*).

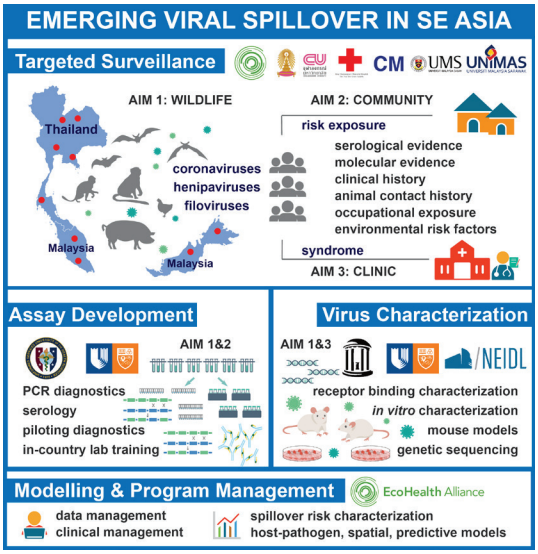


Fig. 3: EID-SEARCH scope, core institutions, and roles.

The administration of this center **(Section 4.1.)** will be centralized at EcoHealth Alliance (EHA) led by PI Daszak, who has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5-20 yrs on NIAID- and USAID-funded research. He will oversee all activities in this EIDRC, supported by Deputy lead, Dr. Olival – who has managed human and wildlife disease surveillance projects in SE Asia for >10 years, and a **Core Executive Committee (Section 4.1.a)**. Co-Is Olival and Zambrana are leaders infectious disease analytics, and head the modeling and analytics work for USAID-PREDICT. Co-Is Wang, Baric, Broder, Anderson, and Laing have developed a unique array of *in vitro* cell culture, serological and molecular reagents to identify and characterize emerging viruses in people and animals (henipaviruses and other PMVs, CoVs, FVs and many others). Co-Is Wacharapluesedee, Hemachudha, and Hughes and collaborators have coordinated clinical and community surveillance of people, and of wildlife and livestock within Thailand and Malaysia for the past 15 years, in collaboration with Ministries of Health, Agriculture and Environment. Finally, this consortium has extensive external funding from NIH, DoD, USAID, DHS and other USG agencies supporting laboratory, analytical and field studies directly related to the current proposal, and will bring substantial leverage to our EIDRC.

3.2. Geographical focus: The three core (hub) countries for this proposed EIDRC are Thailand, Malaysia and Singapore, three contiguous EID hotspot countries that stretch through one of the most significant foci of EID origins globally. Our proposed work includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sarawak and Sabah, and thus contains overlapping wildlife biogeography with India, Bangladesh, Myanmar, SW China, Laos, Cambodia, Vietnam, Philippines, and Indonesia. It encompasses diverse cultural and socioeconomic backgrounds which have correspondingly diverse and intensive interactions with wildlife reservoirs and the global travel network. We have already begun sampling of wildlife hunters, livestock workers, wildlife farmers and market workers, rural villagers living close to high risk wildlife species, people otherwise highly exposed to animals in these countries, and clinical cohorts, as part of current/prior NIH, DTRA, and USAID funding. Our core member's extensive network of collaborators in clinics, research institutes and public health laboratories in almost every other Southeast Asian country positions the EID-SEARCH within a much larger catchment area for emerging zoonoses and will allow us to rapidly scale up to include additional sites in response to an outbreak or shift in research priorities. We will conduct regular meetings with key research, public health and community leaders, including >50 leading laboratories/institutions in 10 countries **(Section 4.2)** to maintain these collaborative relationships with the core members of our consortium (**Fig. 4**). We will use these, and our annual meetings, to share information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks across the region.

Fig.4 : Map of Southeast Asia indicating three hub countries for this proposed EIDRC (**Red**: Thailand, Singapore, Malaysia) and countries in which Key Personnel have existing collaborating partners via other funded work (**Green**), indicating that EID-SEARCH provides access to key collaborators and sites throughout the region.

3.3. Capacity for linkage with other NIAID studies, and scaling up to respond to outbreaks: EID-SEARCH US-based partners include senior scientists with significant experience on NIAID-funded projects, extensive collaborations with other NIAID-funded scientists, and previous experience working with NIAID leadership through workshops, review of programs (e.g. PI Daszak's role in NIAID CEIRS external review), and work on study sections. These contacts ensure that the EID-SEARCH senior leadership will be ready and able to rapidly set up data, sample and reagent/assay sharing protocols with NIAID researchers, the other EIDRCs, the EIDRC CC and other NIAID research centers. EID-SEARCH in-country partners include senior medical doctors and infectious disease specialists at national and regional hospitals in each country and administrative state in this proposal. On news of potential outbreaks, **EID-SEARCH will rapidly mobilize its core staff and their extensive collaborative network across almost all SE Asian countries (Fig. 4) (Sections 4.1.a, 4.2. 4.3).** The core analytical approach can be used to rapidly identify sampling sites and targets to harvest vectors, vector borne arboviruses, birds and mammalian species that harbor most other viral threats. Through existing projects such as the USAID PREDICT Project and DTRA-funded biosurveillance projects, EHA is currently partnered with hospitals and researchers across South and Western Asia, West and Central Africa, and Latin America. Our partners already have capacity to conduct human and wildlife sampling and testing for novel viral agents, so expanding in geography or scale is readily achievable given sufficient resources.

Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife.

1.1 Rationale/Innovation: The vast majority of emerging zoonotic pathogens are linked directly or indirectly to wildlife host, particularly from important mammalian groups like bats, rodents and NHPs (*3*). Our previous work on wildlife origin viruses demonstrates that zoonotic viral emergence tends to occur in specific regions with high diversity of wildlife that harbor viruses of zoonotic potential, and human populations that have close contact to these wildlife **(Fig. 1)** (*2, 3*). In Aim 1 **(see Fig. 9 for overview)**, we will strategically conduct EID surveillance by applying risk analytics to prioritize the regions, wildlife species, and human populations that

have a particularly high risk of viral spillover. We will target high-risk zoonotic host reservoirs (bats, rodents and NHPs) in these regions and apply novel predictive models to estimate viral diversity and host range to identify wildlife species that have been ignored or under-sampled by previous zoonotic disease surveillance efforts. We will strategically collect specimens from these species and screen them, together with some of the tens of thousands of recently collected samples we have archived, for known and novel agents. We will begin screening using conserved PCR assays to identify known and novel viruses in high risk RNA viral families, *Coronaviridae*, *Paramyxoviridae*, and *Filoviridae*. We will expand to other groups of pathogens, including arboviruses, pending available resources and priorities established with the EIDRC coordinating center (EIDRC-CC) and other regional EIDRCs. Using risk ranking algorithms and a series of ecological and virological risk factors, we will triage the wildlife viruses we discover for further genomic characterization and viral isolation. On this subset of viruses, we will then use *in vitro* and *in vivo* approaches (viral rescue, cell lines, gene edited humanized transgenic mice and the collaborative cross genetic reference mouse population) previously developed and widely used by our team to predict capacity of novel viruses to infect people and spillover (*63, 85-88*). These high-risk viruses and their close relatives will be targets for human community serosurveys and clinical sampling in Aims 2 and 3, respectively.

1.2 Preliminary data: 1.2.a. Geographic targeting: EHA has led the field in analyzing where the highest risk of zoonotic spillover is across the globe, identifying SE Asia as a major EID hotspot (*1, 2*). These hotspots are sites where high wildlife biodiversity, human population density and rapid ecologic change collide **(Fig 1)**. Using host and ICTV viral data for all known mammalian viruses, and generalized additive models to correct for sampling bias, we are able to predict the relative number of yet-to-be-described viruses that a species harbors (*3*). This demonstrates substantial undiscovered viral diversity in mammals across Southeast Asia, and particularly in regions of Thailand, Singapore and Malaysia **(Fig. 1)**. Separately, we found that factors driving spillover (host diversity, climate) differ from those driving spread in human populations (population density, bushmeat hunting, livestock production) (*89*). We therefore analyze capacity for viral spread as a separate risk factor, using our unique demographic and air travel and flight data modeling software (*90, 91*). In the current proposed work, we will use all the above approaches to target wildlife sampling (archival and new field collections) in Aim 1, to inform sites for human sampling in Aim 2, and to assess risk of spread in Aim 3.

1.2.b. Reservoir host species targeting: Our analysis of all known mammalian host-viral associations demonstrates that bats, rodents and NHPs represent the three most important reservoirs for zoonoses, responsible for the highest proportion of known and predicted viral diversity of zoonotic potential (*3*). Thus, the bulk of our proposed wildlife sampling and testing will consist of these three taxa with a focus on bats, because this group is the known or hypothesized reservoir for the majority of high-impact zoonotic CoVs, PMVs and FVs (*50, 92-94*). Bats have the highest proportion of predicted zoonotic viruses in any mammalian group (*2*), and are exceedingly diverse in Southeast Asia (~500 species). To further prioritize taxonomic sampling targets within these diverse geographic hotspots, we used a novel Bayesian phylogeographic algorithm to identify host genera and species that are the most important centers of evolutionary diversity for a given viral group, e.g. bat genera for β-CoVs **(Fig. 5)** (*51*). This approach will be extended to other mammal taxa and viral families of interest, allowing us to identify the reservoir hosts of specific zoonotic viral groups, where viral diversity is likely highest.

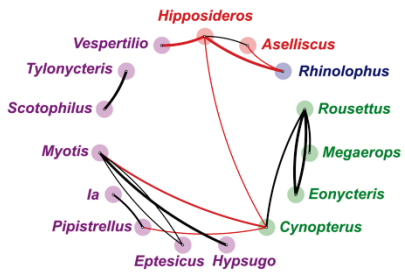


Fig 5: Preliminary analysis of sequence data collected under prior NIH funding, identifying SE Asian bat genera with the highest β-CoV evolutionary diversity. We will apply this Bayesian discrete phylogeographic model to identify taxonomic and geographic centers of viral diversification and sharing for relevant PMV, CoV, FV and other virus clades. Line thickness proportional to probability of virus sharing between two genera. Inter-family switches in red.

Finally, we have designed a strategy to estimate sampling gaps, using a viral 'mark-recapture' approach we previously published (*95, 96*) **(Fig. 6)**. Using prior data for bat, rodent and NHP viral discovery vs. sampling rates, we can identify the likely total viral diversity for specific host groups in a region, and use this to estimate sample sizes to discovering the majority of unknown viruses within a specific family or genus. **In the current**

proposal, we will use these analyses to estimate geographic and species-specific sampling targets **to more effectively identify new strains to support experimental infection studies and risk assessment.**

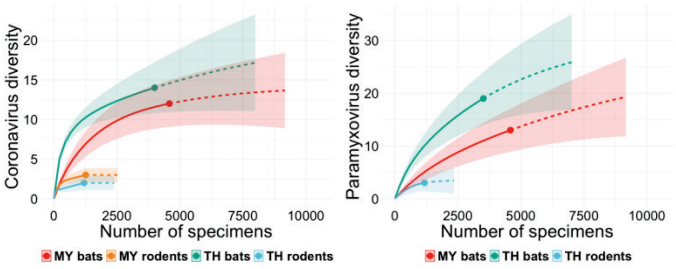


Fig. 6: Estimated CoV **(left)** and PMV **(right)** diversity in bats and rodents from Thailand and Malaysia, using data from PCR screening and RdRp sequences from >10,000 specimens in bats and 4,500 in rodents. Bats have 4X more viral species than rodents, controlling for sampling effort. We estimate that additional collection of 5k-9k bat specimens and testing of our archived bat and rodent specimens alone will identify >80% of remaining CoV and PMV viral species in these key reservoirs, yielding >800 unique viral strains.

1.2.c. Sample testing to identify known and novel viruses: We have conducted extensive wildlife surveillance for novel viruses across Southeast Asia in prior NIAID funded work and as part of the USAID PREDICT project. Under the 10-year PREDICT program globally, we identified 912 novel and 210 known viruses, more than the total number of viruses previously recognized in mammals by the ICTV (*97*). This work included collecting nearly 300,000 individual mammal samples from 14 EHA-led PREDICT countries, and PCR-screening of more than 60,000 individual specimens (*53*). In southern China alone, we identified 178 β-CoVs, of which 172 were novel, discovered a new β-CoV clade, "lineage E" (41), diverse HKU3r-CoVs (179 sequences) within a 'sister' clade to the SARS-CoV lineage, and a new bat-origin SADS-CoV, responsible for killing >25,000 pigs in Guangdong Province (*17*). We have collected 28,957 samples from bats, rodents and NHPs in Thailand and 47,178 in Malaysia, **but have only tested a minority of these using PCR.** We have identified 100 novel viruses in Thailand and 77 in Malaysia. **Furthermore, we have only sequenced a small segment of one gene for all novel viruses found. We have archived duplicate samples which are now available for use in this project**, including fecal, oral, urogenital, serum samples, and biopsies from bats, rodents, and NHPs. In the current proposed work, we will attempt to sequence, isolate and characterize those viruses that our analyses below suggest are most likely to be able to infect humans.

1.2.d. In vitro & in vivo *characterization of viral potential for human infection:* Using Coronaviridae as an example, we have conducted *in vitro* and *in vivo* experiments to characterize the pathogenic potential of novel SARSr-CoVs from bats. We isolated three SARSr-CoVs from bat feces: WIV1, WIV16 and Rs4874, with Spike (S) protein sequences that diverged from SARS-CoV by 3-7% (*24, 61, 98*). We conducted full-length genome sequencing of 12 other novel SARSr-CoVs, some highly similar to outbreak SARS-CoV in the most variable genes (*61*). Using our reverse genetics system we constructed chimeric viruses and rederived full length recombinant SARSr-CoV from in silco sequence. All 3 SARSr-CoV full length isolates and the two chimeric viruses replicated efficiently in Vero E6 cells and in HeLa cells expressing hACE2, civet and bat ACE2, but not in those without ACE2 (*24, 61, 98*). We used the SARS-CoV reverse genetics system (*72*) to generate a chimeric virus with a mouse-adapted SARS-CoV backbone expressing SHC014 S protein with 10% sequence divergence from SARS-CoV S. This and the other full length and chimera viruses replicated in primary human airway epithelium, using the human ACE2 receptor to enter into cells (*62*). Thus, **SARSr-CoVs with diverse variants of SL-CoV S protein without deletions in their RBD can use human ACE2 as receptor for cell entry.** The Baric lab has a well-established hACE2 and DPP4 transgenic mouse model that **we used to assess the capacity of novel SARSr-CoVs and MERS-CoV to infect humans and cause disease** (*85, 99*). Infection of bat SARSr-SHC014 or WIV1 caused SARS-like clinical signs in the transgenic hACE2 mouse model **that weren't reduced by immune-therapeutic monoclonals that attenuate SARS-CoV pathogenicity, nor after challenge following vaccination against SARS-CoV** (*62*) **(Fig. 7)**. We repeated this virus characterization approach with chimeras using HKU3r-CoV S proteins that are ~25% divergent from SARS-CoV S, **and found that they are unable to use the ACE2 receptor**. Additionally, we were unable to culture HKU3r-CoVs in Vero E6, or human cell lines.

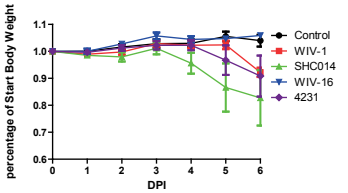


Fig. 7: Body weight change in hACE2 transgenic mice after SARSr-CoV infection, demonstrating that bat-origin SARSr-CoVs cause similar clinical signs to outbreak SARS-CoV strain (4231) (*62, 63*).

A similar approach to the above pipeline for CoVs will be applied to novel henipaviruses we discover during our research. The broad mammalian tropism of HeV and NiV (*68*) is likely mediated by their usage of highly conserved ephrin ligands for cell entry (*19, 100*). A third isolated henipavirus, Cedar virus does not cause pathogenesis in animal models. **Co-Is Broder and Laing** have developed a reverse genetics system and rescued a recombinant CedV to test ephrin receptor usage/tropism and pathogenicity during henipavirus infections (*77*). CedV is unable to use the Ephrin-B3 receptor (*77, 101*) which is found in spinal cord and may underlie NiV encephalitis (*102*) and that pathogenesis also involves virulence factors V and W proteins. The putative henipaviruses, Ghana virus and Mòjiāng virus, predict V and W protein expression, with GhV able to bind to ephrin-B2, but not -B2 (*103*), and the receptor for MoJV remains unknown (*104*) but is likely ephrin-B2 (*105*). Our group has also used similar methods to test the hypothesis that novel Filoviruses discovered in wildlife are able to infect people. NiV, EBOV and MERS-CoV all infect primary microvascular endothelial cells, which are available at Co-I Baric's lab for EID-SEARCH collaborators (*71, 106*). Primary human lung endothelial cells are highly susceptible to Ebolavirus infection (*107*), and Huh7 cells can be used to isolate virus from clinical samples (*108*) offering advantages for reverse genetic recovery of novel FVs (*109*). The three filovirus genera, *Ebolavirus*, *Marburgvirus* and *Cuevavirus*, use Niemann–Pick type C1 (NPC1) protein as cell entry receptor (*110*). For novel filoviruses, cell culture would be carried out under BSL-4, however they can readily be characterized following identification of their encoded GP sequences which can be pseudotyped into recombinant GP-bearing vesicular stomatitis virus (VSV) pseudovirions (*111*). These pseudovirions can be safely used to characterize the tropism and entry filoviruses mediated by GP without a need for BSL4 containment. **Co-Is Wang and Anderson** used cells originating from various mammalian species to determine spillover and/or zoonotic potential of MLAV, a newly discovered Asian filovirus (*47*). Despite the low amino acid sequence identity (22–39%) of the glycoprotein with other filoviruses, MLAV is capable of using NPC1 as entry receptor. Cells from humans, monkeys, dogs, hamsters and bats were able to be infected with a MLAV pseudovirus, implying a broad species cell tropism with a high risk of interspecies spillover transmission.

Mouse models. For novel viruses across all families, we will use the UNC collaborative cross mouse that captures 90% of the diversity within all mouse groups to assess the most suitable model for further experiments (*112*). We have used this model for CoV, FV (Ebola), Flaviviruses, and alphaviruses infections. Clinical signs such as weight loss, hemorrhage, encephalitis, and, acute or chronic arthritis were recapitulated in these mice following SARS, EBOV, West Nile virus and Chikungunya infections, respectively (*86-88, 113-116*). Note that CC OR3015 shows hemorrhagic disease phenotypes after EBOV infection **(Fig. 8)**. *Bat Models.* Duke-NUS has developed two models for bat *in vivo* studies: a colony of *Eonycteris spelaea* (cave dwelling small fruit bat) and a bat mouse model, which contains the bat immune system in an immunodeficient mouse (*117*). In a proof of concept study, **Co-Is Wang and Anderson** infected *E. spelaea* bats with MERS-CoV to demonstrate their susceptibility and suitability for infection with a bat borne virus under BSL3 containment. These bats and the bat mouse model will serve to complement and expand studies from the UNC collaborative cross mouse, pending further funding from EIDRC CC. Recombinant viruses, transgenic mouse models and experimental recombinant protein constructs described above will be made available to the EID-SEARCH consortium and other EIDRCs following standard procedures **(see Resource Sharing Plan)**.

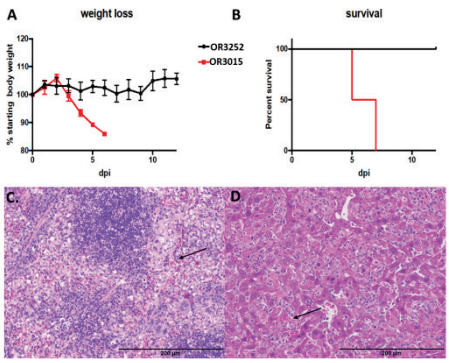


Fig. 8: EBOV Infection in Collaborative Cross Mouse. **Panel A/B:** Wt EBOV in two different CC lines demonstrate different disease patterns. **Panel C/D:** Hemorrhagic phenotypes on d. 6 (arrow) in spleen and liver, resp. From (*86*).

1.3. General Approach: We will conduct analyses to geographically- and taxonomically-target testing of samples from wild mammals that are most likely to harbor known high-profile zoonotic pathogens, or close relatives with potential to infect people. This includes selecting from tens of thousands of samples collected on recent projects and stored in freezers in our laboratories, and new sampling of wildlife in high risk locales. We will use viral family level PCR assays to identify and partially characterize viruses, then follow up sequencing of relevant receptor binding proteins to assess homology and predict binding affinity, then attempt to isolate and

biologically characterize viruses that phylogenetic and other data suggest have high spillover and pandemic potential. We will follow this with *in vitro* binding assays and cell culture experiments, and *in vivo* animal model infections to assess their potential to infect people and cause disease **(Fig. 9)**. EHA will lead the study design, targeted sampling, and data analysis; Co-Is Hughes and Wacharapluesadee will lead field sampling and in-country testing; Duke-NUS, UNC, USUS will lead the serological and PCR diagnostic development and the *in vitro* and *in vivo* analyses, as well as cross-training and tech transfer to the hub labs in Thailand and Malaysia. This will include via support of one Malaysian Ph.D student, Mei-Ho Lee (CM Ltd.) who has worked with the EID-SEARCH collaborators for 9 years. The focus of her study will be infection potential of novel CoVs and PMVs already found in bats in Malaysia under preliminary data for this proposal.

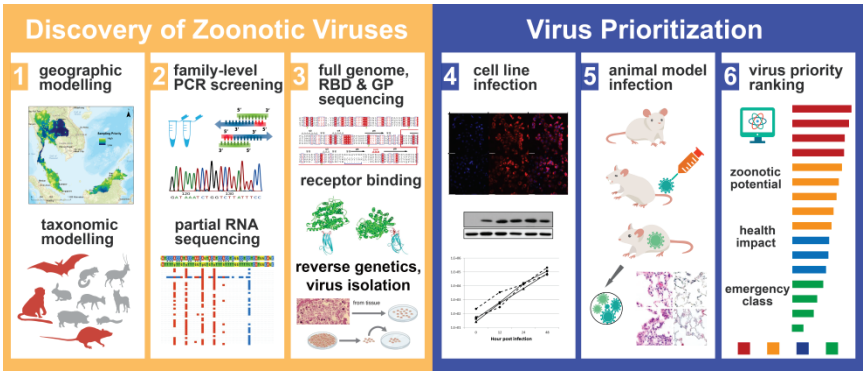


Fig. 9: Aim 1 will use analytical tools to target selection of archived and newly collected samples, conduct PCR and sequencing of novel viruses, recover by reverse genetics, and assess zoonotic potential using *in vitro* and *in vitro* models and analyses.

1.4 Wildlife samples: 1.4.a. Geographic and taxonomic targeting for newly collected wildlife samples: We will prioritize sites within each country that rank highest for both the risk of zoonotic disease emergence (*2*) and the predicted number of 'missing' zoonotic viruses (*3*). Our preliminary analysis **(Fig. 1)** suggests priority areas include: the Kra Isthmus (S. Thailand) and adjacent forests in N. Peninsular Malaysia, NW Thailand (near the Myanmar-Laos border), and lowland rainforests of Sarawak and Sabah. In each of these 'hottest of the hotspots' regions, we will identify 2-4 distinct sampling sites based on the geographic distribution of key hosts we prioritize for additional wildlife sampling. We will priority rank bat, rodent, and NHP species using host trait-based models (*3*). We will also identify species predicted to be centers of evolutionary diversity for CoVs, PMVs, and FVs using ancestral host reconstruction of available sequence data and Bayesian discrete phylogeographic models (*118-120*). We will leverage our strong collaborations with wildlife experts in Malaysia and Thailand, including many of whom are based at our core partner institutions, to ground-truth geographic and taxonomic model outputs with local expert knowledge to ensure the sampling plan is realistic based on current species distributions, population size estimates, seasonality, and accessibility to sites. Most zoonotic viruses are naturally carried by multiple wildlife host species, and there are strong, predictable relations between host phylogeny and viral taxonomy (*3*). We will use a combined network analysis and phylogeographic model to rapidly predict and prioritize new (unsampled) hosts for important, novel viruses we discover during our research. We have shown this relatively simple model (using just wildlife species range overlap and phylogenetic similarity between host species) is both generalizable to estimate host range for all mammalian and avian viruses *and* robust – accurately predicting hosts 98% of the time when cross-validated using data from known viruses (*121*).

1.4.b. Sample size justifications for testing new and archived wildlife specimens: We calculate initial sample sizes targets using our preliminary data on average PCR prevalence from screening tens of thousands of bat, rodent and NHP specimens for CoV and PMVs based on previous studies in Malaysia and Thailand. We will refine these sample size targets, in real-time, over the course of our project using viral discovery curve analyses **(Fig. 6)** to either scale-up or scale-back new sampling and testing of archived specimens for each species depending on viral capture rates. We will only collect additional specimens projected from each prioritized taxa to achieve statistically robust sample sizes and to complement our archived specimens. For example, *Rousettus* spp. bats, known FV reservoirs, were not adequately sampled under PREDICT research in Thailand. Preliminary analysis indicates that, for all viral target families, we will require ~14,000 new samples to identify 80% of remaining viral species diversity in our study region, ~5,000 samples from Thailand and ~9,000 samples from Malaysia **(Fig. 10).** Viral strain diversity from a sampling effort this size is expected to number in the hundreds of strains. For example, given ~6% prevalence of CoVs in bat species previously sampled under the PREDICT project, a target of 14,000 individuals would yield ~800 PCR positive individuals,

representing, an estimated ~600 novel sequences/strains. Similarly, for PMVs, which have an average PCR detection prevalence of ~1.5%, sampling of 14,000 individuals would yield ~200 positives and an estimated 150 unique strains. Filovirus estimates are not feasible yet due to the small number of positive samples in prior studies, but will be estimated as the project begins. From each mammal, we will collect serum, whole blood, throat swabs, urine and fecal samples or rectal swab using our previously-validated nondestructive methods. We will sample sites at different time-points throughout each year, and sample an equal number of males and females to account for seasonal or sex-specific differences viral shedding **(See Vertebrate Animals)** (*122, 123*). Dead or animals euthanized due to injury will be necropsied.

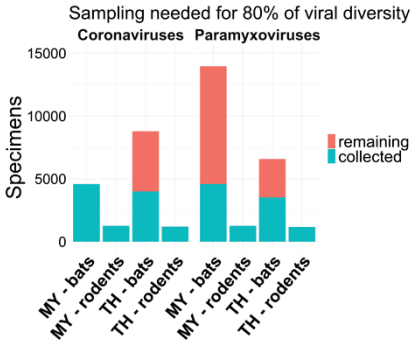


Fig. 10: Stacked bar plot showing number of currently-archived specimens **(blue)** and new specimens required **(orange)** to discover 80% of predicted number of CoVs and PMV species from high-risk bat and rodent taxa in Malaysia and Thailand.

1.4.c. Sample collection, testing, viral isolation: Wildlife will be captured and sampled using previously published techniques, by personnel wearing appropriate personal protective equipment (Tyvek suits or dedicated long external clothing, double nitrile gloves, leather gloves, an N95 or p100 respirator, and safety glasses) (*53, 93, 95, 96, 122, 124*). All samples will be placed in a vapor phase liquid nitrogen dry shipper immediately upon collection, and then transferred to a -80C freezer once back in the lab, until testing. Viral RNA will be extracted from bat fecal pellets/anal swabs. RNA will be aliquoted, and stored at -80C. PCR screening will be performed with pan-coronavirus (*125, 126*), filovirus (*127*) and paramyxovirus primers (*128*), in addition to virus specific primers where additional sensitivity is warranted in key viral reservoirs, i.e. Nipah virus or MERS virus specific primers (*129, 130*). PCR products will be gel purified and sequenced. We will attempt isolation on samples that contain viruses of interest (determined in **Aim 1.5 below**), using Vero cells and primary cell culture from the wildlife taxonomic order (bat, rodent and NHP cell lines). Over 70 bat cell lines are maintained at Duke-NUS from five different bat species. These include primary lung, brain, bone marrow, heart, kidney, intestine, liver, lymph node, muscle, spleen, PBMC and ovary/testes from *Eonycteris spelaea, Cynopterus bracyhotis, Rhinolophus Lepidus, Myotis muricola* and *Pteropus alecto* bats. In addition we have 8 immortalized cell lines, including those derived from lungs, therefore suitable for the isolation of respiratory viruses.

1.4.d. Moving beyond RNA viruses: To detect pathogens from other families, such as non-RNA viruses, we will combine conserved PCR assays with NGS. We will perform either unbiased NGS, or use a pan-viral enrichment library to sequence full-length genomes (*131*). We will conduct this work via EID-CC research projects or as part of outbreak detection/investigation in the region from DNA viruses or other agents.

1.4.e. Sequencing Spike Glycoproteins: Based on earlier studies, our working hypothesis is that zoonotic CoVs, FVs and henipaviruses encode receptor binding glycoproteins that elicit efficient infection of primary or continuous human cell lines (e.g., lung, liver, etc.) (*16, 61, 62*). Characterization these for SARSr-CoVs suggest that viruses up to 10%, but not 25%, different in the spike glycoprotein use human and bat ACE2 receptors for docking and entry. Uneven sampling gaps (e.g., no strains were found with 10-25% spike variation) prevent a thorough understanding of the transition point where the most divergent strains lose human ACE2 receptor usage. Our viral discovery curves **(Fig. 6)** suggest further sampling will reveal a rich diversity of as-yet-undiscovered SARSr-CoVs and strains of other viruses that fill in the phylogenetic tree between currently-described viruses. In this aim we will use phylogenetic and viral discovery analysis to specifically target bat species and regions that are under-sampled to allow sufficient power to identify and characterize missing strain diversity for potential zoonoses. We will sequence the S proteins of novel SARSr-CoVs to prioritize viruses for experimental work in Aim 3 to test this hypothesis. Likewise, we will test the hypothesis that phylogenetic related zoonotic receptor binding protein genes of MERS-related, Ebola-related and NiV/HeV-related viruses will also program efficient entry via human and ortholog receptors. For all novel virus strains, we will sequence the complete entry spike genes by amplifying overlapping fragments using degenerate primers as shown previously (*24, 61*). ***Reverse Genetics:*** Full-length genomes of selected CoVs, FVs or henipaviruses (representative across subclades) will be sequenced using NEBNext Ultra II DNA Library

Prep Kit for Illumina and sequenced on a MiSeq sequencer. PCR or MinIon sequencing will be performed to fill gaps in the genome. The full length spike gene sequences, including the amount of variation in the receptor binding residues that bind the appropriate human receptor will be used to select strains for Aim 3 experiments. We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-CoV cross neutralization, synthetically reconstruct a small subset (1-2) and evaluate growth in Vero, BHK and Huh7 cells (*63, 132*). From full length sequences, synthetic clones will be ordered to recover recombinant viruses using reverse genetics as described by our groups (*62, 63, 77, 109, 133, 134*). Recombinant virus growth will be accessed in Vero, BHK, Huh7 and select bat cells and recovered viruses used for downstream studies. Virus strain prioritization is described below.

1.5. Assessing risk for spillover. 1.5.a. Capacity to infect human cells: We will use a series of cell cultures to assess the capacity of target viruses to enter human cells. For CoVs, FVs and henipaviruses, we will use primary human ciliated airway epithelial cells (HAE) and lung endothelial cell cultures derived from the lungs of de-identified transplant recipients. HAE are highly differentiated and grown on an air-liquid interface for several weeks prior to use (*62, 63, 132*). We will prepare HAE and lung endothelial cultures from three different patient codes in triplicate in collaboration with the tissue procurement facility at the Cystic Fibrosis Center at UNC. Cultures will be inoculated with chimeric bat SARSr-CoVs or novel FVs or henipaviruses to assess efficient replication. At 72 hpi, cultures will be fixed for immunofluorescent staining using antisera to the SARS-CoV conserved nucleocapsid protein (N) or to structural genes of the other viruses (*64, 71*). SARSr-CoVs that differ significantly in S protein sequence (11-24%) from epidemic SARS-CoV yet replicate *in vitro*, will also be evaluated for sensitivity to neutralization in Vero cells by PRNT50 assays using broadly SARS-CoV cross reactive human mAbs S227.14, S230.15, S215.17, and S109.8 (*63, 135*) or monoclonal antibodies targeting the Ebola or Marburg E glycoprotein (Available through BEI), or NiV/HeV glycoproteins (*136*). As controls or if antisera is not available, the S genes of novel SARSr-CoV or spike entry genes of other novel viruses under investigation will be inserted into VEE 3526 replicon vectors (VRP3526-S), packaged and used to vaccinate mice (*137*). Polyclonal sera against test bat-SARS virus or other spike genes will be harvested and tested for ability to cross neutralize SARS-CoV, GD03, WIV-1, SHC014, WIV-16, other novel SARSr-CoV and HKU3-SRBD by PRNT50 assay (*63, 138, 139*). Using PRNT50 titers with sera (n=4 each) among these viruses, antigenic cartography (*140*) will allow comparison of antigenic vs. phylogenetic distance, identifying the transition at which SARSr-CoV strains escape SARS-CoV based vaccines, informing immunotherapeutic and vaccine design strategies (*141-143*). Similar approaches will be applied to novel MERS-related viruses, other CoV, FVs or henipaviruses. While most wildlife-origin viruses with unknown transmission potential or pathogenicity in people uncategorized, we use BSL-3 for isolation as standard, and cell culture for potentially BSL-4 pathogens will be undertaken at collaborating institute NEIDL. To facilitate this, duplicate samples in culture medium from animals PCR +ve for FVs and very close relatives of NiV or HeV will be shipped to NEIDL **(See letter of support)** for culture, characterization and sharing with other approved agencies including NIH Rocky Mountain Laboratories where PI Daszak and Co-Is Baric have ongoing collaborations. Similarly, some duplicate samples of animals PCR +ve for CoVs will be shipped to UNC for further characterization by Co-I Baric. Where feasible and appropriate, training opportunities at NEIDL and UNC for in-country staff will be given, thereby enriching hands-on collaboration across sites.

1.5.b. Host ACE2, DPPR, Niemann-Pick C1 (NPC1) and EphrinB2/EphrinB3 receptors: We will sequence relevant host receptors for select mammalian wildlife species we find positive for strains of high-priority CoV clades, and all new henipaviruses and FVs. Of particular interest is homology across 18 bat and human orthologue ACE2 contact interface residues that engage the SARS RBD as a potential indicator of SARSr-CoV cross species transmission potential and growth in human cells (*70*). Likewise, variation in the NPC1 receptor has been shown to alter EBOV ability to infect in humans and bats, so this can be structurally mapped for prediction and downselection of strains (*144*). Signatures of positive selection in bat NPC1 were concentrated at the virus-receptor interface, with the strongest signal at the same residue that controls EBOV infection in *Eidolon helvum* cells. Thus NPC1 is a genetic determinant of FV susceptibility in bats, and some NPC1 variations likely reflect host adaptations to reduce FV replication and virulence (*145*). NiV and HeV use ephrin B2 or ephrin B3 as a receptor, and variation affects its ability to use human vs bat receptor molecules (*103, 146*).

1.5.c. Host-virus evolution and predicting receptor binding: We will use Bayesian and Maximum Likelihood phylogenetic analyses of RNA-dependent RNA polymerase (RdRp) sequences from PCR screening, receptor binding glycoproteins, and/or full genome sequence data (when available) to reconstruct the evolutionary history of the novel bat SARSr-CoVs, bat MERSr-CoV, FVs and henipaviruses that we identify. We will run co-phylogenetic analyses to map out instances of host-switching (*147, 148*) and ancestral state reconstruction analyses **(Fig. 4)** to identify the most important species for shaping evolutionary diversity for these viruses (*51, 149*), allowing for more targeted future surveillance and spillover mitigation interventions. For receptor binding glycoprotein sequence data from characterized viruses, we will apply codon usage analyses and codon adaptation indices, as used recently on henipaviruses (*150*), to assess the relative contributions of natural selection and mutation pressure in shaping host adaptation and host range.

1.5.d. Viral strain prioritization: Of the expected 100-200 novel SARSr-CoV strains, and approximately 600 total CoV strains, we will down-select to prioritize for further characterization based on S genes that are: i) different from SHC014, WIV1, SARS-CoV with diversity ranges of 10-25%; ii) have virus S RBD that could use human/bat receptors; iii) have recombinant chimeric spikes indicative of gene flow between clade I and II strains; iv) have bat ACE2 or DPP4 receptors that might select for spike RBDs that can use human receptors for entry (15/18 conserved residues in human/bat ACE2 molecules that bind SARSs-CoV S RBD domains are likely more efficient receptors than 3/18 conserved sites). Using structural models based on the SARS S glycoprotein, the extent and location of antigenic variation will be annotated onto the structure, further resolving the locations of highly evolving and conserved structural motifs/epitopes that function in protective immunity (*135, 151-153*). For novel henipaviruses, we will examine the receptor-binding proteins (e.g. envelope attachment (G) proteins) for sequence and structural homology with particular attention to conversation of amino acids that mediate ephrin receptor binding. We will prioritize strains that are predicted to interact with ephrin-B2 and expression V/W proteins to determine whether these viruses represent zoonotic threats. Using the recombinant Cedar virus (rCedV) platform, we will be able to rescue rCedV that express heterotypic G proteins to explore receptor utilization and tissue dissemination in animal models. We will assess ability to infect human cells using a continuous lung epithelial cell line, Calu3, which our group has previously used to generate data on NiV and HeV growth curves and a variety of host expression changes after infection (*154*). We will also use primary human airway cultures and microvascular endothelial cells to assess human infection for FVs, henipaviruses and MERSr- and SARSr-CoVs (*155*) (*156*) . There is some evidence for NiV and HeV replication in mouse lung, low level titers in young animals, 10 fold higher titers in aged mice (*157, 158*). Thus it is likely these models could be improved in the collaborative cross mouse resource at UNC. We have identified CC lines that are highly vulnerable to wildtype SARS and others that develop hemorrhagic disease following mouse adapted Ebola infections (*86-88*).

1.5.e. Animal models: We will use transgenic mouse models and the UNC Collaborative Cross model to assess spillover potential of viruses. For work on CoVs, we will follow the protocols in the transgenic mouse model used by the Baric lab previously. Briefly, in the BSL3 lab, 10- to 20-week old hACE2 or hDPP4 transgenic mice will be intranasally inoculated with 5 x 10^4 PFU of full length wildtype rbat CoV, or chimeric SARSr-CoV or MERSr-CoV encoding different bat CoV spike proteins respectively, then monitored daily for weight loss, morbidity, and clinical signs of disease. Mice will be euthanized at 2, 4, and 6 dpi (endpoint of 14 dpi), organs harvested and virus quantified by plaque assay or genome (mRNA1) RT-PCR. After 7 days in formalin, tissues for histopathology will be removed from the BSL3 and stained with H&E, and for immunohistochemistry using polyclonal antibodies against the N protein. We will conduct limited evaluation of existing countermeasures using therapeutic monoclonal antibodies *in vitro*. Existing SARSr-CoV or MERS-CoV mAbs (*159, 160*) will be diluted in DMEM starting at 1:20, and serial dilutions mixed with an equal volume of 50 PFU of chimeric bat SARSr-CoVs encoding different spike proteins and RFP indicator genes, then incubated on Vero E6 cells at 37°C for 1 h. Antibody neutralization titers will be evaluated by plaque quantification at 4-5dpi. In select instances, hACE2 transgenic mice will be injected with SARS-CoV mAbs, and infected with chimeric bat SARSr-CoVs. Clinical signs and morbidity will be assessed and tissue pathology examined and compared with mice without treatment of mAbs to determine the therapeutic effect on SARSr-CoV infection, and protection of SARSr-CoV by wildtype SARS-S based vaccines assessed as described (*132, 161*). We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-

CoV cross neutralization, synthetically reconstruct a small subset (1-2), recover full length viruses and evaluate the ability of nucleoside analogues to inhibit growth in HAE cultures and/or *in vivo* (*63, 132*). For the Collaborative Cross model, we will infect six to eight mice from 6-8 Collaborative Cross mouse lines (10 weeks of age) with six test viruses/yr, intransasally or by subcutaneous infection with 1 x 10e4 virus. Animals will be followed for weight loss, morbidity, mortality and other clinical disease symptoms (e.g., hunching, hindleg paralysis). One half the animals will be sacrificed at day 3 and day 7 pi. to evaluate virus growth in various organs, and organ pathology. In highly vulnerable lines, additional experiments will be performed using more animals, evaluating virus growth, clinical disease symptoms, respiratory pathology, tropism and organ pathology through day 28 pi. We anticipate that some CC lines will prove highly vulnerable to select wildtype viruses, because of host susceptibility combinations that promote severe disease outcomes.

1.6. Potential problems/alternative approaches: Challenges with logistics or obtaining permission for wildlife sampling in sites we select. We have a >20-year track record of successful field work in Malaysia, and >10 years in Thailand, and have strong established partnerships with local wildlife authorities and communities to ensure access. We have existing permissions in place which will be renewed at the start of this project. Due to the abundance of wildlife habitats in the region, if access to a site is withdrawn, we will rapidly identify a suitable, complementary site with similar bat species composition and abundance. Finally, we have already collected tens of thousands of wildlife samples from the region and will focus on these while waiting for permission. **We may not identify novel viruses in our sample wildlife species due to seasonality of viral shedding or other factors.** We have calculated sample sizes based on realistic detection rates from our extensive preliminary data, and are confident that we will detect and identify large numbers of potentially zoonotic pathogens, particularly for CoVs and PMVs. We acknowledge that FV strain diversity may be harder to capture, given relatively low seroprevalence in bat populations – suggesting lower levels of viral circulation (*42, 43*). However, our team was the first and only group to sequence FV RNA from Asian wildlife species (*46, 47, 57*), and we are confident that with our targeted sampling and testing strategy offers the best chance globally to identify additional strains of Ebola and related viruses. The sampling regions we have selected are subtropical, and our previous data, and even published studies in temperate regions (*162*), do not suggest a strong pattern of seasonality in CoV shedding, and where seasonal patterns do occur, i.e. for henipaviruses in Thailand (*163*), or can be predicted from wildlife serology data, we will be sure to conduct sampling at different timepoints throughout the year to account for this.

Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays.

2.1 Rationale/Innovation: Rapid response requires early detection. Assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities that interface frequently with wildlife that harbor high risk emerging viral strains. To enhance the low statistical probability of identifying these rare events, In Aim 2 we will target rural human communities inhabiting regions identified in Aim 1 as having high viral diversity in wildlife, that also engage in practices that increase the risk of spillover (e.g. hunting and butchering wildlife). We will initially identify 4 subsites in each of Thailand, Peninsular Malaysia, Sarawak, and Sabah for sampling. We will design and deploy human risk factor questionnaires, and specific and sensitive serological assays to identify the baseline frequency, and causes of, spillover of known and related novel viral pathogens in these populations. **We will use the results to test a key hypothesis: that hunting wildlife increases the risk of exposure to novel viruses, compared to the rest of the community.** The alternative hypothesis is that seroprevalence in the general community is not statistically different to those who hunt and butcher wildlife, because exposure to wildlife pathogens is largely through inhabiting a biodiverse landscape where wildlife make indirect contact with people (e.g. via fomites). For participants who report symptoms related to 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever; or 6) unusual presentation of high fever with severe diarrhea; all within the last 10 days, an additional sample type will be collected, two nasal or oropharyngeal swabs. Samples will be marked for additional PCR-based assays to identify presence of known and novel CoVs, henipaviruses and FVs, and for isolation and biological characterization of potential pathogens, using the approach laid out in Aim 1.

2.2 Preliminary data on high-zoonotic risk human community surveillance: 2.2.a Biological sample collection in SE Asia: Our long-term collaboration in the region has included qualitative and quantitative survey data, and collecting large archives of banked human sera and other biological samples from high zoonotic-risk populations. For example, under the USAID-PREDICT project, EHA has collected 1,400 and 678 specimens in Malaysia and Thailand respectively from people living in zoonotic disease interfaces, all of which will be available for retrospective serological testing by EID-SEARCH which we have ensured is compliant with our existing IRB. Our core group has collected and tested many thousand additional specimens from other important community cohorts **(Table 2)**, and some of these will continue under EID-SEARCH **(Section 2.4)**.

Country, site	Lead	# enrolled, focus	Findings
Peninsular Malaysia	Hughes	9,800+ samples, Orang Asli indigenous pop., for PCR/serol.	25+ novel CoVs, influenza, Nipah ab+ve, FV ab+ve
Malaysia Sabah	Kamruddin	1,283 for serology	40% JE, 5% ZIKV ab+ve
Malaysia Sabah	William	10,800 for zoonotic malaria study	High burden of macaque-origin malaria
Malaysia Sabah	Hughes	150 bat cave workers	Ongoing
Malaysia Sarawak	Siang	500 Bidayuh and Iban people	8% PCR prevalence HPV in women
Malaysia PREDICT	Hughes	1,400 high zoonotic-risk communities for viral PCR, serology	Ongoing. Multiple known and novel CoVs, PMVs, others.
Thailand	Wacharaplu-esadee	100s of bat guano harvesters/villagers	Novel HKU1-CoV assoc. clinical findings
Thailand PREDICT	Wacharaplu-esadee	678 high zoonotic-risk communities for viral PCR, serology	Ongoing. Multiple known and novel CoVs, PMVs, others.
Singapore	Wang	856, for Melaka virus	7-11% MELV ab+ve

Table 2: Biological sample collection from healthy populations conducted by members of **EID-SEARCH** in our hub countries.

Of particular interest is the access to indigenous communities in Peninsular Malaysia (Orang Asli) and Sarawak (Bidayuh, Iban people), and the bat guano harvesters in Thailand. These communities hunt, butcher and eat wildlife daily, and work in supremely high-risk zoonotic interfaces (e.g. bat caves). One of our indigenous community sites is experiencing an ongoing outbreak of unknown etiology that we are investigating currently **(Section 3.2.a)**.

2.2.b Human Contact Risk Factors: EHA is the lead organization across all 27 USAID-PREDICT project countries for collecting data on, and analyzing the human aspects of, zoonotic spillover risk (*164, 165*). We conducted exploratory studies using standardized one-on-one semi-structured interviews and site observation data with people living in hotspot areas or engaged in high-zoonotic spillover risk activities, using these data to assess local social and cultural norms underlying wildlife contact (*165*). We then developed a risk factor survey to accompany human biological sampling to evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. We conducted cross-sectional studies in high risk populations in 27 countries, and study participants provided biological samples (oral/oropharyngeal swab, nasal/nasopharyngeal swab, urine sample/urogenital swab, fecal sample/rectal swab, whole blood, serum, plasma). For example, survey and biological samples were collected from: 1,585 participants from 7 sites in Yunnan, Guangxi, and Guangdong provinces, China; 1,400 participants from 4 sites in Malaysia; and 678 participants from 4 sites in Thailand. Samples were tested with novel serological assays and results analyzed against risk factors from survey data (*16*). In China, CoV-seropositive individuals were mostly farmers/peasants (8/9), living in Yunnan province (7/9), 41-60 yrs old (7/9), with domestic contact with rodents (6/9), that are male (6/9). Although these results are preliminary and don't provide detailed information on routes of exposure, they identify key subpopulations to target that would likely increase the opportunity for identifying evidence of spillover for other CoVs, PMVs and FVs. Data from PREDICT surveys have been used to help identify target populations in Aim 2, identify strategies to better target at-risk people, and conduct focused **human risk factor surveys and serosurveys to produce statistically significant findings for henipaviruses, CoVs and FVs.** In Aim 2, we will use combined biological sampling and risk factor surveillance in targeted populations within the community to 1) test the hypothesis that occupational (i.e. wildlife hunting) risk factors correlate with seropositivity to henipaviruses, FVs and CoVs; 2) assess possible health effects of infection in people; and 3) where recent

illness is reported, obtain biological samples for PCR testing and potential viral isolation and characterization. Obtaining this information could be a significant step in understanding the likelihood of recent 'hidden' spillover events and their public health impacts, as well as the risk of future emergence of viral disease.

2.2.c. Risk factors for illness: Our preliminary data in Thailand, Malaysia and other SE Asian countries revealed that frequent contact with wild (e.g. bats, rodents, NHPs) and domestic animals (e.g. poultry, swine) among local communities was associated with a high percentage of self-reported severe/acute respiratory illness (SARI/ARI) and Influenza-like illness (ILI) symptoms **(Section 3.2.b)**. In addition, people involved in crop production, hunting and butchering of wildlife reported statistically significantly higher SARI and ILI symptoms (Li *et al.*, in prep.). **These preliminary data suggest a target population and sites for current proposal with the correct targets for serological surveillance and risk factor surveys in Aim 2 to produce statistically significant findings**. Coupled with better serological tools from our team **(Section 2.2.d)**, we will be able to identify the likely routes of exposure to known or novel coronaviruses, henipaviruses, and FVs in the study region, as well as build data on the illnesses they cause in people.

2.2.d. Serological platform development: Most emerging viruses produced a short-lived viremia in people so that large samples sizes are required to provide enough PCR-positive individuals to analyze infection patterns. For Aim 2, we will focus on serological sampling in the community, because antibodies are typically long-lived and can be used to assess prior virus exposure or infection with much smaller samples sizes (*123*). Most serological assays target a single protein, and for emerging viruses, it's often unclear which viral antigen will be immunodominant during human infection, and therefore which to target in developing serological assays. Co-Is Wang, Anderson (Duke-NUS) and Broder, Laing (USUS) have developed a series of approaches to deal with this problem, and which will be used in testing human biological samples in Aims 2 and 3. Henipaviruses, FV and CoVs express envelope receptor-binding proteins that are the target of protective antibody responses. Co-I Broder has led efforts to express and purify henipavirus native-like virus surface proteins for immunoassay application (*166, 167*), developing monoclonal antibodies (*168, 169*) and as subunit vaccines (*170, 171*), has developed a novel, multiplex microsphere immunoassay (MMIA) allowing serum samples to be simultaneously tested for antibodies reactive with henipaviruses, FVs and CoVs. This MMIA uses a Luminex-based multiplex platform and custom designed virus receptor-binding proteins expressed in a mammalian cell-culture system and purified as native-like oligomeric proteins. These oligomeric antigens retain post-translational modifications and quaternary structures, allowing capture of conformational-dependent antibodies raised during a natural infection, which represent the majority of the protective humoral response. The known diversity of henipaviruses includes five presently described viruses, NiV, HeV, CedV, GhV and MojV and this assay has been expanded to include all described species **(Fig. 11)**. In the past, our collaborators have used NiV and HeV G protein antigens in a MMIA assay to identify a serological footprint of NiV-related African henipaviruses and exposure to virus(es) antigenically-related to NiV in livestock sampled in Bangladesh (*78, 79*). To strengthen serological data interpretation, the G protein from each virus is integrated into the MMIA so that virus-specific vs. henipavirus cross-reactive antibodies can be detected and address specificity of past virus exposure. Extensive cross-reactivity exists among ebolaviruses and between ebolaviruses and marburgviruses (*172-174*). We are validating the MMIA pan-FV assay for specificity, and as part of this, in collaboration with Duke-NUS, we have found serological evidence of past exposure to a filovirus(es) most antigenically-related to ebolaviruses (e.g. Ebola virus, Bundibugyo virus), but unreactive with Reston virus GP in three under-sampled fruit bat species, suggesting that novel FVs circulate within bat hosts in SE Asia (*43*). **This platform has now been set up for use in Malaysia and Thailand and will be available for use in this proposed work.** Co-Is Hughes, Broder, Laing have conducted initial screening of human, wildlife and livestock samples at high-risk interfaces in Malaysia, finding serological evidence of past exposure to viruses antigenically-related to NiV and EBOV in humans, bats and non-human primates (NHPs).

Fig. 11: Validation of multiplex microsphere immunoassay (MMIA) specificity and identification of immunologically cross-reactive viruses for A) NiV B) EBOV.

Co-Is Wang and Anderson (Duke-NUS) have developed a phage-display method to screen antibodies for all known and related bat-borne viruses, including henipaviruses, FVs and CoVs and have set up a similar Luminex-based assay. Additionally the Duke-NUS team has demonstrated capacity for rapid and cost-effective development of LIPS serological assays against novel viral agents **(Sections 2.6.a, 3.2.a)**. These will be used for verification and expanded surveillance. For molecular investigation (Aim 2 & 3), we will combine targeted PCR with a discovery-driven enrichment NGS strategy to not only survey currently known viruses, but also to discover potential novel viruses. Both the phage-display serological and enrichment platforms can be rapidly updated to include new viruses identified.

2.3 General Approach/Innovation: Our human sampling approach uses two approaches in Aims 1 & 2 (Fig. 12). In Aim 2 we will conduct community-based surveillance using a brief, focused questionnaire to identify the prevalence and frequency of risk-factors for viral spillover in tandem with collection of high quality biological samples to determine the seroprevalence of spillover viruses in at-risk human populations. We will use generalized linear models to analyze correlation between biological results and risk-factors for viral spillover. **In Aim 3**, we will conduct clinic-based syndromic surveillance and biological sampling at clinics/hospitals that are the catchment healthcare facilities for the communities sampled in Aim 2. Both community-based and clinic-based syndromic surveillance programs are cross sectional case-control studies designed with the sample sizes necessary to statistically quantify (with a power of 80%) risk factors and health impacts for viral spillover, linked to serological status and PCR prevalence in symptomatic patients. All human sampling conducted under Aims 2 and 3 will adhere to strict criteria for inclusion and recruitment and protection of human subjects from research risk **(see Human Subjects and Clinical Trials Information)**.

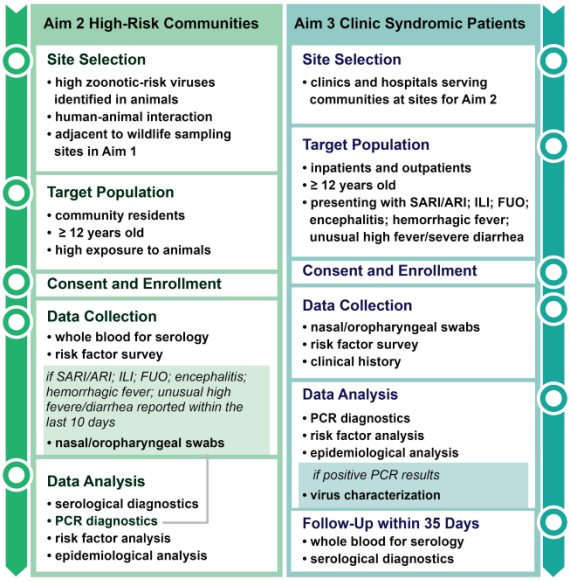


Fig. 12: Human sampling approach. Aim 2 **(left)** focuses on serology in high-risk communities to identify evidence of population exposure to novel zoonotic viruses. Aim 3 **(right)** sets up clinical cohorts at hospitals serving these high-risk communities to conduct syndromic surveillance and identify the etiology of new viral syndromes

2.4 Target population & sample sizes: We will target populations in the same geographic regions as those identified in Aim 1 that harbor wildlife with high potential zoonotic viral diversity, that are EID hotspots, and that are well-connected through regional travel and trade hubs to the global travel network **(Fig 13)**. Within each geographic region we will identify populations at four sub-sites, building on our preliminary data **(Table 2)**. We will target specific communities based on our analysis of previously collected behavioral questionnaire data, and other publicly available data (e.g. wildlife hunting licenses sold in a region, number of guano caves accessed for fertilizer etc.) to identify key at-risk populations and occupations with high exposure to wildlife. We will conduct concurrent sampling trips at visits to sub-sites during which members of the community will be enrolled and wildlife surveillance activities conducted. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock, or slaughtering wildlife will be considered for enrollment. Participants will complete a consent form and questionnaire in addition to having biological samples collected. During these community trips after data collection the field research staff will conduct community meetings to help inform the public health risk of zoonoses. **Target populations:** *Thailand (Co-I Wacharapluesadee):* 100 subjects of healthy high-risk community from 2 study sites, Chonburi (NiV, other PMVs and CoVs found by PCR in bats in our previous work), and Ratchaburi (bat guano harvester villages where we found a MERSr-CoV in bat guano (*50, 175*) and serological evidence of α-CoVs, HKU1-CoV in people (*175*). *Peninsular Malaysia (Co-I Hughes, CM Ltd.):* We will expand our sampling of the Orang Asli indigenous communities to include sub-sites in communities in Districts we have already sampled and

additional Districts in the States of Kelantan Perak, Pahang, and Kelantan all close to bat caves. Samples will be stored and tested at NPHL. *Sarawak (Co-I Tan, Fac. Med. Hlth. Sci. UNIMAS):* We will expand our sampling of the Bidayuh and Iban indigenous people to include the Dayak and Orang Ulu ("people of the interior") because this group has a more intimate connection to remote wildlife-rich areas than the other two, albeit all are hunters. Human sampling will be led by UniMAS in collaboration with CM Ltd / EHA. *Sabah:* We will conduct sampling at the Madai bat and bird cave that has 200 permanent residents who harvest bird's nests for soup, and >1,000 during the harvesting season and at Gomantong cave – one of the largest bat and cave swiftlet nesting sites in the world. *Sabah: (Co-I Hughes):* We will conduct sampling at the Madai bat and bird cave that has 200 permanent residents who harvest bird's nests for soup, and >1,000 during the harvesting season and at Gomantong cave – one of the largest bat and cave swiftlet nesting sites in the world. Protocols follow those for Peninsular Malaysia *Singapore: (Co-I Wang, Duke-NUS):* Active human surveillance will be not be conducted in Singapore, but archived human specimens e.g. from our Melaka virus serosurvey (*8*), will be made available to conduct retrospective serosurveys for specific pathogens that are identified as high interest to the EIDRC, NIH, and EID-CC.

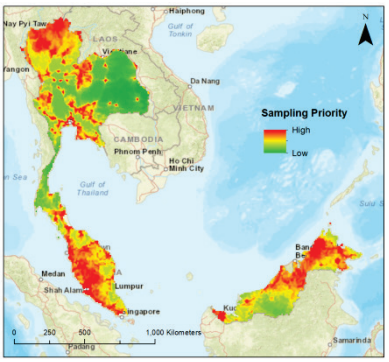


Fig. 13: Targeting high-risk sites for human zoonotic disease surveillance in Thailand and Malaysia. This map plots areas with high numbers of expected viruses in wildlife (*3*), greater spillover probability based on EID risk factors (*2*), and higher levels of connectivity (measured by road density data) that may facilitate person-person spread.

Sample sizes: From our previous work we conservatively anticipate that 10-30% of the community population will have had exposure to wildlife allowing us to capture highly exposed and non-exposure individuals at each site. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock farming, transportation, selling, or slaughtering wildlife in the surveyed areas will be targeted so that they make up ≥30% of the sampled population in each community. We will stratify sampling to ensure appropriate representation of sex, demographic, and socio-economic factors at each community site. In each of our regions, we will conduct sampling at four sub-sites in populations with high prevalence of high occupational and environmental risk of exposure to wildlife populations, sampling 175 persons at each sub-site. We will target populations with, on average, 30% or higher prevalence of occupational or environmental exposures to wildlife based on data from previous studies. This design will give us >80% power to detect 5 percentage point differences in seroprevalence against any of our viral groups between baseline and high-risk subgroups. We conservatively assume 5% base seroprevalence, as well as assuming high spatio-temporal variance amongst sub-sites, with baseline varying 0-25% amongst sub-sties and the fraction of the population at risk varying from 20-40%. (Power calculation conducted by 500 simulations of a generalized linear mixed model (GLMM)). For community-based surveillance, we will enroll 700 individuals per study region, allowing us to make county/province-level comparisons of differing effects; the total sample will be 3,500 participants over 5 years.

2.5 Data & sample collection: Following enrollment, biological specimens (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max 500 µL of whole blood and two 500 µL serum samples) will be collected by locally certified healthcare professional proficient in phlebotomy techniques and a short questionnaire will be administered by trained project staff that speak appropriate local language to all participants. Through the serological findings and the survey we will investigate the details of five risk factors related to high risk wildlife exposure, to maximize the power of the analyses, based on continuing analysis of our previous work, and will focus on: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. For participants who report the symptoms relating to 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever within the last 10 days, an additional sample type will be collected, nasal or oropharyngeal swab (2x). Blood of participants whose serum tests positive for emerging viral pathogens will be taken and Peripheral Blood Mononuclear Cells (PBMCs) frozen for future use.

These will be used for future harvesting of polyclonal and monoclonal antibodies as potential therapeutics/ diagnostics **(see Letter of Support NEIDL)**.

2.6: Laboratory analysis: 2.6.a Serological testing: We will screen all human specimens for henipaviruses, FVs and CoVs using our novel, multiplex microsphere immunoassay (MMIA) **(Section 2.2.d)**. We will use ELISA and serum neutralization tests (SNT) as confirmatory, where feasible and appropriate for the biocontainment level given sensitivity and specificity variation, and the need for live virus for SNTs **(See Select Agent Research)**. Serologic evidence of local spillover will be used to prioritize zoonotic strains for recombinant virus recovery from full length sequences. Preliminary data suggest ELISA will be effective for some viral groups. We previously developed a SARSr-CoV specific ELISA for serosurveillance using the purified NP of a bat SARSr-CoV (Rp3). The specificity of this assay was evaluated using polyclonal antibodies against HKU1, OC43, 229E, NL63, MERS-CoV and EBOV and no significant cross-activity detected (*16*). **This suggests that if we can expand our NP serology tests to cover other bat CoVs, and that doing so may identify many more seropositive individuals.** For CoVs, we will therefore use two serological testing approaches: 1) testing human sera collected from both community- and clinic- based sampling for a panel of bat CoVs that will include SARS-CoV, MERS-CoV, SADS-CoV and a range of bat SARSr-CoVs (*16*); 2) testing for antibodies to common human CoVs (HCoV NL63, OC43 – **Section 2.8**). We will test panels of NP and G recombinant proteins to assess if this approach will act as a comparison to MMIA for FVs and PMVs.

2.6.b RT-PCR testing. Specimens from individuals in the community who reported being symptomatic within the last 10 days **(Section 2.5)** will be screened using RT-PCR initially for CoVs, PMVs, and FVs following published protocols **(Section 1.4.c)**. Positive samples will be subjected to full genome sequencing and RT-PCR amplification of the glycoprotein genes. Samples from the clinic-based syndromic surveillance will also be tested using RT-PCR for Influenza A & B, HCoV NL63, OC43, HKU1, SARS-CoV & 229E, NiV, HeV, Ebola (WHO PCR protocol) based on the symptom as rule-outs.

2.6.c Biological characterization of viruses identified: Novel viruses identified in humans will be recovered by cultivation or viruses re-derived from full length genome sequences using reverse genetic approaches. Viruses will be characterized for growth in primary human cell lines and in mouse models, including the Collaborative Cross Mice or humanized mice expressing human receptors from related strains. The approaches are discussed in more detail under Aim 1.

2.7 Epidemiological analysis: We will conduct a cross sectional case-control study to identify risk factors for Henipavirus, FVs, and CoVs spillover. “Cases” are defined as participants whose samples tested positive for Henipavirus, FVs, or CoVs by serological tests. “Controls” will be selected from the pool of participants admitted to the studies and whose samples tested negative for Henipavirus, FVs, or CoVs. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models and least absolute shrinkage and selection operator (LASSO) regression analyses (*176*) to analyze correlation between biological results, serological/PCR status, and risk factors related to high risk wildlife exposure including: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) home or work proximity to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms lifetime and past 12mo. Additionally, we will use this procedure to determine how clinical presentation differs between Henipavirus, FVs, or CoVs in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms.

2.8 Potential problems/alternative approaches: Rarity of spillover events means it may be difficult to identify sufficient seropositives to statistically analyze human risk profiles. First, we will be targeting our community-based surveillance to subpopulations with high-levels of wildlife exposure, at sites selected for diverse and prevalent viruses to increase likelihood of finding positive individuals. Second, our serology testing will include a multi-plex panel of assays for a large diversity of viruses, increasing likelihood of positives. For example, in previous work our overall bat CoV PCR prevalence in the community was, 11.8%; β-CoV, 3.4%; α-CoV, 9.1%). Thus, using this broad serological panel to screen individuals prone to contact with species of higher zoonotic risk increases the potential for detecting spillover with enough power for statistical analyses,

and will shed light on behaviors that predispose to spillover. Third, we will include common human CoVs, PMVs, and influenza viruses in our panel, so that even if low prevalence of wildlife viruses is found, we will be able to conduct a valuable cross-sectional study of the seroprevalence of more common human viruses. Finally, we will be able to assess relative measures of human-wildlife contact from our survey work. We will analyze intensity of contact with high-risk taxa against other risk factors to provide useful proxy information for spillover risk. **Serological testing may be difficult to interpret in the presence of known and novel viruses.** We will use the three-tiered serological testing system outline in **2.6.a** to try to identify exposure to these 'novel' viruses. However, we know exposure to a range of related viruses generates antisera that are cross-reactive with known, related virus antigens - a serious challenge to serological surveillance and diagnostics. Our collaborations have already demonstrated that SE Asia bat sera samples screened with our pan-filovirus MMIA have been exposed to an Ebola-like virus, that is antigenically-distinct from known Asiatic filoviruses, Reston virus and Měnglà virus (*43*). Using these three serological platforms we will address inherent challenges of indirect virus surveillance through antibody capture by testing multiple binding assay platforms and antigens, using positive control samples to establish serological profiles against known viruses that will aid in our interpretations of cross-reactive antisera responses likely representative of 'novel' viruses.

Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts.

3.1 Rationale/Innovation: Our previous research **(Table 1)** and other data suggests that there are substantial numbers of outbreaks or clinical cases for which the etiologic agent is not diagnosed, e.g. (*177, 178*). To investigate this in SE. Asia, we have assembled a multi-disciplinary team that includes senior infectious disease doctors and clinicians and will work directly with local clinics and regional hospitals to identify the 'cryptic' outbreaks or cases that occur in the region. **In Aim 2, we will conduct serology in high zoonotic-risk communities to find evidence of prior exposure in people,** i.e. evidence of viral spillover into the community. In Aim 3 we identify members of these communities presenting at clinic with undiagnosed illness that may have high-impact zoonotic viral etiology. **Therefore, in Aim 3 we focus on PCR to identify the putative viruses that are replicating in these actively-infected patients (Fig. 12).** We will enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and who present at regional clinics or hospitals with symptoms typical of high impact zoonotic viruses. We will collect detailed survey data to assess their contact type and frequency with wildlife and livestock and conduct molecular and serological diagnostic assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. We will conduct molecular viral discovery assays on patients who present with symptoms indicative of a high-risk zoonotic virus. If novel pathogens are identified by PCR, we will attempt to isolate, obtain full genome sequence and/or biologically characterize the wildtype or molecularly re-derived pathogen, using the *in vitro* and *in vivo* strategy laid out in Aim 1.

3.2 Preliminary data clinical surveillance: 3.2.a. Clinical surveys and outbreak investigations: Our longterm collaboration in the region has included the following activities investigating clinical syndromes: *Peninsular Malaysia*: **At the time of writing, an outbreak of suspected undiagnosed viral illness in the indigenous Batek Orang Asli ("people of the forest") community living at Gua Musang ("civet cat cave") district, in Kelantan, Malaysia, close to Taman Negara – a National Park containing the oldest known rainforest**. This community is one where we have conducted prior routine surveillance and biological sampling, demonstrating proof-of-concept that our two-pronged high-risk community/clinic approach will work. Dozens of people are affected and the outbreak is currently being investigated by our group in collaboration with the Malaysian NPHL. The Orang Asli continue to practice traditional subsistence hunting of wild animals (bats, rodents, primates). These communities are remotely located, in heavily forested areas, with limited access to medical services. This outbreak, and others that will continue to emerge just like it, underscore the critical need for our EIDRC - to improve the technological tools, training, and human capacity for rapid EID surveillance, diagnosis, and response at the frontlines of disease emergence in SE Asia. **Investigating this outbreak is a key priority if EID-SEARCH is funded.** *Sabah*: Co-I Kamruddin (BMHRC) is leading an asymptomatic leptospirosis study on sanitation workers, an ILI study in a Kota Kinabalu clinic and diarrheal study in children under 5 yrs. Co-Is William, Tan and others have conducted clinical observational studies to determine the etiology of central nervous system infections and acute undifferentiated febrile illness in Sabah.

Co-Is William and consultant Yeo have collaborated with Co-I Wang to enroll 90 adult and over 100 pediatric participants with central nervous system infections, negative tests for malaria and dengue from 3 district hospitals in Sabah to determine the various etiologies. While a proportion tested positive for rickettsial agents, the majority had no clear diagnosis. Co-Is William, Rajahram, and Yeo have conducted clinical studies of over 200 inpatients with acute febrile illness with negative tests for malaria and dengue from 3 district hospitals in Sabah to determine the various etiologies. While a high proportion tested positive for rickettsial agents, the majority had no clear diagnosis. *Sarawak:* Co-I Siang (UNIMAS) is conducting a survey of high-risk female Kelabit indigenous people and has found 8% with HPV52 as the dominant strain. He is working on clinical cohorts of hand, foot and mouth disease and will recruit patients from that study in the current proposed work. Finally, Co-Is Siang, Kamruddin are conducting a long-term study of rotavirus and other viral etiologies of acute gastroenteritis in Kuching, Sarawak that will include swine herders and farmers. The Baric lab is a global leader in norovirus VLP diagnostic reagents to detect serologic evidence of norovirus gastroenteritis, as well as RT-PCR detection of novel noroviruses in humans, and has identified novel bat noroviruses, suggesting further opportunities for expansion of this work (*142, 179-181*). This work is particularly relevant in Malaysia because pig farms are shunned by the government and local villagers to they are placed far away from town centers, deep in the forest, and it is this scenario that led to the emergence of Nipah virus in 1998-9 (*6, 22*). *Thailand:* Co-Is Hemachudha, Wacharapluesedee (TRC-EID, Chulalongkorn Univ. Hosp.) work in coordination with the Thailand Ministry of Health to investigate outbreak etiology, including the first case of imported MERS-CoV in Thailand, **for which we produced sequence confirmation within 24 hours from acquiring the specimen**. Three imported MERS cases have been detected in Thailand since 2015, and all cases were confirmed at the TRC-EID. We worked during the Zika outbreak to identify > 500 PCR-positive Zika patients with sequence confirmation. We have also worked on returning and suspected Ebola travelers, syndromic surveillance for SARI, encephalitis, Hand Foot and Mouth disease in children and viral diarrhea. Consultant Hickey (US CDC, Thailand), screened specimens collected between 1998 and 2002 from a large cohort of children from Kamphaeng Phet Province to examine Dengue virus and JE epidemiology (*182, 183*). Among the 784/2,574 convalescent sera henipavirus-specific antibodies were detected in 13 serum samples (1.7% seroprevalence), suggesting prior exposure to endemic henipaviruses or a related PMV in rural Thailand. *Singapore:* Duke-NUS has worked with the Ministry of Health to investigate Zika cases (*184*), and other EIDs. With EHA and others, Duke-NUS has developed novel CoV assays for clinical surveillance, and for expedited analysis during outbreaks. Our pipeline of novel virus detection, reservoir host identification, serological assay development, and serological testing is as rapid as several months, with the development of novel serological assays from sequence data in 7 days (*17*). **For example, with the discovery of SADS-CoV, we identified and fully sequenced a new virus, screened archived wildlife specimens to identify the natural reservoir host species (*Rhinolophus* spp. bats), developed LIPS serological assays, and tested human and swine samples to assess seroprevelance in less than 3 months (*17*).**

3.2.b Analysis of self-reported illness: We have developed a standardized approach for analyzing data from study participants on self-reported symptoms related to target illnesses: fever with cough and shortness of breath or difficulty breathing (severe acute respiratory illness - SARI); fever with muscle aches (fevers of unknown origin (FUO); and, cough or sore throat (influenza-like illness - ILI). For all of our prior human clinical surveillance in 20+ countries, including in Malaysia and Thailand, we used a least absolute shrinkage and selection operator (LASSO) regression to identify associations between participants who reported ILI and/or SARI symptoms and demographics, occupation, and contact with animals in the last year. Results have clear biological relevance. For example, in Yunnan, China, factors strongly associated with prior disease involve animal hunting and consumption **(Fig. 14).** We will expand this approach for all clinical syndromes in Aim 3, and use targeted questions to assess patient's exposure to wildlife in terms that are relevant to each specific country.

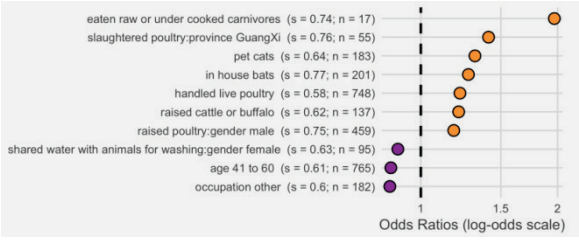


Fig. 14: Factors associated with self-reported ILI & SARI in prior 12 months (s = bootstrap support; n = #+ve out of 1,585 respondents). **Orange circles** = odds ratios > 1 (+ve association); **purple** = odds ratios <1 (-ve association).

3.3 General Approach: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent has not been identified. To capture these, we will expand on our current syndromic surveillance to enroll, and collect high quality biological samples from patients who present at regional clinics and live in rural communities linked to Aim 2. Case definitions and enrollment criteria include: 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever; or 6) high fever/diarrhea with unusual presentation. We will collect survey data to assess contact with wildlife, and occupational and environmental exposures, conduct molecular and serological assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. If novel pathogens are identified by PCR, we will attempt to isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work. We will then use behavioral survey data, ecological and phylogeographic analyses to assess the likely reservoir hosts to inform intervention programs.

3.4 Clinical cohorts. 3.4.a Cohort selection: We will initiate active clinic-based syndromic surveillance at 2 town-level level clinics and 2 provincial-level hospitals in each Thailand, Peninsular Malaysia, Sabah and Sarawak. These serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2 and are inclusive of wildlife sampling sites in Aim 1. Patients ≥ 12 years old presenting at the health facility who meet the syndromic and clinical case definitions above will be recruited into the study after completing a consent form. We will aim to enroll 300 participants per hospital, with that we will have a 95% probability of detecting live virus in patients in each hospital assuming a population prevalence of 1%. This sampling effort will total of 4,800 individuals for clinical syndromic surveillance. We assume up to 40% loss from follow-up, therefore yielding 2880 participants that will be available for follow-up blood sampling **(Section 3.4.b)**. While enrollment is limited by the number of patients that come to the healthcare facility presenting with relevant clinical symptoms, in our work in the PREDICT project we enrolled an average of 100 participants per year, so we are confident this is an achievable enrollment target. Study data will be pooled across country regions, as clinical patients are limited by the number of individuals presenting at hospitals. "Cases" will be determined as participants whose samples tested positive for either CoVs, henipavirus, or FVs, PCR tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. Sites: *Thailand:* We will work with two primary hospital, Phanat Nikhom Hosp., Chonburi district (Key Pers. Dr. P. Hemachudha) & Photharam Hosp., Ratchaburi Province, and a large tertiary hospital, King Chulalongkorn Memorial Hospital, Bangkok Thailand (Co-I Dr. T. Hemachudha). *Peninsular Malaysia:* **Focusing immediately on the current outbreak in Orang Asli, we will work closely with the Malaysian Ministry of Health to support their investigation, and with 6 Orang Asli clinics which focus solely on this indigenous community.** Co-I Sellaran (Lintang Clinic, Kuala Kangsar) refers patients to Sungai Siput Hospital and Gen. Hospital, Ipoh. Key Pers. l Lotfi (Pos Betau clinic, Kuala Lipis) will continue collaboration with Co-I Hughes in the communities at that site. *Sarawak:* Key Pers. Diyana (Director, Bario Clinic) refers patients to Miri Hospital (Co-I Utap) and serves people from the Kelabit, and nomadic tribes in the region, as well as populations within a number of large towns. *Sabah:* We will continue our collaboration with Queen Elizabeth Hospital, QEH (Key Pers. Lee, Rajahram) and Hospital University Malaysia Sabah, HUMS (Co-I Lasimbang, Director). Both clinics include our targeted sites for **Aim 2** in their catchment, and both have ongoing collaboration with the Borneo Medical Health Ctr (Co-I Kamruddin, Director). *Singapore:* Currently, there are no plans to conduct clinic surveillance based on budget constraints. However, in the event that that is an evolving need for clinic surveillance, enrollment, and sampling, we have close working relationships with all hospitals in the country, and the central infectious disease reference lab (key clinicians at which are aware of our proposal). This would allow us to rapidly on-board a hospital based site.

3.4.b Clinic enrollment and follow-up: We will recruit inpatients and outpatients to participate in the study after initial screening to determine if they meet our clinical case definitions. Once consented and enrolled, biological samples will be collected by trained and locally certified hospital staff and a questionnaire administered by trained hospital or research staff that speak appropriate local language. Every effort will be made to take samples concurrently when collecting samples for normative diagnostics. For both inpatients and outpatients, samples will be collected no more than 10 days of reported onset of illness to increase the chance

of PCR detection of viruses (*185*). Where possible, we will follow up 35 days after enrollment to collect an additional two 500 µL serum samples conduct a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. This gives adequate time for development of IgG, which occurs <28 days after onset of symptoms for SARS patients (*186-188*). Serum samples will be used to screen panels of high risk human and local zoonotic Filovirus, Henipavirus and Coronavirus strains as described in Aim 2. These samples may prove valuable to collaborators in that the PBMC could be used in production of therapeutic antibodies.

3.4.c Sampling and clinical interview: Biological specimens whole blood (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples) and two nasal or oropharyngeal swabs will be collected from all participants, and a questionnaire will be administered. We will investigate five risk factors, to maximize the power of the analyses, all related to high risk wildlife exposure, based on continuing analysis of our previous work, and will include: 11) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With additional consent considerations for participants enrolled from clinical settings, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology. Blood will be taken for future harvesting of Peripheral Blood Mononuclear Cells (PBMCs), as per **Section 2.5**.

3.5 Sample testing: The standard syndromic diagnostic PCR assays for common pathogens will be conducted and the results will be shared with the hospital with 48 hours. Specimens will be further tested for 3 viral families; CoV, PMV and filovirus by consensus PCR **(Section 1.4.c)**. Necropsy samples from deceased patients will be further characterized by NGS if previous PCRs are negative and unable to identify the cause of infection. Co-Is Wang, Anderson will conduct a VirScan serological assay using paired serum samples at Duke-NUS that identifies antibodies with a four-fold rise in titer in the convalescent sample compared with the acute sample, thereby indicating a possible etiological agent. A PCR test with the specific primer from each virus antibody positive case will be further tested from acute specimens to confirm infection.

3.6 Viral risk characterization and potential for pandemic spread: We will use statistical models built from collated biological, ecological, and genetic data to assess the likelihood of pathogenicity for the range of diverse viruses we will characterize. The aim is to quickly identify and assess which viruses pose the most significant risk to human health and thus prioritize additional resources to more fully characterize, better understand pathogenicity, and invest in our animal model pipeline and ultimately therapeutic development for those pathogens. We will combine two independent but related statistical models to predict pathogen spillover risk and pathogenicity. First, we will expand a simple generalized additive modeling approach (*3*) to assess spillover risk probability to a new virus that is currently not known to infect people, when we have only limited genetic data from initial PCR screening and sequencing. Additional data from receptor binding protein homology and cell line infection experiments will be incorporated into the model framework as available. Second, given high likelihood to spillover and infect humans, or direct evidence of human infection from our clinical surveillance, we will then assess the likelihood of pathogenicity based on traits for phylogenetically related nearest neighbor viruses as a first approximation and incorporating more detailed data from genomic characterization and animal model experiments (Aim 1). Epidemiological trait data for ~300 viral species known to infect people (e.g. case fatality rate, global estimates of number of human cases per year or decade, R_0, infectious period, and primary symptoms) have been collated by recent studies and will be used as predictor variables (*189*). Thirdly, we will apply tools already developed, and being refined by EHA under DTRA and DHS supported research, to predict pandemic spread for viruses using global flight models, as well as additional datasets on human movement and connectivity across Southeast Asia (*90, 91*) **(Fig. 15)**.

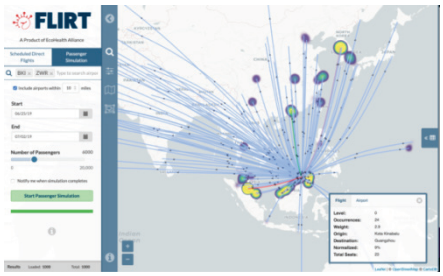


Fig. 15: Probability feed from EHA's Flight Risk Tracker tool (FLIRT). This image is of the likely pathways of spread and establishment for a Nipah-like virus emerging from Sabah, based on human movement and airline flight data (*90, 91*).

3.7 Potential problems/alternative approaches: Patients visiting clinics may have cleared virus, but not yet developed IgG antibodies, reducing seropositive cases. Our 35 day follow-up sampling should account for this because the maximum lag time between SARS infection and IgG development is ~28 days and Ebola virus infection and IgG approximately 20 days (*185-188, 190*). We also expect that patients in rural communities will only visit clinics when symptoms have progressed, likely coinciding with late illness and onset of IgG. We will also have data from our community study that can be analyzed in conjunction with, or in the absence of, clinical data from the hospital data to identify PCR- or seropositives. Finally, the risk of limited detection is outweighed by the potential public health importance of discovering active spillover of new henipaviruses, FVs or CoVs infection.

4. Administrative Plan

4.1. Project management: 4.1.a. Administrative core: The EID-SEARCH organizational partner roles are laid out by Specific Aim above **(see Fig. 3)**. EcoHealth Alliance (EHA) will lead the administrative core of the EID-SEARCH, including: coordination and management of all human and animal research; communication with consortium partners, NIH, other EIDRCs, and the EIDRC-CC; data management and quality assurance; modeling and analysis; and compliance with all US, international, and institutional rules and regulations. The administration will be led by PI Daszak, who has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5-20 yrs on NIAID- and USAID-funded research **(Fig. 16)**. He also has direct experience as one of two external reviewers of one of the CEIRS partners for NIAID (Program officer Diane Post). PI Daszak will be assisted by Deputy Lead, co-I Olival, who has overseen zoonotic disease research in Southeast Asia for >10 years and working directly with our partners in Thailand and Malaysia for 15 years. Senior program manager (Chmura) and full-time program assistant (Hamel) will provide administrative support. The EID-SEARCH data management, epidemiological analysis, and modeling activities will be led by co-Is Olival and Zambrana who have led all modeling and analytics on USAID USAID PREDICT for the last 10 years. They will overse a programmer/data scientist (Mendelsohn), evolutionary biologist/bioinformatician (Latinne), and human epidemiologists (Dawson, Li, Martinez). Our human epidemiology team will work directly with country teams in Malaysia and Thailand to manage human surveillance, IRB applications and approvals, and ensure compliance with all human subjects work. EHA's senior veterinarian (Field) and wildlife field biologist (Phelps) have extensive experience working that bats, rodents, and primates in SE Asia and will coordinate all training and field research in Malaysia and Thailand and ensure compliance with IACUCs.

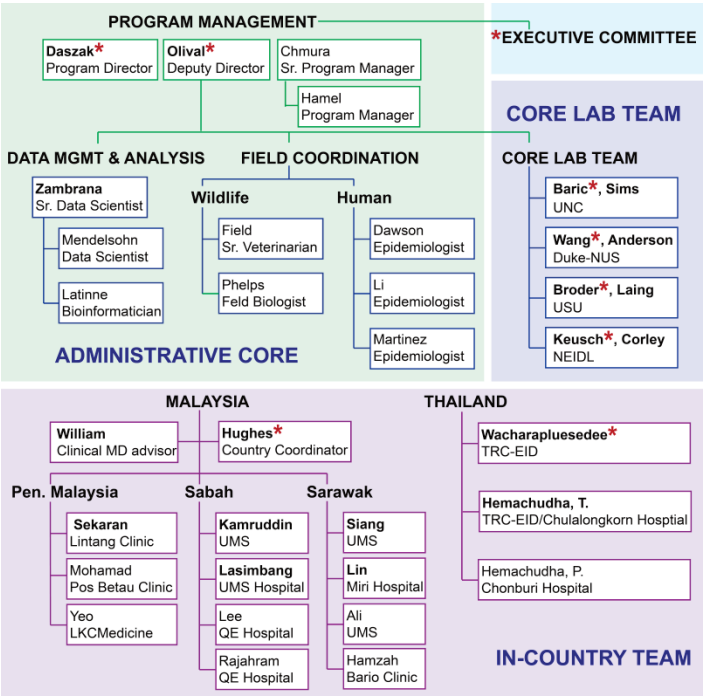


Fig. 16: Administration and program management for the **EID-SEARCH**

The EID-SEARCH core laboratory team includes global experts in virology, molecular pathogenesis, and the development of assays, reagents, and therapeutics for high consequence viral zoonoses (Co-Is Wang, Anderson at Duke-NUS; Baric, Sims at UNC; Broder, Laing at USU; Keusch, Corley at NEIDL). The core laboratory team will work with in-country diagnostic labs in Thailand (Wacharapluesedee, Hemachudha at the TRC-EID, Chulalongkorn University) and Malaysia (Hughes, Lee, CM Ltd.). Wacharapluesedee and Hughes will additionally serve as country coordinators for the EID-SEARCH, liaising directly with EHA on weekly conference calls, and working with in-country partners to ensure the smooth operation and coordination of clinical and community surveillance and wildlife research in Thailand and Malaysia, respectively. **A Core Executive Committee** comprising PI Daszak, Deputy Lead and Co-I Olival, and leads from each core partner and country: Co-Is Hughes, Wacharapleusadee, Baric, Wang, Broder, Keusch (or alternates), will conduct

regular conference calls and in-person meetings to facilitate rapid decision making within the EID-SEARCH. **This committee will also convene to manage EID-SEARCH response to outbreaks.**

4.1.b Project Management in Thailand and Malaysia: Wacharapluesedee and Hughes have collaborated directly with EHA for >15 years, including acting as country coordinators on the USAID PREDICT project for the last 10 years (project end date Sept. 2019). They maintain strong ties with Ministries of Health (MOH), Agriculture and Environment, multiple universities and research institutions, clinics, and hospitals, in their respective countries and across the region. The EID-SEARCH will use these connections to disseminate results, obtain permissions to conduct sampling, and also rapidly respond to and assist with outbreaks as they happen. Peninsular Malaysia, Sarawak, and Sabah are the three main Malaysian administrative regions, and effectively operate as three separate countries, with different regulations and government structures. We therefore provide specific details on the management of EID-SEARCH activities in each:

Coordination among Peninsular Malaysia, Sabah and Sarawak will be led by co-I Hughes (Conservation Medicine Ltd), and follow a successful model we implemented under USAID-PREDICT. **On Peninsular Malaysia** this project will be administered through the Zoonosis Technical Working Committee (ZTWC) established under the PREDICT project with a binding MOU among EHA, CM Ltd. and ZTWC, and including officers from MOH, Dept. of Veterinary Services, and PERHILITAN (the Govt. wildlife agency). EHA will communicate weekly with Co-I Hughes to coordinate and monitor implementation of research and reporting to ZTWC. Co-I Hughes will coordinate activities at all other Peninsular Malaysia institutions: NPHL, the National reference laboratory for diagnostic confirmation of pathogens, will manage molecular and serological screening (BioPlex) of Orang Asli samples, and serological screening of syndromic samples from Sabah and Sarawak; the PERHILITAN molecular zoonosis laboratory will store and conduct molecular and serological screening on wildlife samples; and Universiti Putra Malaysia (UPM) Faculty of Veterinary Medicine will conduct molecular and serological screening (BioPlex) of livestock samples, should these be required. **For Sabah & Sarawak**, work will be administered through the Sabah Zoonotic Diseases Committee (SZDC), a working technical committee comprising appointed and authorized officers from Sabah State Health Dept., Department of Veterinary Services, Sabah Wildlife Dept. (SWD), Universiti Malaysia Sabah (UMS) and EHA, all of which are also committed through a signed MOU. Co-I Hughes will oversee work at all other partners in Sabah, including: the Kota Kinabalu Public Health Lab (KKPHL) for molecular screening of syndromic samples from Sabah and Sarawak; the SWD Wildlife Health and Genetic and Forensics Lab for molecular screening of Sabah wildlife samples; The Borneo Medical Health Research Center (BMHRC) for screening some Sabah wildlife and livestock samples, if required, and human syndromic samples from Sabah and Sarawak. **In Thailand** all human community and wildlife research and testing will be coordinated by co-I Wacharapluesedee from the TRC-EID center. Clinical surveillance will be overseen by senior clinical physician and co-I T. Hemachudha.

4.1.c. Approval and release of results: In our experience, it is critical when working in resource-poor countries, on potentially important pathogens, to strictly adhere to protocols for release of results. EID-SEARCH will liaise with existing points of contact in the Ministries of Health, Environment, and Agriculture in each our administrative areas to approve and release project findings publicly. Results from human screening will be shared with participants when they become available, as per our IRB agreements ensuring no violations to anonymize data requirements (**see Protection of Human Subjects**).

4.2. Flexibility to extend the EID-SEARCH to new sites as needed: The EID-SEARCH consortium partners maintain extensive working relationships with leaders in EID outbreak control, clinical investigations and research at over 50 clinics, research institutes and public health laboratories across Southeast Asia. Due to space constraints, we haven't listed each of these, nor have we solicited >50 Letters of Support for this project. However, each core EID-SEARCH partner has contacted their networks and obtained permission for inclusion in the broader goals of the EIDRC. As examples of these contacts, our core partner, the Thai Red Cross Emerging Infectious Disease Health Science Centre (TRC-EID) at Chulalongkorn University, also serves as the WHO Collaborating Centre for Research and Training on Viral Zoonoses and has ongoing research collaborations across WHO SEARO countries including Bangladesh, Bhutan, Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, (Thailand), Timor-Leste; and has recently served as a training hub for scientists from Malaysia, Myanmar, Laos, the Philippines, and China to learn methods of wildlife sampling and diagnostic screening. Our Thai clinicians (Co-I T. Hemachudha and KP P.

Hemachudha) provide regular case consultations and clinical trainings for doctors across SEARO countries, including with Yangoon General Hospital and the National Health Lab in Myanmar, 2018. To maximize leverage of this broad network, EHA has budgeted for annual meetings in SE Asia, in addition to regular smaller network meetings, with our core team and key public health experts from network labs in each of the 10 SE Asian countries**.** Additionally, we will set up a listserv and an internal communication network to facilitate collaboration and information exchange, including on the first reports of new disease outbreaks. Our annual and smaller network meetings will critically allow face-to-face meetings of the EID-SEARCH that will foster greater sharing of information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks in the region, guided by the PI, Deputy and the Executive Committee.

4.3. Outbreak response: EHA collaboration with expert networks around the world allows us to mobilize and enhance effective One Health response to disease emergencies (*191*), ranging from real-time situation updates and risk analyses to on-the-ground investigations (*192-194*). We will adopt management tools from Emergency Operating Center (EOCs) (*195*) and Incident Management Systems (IMS) (*196*), to shift resources where necessary to help respond to novel zoonotic outbreak events and other public health emergencies. EHA has extensive experience working with governments in low and middle income countries (LMIC) applying these principles of epidemic preparedness during outbreak responses we've been involved with under the USAID-PREDICT project. For example, at the request of the government of Bangladesh, we provided technical field and laboratory support for Nipah virus and avian influenza outbreak investigations, assisting with wildlife sampling as part of the outbreak response alongside human and domestic animal sampling. In India, we provided technical assistance in response to the Nipah virus outbreak in Kerala in 2018. Last month in Indonesia were assisted the Ministry of Health's Center for Health Laboratory in Makassar to provide technical assistance in a mysterious outbreak in a small village in South Sulawesi that killed 4 villagers and infected 72. Our network partners include the key government and govt. approved laboratories that would be directly involved in public health emergency response in their respective countries. The serological and PCR platforms that EID-SEARCH develops will be made available to the main government outbreak investigation teams for clinical work and research during the outbreak. EID-SEARCH will also offer assistance training and conducting animal sampling during an outbreak, epidemiological analysis and modeling to help identify likely reservoirs or likely pathways to spread. Technical and material support for lab, field and analytical activities during an outbreak will be provided by EHA, UNC, USU, Duke-NUS, and NEIDL, as well as in-country partners. Any clinical samples, viral isolates and sequence data will be shared among partners to promote the rapid development of new diagnostic assays, reagents, and therapeutics that can be deployed to the region or other regions as part of the larger NIH EIDRC network.

Finally, while the initial pathogen focus of our group is on CoVs, PMVs and FVs, our broad collaborative group has multidisciplinary expertise on a number of virus-host systems. For example: PI Daszak was PI on a subaward from PI Laura Kramer's U01 on Poxviruses and Flaviviruses, managing a multidisciplinary research project on West Nile virus ecology. He was also co-I on a 5-year NSF-funded project to understand West Nile virus dynamics and risk in the USA (*197-201*); Co-I Baric is a global leader in Norovirus research leading to the development of vaccines and therapeutics (*202-205*); Co-I Wang has conducted significant work on bat immunology, therapeutic, and reagent development, as well as being involved in a range of outbreak investigations, viral discovery programs and other research on a wide diversity of viral groups (*206-215*). Additionally, the serological and PCR-based diagnostic platforms being developed by Co-Is Wang and Broder are adaptable to other viral targets. The modeling tools developed by Co-Is Olival and Zambrana-Torrelio can be used to predict the emergence and spread of diverse viral targets, including influenza, antimicrobial resistance, and vector-borne diseases (*216-221*). Our clinicians working in Thailand and Malaysia have a wide range of infectious disease investigations to adapt to any outbreak situation.

4.4. Communications: EHA will coordinate communication among all co-Is and key personnel, including:

- Multiple meetings per week with PI, Deputy Lead, Senior Program Manager (on project and task status)
- Weekly web/phone meetings between Program Manager and subawardee admin. staff
- Monthly web/phone conferences between EHA PIs and all subawardee PIs.

- Monthly web conferences between key personnel (research presentations/coordination)
- In-person Annual meetings with partner leads, key personnel at EHA and two in-person partner meetings annually between subawardees.
- Annual in-person meeting among all key personnel

4.5. Problem identification and resolution: Regular planning, monitoring, and evaluation meetings will be the primary mechanisms for problem identification. Minor problems (e.g. delays in sample availability or test results) will be dealt with internally through appropriate action and resolution monitored by PI Daszak and co-I Olival, and our Senior Program Manager. In the event of significant problems, such as prolonged poor productivity, inadequate scientific collaboration, or major disputes regarding research direction or resource allocation, EHA will assist with resolving the problem through negotiation with relevant co-Is and consultation with the Executive Committee. Should a resolution not be forthcoming, consultation with the EIDRC-CC, additional external technical advisors, and NIH staff may be warranted.

4.6. Adaptive management and risk mitigation: Maintaining a timeline and meeting milestones will require strict and continuous oversight of all project phases, frequent and regularly scheduled communication, and the ability to make decisions and implement strategies. To maintain our timeline on all projects, including the EID-SEARCH, we use an adaptive management approach to continually evaluate these trade-offs, to make decisions about when iteration is appropriate and when it is necessary to move forward with current information. Our ethos is that regular, scheduled communication among all staff, partners and collaborators will go a long way towards mitigating risks, especially if the process is collaborative and transparent.

5. Data Management Plan

EHA will house the Data Management and Analysis (DMA) team for EID-SEARCH, led by Co-PIs Olival and Zambrana-Torreilo and include Key Personnel Latinne and Mendelsohn. EHA has served as the data and analysis hub for numerous multi-institutional, multi-sectoral, international disease research groups, including acting as Modeling and Analytics lead for the PREDICT project (*122*), the Western Asia Bat Research Network (*222*) and EHA's Rift Valley Fever Consortium. We will leverage our experience and infrastructure from those projects to benefit the EID-SEARCH. **5.1. Project Database:** We will create a dedicated, centralized EID-SEARCH database to ingest, store, link, and provide for analysis all data associated with the proposed study and other expanded projects associated with the research network. The database will be SQL-based and use encrypted, secure cloud hosting services and enable export to archival and platform-independent formats. It will ensure data and metadata compatibility between project components, track data versioning and annotations. The system will be designed to work with both with both paper- and tablet-based field data entry and with the Lockbox laboratory information management systems **(Section 5.2)** in place in individual partner labs. The database will use existing metadata standards, including NCBI standards for genetic and molecular data and Ecological Metadata Language (EML) for field and wildlife data, as well as other standards and formats designated by the EIRDC CC. This will enable rapid publication and deposition of data. Granular security and privacy controls will be applied so that specific expansion projects undertaken in the network may be managed while maintaining data confidentiality as needed.

5.2. Biological Specimen Management: Project laboratories will use the Lockbox Laboratory Information Management System (LIMS), to manage the security, traceability, and quality of biological specimens. The LIMS will support sample barcoding at creation, tracking through transport, storage/inventory, and use via portable scanners. Lockbox supports CLIA and ISO 17025 as well as direct export to NCBI formats such as Sequence Read Archive. We will use the Lockbox LIMS application programming interface (API) to link to the central project database and associated samples with field and ecological data. We note that the project focuses on highly pathogenic viruses, including select agents; Lockbox LIMS supports sample tracking and movement compliant with US Select Agent Regulations and US Department of Commerce Pathogen Import and Export Control Regulations, and includes all necessary encryption, security, and backup protocols.

5.3. Training: Members of the DMA team will team will develop documentation and provide training for field and laboratory teams at all partner institutions in data management, metadata standards and data hygiene best practices. The DMA team will act as trainers and consultants for partner institutions in experimental

design, power analysis, data analysis, and computational and reproducibility issues, and visit each partner institution and/or field team base for training workshops and analysis consultations.

5.4. Data Identification and Privacy: For human clinical data and questionnaires, data will be identified by a unique identification code assigned to each individual and only this, de-identified code will be accessible in the project database, and destroyed at the end of the project - as per details provided in the Clinical Management Plan and Protection of Human Subjects forms.

5.5. Computing Resources: EHA operates a cluster of high-performance servers for data analysis activities, as well as infrastructure to launch cloud-based computing environments (**see EHA Facilities**). Our servers host all necessary software for statistical and bioninformatics work that is available to the DMA and partners anywhere in the world. We use a mixture of cloud services (AWS, Azure, Backblaze, GitHub) to provide redundancy, backup, version control, and rapid post-disaster recovery, and will be available to all project partners for analysis and training.

5.6. Data and Code Sharing: See details provided in the **Resource Sharing Plan.**

6. Clinical Management Plan

6.1. Clinical site selection: Our consortium partners have been conducting lab and human surveillance research, including during outbreaks, for >20 years and have developed strong relationships with local clinical facilities and processes in SE Asia and in LMIC globally. The plan for selection of clinical sites will begin in the same geographic regions as those identified in Aim 1 with high zoonotic viral diversity. Clinical sites will additionally serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2. We have already developed successful working relationships with the major healthcare facilities in Thailand and Malaysia and will use these established partners to rapidly gain appropriate permits and begin data collection quickly. Focusing on these EID hotspots in select biogeographic areas (see **Fig 13**) also reduces the number of additional sites needed to meet our goals within our geographic range. The capacity and capability requirements for clinical sites are fairly minimal, and include ability to enroll patients that meet the clinical case definitions of interest, collect and temporarily store biological samples, and follow standards for data management and subject protection with locked filing cabinets to store all paper records and an encrypted computer. We will be enrolling inpatient and outpatient participants within 10 days of the presentation of symptoms to increase the chance of PCR detection of viruses so we will not wait for advanced normative diagnostic tests, that are likely unavailable in the more remote sites, to be completed. We will work with clinical sites to determine the best process for project staff, in most cases supporting the time of currently hired staff at each site. We will recruit and train hospital staff in project-specific procedures including enrollment of patients, sample types to be collected, storage of samples, administration of the questionnaire, and data management plans. During the development and onboarding of new clinical sites we will assess the physical needs of the site and what supplies will be required for collection of data.

6.2. Standardized approach, oversight, and implementation: Management and oversite for all study sites will be undertaken by the local country coordinator with support from our Core Administrative team at EHA. Our research team has over 10 years of experience building capacity on human subjects' research and has developed training resources and materials for standardized implementation of community and clinical research and SOPs for screening, enrollment, and retention of participants. The country coordinator will conduct regular site visits to the clinical sites and annual visits to observe, monitor and evaluate the research process, and conduct follow-up training if required. Through our work with clinical sites under the USAID-PREDICT project we have developed culturally appropriate screening measures for clinal sites that do not disrupt the flow or quality of medical treatment received by the patient. This was done by working collectively with clinical staff to evaluate current procedures and patient flow at the site to determine the most efficient while minimal invasive inclusion of our study into the daily working of the clinal site. Most efficiently this was done by adding minimal basic screening questions to the current clinal intake forms, which allows the clinal research officer to quickly scan charts or logs for potential patients to enroll avoiding potential enrollees from being overlooked if staff are too busy or not on duty. Patients will be enrolled following established clinical criteria (**see Section 6.3**), samples collected and brief surveys conducted to assess the participants contact with wildlife through: 1) occupational exposures; 2) animal contact; and 3) the environment. With permission

from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology. Additionally, we will be following up with clinical participants to determine how clinical presentation differs between CoV, henipavirus, or FV in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. The country coordinator will be continually monitoring the project database to ensure we hit target sample sizes. While patient's enrollment is limited by the number of individuals presenting at hospitals, in previous research we enrolled an average of 105 patients per year, ranging from 77-244.

6.3. Clinical cohort setup, recruitment, enrollment: We will recruit inpatients and outpatients from clinical sites to participate in the study after initial screening to determine if they meet the clinical case definitions for 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever, of unknown etiology or severe diarrhea with unusual presentation for symptoms to increase the chance of PCR detection of viruses. Once enrolled, biological samples will be collected by trained hospital staff. When possible, samples will be taken concurrently with samples being collected for normative diagnostics. We will collect 5mL of blood for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples and two nasal or oropharyngeal swabs will be collected. Controls who test positive for CoVs, FVs, or Henipaviruses will be selected from the pool of participants admitted to the studies and whose samples tested negative for coronaviruses, henipavirus, or filoviruses. We will follow up 35 days after enrollment to collect an additional 5mL blood draw for collection of two 500 µL serum samples and a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. Following up 35 days post initial collection gives adequate time for 1) development of IgG and IgM, which occurs <28 days after onset of symptoms to evaluate the immunological progression of disease and 2) further the risk assessment of the participants to monitor contact with wildlife, people, and to assess the likely reservoir hosts to collect information to inform potential intervention programs.

6.4. Utilization of collected data: Sampling of wildlife and the enrollment of community surveillance participants and a clinical cohort of participants give us the ability to assess the viruses that are circulating in each of the three populations in a similar geographic region. We will use phylogenetic analysis to compare the relationship between wildlife viruses found and viruses fond in human participants. Additionally, the questionnaire data will allow us to assess relative measures of human-wildlife contact that we will analyze the intensity of contact with species known to be at a higher risk for spillover against other risk to provide useful proxy information for spillover risk. The clinical cohort will be split into cases and controls. "Cases" are defined as participants whose samples tested positive for either CoV, henipavirus, or FV via PCR tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. In clinical study, we will use this procedure to determine how clinical presentation differs between virus-exposed and -unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. We will model the outcomes to analyze correlation between biological results and risk factors related to high risk wildlife exposure. With this collective data set we aim to quantify and detect novel viruses likely to spillover regularly to people, are often unreported or misdiagnosed as their clinical manifestations, and are unknown to detect cryptic outbreaks causing previously 'hidden' clinical syndromes in people. Our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence.

6.5. Development of reagents of value to the community. Members of the EID-SEARCH consortium have substantial experience producing reagents, assays, and other products that are used widely by the clinical and research community, and some of which are on a pathway to commercialization. These include: PIs Daszak and Co-I Olival have produced software for analyzing the spread of novel viral agents through air travel networks; Co-I Baric has collaborated with a Norovirus surveillance collaboration with surveillance cohort at CDC and has developed therapeutics that have reached phase 2 and 3 clinical trials, He is currently working with Takeda Sanofi Pasteur on a Dengue therapeutic and with NIH on a tetravalent vaccine; Co-I Broder

developed a Hendra virus subunit vaccine that was commercially produced by Zoetis for horses and is labeled for human use under compassionate circumstances during outbreak situations.

6.6. Potential expansion: Expansion of the research project to other clinical sites or research areas could happen as new information is gathered, either through our research, the EID-SEARCH information network, or an outbreak being identified in the region by other organizations. If expansion is required we would rapidly shift research activities towards the clinical or community sites where the outbreak is active, using the same process we used to set up initial research locations. First, by working with local stakeholders to create a working relationship and on-boarding new clinical research staff through advanced trainings of project aims, SOPs on data collection and storage, and ethical guidelines. The network of infectious disease researchers in the community and hospital settings would allow for an accelerated response to any potential infectious disease outbreaks as staff are well trained on ethical guidelines for working with human subjects, data and specimen collection, storage, and transpiration, project staff would be on the front lines ready for the call to action. Ideally, our testing for viruses in priority viral families may lead to the discovery cryptic outbreaks before it spreads in the general public. Biological results coupled with survey data on key risk factors will provided necessary information for granular understand of disease spread.

7. Statistical Analysis Plan:

7.1. Framework: Statistical analyses across the project will be conducted under a common Bayesian framework. These models provide a unified, probabilistic approach best-suited for estimating effect sizes in heterogenous populations of human clinical and wildlife subjects in observational studies. Within this Bayesian framework, we will use generalized linear mixed models to estimate population prevalences and seroprevalences, and estimate the effects of demographic, occupational and environmental factors affecting these. We will use occupancy models (*223*) to estimate total viral species and strain diversity and completeness of sampling within the human and wildlife sub-populations, and discrete phylogeographic models to identify taxonomic and geographic centers of viral diversification. All statistical analyses will be performed reproducibly using scripted, programmatic workflows (e.g., the R and Stan languages) and maintained under source code version control (git). As with data management, the DMA team will act as trainers and consultants for exploratory data analysis, power analysis, and study design with project partners, and the EHA computing cluster will be available for partners undertaking additional or expansion studies. Power analyses, current and expansion, are performed via simulation approaches allowing planning for complex, hierarchical variation in study populations. Power analyses and specific analytical components of this study are detailed under each Specific Aim.

7.2. Data Quality Control and Data Harmonization: All data will be are examined at entry by field and lab teams upon data entry, followed by examination by DMA team members at upload and integration, for complete de-identification, completeness, accuracy, and logical consistency. The DMA will provide field and lab teams with reports, produced automatically, of data summaries, including aggregates, distribution, detected outliers and possible mis-entries. On a regular basis (quarterly or as-needed during data collection), DMA team members will review reports with field and lab teams to identify errors and update collection and entry procedures as necessary.

7.3. Statistical Considerations for Behavioral Questionnaires and Clinical Metadata: The data collected from the questionnaire will be analyzed to assess the reported measures of contact for each risk group under study, related to1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, rodents, and primates in/around house and other livestock animals (e.g. farming, butchering, slaughtering); 3) proximity of residence or workplace to environments of increased risks (e.g. nearby bat roosts); 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12 months and lifetime. Specific measures we are interested in are the proportion of respondents indicating they consume wildlife, where wildlife is obtained for consumption, have hunted wildlife, butchered or slaughtered live animals, seen wildlife in their homes, been bitten or scratched by animals, etc. Comparisons of measures of exposure contacts and types between men and women, children and adults, different study regions will be conducted in order to explore the occupational, environmental, and demographic factors (gender, age, socioeconomic status (SES)) that influence contact with animals and to determine who is

most at-risk. Statistical analysis will be employed to identify differences between groups with a 95% probably of detecting a difference. Measures of contact related to different activities within specific groups will be compared to determine the activities that put groups at most risk. As appropriate multivariate analysis (e.g. ordinary linear regression, logistic regression, non-normal distributions of outcome, least absolute shrinkage and selection operator (LASSO) regression, etc.) will be utilized to evaluate the relationship between the outcome variables, positive biological results (PCR or serology) key measures of contact and the factors that influence frequency and types of human-animal contact.

8. Project Milestones and Timelines

8.1 Milestones: <u>End of Year 1:</u> Aim 1: Sample targeting locations, species (for wildlife), sample size justifications completed for whole project and reported to in-country teams; Sample testing, viral isolation, NGS, glycoprotein sequencing begun for all archival and some newly-collected samples; *in vitro* work begun; host-pathogen dynamic analyses; animal model work begun. **Aim 2:** Target human community populations identified and sample sizes calculated for some sites in each country; Community data collection, serological testing and RT-PCR testing begun; first epidemiological analyses of data begin in last quarter. **Aim 3:** Clinical cohort selection underway; clinical enrollment, data collection and sample analysis begun. First Annual meeting in last quarter. First publications submitted by end of year, summary overview papers or reviews.

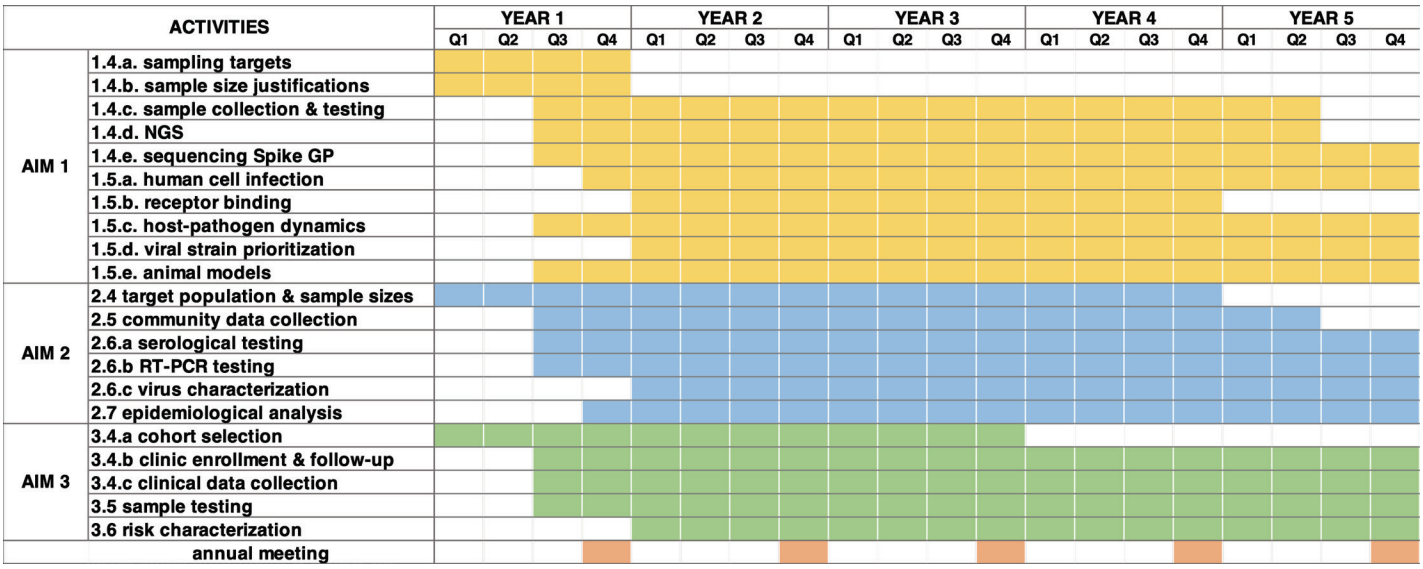
<u>End of Year 2:</u> Aim 1: No sample targeting or sample size justification analyses needed. All other aspects underway **Aim 2:** All aspects underway **Aim 3:** All sub-aims underway. Second Annual meeting in last quarter. Further 2 publications submitted by end of year, including first data papers.

<u>End of Year 3:</u> Aim 1: No sample targeting or sample size justification analyses needed. All other aspects underway **Aim 2:** All aspects underway **Aim 3:** All sub-aims underway. Third Annual meeting in last quarter. Further 3 publications submitted by end of year, largely data papers.

<u>End of Year 4:</u> Aim 1: No sample targeting or sample size justification analyses needed. All other aspects underway **Aim 2:** All aspects underway. Receptor binding work completed. **Aim 3:** No further cohort selection required; all other sub-aims underway. Fourth Annual meeting in last quarter. 3 further publications submitted, including first papers analyzing risk factors, pathogenic potential of novel viruses submitted.

<u>End of Year 5:</u> Aim 1: No sample targeting or sample size justification analyses needed. No receptor binding assays continuing. Serological and PCR testing completed end of 2nd quarter. Glycoprotein, *in vitro* and *in vivo* analyses, analysis of viral risk continue to end of project. **Aim 2:** No further community targeting or sample size work. Community data collection completed at end of 2nd quarter. All other aspects continue to end of project **Aim 3:** All sub-aims underway. Final Annual meeting in last quarter. Further 3 publications submitted.

8.2. Timeline:



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From:	Peter Daszak on behalf of Peter Daszak <daszak@ecohealthalliance.org>
To:	Laing, Eric; Thomas Hughes
Cc:	Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Supaporn Wacharapluesadee; Aleksei Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross; Kevin Olival
Subject:	EIDRC impact scores from NIH
Date:	Monday, November 18, 2019 9:26:16 PM
Attachments:	Study section roster 1573258030732.pdf EIDRC Southeast Asia v7 FINAL_FINAL.pdf
Importance:	High

Dear All,

Just to let you know that we've got the results back from NIH. EHA submitted two EIDRC proposals, and we were linked to a third one as a subcontract. Out of these, the only one to get a score was the Southeast Asia EIDRC (EID-SEARCH) which you are all collaborators on. I also know of another good group that wasn't scored, so just to get a score is actually a good result…

Our 'impact score' was 32, which normally would put us right on the edge of being funded. I've already contacted the NIAID program officer (Jean Patterson) to ask about the likelihood of being funded. She told me that NIH staff are currently working out the structure of the network and a funding plan. She felt our proposal was well-received and scored well enough to be considered, and she asked me to hold off for a week or two and then they'd know more.

My take on this is that they're now deciding what the geography and scope of the network should be, how many EIDRC centers they can afford to fund, and then they'll decide priorities for funding. With this being strongly driven by NIAID's internal concerns about their reach into different geographies, it's really going to be a decision driven by their own considerations of who else is in the mix and what strengths/weaknesses different proposals have. That means we're in with a chance, but we don't really know the factors driving a decision.

I'll contact NIAID again in a few days, but in the meantime, please do the following: 1) try not to share this information with colleagues because we don't want to have a situation where they scored better than us and we got funded, leading to a bad feeling (or worse, the other way round!); 2) if you meet people from NIAID, tell them how keen and enthusiastic you are about the proposal and the work we're conducting (I've attached the final text again as a reminder) – you never know how this might help. Key people at NIAID who will be making this decision are: Tony Fauci (head of NIAID), Cristina Cassetti (Deputy Head, and in charge of this line of work), Jean Patterson (Program Officer directly in charge of the EIDRCs), Eun-Chung Park (also directly in charge of this work), Pat Repik (I think she's involved).

Finally – I've attached the Review Committee Roster for you to look at who the people are who either liked or disliked our proposal! I know a few of them, and I'm sure you know the others…

Will be in touch as soon as I hear more.

Cheers,

Peter

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President

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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

From: Peter Daszak
Sent: Friday, June 28, 2019 8:59 PM
To: 'Laing, Eric'; Thomas Hughes
Cc: 'Wang Linfa'; 'Danielle E. ANDERSON PhD'; 'Ralph S. Baric'; 'Baric, Toni C'; 'Sims, Amy C'; 'Chris Broder'; 'Supaporn Wacharapluesadee'; Aleksei Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross; Kevin Olival
Subject: EIDRC grant submitted
Importance: High

Dear All,

Just to let you all know that the grant was successfully submitted today with time to spare, and no errors. It's in the system, and it's all down to the reviewers now! Thanks to all of you for your help and support in getting this finalized and completed. Please pass on my personal thanks to the Co-Investigators, Key Personnel and Consultants who you also brought into the team.

I've attached a pdf of the final proposal text and will send the Word version in a few minutes. It's actually a good read, and I'm especially grateful to Hongying who generated all the cool graphics, based largely on Ralph's previous NIH grant proposals.

As you read the text, please remember that the wording is very carefully targeted to a typical US-based NIH reviewer, and to the Program Officers, with the sole purpose of trying to win the grant. If I've exaggerated or made mistakes, or used language that isn't quite right, I apologize, but I did it for the key goal of getting funded.

In the meantime, please don't share this proposal beyond our group on this email chain, so we don't give our competitors an edge!

Good luck, and I'll be keeping my fingers crossed all summer in the hope that we win this…

Cheers,

Peter

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President

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From: Peter Daszak
Sent: Wednesday, June 26, 2019 5:19 PM
To: 'Laing, Eric'; Thomas Hughes
Cc: Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Supaporn Wacharapluesadee; Aleksei Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross; Kevin Olival
Subject: EID-SEARCH Comments

Thanks everyone for sending the comments back. Working on v5 now, while others in the office are cranking out sample size calculations, new figures, human and vert. subjects and all the additional information needed after the research plan.

As I go through all your comments, I'll send the occasional email with questions to clarify specific points, so please be on standby for a quick turnaround.

Cheers,

Peter

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From: Laing, Eric [mailto:eric.laing@usuhs.edu]
Sent: Wednesday, June 26, 2019 12:59 PM
To: Thomas Hughes
Cc: Peter Daszak; Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Supaporn Wacharapluesadee; Aleksei Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross; Kevin Olival
Subject: Re: EID-SEARCH v4

Hi Peter,

Built on top of Chris' suggestions. Included a prelimin data figure from Hughes et al, in prep. Double check that is ok with Tom?

- Eric

Eric D. Laing, Ph.D.
Research Assistant Professor
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On Wed, Jun 26, 2019 at 11:26 AM Tom Hughes <tom.hughes@ecohealthalliance.org> wrote:

> Hi Peter,

I have added my edits and comments to Kevin's.

Please let me know if you ave any questions or need more details.

Thanks.

Tom

From: Kevin Olival <olival@ecohealthalliance.org>
Sent: 26 June 2019 2:51 PM
To: Peter Daszak
Cc: Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Eric Laing; Thomas Hughes; Supaporn Wacharapluesadee; Aleksei Avery Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross
Subject: Re: EID-SEARCH v4

Peter, v4 with my edits, some revised figs, and additional comments.

Cheers,
Kevin

Kevin J. Olival, PhD
Vice President for Research

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On Jun 25, 2019, at 9:26 AM, Peter Daszak <daszak@ecohealthalliance.org> wrote:

Dear All,

Here's version 4. Apologies for the delay. It's in better shape now, but there are still lots of areas that need your attention. Please put in time today, and tomorrow to edit and firm up the text.

I've put your names in some of the comment boxes where I need specific help but I also need you to read this critically now and edit and tighten it up. We have 30 pages of space, and our research plan is now 21 pages, so we need to lose maybe 3 or 4 pages. There are also some weak points, specifically on Filoviruses, on the details of where we'll do our clinical work, and on what we'll do with the animal models for henipas and filos.

Please get comments back with me before Wednesday 26th 4pm New York time, so that I have Wendesday evening and all day Thursday to incorporate them. In the meantime, I'll be working with folks here on better figures, and all the extra sections that come after the research plan. I'll need you all to help with those on Thursday while I'm finishing off the draft.

Thanks to all of you in advance and look forward to the next round.

Cheers,

Peter

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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

From: Peter Daszak
Sent: Thursday, June 20, 2019 9:41 PM
To: Wang Linfa; Danielle Anderson (danielle.anderson@duke-nus.edu.sg); Ralph Baric (rbaric@email.unc.edu); Baric, Toni C; Sims, Amy C; Christopher Broder (christopher.broder@usuhs.edu); Eric Laing; 'Tom Hughes'
Cc: Aleksei Chmura (chmura@ecohealthalliance.org); Alison Andre; Luke Hamel (hamel@ecohealthalliance.org); Kevin Olival, PhD (olival@ecohealthalliance.org)
Subject: EIDRC-SEA v.3
Importance: High

Dear all,

Thanks to everyone for comments and text for the earlier draft at the beginning of the week. Kevin's spent the last couple of days pulling all the comments together into one draft and inserting his changes, and I've now attached a v3 of the draft. It's still rough, and now too long, but it's getting there. I wanted you to look at this rough version because I'm stuck in Liberia in meetings Friday, then flying back home Saturday. I'll continue editing this draft on the plane and will be working solely on this grant from home on Sunday, Monday, Tues, Wed, Thurs.

Please work on this rapidly if possible. If you can all push your comments through, using track changes, and get them back to me **by Sunday evening New York Time**, I will turn round a polished draft by Tuesday, giving us time for significant last go-over on Wed, Thurs. It's tight, but unfortunately, it is what it is, so please commit the time if you can!! As well as general edits (using track changes, with references in comment boxes), please do the following…

Linfa/Danielle – We especially need your input in section 1.1, 1.3, 1.4 and 2.5

Ralph/Amy - Please work your magic on this draft, inserting text, editing and reducing text, and making sure the technical details of the characterization are correct and fit the proposed work on CoVs, PMVs and Henipaviruses

Chris/Eric – Eric – congratulations on your (b) (6) and thanks for working on it at this time! Chris – please help and edit/finesse the language in the relevant sections for you

Tom – Your edits crossed over with Kevin's changes, so please insert all the bits that were new in the last version you sent to me, into this version. I think these are mainly the sections and comments from the other Malaysian colleagues, but please check and re-insert all key text. We especially need to be specific about which partner in Malaysia is doing what cohorts, and where, and about who's doing the testing

Thanks again all, and I'm looking forward to non-stop work on this as soon as I get on my flight in 24 hours…

Cheers,

Peter

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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

<EIDRC Southeast Asia v4.docx>

SUMMARY STATEMENT

PROGRAM CONTACT:
Eun-Chung Park
240-627-3338
epark@niaid.nih.gov

(Privileged Communication)

Release Date: **03/10/2019**
Revised Date:

Application Number: **1 R01 AI143978-01A1**

Principal Investigator

EPSTEIN, JONATHAN H

Applicant Organization: ECOHEALTH ALLIANCE, INC.

Review Group: **CRFS**
Clinical Research and Field Studies of Infectious Diseases Study Section

Meeting Date: **02/14/2019**
Council: **MAY 2019**
Requested Start: **07/01/2019**

RFA/PA: **PA18-484**
PCC: **M32A B**

Project Title: **Study of Nipah virus dynamics and genetics in its bat reservoir and of human exposure to NiV in western and eastern populations in Bangladesh to understand patterns of human outbreaks.**

SRG Action: **Impact Score:33 Percentile:22**

Next Steps: **Visit https://grants.nih.gov/grants/next_steps.htm**

Human Subjects: **30-Human subjects involved - Certified, no SRG concerns**

Animal Subjects: **30-Vertebrate animals involved - no SRG concerns noted**

Gender: **1A-Both genders, scientifically acceptable**

Minority: **5A-Only foreign subjects, scientifically acceptable**

Children: **3U-No children included, scientifically unacceptable**

Project Year	Direct Costs Requested	Estimated Total Cost
1	479,076	607,258
2	478,757	606,854
3	477,744	605,570
4	478,484	606,508
5	472,595	599,043
TOTAL	2,386,656	3,025,232

ADMINISTRATIVE BUDGET NOTE: The budget shown is the requested budget and has not been adjusted to reflect any recommendations made by reviewers. If an award is planned, the costs will be calculated by Institute grants management staff based on the recommendations outlined below in the COMMITTEE BUDGET RECOMMENDATIONS section.
EARLY STAGE INVESTIGATOR
NEW INVESTIGATOR

1R01AI143978-01A1 EPSTEIN, JONATHAN

INCLUSION OF CHILDREN PLAN UNACCEPTABLE
EARLY STAGE INVESTIGATOR
NEW INVESTIGATOR

RESUME AND SUMMARY OF DISCUSSION: This application focuses on Nipah virus outbreaks in Bangladesh and seeks to understand why outbreaks appear to only occur in the western part of Bangladesh despite the virus, host (Pteropus medius fruit bats), and the primary route of transmission (date palm sap consumption) being present throughout the country. Given the virulence, the increase in outbreaks and the limited knowledge of the driving factors of this virus transmission, the significance is very high. The revised application is responsive to prior critiques and is more focused. Strengths are the significance, the expertise of the impressive investigative team, the field site, the multidisciplinary approach of integrating field studies, animal models and modeling, and the feasibility of the approach. Notwithstanding these strengths, the following weaknesses in the approach slightly detract from the impact of this application. Some weaknesses are the consideration of sap consumption as a proxy for exposure, the use of increase in seroprevalence as a proxy for the prevalence of viral carriage, the insufficient number of subjects for risk factor analysis, and the small number of hamsters for transmissibility studies. Other weaknesses are the exclusion of children and the lack of consideration of the confounding effect of recency of Nipah introduction in the East.

DESCRIPTION (provided by applicant): Nipah virus is a zoonotic paramyxovirus, carried by old world fruit bats across Africa and Asia, which causes severe, fatal encephalitis in humans and can be transmitted from person to person. In Bangladesh, where outbreaks in people occur annually, the primary route of transmission is the consumption of raw date palm sap. Date palm sap is harvested and consumed most intensively in western Bangladesh, an area referred to as the “Nipah belt,” however, date palm sap consumption, NiV, and its bat reservoir, Pteropus medius, are present throughout Bangladesh. It is unclear why outbreaks have not been detected in eastern Bangladesh. Cryptic spillover of NiV creates a significant risk that more pathogenic and transmissible strains will emerge and lead to large epidemics. This multidisciplinary project will determine whether NiV outbreaks have occurred in eastern Bangladesh and how differences human behavior, infection patterns in bats, and the circulation of potentially less pathogenic strains of NiV influence outbreaks outside of the Nipah belt. This project combines human exposure studies with multi-site longitudinal infection studies in bats and in vivo experimental infections comparing clinical outcomes among diverse strains to achieve the following specific aims: 1) To compare NiV exposure and its behavioral determinants among human populations inside and outside the Nipah belt in Bangladesh. We will test the hypothesis that NiV spillover has occurred in Bangladesh in communities outside the Nipah belt. We’ll use behavioral questionnaires and a multiplex Luminex serological assay to screen high risk populations for IgG antibodies against NiV and determine if Nipah exposure has occurred and how behavioral risk varies by locality. 2) To compare Nipah virus temporal dynamics in Pteropus bat colonies inside and outside of the Nipah belt. We will conduct longitudinal bat NiV field studies in six locations (3 western, 3 eastern), characterize local bat demography, measure changes in seroprevalence over time; determine viral shedding patterns and genetic variation among strains. Through experimental bat infections in a BSL 4 lab, we will determine whether bats with antibodies against NiV (previous exposure) may be re-infected and shed virus. This will answer a critical question about transmission dynamics and allow us to test the hypothesis that viral shedding occurs with different frequency in eastern bats compared to western bats, which may influence zoonotic transmission. 3) To compare pathogenicity and transmissibility of diverse Nipah virus isolates from bats inside and outside the Nipah belt, using animal models. Malaysia type NiV and Bangladesh type have different clinical profiles in people. NiV genetic variation may account for lower human infection rates in eastern Bangladesh. To test this hypothesis, we will compare transmissibility and pathogenicity of diverse NiV strains isolated from Pteropus medius in a Syrian hamster model under BSL 4 conditions.

PUBLIC HEALTH RELEVANCE: Nipah virus is an emerging zoonotic virus carried by fruit bats and causes a severe encephalitis with greater than 70% mortality. In Bangladesh, small outbreaks occur annually but only in the western part of the country, however, repeated spillover events from bats provide an opportunity for a genetic strain to emerge that is both more pathogenic and more transmissible, which could cause a much larger epidemic. Through a combination of field studies, laboratory experiments, and mathematical modeling of Nipah virus in bat populations, this project aims to understand why Nipah virus outbreaks appear to only occur in the western part of Bangladesh despite the virus, host (Pteropus medius fruit bats), and the primary route of transmission (date palm sap consumption) being present throughout the country.

CRITIQUE 1

Significance: 2
Investigator(s): 1
Innovation: 2
Approach: 5
Environment: 1

Overall Impact: This resubmission application from a New Investigator, proposes to address why outbreaks of Nipah virus occur in one region of Bangladesh and not in others. In doing so, the proposal will provide key information on the transmission dynamics and natural history of Nipah virus and provide insights on the reservoir, viral pathogen and exposure factors that may influence spillover infections. This application is bold and exciting and has many strengths which include the PI and study team who are leaders in the field of Nipah research, the field and laboratory capacity that has been established to implement the project and the highly innovative approach which integrates field investigations of humans and reservoirs, experimental animal models of infection and modeling. There are however major weaknesses with respect to the scientific rigor of Aim 1. Nevertheless, the application generated significant enthusiasm which was reflected by the overall impact score.

1. Significance:

Strengths

- The proposed studies will provide important descriptive information on the spatial and temporal distribution of seropositivity in bats, which may provide insights into the underlying transmission dynamics.
- Likewise, the experimental bat infection addresses key knowledge gaps in the natural history of Nipah in the primary reservoir.
- If successful, Aim 3 will set the stage to identify pathogen-specific factors that influence disease severity and transmissibility.
- Many parts of the proposed studies are supported by strong scientific premise.

Weaknesses

- The rigor of proposed Aim 1 studies is a concern since the factors that influence a spillover infection are complex (bat abundance, frequency and degree of source contamination, etc.) and extend beyond sap consumption.

2. Investigator(s):

Strengths

- The PI, although a new investigator, has an impressive track record of accomplishments in the field of emerging infectious disease.
- The study team are leaders in the field of Nipah virus research and one of the few groups who can implement this ambitious project

Weaknesses

- None noted

3. Innovation:

Strengths

- The integration of multidisciplinary field, laboratory and modeling approaches to study the natural history and transmission of Nipah is an exciting and highly innovative feature of the application.
- The proposed experimental bat infection studies, as well as the hamster transmission model are novel.

Weaknesses

- None noted.

4. Approach:

Strengths

- The field sites distributed in regions of differing outbreak risk is a strength.
- The serial trapping surveys of bats are well designed and should yield sufficient numbers to delineate the dynamics of seropositivity.
- The proposed experimental bat and hamster infection models and bank of well-characterized viral isolates is another strength of the proposal.

Weaknesses

- Sap consumption in Aim 1 may not be a robust proxy for exposure and details are not provided on how this exposure will be measured and evaluated in the models. Alternative hypotheses for sporadic spillover infection (in contrast to disease during outbreaks) are not considered.
- Although 3,000 participants will be recruited, seroprevalence may be low and even if it does reach 2%, there may not be sufficient numbers to perform robust risk factor analyses.
- The use of increase in seroprevalence as a proxy for the prevalence of viral carriage/shedding is a potential weakness. The proposal does not describe how criteria for seropositivity in bats was established.
- The description of the SIRS model is sparse and how uncertainty will be addressed
- Although the proposal states at power of 81%, the sample of three naïve hamsters per group seems small for the proposed transmissibility studies.

5. Environment:

Strengths

- Outstanding field and laboratory capacity which include BSL4 facilities.

Weaknesses

- None noted.

Study Timeline:

Strengths

- NA

Weaknesses

- NA

Protections for Human Subjects:

Acceptable Risks and/or Adequate Protections

Inclusion of Women, Minorities and Children:

- Sex/Gender: Distribution justified scientifically
- Race/Ethnicity: Distribution justified scientifically
- For NIH-Defined Phase III trials, Plans for valid design and analysis: Not applicable
- Inclusion/Exclusion of Children under 18: Excluding ages <18; not justified scientifically
- Children are at risk for Nipah as evidenced by prior outbreaks

Vertebrate Animals:

YES, all criteria addressed

Biohazards:

Acceptable

Resubmission:

- The resubmission has responded to many of the critiques, particularly those raised about the ambitious nature and the lack of scientific rigor.

Applications from Foreign Organizations:

Justified

Select Agents:

Acceptable

Resource Sharing Plans:

Acceptable

Authentication of Key Biological and/or Chemical Resources:

Acceptable

Budget and Period of Support:

Recommend as Requested

CRITIQUE 2

Significance: 1
Investigator(s): 1
Innovation: 2
Approach: 4
Environment: 1

Overall Impact: This ambitious proposal by Dr. Epstein and colleagues proposes to use multidisciplinary approach to examine drivers of Nipah virus spillovers and outbreaks. The team has the necessary expertise and a suitable environment for doing the proposed research. The topic is important especially given the fatality rate of Nipah virus infections and the potential to cause larger outbreaks or epidemics. Overall there is enthusiasm, however there are some significant concerns around the design of the human serosurvey. In particular, the investigators propose to match on date palm sap consumption a cross east-west sites which is inappropriate given that is a main exposure they would like to examine. Some concern also exists around the exclusion of children from the study population and the possibility of confounding by recentness of Nipah introduction given the study design.

1. Significance:

Strengths

- Nipah virus is a high priority pathogen with a high case fatality rate and no effective interventions
- Repeated spillover events and the potential for human-to-human transmission highlight the need to fill critical gaps in knowledge about factors contributing to outbreaks

Weaknesses

- None noted.

2. Investigator(s):

Strengths

- The PI, Dr. Jonathan Epstein, is well-qualified to lead this study based on his history of conducting similar studies on emerging pathogens and on his expertise in Nipah virus
- Investigators have a history of working together productively

Weaknesses

- None noted.

3. Innovation:

Strengths

- Multidisciplinary approach to understanding Nipah virus spillover and transmission.
- Use of bats as a lab animal model to inform parameters for mathematical models that are not possible/extremely difficult to measure in the wild.

Weaknesses

- Human study proposed is not particularly innovative.

4. Approach:

Strengths

- Proposed studies seem to be appropriately powered, assuming the 2% seroprevalence in the East in Aim 1 which is impossible to predict without a serosurvey.
- Bat studies will provide valuable information on viral dynamics and seasonality of Nipah.
- Modeling approaches are appropriately sophisticated.

Weaknesses

- Since the authors plan to look specifically at date palm consumption as one of their primary exposures of interest, it is highly inappropriate to match on date palm consumption. The matching proposed is at the village level and by household consumption categories (high, medium, low), however, matching even roughly on an exposure of interest will bias that point estimate.
- Study is limited to adults; however, children may consume date palm (and perhaps Tari?) or participate in production and there have been a number of documented cases of Nipah in children in Bangladesh including at least one documented outbreak where nearly all cases were boys <15 years of age. Additionally, seropositivity in children would be more likely to indicate recent infection which would likely better match with exposure data collected through the surveys.
- Given the focus of IgG antibodies and the limitation of the studies to adults who have had many years to become seropositive, it is possible that findings may be confounded by when the virus was first introduced into the area. For example, if Nipah circulation has begun more recently in bats in the East.

5. Environment:

Strengths

- Institutions in US, and particularly the institutions in Bangladesh provide a great environment for the proposed research.

Weaknesses

- None noted.

Study Timeline:

Strengths

- Not Applicable

Weaknesses

- Not Applicable

Protections for Human Subjects:

Acceptable Risks and/or Adequate Protections

Data and Safety Monitoring Plan (Applicable for Clinical Trials Only):

Not Applicable (No Clinical Trials)

Inclusion of Women, Minorities and Children:

- Sex/Gender: Distribution justified scientifically
- Race/Ethnicity: Distribution justified scientifically
- For NIH-Defined Phase III trials, Plans for valid design and analysis: Not applicable
- Inclusion/Exclusion of Children under 18: Excluding ages <18; not justified scientifically
- Study limited to adults and exclusion of children is not justified by stating that children are not disproportionately affected by Nipah. Human subjects' sections have not been fully updated to account for changes in the study design between the initial submission and the resubmission.

Vertebrate Animals:

YES, all criteria addressed

Biohazards:

Acceptable

Applications from Foreign Organizations:

Justified

- Nipah virus doesn't circulate in the US

Select Agents:

Acceptable

Resource Sharing Plans:

Unacceptable

- Viral sequences should be made available on GenBank or another appropriate site rather than github.

Authentication of Key Biological and/or Chemical Resources:

Acceptable

Budget and Period of Support:

Recommend as Requested

CRITIQUE 3

Significance: 3
Investigator(s): 1
Innovation: 2
Approach: 3
Environment: 1

Overall Impact: The previous criticisms were acknowledged, and the PI has presented a more focused and less extensive proposal that is judged to have many strengths. Accordingly, the problem being investigated has high relevance, the investigative team is strong, and they have a compelling approach to understanding the relative importance of infection derived from spillover from bats compared to direct human to human transmission. Their idea that viruses cause outbreaks in bats more frequently in the Nipah belt because the strains are more virulent than in the east. This sets the stage for more likely spillover in the west than in the East. It will not explain, however, how the differences arose in the first place or if the pattern will be retained and will not be recapitulated in the East. Their plans to compare strain pathogenicity differences by animal studies in BL4 facilities with bats (Australia) and hamsters (Montana) with strains collected from the different regions of Bangladesh are logical and should evaluate their ideas. However, such studies may not reveal how to limit the problem in the Nipah belt. Overall, this improved proposal has excellent potential to better understand the epidemiology of a most relevant zoonotic disease.

THE FOLLOWING SECTIONS WERE PREPARED BY THE SCIENTIFIC REVIEW OFFICER TO SUMMARIZE THE OUTCOME OF DISCUSSIONS OF THE REVIEW COMMITTEE, OR REVIEWERS' WRITTEN CRITIQUES, ON THE FOLLOWING ISSUES:

PROTECTION OF HUMAN SUBJECTS: ACCEPTABLE

INCLUSION OF WOMEN PLAN: ACCEPTABLE

INCLUSION OF MINORITIES PLAN: ACCEPTABLE

INCLUSION OF CHILDREN PLAN: UNACCEPTABLE
Justification for excluding children under 18 is unacceptable.

VERTEBRATE ANIMALS: ACCEPTABLE

COMMITTEE BUDGET RECOMMENDATIONS: The budget was recommended as requested.

Footnotes for 1 R01 AI143978-01A1; PI Name: EPSTEIN, JONATHAN H

NIH has modified its policy regarding the receipt of resubmissions (amended applications). See Guide Notice NOT-OD-14-074 at http://grants.nih.gov/grants/guide/notice-files/NOT-OD-14-074.html. The impact/priority score is calculated after discussion of an application by averaging the overall scores (1-9) given by all voting reviewers on the committee and multiplying by 10. The criterion scores are submitted prior to the meeting by the individual reviewers assigned to an application, and are not discussed specifically at the review meeting or calculated into the overall impact score. Some applications also receive a percentile ranking. For details on the review process, see http://grants.nih.gov/grants/peer_review_process.htm#scoring.

MEETING ROSTER

Clinical Research and Field Studies of Infectious Diseases Study Section
Infectious Diseases and Microbiology Integrated Review Group
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Notice of NIH Policy to All Applicants: Meeting rosters are provided for information purposes only. Applicant investigators and institutional officials must not communicate directly with study section members about an application before or after the review. Failure to observe this policy will create a serious breach of integrity in the peer review process, and may lead to actions outlined in NOT-OD-14-073 at https://grants.nih.gov/grants/guide/notice-files/NOT-OD-14-073.html and NOT-OD-15-106 at https://grants.nih.gov/grants/guide/notice-files/NOT-OD-15-106.html, including removal of the application from immediate review.

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Consultants are required to absent themselves from the room during the review of any application if their presence would constitute or appear to constitute a conflict of interest.

Introduction to Resubmission: We thank the reviewers for their helpful comments and note their enthusiasm for the overall project, its significance, innovation, impact on health and our multidisciplinary team, including: "…the PI has a very impressive track record in this area that give confidence the scientific aspects of the project can be successfully undertaken. The premise is supported by strong preliminary data"; "the science of the application is well-structured to investigate the hypothesis and is highly likely to generate important data on Nipah virus;" the "combination of epidemiological and basic research approaches is a strength;" and that "bat infection experiments to understand viral infection and potential for re-infection of seropositive bats from the region is highly innovative." We have significantly changed our resubmitted research plan in response to their criticisms, as follows:

1) *The proposal is overly ambitious.* We have significantly reduced the scope and complexity of the project by: (1) removing the targeting of hunters from Aim 1. This simplifies the protocol, reduces the need to find this rarer population, and improves the analytical power of the core aim of understanding palm sap consumption effects; (2) removing the generation of bat cell lines from Aim 2. We considered reducing intensity of bat sampling but find that our design is necessary for adequate power. We are confident we can implement this study, having a field team in Bangladesh that is experienced in frequent sampling at multiple sites.

2) *The project team is large and activities complex, making management difficult / confusion about who is PI and project workflow.* The PI (Epstein) has 10 years of experience managing projects of similar scope and complexity in Bangladesh (and four other countries) under USAID PREDICT, which includes repeated wildlife (bat, rodent, primate) and human sampling at multiple study sites in Bangladesh working with the co-investigators and field team listed in the current proposal. We have added a new figure detailing management structure and workflow by aim. In addition, we increased Dr. Flora's time from 2 to 3 months per year to give her more time to coordinate human surveillance activities.

3) *Concern about not obtaining bat viruses*. We have already successfully obtained 11 bat Nipah virus isolates from *P. medius* from two locations in Bangladesh, now published [1], which were from roost urine collection and bat swabs. Also, while our data suggests extended inter-epidemic periods, they are shorter than our than our sampling period. We expect all six bat study populations to be shedding at different points during the study period. Our sampling strategy also includes intensive bat urine collection at these sites and during human outbreaks which predictably happen each winter and which have yielded most of our isolates, to date.

4) *The power calculations are not well described.* We have detailed the analytical approach and power calculations for each aim.

5) *Clarify human clinical expertise / bat experiment experience:* Co-Is Mahmud Rahman and Merjady Flora have extensive human clinical/epi expertise. As former and current director of IEDCR they have overseen human surveillance and multiple Nipah outbreaks. Experienced medical officers from IEDCR will perform all blood draws on human subjects. Vincent Munster at NIH RML has previously designed and performed bat and hamster BSL-4 infections (see [2-5].)

6) *Confounding effects of human-to-human transmission*: **Aim 1** now more clearly tests the hypothesis that spillover has occurred in the East. Reviewer 1 raises a valid point about serology being unable to differentiate between an exposure from bats (spillover) and from other Nipah cases (human transmission). However, 1) our goal is to determine whether people have been exposed to Nipah virus outside the Nipah Belt, so in either case, a seropositive individual signifies local exposure that was previously undetected; and 2) we will incorporate village and household-level clustering into our models, to control for human transmission effects and measure the effect of zoonotic exposure via palm sap. Distant cases = separate exposure events likely linked to a local spillover event.

7) *The bat experiments do not test the stated hypothesis / don't address sex-based differences.* The bat experiment, which the other reviewers found "highly innovative" and had enthusiasm for answers a critical question about susceptibility after prior exposure and directly informs the dynamic models that will test our hypothesis under **Aim 2**. We have kept this in the resubmission because it is vital to our main goals and a novel approach. It is not feasible to house multiple male hamsters or bats together without risk of injury or death from fighting. Therefore, we have predominantly female cohorts and will not assess sex-based effects.

RESEARCH STRATEGY

A. SIGNIFICANCE

Nipah virus (NiV) is an emerging zoonotic paramyxovirus (genus *Henipavirus*) that has caused repeated outbreaks in Bangladesh and India with mean case fatality rates greater than 70% [6-8]. *The World Health Organization has recently prioritized NiV and related henipaviruses among the ten most critical pathogens that threaten global health and for which countermeasures must be urgently developed [9]*. Old world frugivorous bats (*Pteropus* spp.) are natural reservoirs of henipaviruses in Asia and Australia [10, 11]. Nipah virus has several characteristics that make it a global health priority. 1) Its bat reservoir occurs throughout Asia, overlapping human and livestock populations, giving it geographically broad opportunity to cause outbreaks [10]; 2) henipaviruses can be transmitted directly from bats to people or via domestic animals [10]; 3) NiV can be transmitted from person to person [12]; 4) spillover has repeatedly occurred in highly populous and internationally connected regions; 5) repeated spillovers of strains with varying person-to-person transmission rates indicate the ability to evolve **pandemic potential** [13]; and 5) it is associated with a high mortality rate in people and currently has no vaccine or treatment [10, 13]. In 2018, NiV outbreaks occurred in Bangladesh and southern India, approximately 1100 miles apart. While NiV outbreaks in Bangladesh occur seasonally, there are regions in Africa and Asia where bats and henipaviruses occur but where no human cases have been identified, leaving open the possibility that cryptic spillover is occurring. A major gap in assessing NiV risk is a lack of understanding of why outbreaks happen in some locations where there are bats and virus, and not others. Outbreaks in Bangladesh have primarily been associated with date palm sap consumption, followed by human-to-human transmission [14]. However, outbreaks in Malaysia and the Philippines have demonstrated alternate routes of infection: aerosolization of virus by infected pigs with respiratory disease and exposure to bodily fluids or infected tissue from infected horses via butchering or meat consumption, respectively [15] Unrecognized human or animal case clusters increase opportunity for the emergence of more virulent and transmissible strains [16].

Within Bangladesh, there is a strong spatial clustering of observed NiV cases in the western part of the country, termed the *Nipah belt* [8]. While sap consumption is most intensive in the Nipah belt, the bat reservoir, NiV, and sap consumption occur throughout Bangladesh [16]. No human clusters of NiV encephalitis have been detected in Bangladesh outside of the Nipah belt. **The major goal of this proposal is to determine whether sap-consuming populations in eastern Bangladesh have been exposed to NiV, and to determine how ecological dynamics and viral genetics each contribute to spatial differences in exposure and outbreak risk.**

This proposed project has broad significance. It will determine whether zoonotic transmission ("spillover") of NiV has occurred in regions of Bangladesh beyond where outbreaks have been observed (**Aim 1**). It will broaden our understanding of what factors may influence human outbreaks by 1) measuring differences in viral dynamics within bat reservoir populations in locations where NiV outbreaks have and have not occurred (**Aim 2**) and 2) by comparing genotypic and phenotypic characteristics of bat viral isolates from different areas under controlled experimental conditions (**Aim 3**). These studies will also improve our understanding of transmission patterns in other geographies where henipaviruses occur. This broader understanding of NiV risk aligns with WHO's prioritization strategy [9], and may be used to better target interventions such as vaccine deployment. (The Coalition for Epidemic Preparedness and Innovations (cepi.net) has very recently funded the development of a NiV vaccine) and educational outreach campaigns in resource limited settings.

B. INNOVATION.

This project is innovative in its **multidisciplinary approach** to understanding NiV spillover. **We will test complementary hypotheses** on the drivers of NiV spillover, using a combination of human surveillance and behavioral studies, ecological field studies and mathematical models, and viral phylogenetics and infection studies using **novel bat lab animal models.** Combining field and laboratory methods with mathematical modeling will allow us to gain a far deeper system-wide understanding of what drives NiV spillover. For the first time, we will apply a state-of-the art, viral genus-wide serological multiplexed assay to wildlife and human sera to screen for IgG antibodies specific to each known henipavirus. Our study will conduct, for the first time, experimental infections using multiple, diverse, wild-type bat viral isolates in the natural NiV host *Pteropus medius*. This will allow us to determine how these bats respond immunologically when infected or re-infected with NiV, and better explain henipavirus dynamics in wild bats to predict seasonal and interannual risk of NiV spillover. This will help advance the science of comparative immunology and viral tolerance in bat hosts by establishing a new host-virus model, while answering specific and fundamental questions about henipaviruses

in their natural reservoir.

C. APPROACH

The question of why outbreaks of Nipah virus occur in certain locations and not others has broad implications for zoonotic viral epidemics: henipaviruses exist in bat reservoirs across Africa and Asia, where bats, people and livestock interact in different ways and the pathogenicity of these viruses in humans is unknown. The recent outbreak in Kerala, India is a reminder that spillover is possible wherever Nipah virus, its bat reservoir, and people interact [17]. A better understanding of why Nipah might emerge in one location versus another could allow deployment of effective interventions where they're most needed. **Our overall hypothesis is that spillover occurs across Bangladesh, and is driven by differences in human behavior and dynamics of viral circulation in local bat populations, and likelihood of a subsequent outbreak is influenced by viral strain.** We test this hypothesis by 1) using serology to compare NiV exposure in people inside and outside the Nipah belt and across different sap consumption profiles (**Aim 1**); conducting longitudinal studies of multiple pteropid bat populations inside and outside the Nipah belt and using dynamic models to analyze timing, intensity, and drivers of viral shedding (**Aim 2**); and conducting BSL-4 experimental animal infections to compare genotype and phenotype of diverse NiV isolates from bats. By understanding behavioral, ecological, and virological drivers of Nipah virus spillover, public health authorities can more accurately target surveillance and intervention strategies (e.g. vaccination) to control outbreaks and prevent a pandemic.

C1. INTRODUCTION

Nipah virus epidemiology and ecology. In Bangladesh, there have been more than 15 NiV outbreaks, occurring nearly every year since 2001 [18]. Human cases have exclusively occurred November to April, within the western part of the country, referred to as the "Nipah belt" [13]. Date palm sap has been the predominant route of transmission between bats and people [8]. We recently showed that the bat reservoir of NiV in Bangladesh, *P. medius*, is infected throughout the country [19] and that people outside the Nipah belt also drink date palm sap [16]. Surveillance has been concentrated within the Nipah belt [20], and proximity to hospitals is a strong predictor of case reports [21], but broad awareness of NiV among clinics across the country and nationwide surveillance through media reports suggests that a cluster of cases would likely be reported [21, 22].

Differences in human behavior are known to drive exposure to wildlife reservoirs of emerging viruses (e.g. Ebola, SARS CoV, MERS CoV) and risk of spillover [23-25]. Sap consumption occurs across Bangladesh, but is more intensive in the western region [16]. In May 2018, a NiV outbreak was reported in Kerala, southern India – a completely new location more than 1100 mi from previous outbreaks in West Bengal and Bangladesh. The outbreak in Kerala, India, occurred in an area where date palm sap is not cultivated and spillover may have occurred through another route [17]. A case control study of risk factors other than sap found 40% of exposure risk of Nipah cases was associated with observing bats feeding in trees around houses at night [26].

Little is known about Nipah virus dynamics in *P. medius* in Bangladesh. While human date palm sap consumption explains a significant portion of NiV spillover risk, and consumption is generally higher in the western part of the country, there is sap consumption across Bangladesh and some cases would be expected in eastern Bangladesh[16]. Viral dynamics within bats may also influence the number and location of spillover events in a given year [21]. Zoonotic bat-borne viruses such as Ebola, Marburg, Nipah, Hendra and coronaviruses are thought to have seasonal spikes in infection that coincide with annual or semi-annual synchronous birth pulses [27-32]. Seasonal NiV shedding patterns have been observed in *P. lylei* in Thailand, but studies are limited to viral detection in pooled urine samples from underneath roosts, so there is no information about which individual bats are shedding. In Malaysia, we found serological evidence of Nipah virus infection in both *P. vampyrus* and *P. hypomelanus* throughout the country, with mean seroprevalence of 32.8% and 11.1%, respectively however, viral infection rates were estimated to be low and no clear seasonal infection pattern was observed [33]. *Pteropus* species are highly mobile and colonies are interconnected through bat immigration, forming a metapopulation structure [33-36]. Human NiV outbreaks occur almost annually and have all occurred in winter months (Nov-Apr), which is when sap is harvested. However, there is variability in the number, location and size of outbreaks that occur year-to-year, and some years have had none [18]. This may be due to variability in the timing of viral shedding within local bat populations. Comparing the periodicity and intensity of Nipah virus infection among different bat populations across Bangladesh is important for understanding drivers of spillover and the observed spatial and temporal patterns of human outbreaks.

Viral genetics may also influence observed outbreak patterns, particularly when it is only severe cases are detected through existing surveillance mechanisms [26]. Certain strains of Nipah virus may be more transmissible among people, and superspreaders have been identified in several outbreaks [14, 17]. Two distinct clades of NiV have been described using partial gene sequences from humans and animals: a Bangladesh clade, which includes sequences identified in India and Bangladesh; and a Malaysian clade, which comprises sequences from Malaysia, Cambodia, and Thailand [37]. Strains of NiV from these two clades are associated with differences in pathogenesis, epidemiological and clinical profiles in humans and animal models [38-42]. Primate models and human clinical data indicate that the Malaysia genotype is less pathogenic than the Bangladesh genotype, having less upper respiratory tract involvement, which may influence transmission [42]. Ferret models also showed less oral secretion when infected with a Malaysia strain than a Bangladesh strain [43]. Nipah virus cases in Bangladesh have shown more strain diversity than in the Malaysia outbreak [14], and local human infections likely reflect the strain circulating in local bat populations at the time of spillover. Comparing pathogenicity among viral strains from different regions has been challenging because there have been few NiV isolates obtained from bats or people [11, 44]. Recently, whole genomes from 10 isolates from *Pteropus medius* in Bangladesh were characterized by our group [1]. **The objective of this proposal is to identify how human behavior, viral ecology and viral genetics in bats drive Nipah virus spillover across Bangladesh.**

C2. AIM 1: Compare NiV exposure and its behavioral determinants among human populations inside and outside the Nipah belt in Bangladesh. C2.1 *Rationale and preliminary data.* Our group has studied Nipah virus epidemiology in humans, bats and livestock **in Bangladesh since 2006**, including leading an R01 (TW05689 Daszak, PI), a K08 (AI067549, Epstein, PI) and under USAID PREDICT in collaboration with icddr,b and the Institute for Epidemiology, Disease Control & Research (IEDCR)**. PI, Epstein has managed wildlife and human zoonotic virus surveillance (including Nipah virus), behavioral studies, and laboratory work with the coinvestigators named in this current proposal under USAID's PREDICT project since 2009**. We examined how date palm sap consumption influences NiV exposure by conducting a country-wide case-control study, surveying villages inside the Nipah Belt that either had (case) NiV cases or did not, and villages outside the Nipah Belt [16]. Data related to number of date palm trees present, household consumption, *Pteropus* bat abundance, and hunting was collected from 207 villages (**Figure 1A-C**). Our finding that date palm cultivation and consumption was more intensive in the Nipah belt (**Figure 1A**), but that bats and sap consumption occur throughout the country (**Figure 1C**), led to our hypothesis that undetected NiV spillover occurs beyond the Nipah belt.

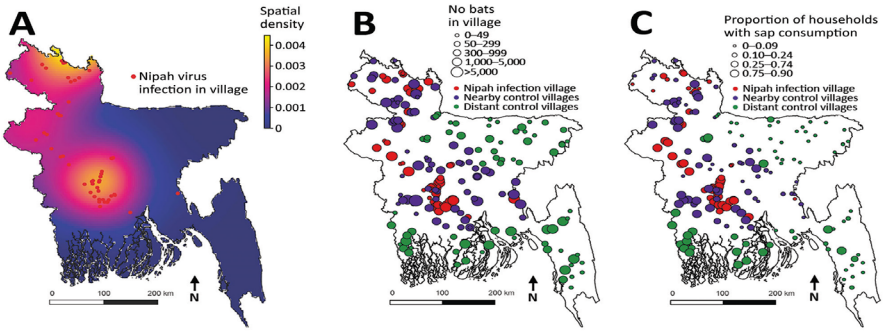


Figure 1. A) Locations of identified bat-to-human transmission of NiV and spatial intensity of transmission events, Bangladesh, 2001–2012. B) Relative sizes of the *Pteropus medius* bat populations in case and control villages (including within 5 km of each village). C) Proportion of households in case and control villages with persons who regularly consume fresh date palm sap.

Viral surveillance of people exposed to wildlife. Our team is experienced with human surveillance and includes medical officers from IEDCR, the national public health agency responsible for Nipah virus outbreak investigations. Our group is currently conducting human surveillance for known and novel zoonotic viruses in three of our proposed study locations: Faridpur, Dinajpur, and Rajshahi, where there have been prior NiV outbreaks. Under our current USAID-funded PREDICT project, **we have already collected biological samples (blood, oropharyngeal and urine samples) and detailed behavioral questionnaires from 500 people**, including people in rural villages. The work proposed here will allow us to determine whether eastern populations with known date palm sap consumption rates have been exposed to Nipah virus, how exposure

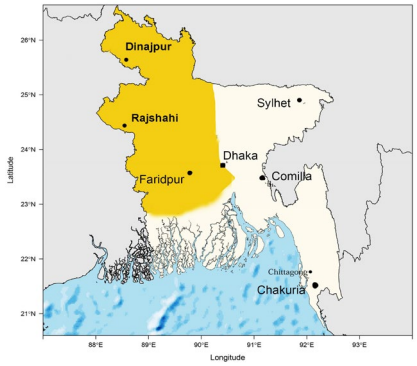


Fig 2. Map of Bangladesh study sites

rates compare to western populations, and whether different risky behaviors occur in eastern and western Bangladesh.

C2.2. *Research design*. We propose to conduct a **serological and behavioral survey of individuals in six locations across Bangladesh to determine whether people in rural populations outside the Nipah belt have been exposed to Nipah virus and how palm sap consumption, geography, and other behaviors influence exposure.** This work will be done with our partners at the Institute for Epidemiology, Disease Control & Research (IEDCR) within the Ministry of Health, and icddr,b (**see letters of support**). We will identify six study locations: three within the Nipah belt (in Faridpur, Rajshahi, and Dinajpur districts) and three outside the Nipah belt (in Sylhet, Comilla, and Chittagong districts) that represent a range of household sap consumption rates based on our previous findings (**Figure 1C and 2**) that 50% to 90% of households had someone who drinks 1 or more glasses of date palm sap per day [16]. We will use demographic data from our previous case control study to select sites in each district, capturing the range of intensity of sap consumption from low to high [16]. Villages will be paired west-east by high, medium, and low household consumption. We will randomly select households within 50km radius of a central point at each site and enroll each adult member within the household until we reach a sample size of 500 individuals per site. This gives a study cohort that includes both genders and a range of ages. Fifty km is estimated to be the distance people may reasonably travel within a day, as well as bat foraging range.

Trained medical officers from IEDCR will collect up to 10mL blood from each enrolled subject for serological testing. Serum will be serparated on ice overnight, transferred to cyrovials and transported to IEDCR in liquid nitrogen dry shippers. We will screen serum from all subjects for IgG antibodies against Nipah and all other known henipaviruses (Hendra, Cedar, & Mojiang) using a Luminex multiplex assay developed by the Broder lab and the CSIRO Australian Animal Health Laboratory in Geelong [45]. The assay will be performed at icddrb, which has a Luminex machine. This assay has previously been used to screen human, livestock, and bat sera in Africa [46-48], Australia [44, 49], and Bangladesh, including our 6-year longitudinal study of bats and a livestock survey (**C2, Fig 3 and [50])**. Positive controls for the Luminex-based and confirmatory screening assays will be monoclonal antibodies (mAbs) and polyclonal antisera generated using the recombinant viral protein antigens as well as known positive bat and human serum samples. *Confirmatory assays.* The first line confirmatory tests for our Luminex-based assay will be a Nipah virus IgG ELISA [51]. IgG-negative serum samples will also be screened by an IgM Nipah ELISA at IEDCR, which is already standard practice during Nipah outbreak investigations [52]. This activity will build technical capacity for NiV detection within partner labs that are part of the national One Health Lab network and that routinely perform NiV diagnostic testing for the Government of Bangladesh (**see icddr,b and IEDCR letters of support**).

Our social scientists will administer a questionnaire to enrolled subjects to capture history of encephalitis, confirmed NiV diagnosis or contact with a suspected NiV case, contact with bats, hunting and eating bats, household proximity to a bat roost, frequency and volume of drinking date palm sap, collecting or eating bitten fruit from the ground, and exposure to pigs, cattle and goats [53-56]. These behavioral data will be analyzed with serological data to determine behavioral risk for NiV exposure.

Data Analysis. We will use generalized linear mixed models (GLMMs) to estimate effects of region, sap consumption, and other potential risk factors, clustering observations at site and household level to control for local effects of person-to-person transmission. Assuming a conservative background seroprevalence of 2%, a sample size of 3000 individuals (500/site), allows us to a) detect the presence of any NiV IgG antibodies in the population at each site with >95% confidence; b) identify differences in seroprevalence based on region with 78% power and 95% confidence; and determine effects of sap consumption (80% power, 95% confidence) on seroprevalence. (Power calculated via 1000 simulations [57] from a GLMM with effect sizes (odds ratios) of 2 and 10% standard deviation of variance among sites).

C2.3) *Expected outcomes.* Luminex provides a continuous quantitative output (mean fluorescence index, MFI) which can be used to detect reactive antibodies in a samples and compare strength of reactivity against multiple antigens using a single sample [45]. We will compare the MFI across all specific henipavirus antigens to assess which samples are NiV positive and which may be reacting to an antigenically-related henipaviruses [46, 50, 58]. In the absence of a definitive “positive” specific reaction from Nipah soluble glycoprotein, we would

interpret reactivity from the N protein and other henipavirus glycoproteins as an indication of antibodies against an unknown henipavirus.

C2.4) *Potential problems and solutions*. There is a possibility that we will not find anyone with IgG antibodies in some of the locations of our study, particularly in the eastern region. We used a very conservative assumption of low baseline seroprevalence (2%) and high variance to calculate a sample size that gives us confidence that we will identify seropositive individuals. While there is no published data describing baseline henipavirus exposure rates in areas not associated with outbreaks, we have based our estimates on Ebola virus (also bat-borne Ebola seroprevalence in populations not associated with EVD outbreaks have been reported between 0% and 24% [59], and our own work has found seroprevalence of 1-5.5% of antibodies against SARS-like coronaviruses in populations without SARS cases. Ultimately, our sample size would allow us to be confident that a negative finding was real, and so we could look comparatively at-risk factors in western and eastern locations and identify behavioral, bat, or virus-related differences (**from aims 2 and 3**).

C3. AIM 2: Compare Nipah virus temporal dynamics in *Pteropus* bat colonies inside and outside the Nipah belt, Bangladesh. C3.1. *Rationale and preliminary data.* NiV and Hendra virus infections are relatively rare in pteropid bats [19, 33, 60]. In Australia, Hendra virus has been detected in 1%-3% of bats sampled and 2.5% of pooled urine samples sampled [44]. In Malaysia, we detected NiV in less than 1% of bats [61]. However, serology data indicates that NiV circulates broadly within and among bat colonies. In Bangladesh, we screened more than 3,000 *P. medius* for NiV over 6 years and found 20-80% seroprevalence across different sites and periods, including a 6-year longitudinal study of a single population in Faridpur (**Figs 3&4**); Our work on spatial and temporal patterns of bat henipavirus infections has generated hypotheses on drivers of spillover risk [33, 36, 44, 62], and preliminary data for this proposal, including: **Location-based differences in Nipah virus seroprevalence in bats.** We detected anti-Nipah IgG antibodies in each of eight bat colonies sampled across Bangladesh between January 2006 and November 2012 ranging from 20% (95% CI: 10%-30%; n=99) in Rajbari to 56% (95% CI: 49%-63%; n=100) in Tangail. Seroprevalence varied significantly among locations (χ^2 = 55.61, p<.001), but we did not detect differences between sites within and outside the Nipah belt. Timing of sampling could explain some of the differences, but we also found that the population structure, which can influence viral dynamics, varied across space [63, 64]. We previously used satellite telemetry to show that *P. medius* exists as a metapopulation, and that connectivity among susceptible bats across multiple roost sites may allow henipavirus persistence even at low incidence [19, 36]. However, the more sedentary nature of *P. medius* in Bangladesh compared to *P. vampyrus* in Malaysia, and the short infectious period for NiV may create a patchy landscape of viral dynamics and diversity across bat populations in Bangladesh. This may explain the high viral diversity in disparate bat populations in Bangladesh (e.g. Faridpur vs. Comilla) and, by proxy, in human cases at different sites [36]. We also found that *P. medius* prefers to roost close to human settlements, rather than in intact forest [64]. Availability of roosting habitat and landscape types, including urban centers, as well as individual variation in viral shedding (e.g. bat superspreaders) can influence henipavirus dynamics [35, 65].

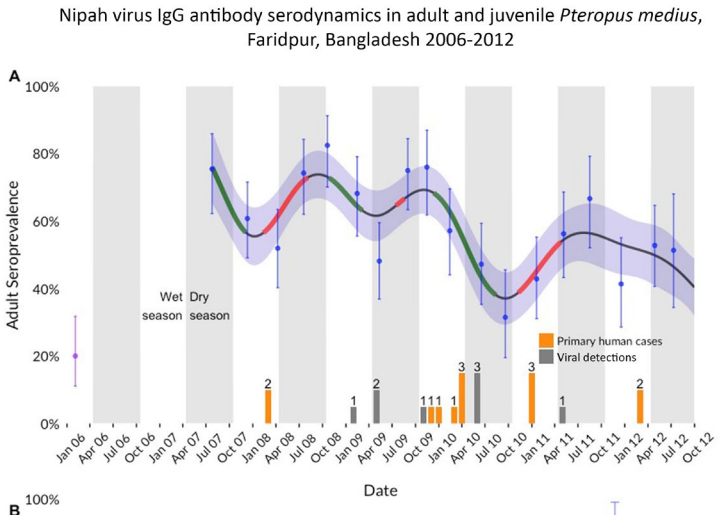


Fig 3. Seroprevalence fluctuations in the Fairdpur bat population. Observed data (blue points ±1 SE) and GAM time-sereis fit, with periods of significant increase (red), and decrease (green). Bars indicate viral detections and local human cases.

Evidence for NiV in bats outside the Nipah belt. Between 2006 and 2014, we detected NiV RNA in individual bats and pooled roost urine both inside (Rajbari, Thakurgaon, and Faridpur) and outside (Comilla and Sylhet) the Nipah Belt. **Of the pooled urine samples, three positive samples from Bhanga (n=19) and 16 from Joypurhat (n=19) were collected during active human outbreaks.** The estimated viral prevalence in individual bats from Rajbari in January 2006 was 3.8% (95% CI: 0% -11%; n=78). Viral prevalence estimates (18 sample points, 100 bats each) during the longitudinal study in Faridpur ranged from 0% to 3% (95% CI: 0%-10%; n=100) (**see C3.1c**). **The estimated detection rate from pooled urine samples across the entire**

study was 2.7% (+/- 1.6%; n=829).

Temporal fluctuations in bat NiV seroprevalence are equal to or larger than location-based differences. We found significant fluctuations in adult and juvenile seroprevalence in a single bat population sampled repeatedly over a six-year period (**Figs 3 & 4**). Juvenile seroprevalence ranged from 0% to 44% (95%CI 37%-51%), and decreased over the first year of life, with significant lower prevalence from mid-October to mid-December. Adult seroprevalence ranged from 31% (95% CI: 20%-46%) to 82% (95% CI: 77%-87%) and went through three periods of significant increase during the study, approximately two years apart (**Figure 3**). These periods did not occur in the same season each year. Each period was preceded by significantly decreasing seroprevalence [19]. **Sporadic human outbreaks may be explained by fluctuations within an approximate two-year NiV Inter-epidemic period in bats.** We developed a parameterized mechanistic model of NiV transmission using demographic, serological, immunological, and virological data from our longitudinal study in Faridpur and published data (**Figure 4**). Model simulations suggest henipaviruses require periodic re-introduction via recrudescence or immigration to persist. Persistent henipavirus infection was observed in an isolated island population of *E. helvum* [58, 66], and NiV recrudescence has been observed in *P. vampyrus* [67] and in humans [68], indicating that **recrudescence leading to outbreaks within colonies is a likely mechanism of viral persistence.** However, re-introduction or recrudescence of NiV may not guarantee viral circulation in bats and depends on the proportion of susceptible adult bats at the time. Further, our longitudinal data was collected from a single bat population within the Nipah belt, it is unknown whether this is representative of bat viral dynamics across Bangladesh. **The high spatial variation in seroprevalence we found across sites may be due to either differing timing of viral circulation or differences in overall circulation rates.** Bat populations in other parts of Bangladesh may have different viral dynamics due to their size, connectivity, environment, or the genotypes of henipaviruses circulating locally. **We propose to conduct six parallel longitudinal studies of additional bat populations and model the viral dynamics, and experimental tests of reinfection as a driver of circulation, to test the hypothesis that transmission dynamics are heterogeneous across space, which may influence the frequency of spillover into human populations.**

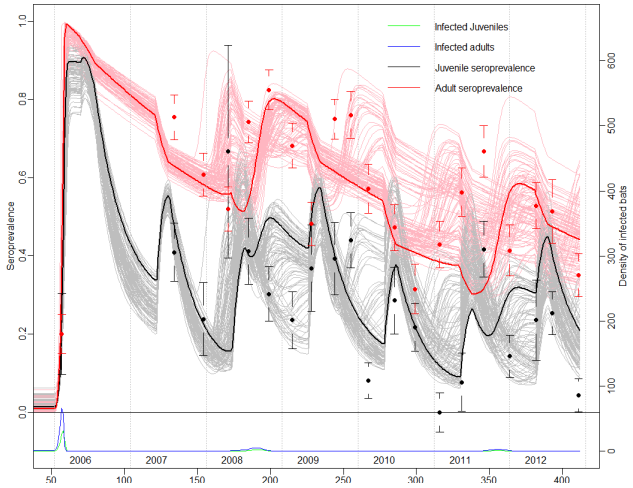


Figure 4. Mechanistic model simulations of NiV serological dynamics in adult and juvenile bats in Faridpur. The observed data (red and black points ±1 SE) and model fit (solid lines) for the fraction of adults and juveniles seropositive for NiV (left axis), and the model estimated density of infected adult and juvenile bats (bottom panel and right axis).

C3.1b *Research Design: field sampling and modeling bat viral dynamics*: We will conduct six parallel 36-month longitudinal studies of NiV infection in *P. medius* at three locations within the Nipah belt, including the Faridpur population previously studied, Rajshahi, and Dinajpur, in western Bangladesh; and three locations in eastern Bangladesh: Sylhet, Comilla, and Chakuria (**Figure 2**). We will select bat roosts that are comparable in size and structure to the Faridpur colony (e.g. number of bats, area of trees containing the roost, proximity to village center and closest urban center), which had ~1,000 bats at its maximum size. We will capture and sample 60 bats at each site every two months for 36 months. We will perform roost counts at flyout (dusk) on the first night of each sampling trip. We will capture adult and juvenile bats, but sample no more than 10 juveniles per period. Given the range of seroprevalence we observed previously (20% - 56%), 50 adult bats will allow us to determine seroprevalence differences of at least 20% with >95% confidence. Bats will be captured using mist nets, and anesthetized using isoflurane gas and a portable vaporizer as previously described [69]. Field personnel will wear appropriate personal protective equipment, including dedicated long clothing, an N95 or P100 respirator, nitrile gloves and safety glasses [69]. We will record each bat's age class (juvenile or adult), sex, mass, and body condition, and collect up to 3.0ml blood, and either urine or urogenital swabs. All bats will be released at the site of capture following sampling. One set of urine samples will be placed in Trisol and the other in viral transport medium and immediately stored at -80C in a liquid nitrogen vapor cooler (dry shipper). Blood will be collected in 3.0ml vacutainer tubes with clotting factor (red top) and allowed to stand vertically on ice overnight so that serum separates. The following morning, serum will be aliquoted into two cryovials and frozen neat at -80C.

To maximize detection of viral RNA and virus isolation when bats are shedding, a locally resident team of

field technicians will collect pooled urine samples twice per week from underneath the study colony at each site using 2x3m plastic sheets while bats return from foraging at approximately 0600h as conducted previously [70-72] and (**section C4.1**). Technicians will wear full PPE as described above but with hooded Tyvek suits and face shields. Pooled samples from each sheet will be split and aliquoted into lysis buffer and VTM, placed into a liquid nitrogen dry shipper and sent to icddr,b every 15 days. Urine samples will be tested by experienced technicians, trained in biosafety, at icddr,b using a real-time PCR assay that targets the N gene region of NiV [73]. When a positive PCR reaction is discovered, urine samples will rapidly be collected from individual bats in the colony to maximize opportunity for isolating multiple viral strains. We will also test urine samples using a consensus RT-PCR paramyxovirus assay that targets the nucleocapsid gene region, and has previously detected novel Nipah-related viruses in *P. medius* under our USAID-EPT PREDICT funding [74, 75]. Samples will be stored in a -80C freezer at icddr,b or IEDCR until tested.

Data Analysis: We will characterize and compare bat-viral dynamics using two modeling approaches: a nonparametric model to measure the periodicity and seasonality of NiV circulation events in the bat populations; and a mechanistic, stochastic, model to derive epidemiological parameters to compare across populations. For the nonparametric approach, we will use Generalized Additive Mixed Models (GAMs) [76], to fit curves estimating the time-varying seroprevalence at all sites. Seasonal and inter-annual effects will be estimated separately. This model will allow us to determine (a) periods with significantly increasing seroprevalence from which we can infer active viral circulation in the bat population, (b) the degree to which seasonality and periodicity of viral circulation are consistent and predictable within sites, (c) the degree to which seasonality and periodicity are common among sites in the region, and (d) across all sites. We estimate that we will be able to detect differences between eastern and western sites in overall seroprevalence of 20% with >95% power, and differences in timing of one quarter with 75% power. (Estimated via 1000 GAMM simulations with site-to-site standard deviation of seroprevalence 20% and timing of 1 month, 95% confidence).

To estimate epidemiological parameters we will fit a stochastic, age-structured, Susceptible-Infected-Recovered-Susceptible (SIRS) model which includes bat demographic processes. We will use partially observed Markov Process framework (POMP) [77] to fit this model. This will allow us to estimate the number of infected individuals based on the recovered (seropositive) dynamics, as well as a time-varying R_0 value in the bat population, transmission rates among different age classes, infectious periods, and re-infection rates. As with GAMs, parameters from captive bat experiments will be incorporated resolve key ambiguities that cannot be resolved from the time-series data, primarily bounding recrudescence and antibody waning parameters.

C3.1d *Expected outcomes.* We expect that there will be differences in the temporal dynamics of each bat population. We do not expect significant differences in roost demography based on location (adults vs. juveniles, male vs. female). **Our preliminary data suggests that we will see at least one episode of viral circulation within a three-year period at each field site**. We are confident that we will be able to capture differences in infection dynamics among our study sites. Our previous experience is that viral isolations were infrequent but high during high circulation events in bats, including around human outbreaks. **We estimate we will generate 15 new isolates if each site has only a single high-circulation event over the course of the study**, in addition to those collected at outbreak events. (Assuming 50% positive during such events, which were 84% in our previous work).

C3.1e *Potential Pitfalls and Solutions.* It is possible that our selected bat roost will be disturbed or destroyed during the study. Bats utilize multiple roost sites within at least a 10km radius and regularly shift among these sites [19]. Therefore, we would be able to identify nearby roosts that represent the same local bat population. We will microchip bats at each site to determine whether the new site includes bats from the initial site.

C3.2 **Experimental wild-type NiV infection of *P. medius* with and without anti-NiV IgG antibodies.**
C3.2a. *Rationale and preliminary data*. Studies of NiV transmission in free-ranging bats are limited by low infection rates. Serology data, while more readily obtained, is an indirect measure of infection. For example, IgG antibodies indicate past infection, but it is difficult to know exactly when, or to which virus a bat was exposed, **and it is unknown whether IgG positive bats can recrudesce, be re-infected by, or shed virus.** Experimental infections will provide fundamental information about henipavirus pathogenicity and transmission that is impossible to obtain from sampling wild bats. Previous studies of NiV infection provided data about viral replication, shedding and host immunological response in pteropid bats, but relied on a human NiV isolate for inoculation [11], which limited the interpretability of results [11]. Recently, our group has obtained 11 viral isolates from *P. medius* (**Section C4**), allowing us to study wild-type NiV in its appropriate host under

controlled experimental conditions. Very few laboratories have experience conducting experimental bat infections using zoonotic viruses – especially viral select agents. Among them are AAHL in Geelong, with whom we've conducted Nipah and Hendra infections in *Pteropus* species [11] and Rocky Mountain Laboratories in Montana [4], with whom we are currently collaborating under this proposal and who now have facilities designed to house pteropid bats and will receive our Nipah isolates (see **letter of support & MTA**). **Our aim is to conduct an experimental infection at NIH RML to determine whether seropositive *Pteropus* bats can be infected by NiV and sustain a productive viral infection.** This will allow comparison of the duration and intensity of viral shedding in bats with no detectable antibodies to Nipah, representing susceptible members of a colony, and those with MFI readings equal to a positive IgG titer that represent "recovered" or resistant individuals. These empirical data cannot be obtained through studies of free-ranging bats and is critical for parameterizing our models so as to be able to compare viral dynamics across sites.

PI Epstein and Co-I Daszak are among the few **that have experience internationally transporting *Pteropus* bats.** We organized successful capture, quarantine, and export of *Pteropus vampyrus* from Malaysia to CSIRO AAHL, Australia for experimental infection studies [11]. We will catch and quarantine 60 adult female *Pteropus medius*. Bats will be sourced from Raipur District, where we have previously isolated NiV [1](**section C4.1**: **Aim 3**). We will house them humanely in a closed facility that has double fencing and prevents exposure to wild bats as we have done previously [11, 67], and test all 60 bats for NiV IgG antibodies using a Luminex assay and IgG ELISA and for viral RNA in urine and saliva using a real-time NiV PCR weekly for three consecutive weeks. We will select 34 individuals for shipment: 16 without detectable IgG antibodies; and 16 with IgG antibodies of a similar MFI level (within 10% of mean MFI) using the Luminex assay described above (**section C2.2**), plus two additional controls for histopathology. Our experienced team will handle bats while sampling, and animal care staff will also be trained in biosafety and use of PPE when feeding/cleaning quarantine cages (dedicated clothing, nitrile gloves, eye protection and N95 respirators. The remaining bats will be released by our team at the original roost site. The seropositive and seronegative groups will be separated after the initial test and held in quarantine until all individuals have three successive negative PCR tests. Bats will then be exported from Bangladesh, with all appropriate CITES and export permits from the Bangladesh Forest Department, and US import permits from US Fish and Wildlife and US CDC, and sent to NIH RML where they will be housed as two separate groups under BSL 2 conditions until the start of the experiment.

C3.2b. *Research design: experimental infection of wild caught* Pteropus medius *with wild-type NiV.*
Bats will be moved into the BSL 4 lab 1 week prior to NiV inoculation so that they become acclimatized. We will then inoculate 32 bats (16 seropositive and 16 seronegative) with 10^5 $TCID_{50}$ NiV_{Ray} which is currently in process of being transferred from AAHL to RML (**see MTA**). Bats will be inoculated with a combination of intranasal (25ul each nostril) and intraperitoneal (100ul) routes. Two mock inoculated bats will be included as controls and used for histopathology and DNA genomic sequencing. Mock inoculated bats will be inoculated with standard tissue culture media via the same routes and volumes described above. Animals will be monitored daily for signs of disease, including change in temperature and weight. Blood, oropharyngeal and urine samples or urogenital swabs and rectal swabs will be collected on days 1-15 PI. The experiment will run for 56 days. On days 4, 8, 16, 20, and 32, 48, and 56, 2 bats will be euthanized and we will collect trachea, heart, lung, liver, spleen, kidney, duodenum, coon, bladder, nasal turbinates, and brain for virological, clinical chemistry, gene expression, histopathological analysis. Throughout the experiment shedding from the respiratory and urogentical tract will be analyzed by qRT-PCR and virus titration. Pre-inoculation and end-date blood draws will be used to isolate PBMCs. An Elispot assay will be used to determine the level of pre-exposure to Nipah and the development of interferon-γ secreting CD4 and CD8 T-cells after infection.

Data Analysis: We will us GLMs to compare outcomes between groups and GAMs to conduct area-under the curve analysis to measure viral shedding. We will have at least 89% power to detect infection rates as low as 10% in the seropositive bats as late as the last day of the experiment when the fewest of each group (9) will be available for testing (95% power, Wald exact binomial test).

C3.2c *Expected outcomes.* **This experiment will provide critically important information missing from mechanistic models of Nipah transmission within bat populations**, and would allow us to more accurately understand viral outbreaks in bats and spillover to humans. We expect to see the seropositive group have limited or no viral replication post inoculation. We may see viral replication in endothelial cells and in certain organ tissue, but without viral excretion. If the seronegative bats are truly naïve, we would expect to see a

productive infection over a 10-18 day period that includes shedding of virus in urine and saliva [78]. The duration and intensity of viral infection in the seropositive individuals will be an important outcome and will be highly informative.

C3.2d *Potential problems and solutions.* One limitation of this experiment is that we may not be able to differentiate between seronegative bats that have never been exposed to NiV (naïve) and those with prior exposure but with titers below the Luminex detection threshold. We have observed high (>70%) seroprevalence and individual seroreversion (change from seropositive to negative) in individual recaptured bats, and in experimental studies, so we know that individual titers wane over time [19, 79]. This suggests that most bats are exposed to NiV at some point. To assess the historic exposure status of the animals we will develop a bat interferon-γ Elispot assay to detect IFN-γ secreting CD4 and CD8 T-cells. **This experiment reflects a natural state where most adult bats in a colony are either seropositive *or have been previously exposed and are susceptible***.

C4. AIM 3: Compare pathogenicity and transmissibility of diverse Nipah virus isolates from bats inside and outside the Nipah belt, using animal models. C4.1 *Rationale and preliminary data*. Nipah virus genetics appears to influence its pathogenicity and transmissibility, but the relationship between genotype and phenotype has not been adequately studied. In the first recognized NiV outbreak in Malaysia 1998-99, person-to-person transmission was observed rarely [80-82], but in Bangladesh person-to-person transmission has accounted for over one-third of recognized cases [53]. Only 14% of patients infected during the Malaysian outbreak presented with cough, and only 6% had abnormal chest radiographs [83]. By contrast, 62% of confirmed Nipah patients in Bangladesh had cough, 69% developed difficulty breathing with chest radiographs often showing substantial pulmonary involvement [84]. Differences in human-to-human patterns of spread and mortality rates have been observed in different outbreaks in Bangladesh, and superspreaders identified in some [8, 85]. Further, we detected non-neutralizing antibodies against NiV in 44% of 138 pigs in Bangladesh with no history of severe respiratory or neurologic disease, suggesting that they may have been exposed to a less pathogenic strain of NiV [50, 54 , 86].

Animal models have shown differences in pathogenicity, disease progression, and transmissibility among NiV strains. In nonhuman primate models, the Bangladesh (NiV-B) strain was more pathogenic than the Malaysia strain, NiV-M [42]; in Syrian hamsters, NiV-M caused more rapid onset of disease [40] and ferrets had significantly higher viral load in oral secretions when infected with NiV-B suggesting a greater likelihood of NiV-B being transmitted through close contact [38]. Syrian hamsters are a lab animal model that replicate human respiratory and neurological disease when infected with NiV [2, 87, 88]. Our group has extensive experience performing transmission and pathogenicity experiments with NiV using a hamster model, under BSL 4 conditions [2, 3, 5, 40]. **Our aim is to test the hypothesis that diverse bat Nipah virus strains from different locations in Bangladesh have differences in pathogenicity and transmissibility in the hamster model (at NIH RML).** If full genome human NiV sequences are obtained by IEDCR concurrently with bat NiV sequences during an outbreak, we will also compare them to assess genetic differences between local bats and humans and the clinical outcomes observed in the outbreak.

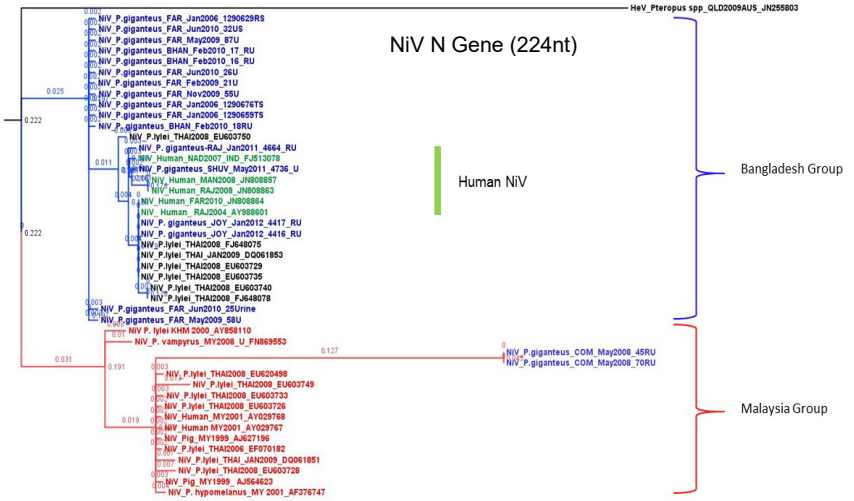


Fig 5. **Nipah Virus phylogenetic tree:** Clustal W alignments on N (224nt), G (1826 nt) and P (2174 nt) gene sequences. *P. medius* NiV sequences shown in blue and human sequences from Bangladesh shown in green. Other sequences from bats, pigs and humans in Malaysia, Thailand, and Cambodia are in black or red. Sequences from Comilla cluster within the Malaysia NiV group.

Evidence for NiV diversity in bats in different parts of Bangladesh. We have strong evidence for naturally-occurring NiV strain variation and have detected more than 15 different NiV variants, including a **highly differentiated strain from eastern bat populations in Comilla** (**Figure 5**). The N gene sequences from bat colonies in Faridpur collected between 2006 and 2011

were identical in the N gene region, showing stability over time. Human NiV N gene sequences collected from various locations within the Nipah Belt over the same time period show more nucleotide diversity than those from a single *P. medius* population over multiple years (Faridpur), but across the Nipah belt, human NiV sequences were nested within the larger diversity found in *P. medius*. By contrast, **the viral N-gene sequence detected from bats in Comilla, 150km east of Faridpur, was significantly (~20%) divergent from those in bats and humans in the western part of Bangladesh, and more closely related to Malaysia than Bangladesh NiV strains.** *These findings suggest that geographic, not temporal, variation is the primary determinant of NiV natural sequence variation.*

Genetic differences in NiV strains found in the East may, in part, explain why human clusters have not been observed. NiV Malaysia and NiV Bangladesh are about 91-94% similar. If a difference of 6% nucleotide diversity can affect the clinical phenotype (i.e. Malaysia vs. Bangladesh strains), then it is plausible that there would be phenotypic differences in a NiV strain that was 20% divergent from known strains.

Bat viral isolates obtained during outbreak investigations. We currently have a partnership with IEDCR, via an MOU, through which we participate in outbreak investigations where we can locate and intensively collect roost urine from bats at the site and time of human cases, as close in time as possible to the initial spillover event. **This approach has resulted in us obtaining 12 viral isolates from bats in a 2013 outbreak**, which we can use in animal model experiments at RML. In April 2013, we collected urine samples under *P. medius* roosts during an outbreak investigation in Raipur, Pabna. Full-length genome sequences were characterized from 10 of the bat NiV isolates [1]. All bat isolates had an almost identical genome sequence with 99.9% conservation among all ten genomes characterized in this study. The complete sequence identity match among the isolates obtained during the outbreak investigation in Raipur suggests there were not multiple strains co-circulating in the Raipur bat population at the time of sampling, supporting our previous study in Faridpur where prevalent strains may persist over time in a population. In addition to the outbreak-associated isolates, we isolated virus from an individual bat in Sylhet (eastern Bangladesh) during normal sampling activities[1]. There has not yet been any experimental study using outbreak-associated bat viral strains *in vivo that* links viral genetic characteristics with clinical and epidemiologic outcomes. Previous experiments with NiV have relied on using highly passaged viral cultures from Malaysia, which were derived from pigs and passaged through many cell lines; or Bangladesh strains from humans which were also passaged through cell lines [11, 43]. **Our group has the only set of viral isolates from *Pteropus medius*, the actual natural reservoir and source of human infection in Bangladesh. We also now have a proven methodology for sampling bats that has significantly increased our NiV detection and isolation rate.** Our isolates from Raipur and Sylhet are currently being transferred from AAHL (Geelong) to NIH RML (**see MTA**) which will allow us to use them in the proposed animal model experiments. **RML will conduct these experiments as a collaborative endeavor, (charging only for supplies) which makes them achievable under this proposal timeline and budget.**

C4.2. *Research Design*

C4.2.a *Experimental infection of hamsters with bat NiV strains to assess pathogenicity and transmissibility.* We will select six of the most genetically diverse bat NiV isolates obtained from *P. medius* and use them in Syrian hamster experimental infections to compare transmissibility and pathogenicity. **This will be conducted in two separate experiments – one to compare pathogenesis among viral strains, and a second to compare transmissibility.** The experimental design for the analyses of differences in pathogenicity and transmissibility between different isolates of Nipah virus has previously been used by our group to determine difference between Nipah virus Malaysia and Nipah virus Bangladesh [2, 5, 40, 89, 90]. We will use six different isolates, including two already obtained (**see section C4.1)** to infect Syrian hamsters. All experimental animal studies will be conducted by Co-I Munster under BSL 4 conditions at NIH NIAID Rocky Mountain Lab (**see RML letter of support**). We will use animal models to compare the transmissibility and pathogenicity of diverse NiV **wild-type** strains obtained from bats. We will select candidate strains for use in the experiment by conducting a whole genome phylogenetic analysis for each isolate and look at % similarity of each gene region to a base genome, which will be the genome from our isolate in Raipur. We will examine nucleotide-level nonsynonymous mutations across genomes for evidence of positive selective pressure, and identify the % nucleotide and amino acid differences between each isolate and the Raipur genome. We will select 2 additional isolates from within the Nipah belt and 2 from outside the Nipah belt that have the greatest phylogenetic distance from the basal (Raipur) genome. Since it is currently unknown exactly how genetic differences correspond to phenotypic differences, this approach maximizes our opportunity to look at diversity and disease outcome and whether location influences genetic diversity (e.g. the 20% partial N-gene divergent

NiV we found in Comilla). We will use an adaptive selection strategy should new information arise during the project period that would allow us to more effectively choose isolates for the experiments.

Bat Nipah virus isolation. As soon as a suspected Nipah virus cluster is identified by IEDCR, and an investigation initiated, we will travel to the outbreak site and identify 3 bat roosts located within 5km of the index case's village. We will collect urine under each roost daily for 10 consecutive days by laying down 4 2mx3m plastic sheets under each roost daily, and pooling all urine droplets on each tarp to make one sample. Each sample will be divided evenly and placed in a cryotube with Trisol for testing by real-time PCR at icddr,b [73] and another tube with VTM for viral culture which will be stored at -80C until testing. PCR-positive bat samples and their paired VTM sample will be sent to NIH RML for whole genome sequencing and viral culture under BSL 4 conditions [37]. Briefly, vero cells in 96-well plates will be prepared in EMEM containing 10% fetal bovine serum and 1x Antibiotic-Antimycotic. 50 µl of each individual sample will be added to two wells and incubated for 90 min. Inoculum will be removed and 200 µl EMEM was added to each well. At 7 days post infection (dpi) at 37°C the plates were checked for cytopathic effect (CPE) and subsequently frozen at -80°C. Putative virus culture from wells showing CPE will be passaged a second time by inoculation of a 24-well plate containing 80% confluent Vero cells in EMEM with 80 µl culture supernatant from each of the positive wells. Plates will be incubated at 37°C for 90 min, inoculum removed and 1 ml EMEM added. Plates will then be incubated at 37°C for 5 days. For a third passage, 300 µl of culture supernatant will be transferred to a 25 cm-flask containing 80% confluent Vero cells. The flasks will be incubated for 90 min at 37°C then the inoculum will be removed and 5 ml EMEM added. The flasks will be incubated at 37°C and viral supernatants harvested at 2-3 dpi. Supernatants will be clarified by centrifugation at 10,000 RCF for 5 min and frozen at -80°C.

Whole genome sequencing for phylogenetic comparisons. A virus enrichment strategy [91] will be used at RML for bat NiV samples to obtain the full genome sequence for the NiV isolates. All reads will be mapped to NiV sequence JN808863 using the Map to Reference function in Geneious (Geneious version 7.1.6). The consensus nucleotide sequence will be selected by majority rules at each site. To ascertain the 5' and 3' ends due to low coverage and PCR errors and chimeras, full genome Nipah virus sequences from GenBank will be aligned using MAFFT in Geneious. A 90% similarity threshold will be used to generate consensus 5' and 3' sequences of 120 nucleotides. Full genome henipaviruses will be downloaded from GenBank and an alignment generated with the MAFFT plugin in Geneious. Node robustness will be tested using 1,000 bootstrap replicates under the GTR+ Γ model in PHYML [92].

Experiment 1: Comparing pathogenicity of NiV strains from inside and outside the Nipah belt.
In this study, we will analyze the differences in NiV phenotypes in the Syrian hamster model. We will use six experimental groups of 10 animals – one for each selected bat viral isolate, and a control group with 5 animals. Animals will be challenged intranasally with 10^{3-5} $TCID_{50}$ of NiV. Control group will be inoculated with uninfected cell supernatant. A single intranasal inoculation will be delivered in a 60 µL dose (30 µl/nare). Oral and nasal swabs will be taken daily following infection. On day 5 after challenge, four animals from each group will be euthanized to monitor virus replication. The remaining six animals will then be monitored for survival for up to 28 days. All manipulations will be conducted on animals anesthetized with inhalational isoflurane. Blood samples will be collected from the hamsters before inoculation and at the endpoint. No more than 10% of the blood volume in a two-week period will be collected. A single, terminal blood sample will be collected at the end of the experiment. Animals will be anesthetized and bled via cardiac puncture. Virus titers and viral RNA load will be measured from swabs obtained from the nose and throat. Virus titers and viral RNA load will be measured in target organs, such as the lung, brain and spleen. Survival rates will be analyzed for all groups.

Experiment 2: Comparing transmissibility of NiV strains from inside and outside the Nipah belt.
In this experiment, we will analyze the contribution of shedding and contact transmission using the same viral isolates as in experiment 1. We will use 6 groups of 12 hamsters (70 total). Each group will have two sets of 6 hamsters. For each group of six, three will be inoculated with NiV, then on day 1 after inoculation, an additional set of 3 naïve hamsters will be put together with the inoculated animals. All naïve animals will be monitored for clinical signs and survival for up to 28 days. In order to obtain consistent results we will do 2 sets of six animals per NiV strain. Video of each experimental group will be collected for 3 hours on day 1-4 post inoculation and contact behaviors characterized. As in Experiment 1, nasal and oral swabs will be collected daily and a pre-inoculation and terminal blood sample will be collected to screen for antibodies. Several different parameters will be investigated in the experiment outlined above. Virus titers and viral RNA load will be measured in target organs, such as the lung, brain and spleen from animals reaching end-point criteria. Survival rates will be analyzed for all groups. Seroconversion will be analyzed using a NiV G specific ELISA assays.

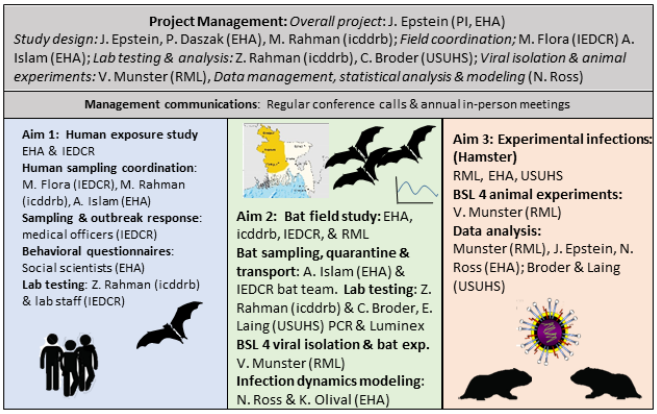
Data Analysis: We will analyze outcomes of hamster model infections using GLMs, with strain being the main effect and survival rate, viral titer, and presence of clinical signs as the outcome. To avoid multiple comparison issues, we will model viral titer across tissue types and clinical signs as a single, multivariate outcomes rather than a series of individual measures. We expect to capture large, qualitative phenotypic differences between strains similar to those between Bangladesh and Malaysia strains measured in experiments with comparable sample sizes [39, 93], where differences in viral titer in organs between strains were an order of magnitude - essentially binary responses. For such binary responses, assuming 3X difference in probability of outcomes (0.75/0.25), we will detect differences in symptoms measured in euthanized animals (4) with 68% power, differences in remaining animals (6) with 81% power, and differences measured in all animals (10) with 94% power. We will detect differences in transmission (also a binary outcome) with 81% power. (Exact binomal Wald statistics used in all cases). To determine whether there will be significant differences in the time to death between viruses, a log-rank test will be performed. In addition, area under curve analyses will be performed to determine difference in magnitude of shedding.

C4.2.b *Comparing human and bat NiV sequences obtained during human NiV outbreaks.* IEDCR has agreed to send PCR-positive human samples to RML for whole genome sequencing. If we are able to sequence full Nipah virus genomes from humans and bats within the same outbreak, we will conduct comparative phylogenetic and functional genomic analyses to look at mutations that have occurred after spillover and also to test the hypothesis that human viral genomes are most similar to the virus circulating in local bats compared to virus in bats from other locations.

C4.2c *Expected outcomes.* **We have been successful in isolating NiV from bats**. The hamster experiments will provide key information about differences in tissue tropism, clinical signs, and how strain genetics may influence transmission and epidemic profiles. Our whole genome phylogenetic analysis will provide new insight into which gene regions show greatest positive selective pressure, and how genetics correlates with epidemiologic and clinical profiles.

C4.2d *Potential problems and solutions.* Challenges in isolating virus from bats: **First, we already have 12 bat NiV isolates in hand from 1 location within and 1 outside the Nipah belt [1] demonstrating that both collection methods (outbreak urine collection and individual bat sampling) have been successful**. We are confident that we will obtain at least four additional isolates necessary to fulfill the requirements for this aim. Human Nipah outbreaks occur annually. There were an estimated 57 NiV spillovers (bat-to-human transmission) detected in Bangladesh between 2007 and 2013 [21], an average of more than 9 spillovers per Nipah season. We conservatively estimate that IEDCR will detect and investigate three outbreaks per year, from which we may obtain viral isolates from associated bats. We expect to have at least 15 opportunities to isolate virus (3 from outside the Nipah belt, 3 from within, plus at least 9 outbreaks).

Project management and timeline.



Project Timeline					
Aim - Activity	Y1	Y2	Y3	Y4	Y5
AIM 1 - Establish sites / IRB / human sampling	3.25 y				
AIM 1 - Luminex testing, human samples		3.5 y			
AIM 2 - Establish sites / IACUC / bat sampling	4.0 y				
AIM 2 - Outbreak associated bat sampling	3.0 y				
AIM 2 -Luminex & PCR testing bat samples		4.0y			
AIM 2 - Permits; quarrantine bats & ship to RML		1.0 y			
AIM 2 - Bat experiment at RML			<1 y		
AIM 3 - Screen bat samples (icddrb) & culture (RML)		3.5 y			
AIM 3 - Hamster epxeriments at RML				<1 yr	
Data Analysis and Modeling		4.0 y			
Communication plan (video or in-person mtgs)					
Reporting and scientific publication					

From:	Jon Epstein on behalf of Jon Epstein <epstein@ecohealthalliance.org>
To:	Christopher Broder
Subject:	Nipah R01
Date:	Tuesday, September 24, 2019 6:01:09 PM
Attachments:	Reviewer summary 1R01AI143978-01A1.pdf Nipah virus R01 Research Strategy 2018_12p_R1_Dec 7_final_no refs.docx

Chris,
I'm going to resubmit our Nipah R01 on Oct 7th. I've had extensive discussions with EunChung about it - it got really close to being funded, but now it's got to go back in as a new submission.

Given your stellar track record with NIH, would you be willing to go through it and the reviewer comments with me and let me know what you think we could change to get it funded?

-Jon

--

Jonathan H. Epstein DVM, MPH, PhD

Vice President for Science and Outreach

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SUMMARY STATEMENT

PROGRAM CONTACT: **(Privileged Communication)** ***Release Date:*** **04/29/2019**
Revised Date:

Application Number: **1 I80 VP000180-01**

Principal Investigator

OLIVAL, KEVIN J.

Applicant Organization: ECOHEALTH ALLIANCE, INC.

Review Group: **ZRG1 ETTN-U (80)**
Center for Scientific Review Special Emphasis Panel
Special Topics: Global Health Engagement in the Military

Meeting Date: **04/10/2019** ***RFA/PA:*** **VP18-011**
Council: **AUG 2019**
Requested Start: **11/01/2019**

Project Title: **Strengthening biosurveillance and early detection capabilities for MERS-CoV and other coronaviruses in USCENTCOM and USEUCOM**
SRG Action: **Impact Score:39**

Human Subjects: **X8-Human subjects involved - Exemption #8 designated**

Project Year	Direct Costs Requested	Estimated Total Cost
1		928,626
2		951,728
3		968,751
TOTAL		2,849,106

1I80VP000180-01 Olival, Kevin

RESUME AND SUMMARY OF DISCUSSION: This proposal plans to improve the diagnostic capability for detection of coronaviruses (CoV). Coronaviruses are responsible for Severe Acute Respiratory Syndrome, Middle East Respiratory Syndrome, and perhaps other severe and acute infections. The intent in this proposal is to leverage existing relationships in Jordan and the Republic of Georgia to develop a new assay system for coronavirus surveillance based on microsphere luminescent multiplex technology in animals and humans and to detect disease.

The discussion raised several questions with regard to the stage of development of the assay, the strength of the preliminary data to date, the selection of geographic areas for field testing, and additional issues. Specific points included:

- The assay system is at a preliminary stage of development and still requires validation.
- The preliminary data are not sufficiently robust to proceed with field testing and the time required to have sufficient confidence in the multiplex read out may be more than currently estimated
- A performance comparison of the candidate assay with other existing methods is not described
- Other parts of the world with a U.S. military presence have higher endemic rates of coronavirus infections and may harbor larger reservoirs. The selected areas may have limitations to fully test the system and assess its utility and potential impact.
- A method to distinguish samples from people that were exposed from samples from infected individuals is missing.
- The human subject protection excludes children, who well may be a significant at risk population and additional reservoir for infection. An approach to include children would form a more complete description of the ecology and risk factors for infection.
- The disposition of people that have a positive signal with regard to treatment and follow up is not described.

DESCRIPTION (provided by applicant): Emerging coronaviruses (CoVs), e.g. Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) and Middle East Respiratory Syndrome coronavirus (MERS-CoV), represent a significant threat to global health and security. EcoHealth Alliance, together with partners from Uniformed Services University, propose to increase trans-regional medical readiness for emerging CoVs by strengthening early detection and biosurveillance capabilities where they are needed most – in partner nation laboratories in the Middle East (Jordan) and Europe (Georgia) at the front line of disease emergence. Our multi-disciplinary project integrates novel diagnostic assay development, field surveillance at the human-animal interface, training and laboratory capacity strengthening, and data analytics to identify risk factors for CoV emergence and inform strategies for better force health protection. We propose five specific aims: Aim 1) Develop recombinant MERS-CoV and SARS-CoV protein antigens for a multiplex microsphere-based serological assay. Aim 2) Determine the seroprevalence of MERS-coronavirus across the human-camel value chain in Jordan to inform locations, seasons, and epidemiological interfaces of greatest risk. Aim 3) Transfer cost-effective Luminex-technology and multiplex serological assays to partner nation laboratories to accelerate early detection capabilities for emerging zoonotic diseases. Aim 4) Leverage an ongoing DoD-funded bat-borne disease surveillance project in CENTCOM and EUCOM countries to screen specimens for coronaviruses using multiplex serological assays. Aim 5) Develop and validate coronavirus `hotspot' risk maps to improve situational awareness and force health protection. Modeling outputs and timely project results will be synthesized for policy makers in short reports throughout the project. Our proposed activities meet the outlined Global Health Engagement objectives and CENTCOM Demand Signal as follows: Novel research that leverages ongoing efforts focused on MERS-CoV; Develop, optimize, and transfer low-cost, multiplex technologies to strengthen partner nations' lab capabilities and early detection for emerging pathogens; and combine human and

animal surveillance data with advanced disease risk mapping analytics to prioritize warfighter and force health protection. Together, our proposed research program will integrate the development, optimization, installation and application of a robust and cost-effective platform for CoV biosurveillance covering a large trans-regional footprint within CENTCOM and EUCOM. 1

PUBLIC HEALTH RELEVANCE: Emerging viruses represent a significant threat to global health and security in the US and globally. One group of viruses of particular concern are Coronaviruses – which include SARS coronavirus that caused a pandemic in 2002-03 killing 10% of people infected, and MERS coronavirus continuing to infect people in the Middle East with a mortality rate of 35%. This project will develop new, cost- effective tools for the early detection of coronaviruses, and transfer those diagnostic platforms where they are needed most – in partner nation laboratories at the front line of disease emergence. We will screen human, camel, and bat samples and use this information to better understand and protect against the risk of coronavirus emergence. ! 1!

CRITIQUE 1

Significance: 3
Investigator(s): 3
Innovation: 4
Approach: 4
Environment: 3

Overall Impact: The investigators propose to develop a Luminex multiplex microsphere serologic assay that will test for 4 MERS-CoV antibodies as well as SARS-CoV antibodies. Using multiple antigens, having the antigens in their native folded shape and using the Luminex platform are expected to increase sensitivity to be able to detect mild or asymptomatic infections. For preliminary, data they describe a 16-glycoprotein antigen that they have developed to detect multiple henipaviruses and filoviruses that are currently being validated and a Western blot showing that they have a stably expressed one SARS antigen and one MERS antigen that will be used in the proposed assay. They state that they will also validate their assay using samples from experimentally infected animals. The assay is expected to be completed by the end of year one. The multiplex assay and equipment and training for the assay will be disseminated in year 1 and 2.

They will then use the serologic assay to evaluate camels and camel workers in Jordan and will also screen archived samples from 1,000 people in Northern and Middle Jordan who have varying degrees of camel contact as well as an additional 1,000 bats from Jordan, Georgia and Turkey. Based on this data they will develop a “hotspot” risk map.

This proposal aligns with the scientific priority to “leverage ongoing efforts focused on MERS CoV.” MERS and SARS are important emerging pathogens that have high mortality and the capacity to cause serious outbreaks. Accomplishing these aims would impact on detection of emerging pathogens in a region where active military personnel are present.

The investigators are experienced in the Luminex multiplex assay development and the epidemiology of emerging viruses. However, the preliminary does not indicate that the multiplex assay will be fully validated by the end of the first year and the approach does not include a comparison to current methods. It will likely take more effort for validation if this is going to be a reliable tool for determining seroprevalence. This is very concerning because all of the other aims of the grant rely on the assay being better than current assays to be successful.

A weakness in the approach is that all of the human samples being tested are samples that have potentially been exposed to MERS, which makes it very difficult to determination of specificity.

The investigators do not plan to use the current serologies as a comparison in samples that they collect from camels and camel workers.

The investigators do not have alternative approaches to the assay not performing well.

There is no plan to refine the assay if individual components are unnecessary or are redundant.

The expression of one of four of the assay antigens is encouraging but leaves some concern about the proposed timeline.

Also of concern, is that the investigators cite a reference that they misinterpret as evidence that current serology is insensitive. In fact, this reference demonstrated that ELISA and plaque neutralization/reduction assays were sensitive in asymptomatic camel workers. The investigators stated that the T-cell assay in the reference were more sensitive than standard serology, which was not the case.

Also of concern is the fact that the incidence of MERS is much lower in Jordan with 28 cases being reported since 2012 compared to 1,854 cases in Saudi Arabia and there is no reason given for why these studies are not being done in Saudi Arabia where 83% of all cases have been reported. This decreases the likelihood that an adequate number of seropositive samples will be found and that it may not reflect the epidemiology of the significant hotspots of transmission. If there are logistical issues that prohibit the study of the disease in the area of greatest risk, then those issues should be stated in the grant.

1. Significance:

Strengths

- MERS CoV and SARS CoV are important emerging pathogens and better methods of surveillance would improve epidemiologic investigations.
- Identifying points with greater levels of infection risk would allow for interventions to decrease transmission to humans.
- Strengthening partner labs in Jordan and Georgia will be beneficial in detection and surveillance.

Weaknesses

- Little evidence is presented that current immunoassays are insufficient.

2. Investigator(s):

Strengths

- The investigators are highly accomplished in all of the areas of research that are outlined and establish collaboration with investigators from Jordan and Georgia.

Weaknesses

- None

3. Innovation:

Strengths

- Development of a novel multiplex assay and potential discovery of MERS-CoV transmission risk.

Weaknesses

- None noted.

4. Approach:

Strengths

- The multiplex immunoassay with antigens in their native conformation may provide a more sensitive and specific test for MERS-CoV.
- The hypothesis that camel crowding in wholesale markets increases transmission is well founded and would be amenable to public health interventions.

Weaknesses

- This proposal is based on the premise that the current serologic assays are not sensitive. The evidence provided does not fully support this and the primary study referenced is misinterpreted. Aims 2-5 are dependent on aim 1. This is concerning because the preliminary data presented for the previous 16 antigen multiplex assay does not demonstrate a field validated assay or preliminary data regarding MERS-CoV validation. And, of the proposed MERS-CoV proteins, only the S2 has been expressed.
- Given that the seroprevalence of MERS-CoV is unknown in the population being tested determining specificity will be difficult to assess without testing in a non-exposed population.

5. Environment:

Strengths

- Substantial resources and collaborations.

Weaknesses

- Jordan's lower incidence of disease may not model risk in areas of higher transmission.

Protections for Human Subjects:

Acceptable Risks and/or Adequate Protections

Data and Safety Monitoring Plan (Applicable for Clinical Trials Only):

Acceptable

Inclusion of Women, Minorities and Children:

- Sex/Gender: Distribution justified scientifically
- Race/Ethnicity: Distribution justified scientifically
- For NIH-Defined Phase III trials, Plans for valid design and analysis:
- Inclusion/Exclusion of Children under 18:

Vertebrate Animals:

YES, all criteria addressed

Biohazards:

Acceptable

Applications from Foreign Organizations:

Justified

Resource Sharing Plans:

Acceptable

Authentication of Key Biological and/or Chemical Resources:

Acceptable

Budget and Period of Support:

Recommend as Requested

CRITIQUE 2

Significance: 2
Investigator(s): 1
Innovation: 1
Approach: 3
Environment: 2
Overall Impact: This project is truly a complete research project; assay development, through to application of this assay to assess epidemiological assessment of risk areas and mapping. This will increase our understanding of the epidemiology of CoVs and inform the risk of future transmission to humans. The team is strong and includes investigators in the study areas and includes investigators from the study country locations and has full support of public health bodies in these places.

1. Significance:

Strengths

- Addresses a pathogen which have /are currently causing large issues
- Understanding more about the possible spillover events is of vital importance
- This clearly addresses a priority area under this grant call.

Weaknesses

- Does not address what could be done with the greater understanding of where spillover could occur- could anything be included that would suggest ways to mitigate transmission?

2. Investigator(s):

Strengths

- Team with experience of successfully implementing this type of project
- Experience of such modelling and mapping work
- Experience with the serological platform
- Local partners in the Middle East

Weaknesses

- None noted.

3. Innovation:

Strengths

- Testing of new multiplex assays
- Application of fairly newly developed modelling techniques
- Combination of all these disciplines is innovative

Weaknesses

- None noted.

4. Approach:

Strengths

- Transfer of technology to partner institutions in Jordan and Georgia
- Development of serological testing
- Testing relevant possible animal reservoirs with this assay
- Application of modelling techniques to determine spillover events- however how these will be validated is not made clear in the application

Weaknesses

- Risk not addressed for what happens if serological test is not sufficiently good for this work
- Does not add to the evidence body about some of the other epidemiological factors that will impact the probability of spillover or the factors that lead to a spillover event that then causes severe disease in humans

5. Environment:

Strengths

- Across multiple excellent diverse institutions
- Leveraged previously collected large banks of serum samples, collected by the PI's organization and other organizations that have provided their support
- All lab resources appear to be available in all locations
- Appear to have full support of ministry of health in Jordan

Weaknesses

- Bat samples are collected by a study with PI not included in this study, however letters of support are included.

Protections for Human Subjects:

Acceptable Risks and/or Adequate Protections

Inclusion of Women, Minorities and Children:

- Sex/Gender: Distribution justified scientifically

- Race/Ethnicity: Distribution justified scientifically
- For NIH-Defined Phase III trials, Plans for valid design and analysis:
- Inclusion/Exclusion of Children under 18: Excluding ages <18; justified scientifically

Vertebrate Animals:

YES, all criteria addressed

Biohazards:

Not Applicable (No Biohazards)

Applications from Foreign Organizations:

Justified

Resource Sharing Plans:

Acceptable

Authentication of Key Biological and/or Chemical Resources:

Acceptable

Budget and Period of Support:

Recommend as Requested

CRITIQUE 3

Significance: 2
Investigator(s): 1
Innovation: 2
Approach: 5
Environment: 1

Overall Impact:

1. Significance:

Strengths

- Little well defined understanding of serology of CoV in camels and spread to humans though they are thought to be primary hosts for these viruses.

Weaknesses

- None noted.

2. Investigator(s)

Strengths

- Well qualified

Weaknesses

- Less medical, may benefit from a clinical medical personnel to add to the extensive lab and veterinary medicine personnel

3. Innovation

Strengths

- Applying new technology to facilitate surveillance and improve laboratory detection facilities in Jordan and Georgia

Weaknesses

- None noted.

4. Approach:

Strengths

- Well spelled out approach to the study and components of the study

Weaknesses

- Protection of people that are deemed + for virus and implications are not delineated.

5. Environment

Strengths

- Partner locations and organizations with the appropriate technology and capacity to conduct study

Weaknesses

- None noted.

Protections for Human Subjects:

Unacceptable Risks and/or Inadequate Protections

- What will happen to participants who have virus detected, especially for pregnant women. What arrangements have been made for care.

Data and Safety Monitoring Plan (Applicable for Clinical Trials Only):

- Not provided. Need to provide

Inclusion of Women, Minorities and Children:

- Sex/Gender: Distribution justified scientifically
- Race/Ethnicity: Distribution justified scientifically
- For NIH-Defined Phase III trials, Plans for valid design and analysis:
- Inclusion/Exclusion of Children under 18: Excluding ages <18; not justified scientifically
- No mention of why not children

Vertebrate Animals:

YES, all criteria addressed

Biohazards:

Applications from Foreign Organizations:

Justified

Budget and Period of Support:

Recommend as Requested

THE FOLLOWING SECTIONS WERE PREPARED BY THE SCIENTIFIC REVIEW OFFICER TO SUMMARIZE THE OUTCOME OF DISCUSSIONS OF THE REVIEW COMMITTEE, OR REVIEWERS' WRITTEN CRITIQUES, ON THE FOLLOWING ISSUES:

PROTECTION OF HUMAN SUBJECTS: ACCEPTABLE FOR ENROLLMENT BUT QUESTIONS WITH RESPECT TO FOLLOW UP CARE

INCLUSION OF WOMEN PLAN: ACCEPTABLE

INCLUSION OF MINORITIES PLAN: ACCEPTABLE

INCLUSION OF CHILDREN PLAN: OPERATIONALLY ACCEPTABLE TO EXCLUDE CHILDREN LESS THAN 18 YEARS BUT NOT SCIENTIFICALLY JUSTIFIED

VERTEBRATE ANIMALS: ACCEPTABLE

COMMITTEE BUDGET RECOMMENDATIONS: The budget was recommended as requested.

From:	Kevin Olival on behalf of Kevin Olival <olival@ecohealthalliance.org>
To:	Chris Broder; Eric Laing
Subject:	Panel Review of our GHERI FY18 submission
Date:	Tuesday, July 16, 2019 1:17:22 PM
Attachments:	GHERI_Olival2018_summarystatement.pdf ATT00002.bin

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Eric and Chris,

Attached is the panel summary statement from our GHERI proposal we submitted on CoV assay development, and MERS and bat borne CoV surveillance.

Disappointing we didn't get this, and unfortunately MERS isn't listed as a priority for FY19, so not sure this will be worth putting back in. Let me know your thoughts after reviewing the FY19 priorities.

In any case, Reviewer 1 was most critical, Reviewers 2 and 3 ranked it quite high overall. The main criticism were related to the assay development, validation, justifying the need for additional MERS/CoV serological assays, and our study design (i.e. not doing it in Saudi Arabia, and sampling unexposed people with an additional assay for validation).

Cheers,
Kevin

II. Research Strategy:

1. Significance: Southeast Asia is a critical hotspot for emerging diseases due to its high biodiversity of wildlife and their viruses, and the presence of key ecological and socioeconomic drivers of emergence **(Fig. 1)** (*1*). These include dense human populations living in networks of rural and urban communities, with strong cultural and occupational connection to wildlife and livestock farming and trading, and intimate connection to global travel networks (*2*). The region is undergoing rapid environmental and demographic change, both of which increase the risk of disease emergence and spread. Pathogens that emerge in this region often spread and sometimes enter the USA (e.g. prior influenza pandemics, SARS) and threaten global health security.

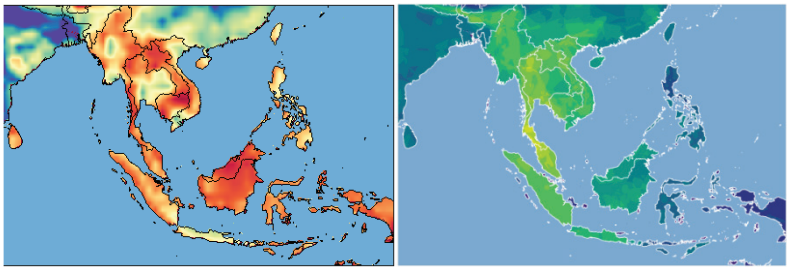


Fig. 1: Southeast Asia, and in particular, parts of Thailand and Malaysia are hotspots of risk for disease emergence **(left)**, and predicted high diversity of 'missing' or as-yet undiscovered viruses, yellow = highest diversity **(left)**. From (*1-3*).

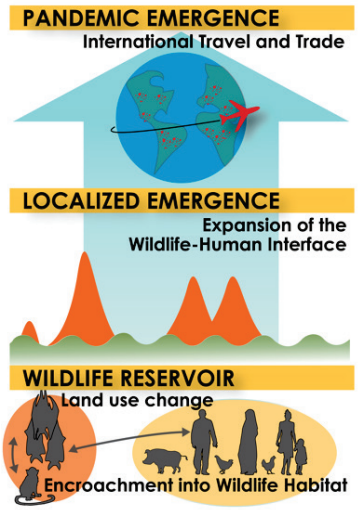
Not surprisingly novel viruses, including near-neighbors of known agents, have emerged in the region leading to often unusual clinical presentations **(Table 1)**.This adds to a significant background burden of repeated Dengue virus outbreaks in the region (*20*), and over 30 known *Flavivirus* species circulating throughout S. and SE Asia (*21*). The events in **Table 1** are dominated by three viral families: coronaviruses (CoVs), paramyxoviruses (PMVs - particularly henipaviruses) and filoviruses (FVs), which are initial examples of our team's research focus and capabilities. These viral groups have led to globally important emerging zoonoses (*4, 5, 22-31*), causing tens of thousands of deaths, and costing billions of dollars (*32-34*). Furthermore, near-neighbors of known pathogens in these families have been reported throughout the region, including by members of our consortium: henipaviruses in bats in Thailand, Cambodia, Philippines, Laos, Indonesia, China, Malaysia, Bangladesh and India (*35-41*); a novel henipavirus, Mòjiāng virus, in wild rats in Yunnan (*14*); serological evidence of FVs in bats in Bangladesh (*42*) and Singapore (*43*); Nipah- and Ebola-like viruses in *Hipposideros*, *Cynopterus* and *Rhinolophus* species in Malaysia (Hughes *et al.*, in prep.); evidence of novel FVs in bats in China (*44-46*), including Měnglà virus that appears capable of infecting human cells (*47*); novel PMVs in bats and rodents consumed as bushmeat in Vietnam (*48*); a lineage C β-CoV in bats in China using the same cell receptor as MERS-CoV to infect human cells *in vitro* (*49*); MERSr-CoVs in bat guano harvested as fertilizer in Thailand (*50*), and directly in bats (Wacharapluesadee *et al.*, in prep.); 172 related viruses.

Viral agent	Site, date	Impact	Novelty of event	Ref.
Nipah virus	Malaysia, Singapore 1998-9	~246 human cases, ~40% fatal	2nd emergence of a zoonotic henipavirus, 1st large outbreak	(*4-6*)
Melaka & Kampar virus	Malaysia 2006	SARI in family group, individual	1st disease due to bat-origin reoviruses, also Singapore, Vietnam etc.	(*7-10*)
Reston filovirus	Philippines 2008	Seropositive people killed pigs,	No prior FVs in pigs	(*11*)
Thrombocytopenia Syndrome virus	E. Asia 2009	100s of deaths in people	Novel tick-borne zoonosis with large caseload	(*12*)
Reston filovirus	Shanghai 2011	PCR positive pigs	Further evidence of pig RESTV infection	(*13*)
Mòjiāng virus	Yunnan 2012	Death of 3 mineworkers	1st evidence of rodent origin henipavirus in people	(*14*)
Nipah-like virus	Philippines 2015	Killed horses	No prior horse infection for NiV, known for HeV	(*15*)
SARSr-CoV & HKU10-CoV	S. China 2015	Seropositive people	1st evidence human infection HKU10 & SARSr-CoV	(*16*)
SADS-CoV	China 2017	>25,000 pig deaths seronegative people	Novel emergence of bat-origin CoV	(*17*)
Nipah virus	Kerala 2018, 2019	Killed 17/19 people 2018, 1 infected 2019	1st outbreak of NiV outside Bangladesh, W. Bengal focus	(*18, 19*)

Table 1: Recent emergence events in SE Asia indicating potential for novel pathways of emergence, or unusual presentations for known or

novel β-CoVs (52 novel SARSr-CoVs) and a new β-CoV clade ("lineage E") in bats in S. China that occur throughout the region (*24, 49, 51, 52*); 9 novel wildlife-origin CoVs and 27 PMVs in Thailand, and 9 novel CoVs in Malaysia reported by us from USAID-PREDICT funding (*51, 53*). These discoveries underscore the clear and present danger of zoonotic events in the region. The wide diversity of potentially pathogenic viral strains also has significant potential use in broadly active vaccine, immunotherapeutic and drug development (*49*).

Most high-impact zoonotic viruses originate in wildlife reservoirs, sometimes spilling over first into livestock 'amplifier' hosts, or directly into localized human populations with high levels of animal contact **(Fig. 2)**. Efforts to prevent emerging zoonoses have targeted these high-risk populations in regions prone to disease emergence (*31, 54*). However, these regions tend to be in the developing world, where nations often lack the resources to investigate novel zoonotic events, or wildlife reservoirs of zoonoses, and prioritize more immediate public health concerns such as Dengue virus or NTDs (*20*). Zoonotic disease surveillance and control is also hampered by inadequate information on basic disease ecology (e.g. range of wildlife reservoirs, risk factors for spillover) and pathogen biology (viral diversity, pathogenesis), and by logistical challenges sharing novel diagnostics, animal models, samples and reagents at sites where outbreaks begin (*55*). Countermeasure and vaccine development is challenged by the small number of isolates available, and the presence of closely-related strains, some with evidence of transmission to people but with unknown pathogenicity. For example, using NIH and other funding, we found serological evidence of exposure to African henipaviruses in 3-4% of a sample of people in Cameroon, with a significant risk factor being butchering of bats; and serological responses to bat SARSr-CoVs in 6/209 (2.87%) and bat HKU10-CoV in 2/412 (0.5%) people living close to a bat cave in Yunnan, all novel viruses with unknown clinical impact (*16, 56*). We discovered two novel bat-borne reoviruses, Melaka and Pulau viruses, and showed that 14/109 (13%) of people living in close proximity to bat roosts on Tioman Island, Malaysia were exposed to these viruses (*7*) as well as 12/856 (1.4%) of a random sample screened in Singapore (*8*). Previous studies in the Philippines found famers and butchers who had contact with sick pigs tested positive for Reston ebolavirus antibodies but had not experienced illness (*11*), and it was our team that discovered Philippine bats as natural reservoir hosts for Reston virus (*57*). In preliminary screening in Malaysia with funding from DTRA we found serological evidence of exposure to henipaviruses (Hendra, Nipah and Cedar) in 14 bats and 6 human samples, and Ebola (Zaire and Sudan)-related viruses in 11 bats, 2 non-human primates (NHP) and 3 human samples (Hughes, in prep.).



Even when spillover leads to outbreaks of illness and mortality, they are often unreported, undiagnosed or misdiagnosed. We initiated targeted syndromic surveillance of encephalitis patients in Bangladesh clinics and showed that Nipah virus (NiV) causes repeated annual outbreaks with an overall mortality rate of ~70% (*58*). NiV has been reported in people in West Bengal, India, which borders Bangladesh (*28*), in bats in Central North India (*59*), and now in people in Kerala, South India in 2018 and 2019, raising the specter of future spillover at other sites in the region (*18, 60*).

Fig. 2: Our Early Warning Approach: Most zoonotic viruses emerge from wildlife hosts, sometimes via livestock **(lower panel)**, to cause small scale outbreaks (green) or larger chains (red) of human-to-human transmission **(middle)**. In some cases, these spread more widely via air travel **(upper)**. Our EIDRC proposal targets each stage: **SA1** examines the diversity and spillover potential of viral threats in targeted SE Asian wildlife; **SA2** seeks evidence of their spillover into focused high-risk human populations; **SA3** identifies their capacity to cause illness and to spread more widely. Figure from (*54*).

Under a current NIAID R01, we have shown that we can use analytical approaches to target surveillance of wildlife and people and identify likely sites of spillover. We used phylogenetic analysis of bat hosts and their CoVs to identify a key cave complex in SW Yunnan Province that harbors all the genetic elements of SARS-CoV (*61*), as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics (*24, 49, 51, 52, 62-64*). We conducted risk factor surveys and biological sampling of human populations living close to this cave and found serological evidence of spillover in people highly exposed to wildlife (*16*). **This work provides proof-of-**

concept for an early warning approach that we will expand in this EIDRC to target other viral groups in one of the world's most high-risk EID hotspots.

The overall premise of this EIDRC proposal is that there is substantial evidence that: 1) diverse CoVs, henipaviruses and FVs related to known human pathogens are circulating in wildlife reservoirs in Southeast Asia; **2)** these viruses likely spillover regularly to people, are often unreported or misdiagnosed, and their clinical manifestations and potential to cause pandemics are unknown and underestimated; and **3)** our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence. To scale-up this approach, we propose to launch **EID-SEARCH** (the **E**merging **I**nfectious **D**iseases - **S**outh **E**ast **A**sia **R**esearch **C**ollaboration **H**ub), to better understand, and respond to, the risk of zoonotic viral emergence in Southeast Asia. We will initially focus on the diversity of known and novel CoVs, henipaviruses, and FVs in wildlife reservoirs. We will use this work to build our center's interactive network throughout the region, bridge barriers, provide early focal point for basic and translational studies, develop sample handling and storage routines, communication networks, cohort management, infrastructure development, and cross-training in surveillance and novel diagnostic platforms. We will develop and transfer technology to test these viruses' capacity to infect human cells and mouse models, develop new specific and sensitive serological and molecular diagnostic tools, conduct surveillance of human communities with high risk of exposure to wildlife, and clinical cohorts to identify evidence of spillover of viruses causing previously 'cryptic' clinical syndromes in people. We will test the capacity of novel viruses to infect human cells and/or mouse models, and thus asses potential for spillover and spread which will feed into our risk models. Our group also has extensive experience in other viruses, including vector-borne flaviviruses, and alphaviruses, and will use this experience to provide robust long-term surveillance, diagnostics, and basic science opportunities. While the current proposal focuses its basic and applied research agendas on this group of viral EID, we note that the EID-SEARCH is poised to rapidly respond to any outbreak. **Thus, FVs, henipaviruses and CoVs are examples of sentinel surveillance systems in place that illustrate EID-SEARCH's capacity to identify and initiate a rapid response targeting any novel microbial pathogen that emerges in this region.**

2. Innovation: Previous work by our consortium has developed strategies to target surveillance in wildlife and people to better anticipate and identify early spillover events and pre-empt outbreaks of emerging viruses. In the EID-SEARCH we scale up this approach in a central research "hub" made up of leading laboratories, clinics and surveillance sites in three critically high-risk EID hotspot countries in SE Asia. EID-SEARCH's reach is expanded through its regional network of collaborators that covers nearly all of the greater SE Asia region. The innovation of the EID-SEARCH is in: **1)** Its multidisciplinary approach that combines modeling to target geographic and taxonomic sampling targets with on-the-ground zoonotic disease surveillance in human and wildlife populations; **2)** the biological characterization approach that identifies how likely viruses are to be able to infect people, and enables evaluation of existing countermeasure technologies; an approach that is grounded in our successful work on SARSr-CoVs; and **3)** the strategic sampling of both high-risk communities with extensive animal contact at the front-lines of viral spillover *and* syndromic clinical cohorts that present with signs of novel viral emergence, to identify risk of viral spillover in an early warning system approach **(Fig. 2)**. **In Aim 1, we will target viruses in archived and newly collected wildlife samples, and characterize their risk of spillover to people**. We will use spatial and phylogeographic analyses to identify the geography and species of wildlife to test samples from, and to further characterize viruses we have recently discovered. We will use *in silico* methods (novel ecological-phylogenetic risk ranking and mapping of receptor binding domains), cell culture, and animal models to assess their potential for spillover into high-risk human populations. **In Aim 2, we will conduct targeted cross-sectional serological surveys of human communities with high levels of animal contact to find evidence of viral spillover, and identify occupational and other risk factors for zoonotic virus transmission.** We will use serological tests targeting viruses identified in Aim 1 to identify the baseline spillover of known or novel CoVs, henipaviruses and FVs in these populations. Serological findings will be analyzed together with subject metadata (including a simple animal-contact survey, location data, viral sequence/isolates etc.) to identify zoonotic risk factors and routes of exposure from specific wildlife or vector species. **In Aim 3, we will use syndromic surveillance of clinical human cohorts to identify evidence of viral etiology for otherwise 'cryptic' outbreaks.** We will enroll and

collect biological samples from patients in local clinics and hospitals who present with syndromes previously linked to known viral agents and live within communities at high risk for disease emergence. We will conduct molecular and follow-up serological diagnostic assays to identify likely viral etiology of syndromes that may represent otherwise cryptic events. Where viruses are identified, we will attempt to isolate or molecularly re-derive them, and use survey data, ecological and phylogenetic analysis to identify likely reservoir hosts and inform intervention programs. Our US partner BSL-4 laboratory, NEIDL, will attempt isolation and characterization of viruses requiring BSL-4 of containment (e.g. novel filoviruses, close relatives of Hev/NiV).

3. Approach: 3.1. Research team: The EID-SEARCH builds on long-term collaboration among world leaders in emerging virus research with proven experience collaborating internationally on field, lab and human surveillance research **(Fig. 3)**. Over the past two decades, our consortium partners have conducted high profile research on EIDs within the region and globally, including identifying the wildlife reservoirs for NiV and Hendra virus (HeV), MERS- and SARS-CoVs (*24, 27, 59, 61, 65-67*), discovering SADS-CoV (*17*), and developing an array of serological, molecular, *in vitro* and *in vivo* approaches to characterizing high-risk CoVs, henipaviruses, and FVs (*62-64, 68-81*). Our team has substantial experience conducting human surveillance in the community and during outbreaks **(Sections 2.2.a., 2.2.b., 2.2.c, 3.2.a, 3.2.b)**, including: USAID-PREDICT, and DTRA-funded viral surveillance of indigenous communities in Peninsular Malaysia and syndromic surveillance of hospital patients in Sabah (Co-I Hughes); collection of >2,000 serum samples from healthy blood donors and sanitation workers, febrile and influenza like illness (ILI), and diarrheal stool samples from children under 5 years of age across Borneo Malaysia (Co-I Kamruddin); collaboration on the MONKEYBAR project with the London School of Hygiene and Tropical Medicine to analyze risk and prevalence of the zoonotic malaria *Plasmodium knowlesi* through case control and cross-sectional studies in Sabah villagers (n=~800, 10,000 resp.) and clinical observational studies to determine the etiology of central nervous system infections and acute undifferentiated febrile illness in Sabah. (Co-Is William, Rajahram, Yeo); and community-based surveillance of hundreds of bat-exposed villagers in Thailand (Co-Is Wacharapluesedee, Hemachudha). As a proof-of-concept of how our team can rapidly scale up resources for diagnostics and surveillance in the face of a new EID, in 2016, we discovered a novel bat-origin HKU-2 CoV (*82, 83*) killing >20,000 pigs in S. China, designed PCR and serological assays, surveyed pig farmers for evidence of exposure, and conducted experimental infections, pathogenesis and molecular studies, **all in the span of 3 months** (*17, 84*).

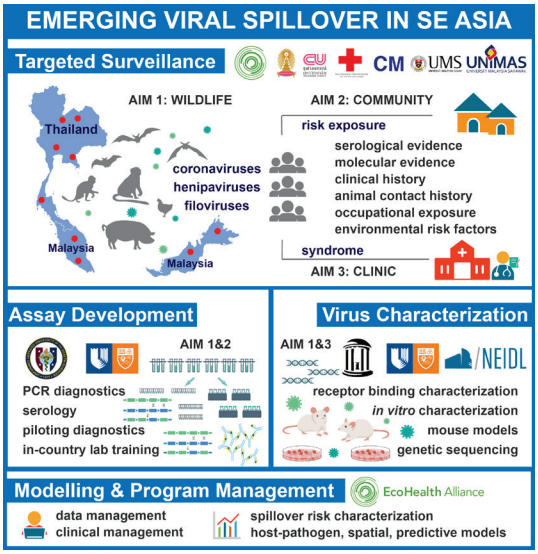


Fig. 3: EID-SEARCH scope, core institutions, and roles.

The administration of this center **(Section 4.1.)** will be centralized at EcoHealth Alliance (EHA) led by PI Daszak, who has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5-20 yrs on NIAID- and USAID-funded research. He will oversee all activities in this EIDRC, supported by Deputy lead, Dr. Olival – who has managed human and wildlife disease surveillance projects in SE Asia for >10 years, and a **Core Executive Committee (Section 4.1.a)**. Co-Is Olival and Zambrana are leaders infectious disease analytics, and head the modeling and analytics work for USAID-PREDICT. Co-Is Wang, Baric, Broder, Anderson, and Laing have developed a unique array of *in vitro* cell culture, serological and molecular reagents to identify and characterize emerging viruses in people and animals (henipaviruses and other PMVs, CoVs, FVs and many others). Co-Is Wacharapluesedee, Hemachudha, and Hughes and collaborators have coordinated clinical and community surveillance of people, and of wildlife and livestock within Thailand and Malaysia for the past 15 years, in collaboration with Ministries of Health, Agriculture and Environment. Finally, this consortium has extensive external funding from NIH, DoD, USAID, DHS and other USG agencies supporting laboratory, analytical and field studies directly related to the current proposal, and will bring substantial leverage to our EIDRC.

3.2. Geographical focus: The three core (hub) countries for this proposed EIDRC are Thailand, Malaysia and Singapore, three contiguous EID hotspot countries that stretch through one of the most significant foci of EID origins globally. Our proposed work includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sarawak and Sabah, and thus contains overlapping wildlife biogeography with India, Bangladesh, Myanmar, SW China, Laos, Cambodia, Vietnam, Philippines, and Indonesia. It encompasses diverse cultural and socioeconomic backgrounds which have correspondingly diverse and intensive interactions with wildlife reservoirs and the global travel network. We have already begun sampling of wildlife hunters, livestock workers, wildlife farmers and market workers, rural villagers living close to high risk wildlife species, people otherwise highly exposed to animals in these countries, and clinical cohorts, as part of current/prior NIH, DTRA, and USAID funding. Our core member's extensive network of collaborators in clinics, research institutes and public health laboratories in almost every other Southeast Asian country positions the EID-SEARCH within a much larger catchment area for emerging zoonoses and will allow us to rapidly scale up to include additional sites in response to an outbreak or shift in research priorities. We will conduct regular meetings with key research, public health and community leaders, including >50 leading laboratories/institutions in 10 countries **(Section 4.2)** to maintain these collaborative relationships with the core members of our consortium (**Fig. 4**). We will use these, and our annual meetings, to share information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks across the region.

Fig.4 : Map of Southeast Asia indicating three hub countries for this proposed EIDRC (**Red**: Thailand, Singapore, Malaysia) and countries in which Key Personnel have existing collaborating partners via other funded work (**Green**), indicating that EID-SEARCH provides access to key collaborators and sites throughout the region.

3.3. Capacity for linkage with other NIAID studies, and scaling up to respond to outbreaks: EID-SEARCH US-based partners include senior scientists with significant experience on NIAID-funded projects, extensive collaborations with other NIAID-funded scientists, and previous experience working with NIAID leadership through workshops, review of programs (e.g. PI Daszak's role in NIAID CEIRS external review), and work on study sections. These contacts ensure that the EID-SEARCH senior leadership will be ready and able to rapidly set up data, sample and reagent/assay sharing protocols with NIAID researchers, the other EIDRCs, the EIDRC CC and other NIAID research centers. EID-SEARCH in-country partners include senior medical doctors and infectious disease specialists at national and regional hospitals in each country and administrative state in this proposal. On news of potential outbreaks, **EID-SEARCH will rapidly mobilize its core staff and their extensive collaborative network across almost all SE Asian countries (Fig. 4) (Sections 4.1.a, 4.2. 4.3).** The core analytical approach can be used to rapidly identify sampling sites and targets to harvest vectors, vector borne arboviruses, birds and mammalian species that harbor most other viral threats. Through existing projects such as the USAID PREDICT Project and DTRA-funded biosurveillance projects, EHA is currently partnered with hospitals and researchers across South and Western Asia, West and Central Africa, and Latin America. Our partners already have capacity to conduct human and wildlife sampling and testing for novel viral agents, so expanding in geography or scale is readily achievable given sufficient resources.

Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife.

1.1 Rationale/Innovation: The vast majority of emerging zoonotic pathogens are linked directly or indirectly to wildlife host, particularly from important mammalian groups like bats, rodents and NHPs (*3*). Our previous work on wildlife origin viruses demonstrates that zoonotic viral emergence tends to occur in specific regions with high diversity of wildlife that harbor viruses of zoonotic potential, and human populations that have close contact to these wildlife **(Fig. 1)** (*2, 3*). In Aim 1 **(see Fig. 9 for overview)**, we will strategically conduct EID surveillance by applying risk analytics to prioritize the regions, wildlife species, and human populations that

have a particularly high risk of viral spillover. We will target high-risk zoonotic host reservoirs (bats, rodents and NHPs) in these regions and apply novel predictive models to estimate viral diversity and host range to identify wildlife species that have been ignored or under-sampled by previous zoonotic disease surveillance efforts. We will strategically collect specimens from these species and screen them, together with some of the tens of thousands of recently collected samples we have archived, for known and novel agents. We will begin screening using conserved PCR assays to identify known and novel viruses in high risk RNA viral families, *Coronaviridae*, *Paramyxoviridae*, and *Filoviridae*. We will expand to other groups of pathogens, including arboviruses, pending available resources and priorities established with the EIDRC coordinating center (EIDRC-CC) and other regional EIDRCs. Using risk ranking algorithms and a series of ecological and virological risk factors, we will triage the wildlife viruses we discover for further genomic characterization and viral isolation. On this subset of viruses, we will then use *in vitro* and *in vivo* approaches (viral rescue, cell lines, gene edited humanized transgenic mice and the collaborative cross genetic reference mouse population) previously developed and widely used by our team to predict capacity of novel viruses to infect people and spillover (*63, 85-88*). These high-risk viruses and their close relatives will be targets for human community serosurveys and clinical sampling in Aims 2 and 3, respectively.

1.2 Preliminary data: 1.2.a. Geographic targeting: EHA has led the field in analyzing where the highest risk of zoonotic spillover is across the globe, identifying SE Asia as a major EID hotspot (*1, 2*). These hotspots are sites where high wildlife biodiversity, human population density and rapid ecologic change collide **(Fig 1)**. Using host and ICTV viral data for all known mammalian viruses, and generalized additive models to correct for sampling bias, we are able to predict the relative number of yet-to-be-described viruses that a species harbors (*3*). This demonstrates substantial undiscovered viral diversity in mammals across Southeast Asia, and particularly in regions of Thailand, Singapore and Malaysia **(Fig. 1)**. Separately, we found that factors driving spillover (host diversity, climate) differ from those driving spread in human populations (population density, bushmeat hunting, livestock production) (*89*). We therefore analyze capacity for viral spread as a separate risk factor, using our unique demographic and air travel and flight data modeling software (*90, 91*). In the current proposed work, we will use all the above approaches to target wildlife sampling (archival and new field collections) in Aim 1, to inform sites for human sampling in Aim 2, and to assess risk of spread in Aim 3.

1.2.b. Reservoir host species targeting: Our analysis of all known mammalian host-viral associations demonstrates that bats, rodents and NHPs represent the three most important reservoirs for zoonoses, responsible for the highest proportion of known and predicted viral diversity of zoonotic potential (*3*). Thus, the bulk of our proposed wildlife sampling and testing will consist of these three taxa with a focus on bats, because this group is the known or hypothesized reservoir for the majority of high-impact zoonotic CoVs, PMVs and FVs (*50, 92-94*). Bats have the highest proportion of predicted zoonotic viruses in any mammalian group (*2*), and are exceedingly diverse in Southeast Asia (~500 species). To further prioritize taxonomic sampling targets within these diverse geographic hotspots, we used a novel Bayesian phylogeographic algorithm to identify host genera and species that are the most important centers of evolutionary diversity for a given viral group, e.g. bat genera for β-CoVs **(Fig. 5)** (*51*). This approach will be extended to other mammal taxa and viral families of interest, allowing us to identify the reservoir hosts of specific zoonotic viral groups, where viral diversity is likely highest.

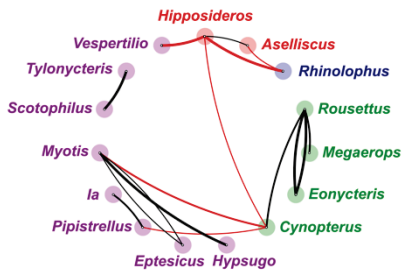


Fig 5: Preliminary analysis of sequence data collected under prior NIH funding, identifying SE Asian bat genera with the highest β-CoV evolutionary diversity. We will apply this Bayesian discrete phylogeographic model to identify taxonomic and geographic centers of viral diversification and sharing for relevant PMV, CoV, FV and other virus clades. Line thickness proportional to probability of virus sharing between two genera. Inter-family switches in red.

Finally, we have designed a strategy to estimate sampling gaps, using a viral 'mark-recapture' approach we previously published (*95, 96*) **(Fig. 6)**. Using prior data for bat, rodent and NHP viral discovery vs. sampling rates, we can identify the likely total viral diversity for specific host groups in a region, and use this to estimate sample sizes to discovering the majority of unknown viruses within a specific family or genus. **In the current**

proposal, we will use these analyses to estimate geographic and species-specific sampling targets **to more effectively identify new strains to support experimental infection studies and risk assessment.**

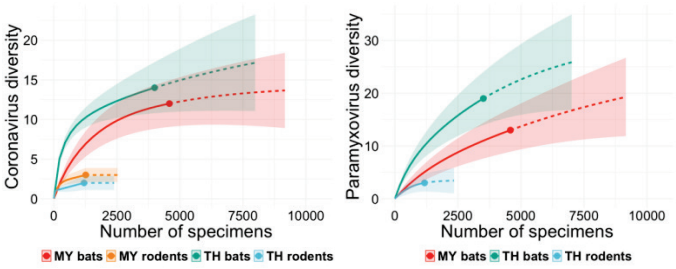


Fig. 6: Estimated CoV **(left)** and PMV **(right)** diversity in bats and rodents from Thailand and Malaysia, using data from PCR screening and RdRp sequences from >10,000 specimens in bats and 4,500 in rodents. Bats have 4X more viral species than rodents, controlling for sampling effort. We estimate that additional collection of 5k-9k bat specimens and testing of our archived bat and rodent specimens alone will identify >80% of remaining CoV and PMV viral species in these key reservoirs, yielding >800 unique viral strains.

1.2.c. Sample testing to identify known and novel viruses: We have conducted extensive wildlife surveillance for novel viruses across Southeast Asia in prior NIAID funded work and as part of the USAID PREDICT project. Under the 10-year PREDICT program globally, we identified 912 novel and 210 known viruses, more than the total number of viruses previously recognized in mammals by the ICTV (*97*). This work included collecting nearly 300,000 individual mammal samples from 14 EHA-led PREDICT countries, and PCR-screening of more than 60,000 individual specimens (*53*). In southern China alone, we identified 178 β-CoVs, of which 172 were novel, discovered a new β-CoV clade, "lineage E" (41), diverse HKU3r-CoVs (179 sequences) within a 'sister' clade to the SARS-CoV lineage, and a new bat-origin SADS-CoV, responsible for killing >25,000 pigs in Guangdong Province (*17*). We have collected 28,957 samples from bats, rodents and NHPs in Thailand and 47,178 in Malaysia, **but have only tested a minority of these using PCR.** We have identified 100 novel viruses in Thailand and 77 in Malaysia. **Furthermore, we have only sequenced a small segment of one gene for all novel viruses found. We have archived duplicate samples which are now available for use in this project**, including fecal, oral, urogenital, serum samples, and biopsies from bats, rodents, and NHPs. In the current proposed work, we will attempt to sequence, isolate and characterize those viruses that our analyses below suggest are most likely to be able to infect humans.

1.2.d. In vitro & in vivo *characterization of viral potential for human infection:* Using Coronaviridae as an example, we have conducted *in vitro* and *in vivo* experiments to characterize the pathogenic potential of novel SARSr-CoVs from bats. We isolated three SARSr-CoVs from bat feces: WIV1, WIV16 and Rs4874, with Spike (S) protein sequences that diverged from SARS-CoV by 3-7% (*24, 61, 98*). We conducted full-length genome sequencing of 12 other novel SARSr-CoVs, some highly similar to outbreak SARS-CoV in the most variable genes (*61*). Using our reverse genetics system we constructed chimeric viruses and rederived full length recombinant SARSr-CoV from in silco sequence. All 3 SARSr-CoV full length isolates and the two chimeric viruses replicated efficiently in Vero E6 cells and in HeLa cells expressing hACE2, civet and bat ACE2, but not in those without ACE2 (*24, 61, 98*). We used the SARS-CoV reverse genetics system (*72*) to generate a chimeric virus with a mouse-adapted SARS-CoV backbone expressing SHC014 S protein with 10% sequence divergence from SARS-CoV S. This and the other full length and chimera viruses replicated in primary human airway epithelium, using the human ACE2 receptor to enter into cells (*62*). Thus, **SARSr-CoVs with diverse variants of SL-CoV S protein without deletions in their RBD can use human ACE2 as receptor for cell entry.** The Baric lab has a well-established hACE2 and DPP4 transgenic mouse model that **we used to assess the capacity of novel SARSr-CoVs and MERS-CoV to infect humans and cause disease** (*85, 99*). Infection of bat SARSr-SHC014 or WIV1 caused SARS-like clinical signs in the transgenic hACE2 mouse model **that weren't reduced by immune-therapeutic monoclonals that attenuate SARS-CoV pathogenicity, nor after challenge following vaccination against SARS-CoV** (*62*) **(Fig. 7)**. We repeated this virus characterization approach with chimeras using HKU3r-CoV S proteins that are ~25% divergent from SARS-CoV S, **and found that they are unable to use the ACE2 receptor**. Additionally, we were unable to culture HKU3r-CoVs in Vero E6, or human cell lines.

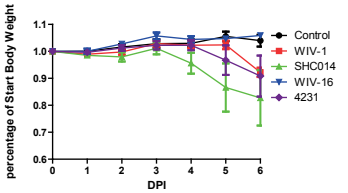


Fig. 7: Body weight change in hACE2 transgenic mice after SARSr-CoV infection, demonstrating that bat-origin SARSr-CoVs cause similar clinical signs to outbreak SARS-CoV strain (4231) (*62, 63*).

A similar approach to the above pipeline for CoVs will be applied to novel henipaviruses we discover during our research. The broad mammalian tropism of HeV and NiV (*68*) is likely mediated by their usage of highly conserved ephrin ligands for cell entry (*19, 100*). A third isolated henipavirus, Cedar virus does not cause pathogenesis in animal models. **Co-Is Broder and Laing** have developed a reverse genetics system and rescued a recombinant CedV to test ephrin receptor usage/tropism and pathogenicity during henipavirus infections (*77*). CedV is unable to use the Ephrin-B3 receptor (*77, 101*) which is found in spinal cord and may underlie NiV encephalitis (*102*) and that pathogenesis also involves virulence factors V and W proteins. The putative henipaviruses, Ghana virus and Mòjiāng virus, predict V and W protein expression, with GhV able to bind to ephrin-B2, but not -B2 (*103*), and the receptor for MoJV remains unknown (*104*) but is likely ephrin-B2 (*105*). Our group has also used similar methods to test the hypothesis that novel Filoviruses discovered in wildlife are able to infect people. NiV, EBOV and MERS-CoV all infect primary microvascular endothelial cells, which are available at Co-I Baric's lab for EID-SEARCH collaborators (*71, 106*). Primary human lung endothelial cells are highly susceptible to Ebolavirus infection (*107*), and Huh7 cells can be used to isolate virus from clinical samples (*108*) offering advantages for reverse genetic recovery of novel FVs (*109*). The three filovirus genera, *Ebolavirus*, *Marburgvirus* and *Cuevavirus*, use Niemann–Pick type C1 (NPC1) protein as cell entry receptor (*110*). For novel filoviruses, cell culture would be carried out under BSL-4, however they can readily be characterized following identification of their encoded GP sequences which can be pseudotyped into recombinant GP-bearing vesicular stomatitis virus (VSV) pseudovirions (*111*). These pseudovirions can be safely used to characterize the tropism and entry filoviruses mediated by GP without a need for BSL4 containment. **Co-Is Wang and Anderson** used cells originating from various mammalian species to determine spillover and/or zoonotic potential of MLAV, a newly discovered Asian filovirus (*47*). Despite the low amino acid sequence identity (22–39%) of the glycoprotein with other filoviruses, MLAV is capable of using NPC1 as entry receptor. Cells from humans, monkeys, dogs, hamsters and bats were able to be infected with a MLAV pseudovirus, implying a broad species cell tropism with a high risk of interspecies spillover transmission.

Mouse models. For novel viruses across all families, we will use the UNC collaborative cross mouse that captures 90% of the diversity within all mouse groups to assess the most suitable model for further experiments (*112*). We have used this model for CoV, FV (Ebola), Flaviviruses, and alphaviruses infections. Clinical signs such as weight loss, hemorrhage, encephalitis, and, acute or chronic arthritis were recapitulated in these mice following SARS, EBOV, West Nile virus and Chikungunya infections, respectively (*86-88, 113-116*). Note that CC OR3015 shows hemorrhagic disease phenotypes after EBOV infection **(Fig. 8)**. *Bat Models.* Duke-NUS has developed two models for bat *in vivo* studies: a colony of *Eonycteris spelaea* (cave dwelling small fruit bat) and a bat mouse model, which contains the bat immune system in an immunodeficient mouse (*117*). In a proof of concept study, **Co-Is Wang and Anderson** infected *E. spelaea* bats with MERS-CoV to demonstrate their susceptibility and suitability for infection with a bat borne virus under BSL3 containment. These bats and the bat mouse model will serve to complement and expand studies from the UNC collaborative cross mouse, pending further funding from EIDRC CC. Recombinant viruses, transgenic mouse models and experimental recombinant protein constructs described above will be made available to the EID-SEARCH consortium and other EIDRCs following standard procedures **(see Resource Sharing Plan)**.

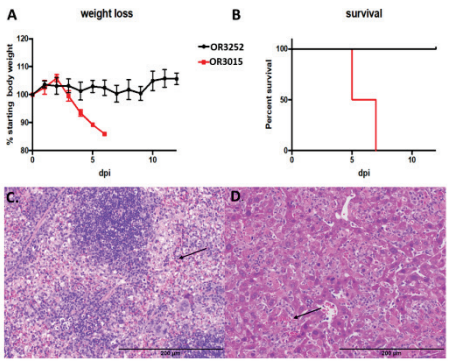


Fig. 8: EBOV Infection in Collaborative Cross Mouse. **Panel A/B:** Wt EBOV in two different CC lines demonstrate different disease patterns. **Panel C/D:** Hemorrhagic phenotypes on d. 6 (arrow) in spleen and liver, resp. From (*86*).

1.3. General Approach: We will conduct analyses to geographically- and taxonomically-target testing of samples from wild mammals that are most likely to harbor known high-profile zoonotic pathogens, or close relatives with potential to infect people. This includes selecting from tens of thousands of samples collected on recent projects and stored in freezers in our laboratories, and new sampling of wildlife in high risk locales. We will use viral family level PCR assays to identify and partially characterize viruses, then follow up sequencing of relevant receptor binding proteins to assess homology and predict binding affinity, then attempt to isolate and

biologically characterize viruses that phylogenetic and other data suggest have high spillover and pandemic potential. We will follow this with *in vitro* binding assays and cell culture experiments, and *in vivo* animal model infections to assess their potential to infect people and cause disease **(Fig. 9)**. EHA will lead the study design, targeted sampling, and data analysis; Co-Is Hughes and Wacharapluesadee will lead field sampling and in-country testing; Duke-NUS, UNC, USUS will lead the serological and PCR diagnostic development and the *in vitro* and *in vivo* analyses, as well as cross-training and tech transfer to the hub labs in Thailand and Malaysia. This will include via support of one Malaysian Ph.D student, Mei-Ho Lee (CM Ltd.) who has worked with the EID-SEARCH collaborators for 9 years. The focus of her study will be infection potential of novel CoVs and PMVs already found in bats in Malaysia under preliminary data for this proposal.

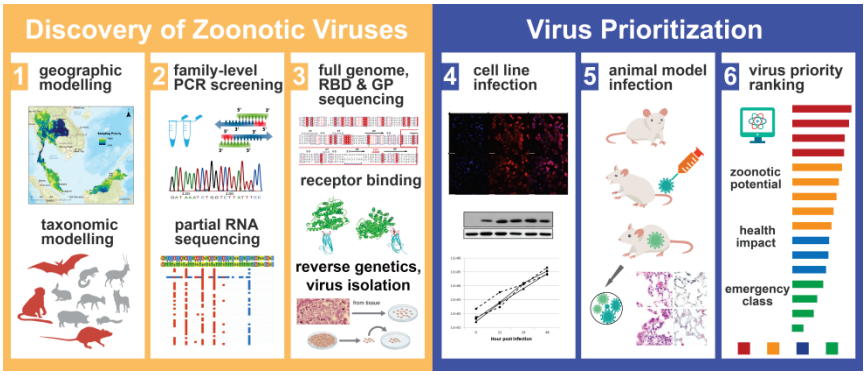


Fig. 9: Aim 1 will use analytical tools to target selection of archived and newly collected samples, conduct PCR and sequencing of novel viruses, recover by reverse genetics, and assess zoonotic potential using *in vitro* and *in vitro* models and analyses.

1.4 Wildlife samples: 1.4.a. Geographic and taxonomic targeting for newly collected wildlife samples: We will prioritize sites within each country that rank highest for both the risk of zoonotic disease emergence (*2*) and the predicted number of 'missing' zoonotic viruses (*3*). Our preliminary analysis **(Fig. 1)** suggests priority areas include: the Kra Isthmus (S. Thailand) and adjacent forests in N. Peninsular Malaysia, NW Thailand (near the Myanmar-Laos border), and lowland rainforests of Sarawak and Sabah. In each of these 'hottest of the hotspots' regions, we will identify 2-4 distinct sampling sites based on the geographic distribution of key hosts we prioritize for additional wildlife sampling. We will priority rank bat, rodent, and NHP species using host trait-based models (*3*). We will also identify species predicted to be centers of evolutionary diversity for CoVs, PMVs, and FVs using ancestral host reconstruction of available sequence data and Bayesian discrete phylogeographic models (*118-120*). We will leverage our strong collaborations with wildlife experts in Malaysia and Thailand, including many of whom are based at our core partner institutions, to ground-truth geographic and taxonomic model outputs with local expert knowledge to ensure the sampling plan is realistic based on current species distributions, population size estimates, seasonality, and accessibility to sites. Most zoonotic viruses are naturally carried by multiple wildlife host species, and there are strong, predictable relations between host phylogeny and viral taxonomy (*3*). We will use a combined network analysis and phylogeographic model to rapidly predict and prioritize new (unsampled) hosts for important, novel viruses we discover during our research. We have shown this relatively simple model (using just wildlife species range overlap and phylogenetic similarity between host species) is both generalizable to estimate host range for all mammalian and avian viruses *and* robust – accurately predicting hosts 98% of the time when cross-validated using data from known viruses (*121*).

1.4.b. Sample size justifications for testing new and archived wildlife specimens: We calculate initial sample sizes targets using our preliminary data on average PCR prevalence from screening tens of thousands of bat, rodent and NHP specimens for CoV and PMVs based on previous studies in Malaysia and Thailand. We will refine these sample size targets, in real-time, over the course of our project using viral discovery curve analyses **(Fig. 6)** to either scale-up or scale-back new sampling and testing of archived specimens for each species depending on viral capture rates. We will only collect additional specimens projected from each prioritized taxa to achieve statistically robust sample sizes and to complement our archived specimens. For example, *Rousettus* spp. bats, known FV reservoirs, were not adequately sampled under PREDICT research in Thailand. Preliminary analysis indicates that, for all viral target families, we will require ~14,000 new samples to identify 80% of remaining viral species diversity in our study region, ~5,000 samples from Thailand and ~9,000 samples from Malaysia **(Fig. 10).** Viral strain diversity from a sampling effort this size is expected to number in the hundreds of strains. For example, given ~6% prevalence of CoVs in bat species previously sampled under the PREDICT project, a target of 14,000 individuals would yield ~800 PCR positive individuals,

representing, an estimated ~600 novel sequences/strains. Similarly, for PMVs, which have an average PCR detection prevalence of ~1.5%, sampling of 14,000 individuals would yield ~200 positives and an estimated 150 unique strains. Filovirus estimates are not feasible yet due to the small number of positive samples in prior studies, but will be estimated as the project begins. From each mammal, we will collect serum, whole blood, throat swabs, urine and fecal samples or rectal swab using our previously-validated nondestructive methods. We will sample sites at different time-points throughout each year, and sample an equal number of males and females to account for seasonal or sex-specific differences viral shedding **(See Vertebrate Animals)** (*122, 123*). Dead or animals euthanized due to injury will be necropsied.

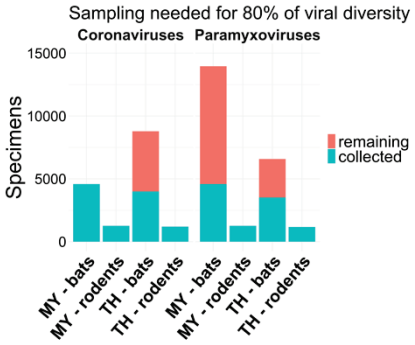


Fig. 10: Stacked bar plot showing number of currently-archived specimens **(blue)** and new specimens required **(orange)** to discover 80% of predicted number of CoVs and PMV species from high-risk bat and rodent taxa in Malaysia and Thailand.

1.4.c. Sample collection, testing, viral isolation: Wildlife will be captured and sampled using previously published techniques, by personnel wearing appropriate personal protective equipment (Tyvek suits or dedicated long external clothing, double nitrile gloves, leather gloves, an N95 or p100 respirator, and safety glasses) (*53, 93, 95, 96, 122, 124*). All samples will be placed in a vapor phase liquid nitrogen dry shipper immediately upon collection, and then transferred to a -80C freezer once back in the lab, until testing. Viral RNA will be extracted from bat fecal pellets/anal swabs. RNA will be aliquoted, and stored at -80C. PCR screening will be performed with pan-coronavirus (*125, 126*), filovirus (*127*) and paramyxovirus primers (*128*), in addition to virus specific primers where additional sensitivity is warranted in key viral reservoirs, i.e. Nipah virus or MERS virus specific primers (*129, 130*). PCR products will be gel purified and sequenced. We will attempt isolation on samples that contain viruses of interest (determined in **Aim 1.5 below**), using Vero cells and primary cell culture from the wildlife taxonomic order (bat, rodent and NHP cell lines). Over 70 bat cell lines are maintained at Duke-NUS from five different bat species. These include primary lung, brain, bone marrow, heart, kidney, intestine, liver, lymph node, muscle, spleen, PBMC and ovary/testes from *Eonycteris spelaea, Cynopterus bracyhotis, Rhinolophus Lepidus, Myotis muricola* and *Pteropus alecto* bats. In addition we have 8 immortalized cell lines, including those derived from lungs, therefore suitable for the isolation of respiratory viruses.

1.4.d. Moving beyond RNA viruses: To detect pathogens from other families, such as non-RNA viruses, we will combine conserved PCR assays with NGS. We will perform either unbiased NGS, or use a pan-viral enrichment library to sequence full-length genomes (*131*). We will conduct this work via EID-CC research projects or as part of outbreak detection/investigation in the region from DNA viruses or other agents.

1.4.e. Sequencing Spike Glycoproteins: Based on earlier studies, our working hypothesis is that zoonotic CoVs, FVs and henipaviruses encode receptor binding glycoproteins that elicit efficient infection of primary or continuous human cell lines (e.g., lung, liver, etc.) (*16, 61, 62*). Characterization these for SARSr-CoVs suggest that viruses up to 10%, but not 25%, different in the spike glycoprotein use human and bat ACE2 receptors for docking and entry. Uneven sampling gaps (e.g., no strains were found with 10-25% spike variation) prevent a thorough understanding of the transition point where the most divergent strains lose human ACE2 receptor usage. Our viral discovery curves **(Fig. 6)** suggest further sampling will reveal a rich diversity of as-yet-undiscovered SARSr-CoVs and strains of other viruses that fill in the phylogenetic tree between currently-described viruses. In this aim we will use phylogenetic and viral discovery analysis to specifically target bat species and regions that are under-sampled to allow sufficient power to identify and characterize missing strain diversity for potential zoonoses. We will sequence the S proteins of novel SARSr-CoVs to prioritize viruses for experimental work in Aim 3 to test this hypothesis. Likewise, we will test the hypothesis that phylogenetic related zoonotic receptor binding protein genes of MERS-related, Ebola-related and NiV/HeV-related viruses will also program efficient entry via human and ortholog receptors. For all novel virus strains, we will sequence the complete entry spike genes by amplifying overlapping fragments using degenerate primers as shown previously (*24, 61*). ***Reverse Genetics:*** Full-length genomes of selected CoVs, FVs or henipaviruses (representative across subclades) will be sequenced using NEBNext Ultra II DNA Library

Prep Kit for Illumina and sequenced on a MiSeq sequencer. PCR or MinIon sequencing will be performed to fill gaps in the genome. The full length spike gene sequences, including the amount of variation in the receptor binding residues that bind the appropriate human receptor will be used to select strains for Aim 3 experiments. We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-CoV cross neutralization, synthetically reconstruct a small subset (1-2) and evaluate growth in Vero, BHK and Huh7 cells (*63, 132*). From full length sequences, synthetic clones will be ordered to recover recombinant viruses using reverse genetics as described by our groups (*62, 63, 77, 109, 133, 134*). Recombinant virus growth will be accessed in Vero, BHK, Huh7 and select bat cells and recovered viruses used for downstream studies. Virus strain prioritization is described below.

1.5. Assessing risk for spillover. 1.5.a. Capacity to infect human cells: We will use a series of cell cultures to assess the capacity of target viruses to enter human cells. For CoVs, FVs and henipaviruses, we will use primary human ciliated airway epithelial cells (HAE) and lung endothelial cell cultures derived from the lungs of de-identified transplant recipients. HAE are highly differentiated and grown on an air-liquid interface for several weeks prior to use (*62, 63, 132*). We will prepare HAE and lung endothelial cultures from three different patient codes in triplicate in collaboration with the tissue procurement facility at the Cystic Fibrosis Center at UNC. Cultures will be inoculated with chimeric bat SARSr-CoVs or novel FVs or henipaviruses to assess efficient replication. At 72 hpi, cultures will be fixed for immunofluorescent staining using antisera to the SARS-CoV conserved nucleocapsid protein (N) or to structural genes of the other viruses (*64, 71*). SARSr-CoVs that differ significantly in S protein sequence (11-24%) from epidemic SARS-CoV yet replicate *in vitro*, will also be evaluated for sensitivity to neutralization in Vero cells by PRNT50 assays using broadly SARS-CoV cross reactive human mAbs S227.14, S230.15, S215.17, and S109.8 (*63, 135*) or monoclonal antibodies targeting the Ebola or Marburg E glycoprotein (Available through BEI), or NiV/HeV glycoproteins (*136*). As controls or if antisera is not available, the S genes of novel SARSr-CoV or spike entry genes of other novel viruses under investigation will be inserted into VEE 3526 replicon vectors (VRP3526-S), packaged and used to vaccinate mice (*137*). Polyclonal sera against test bat-SARS virus or other spike genes will be harvested and tested for ability to cross neutralize SARS-CoV, GD03, WIV-1, SHC014, WIV-16, other novel SARSr-CoV and HKU3-SRBD by PRNT50 assay (*63, 138, 139*). Using PRNT50 titers with sera (n=4 each) among these viruses, antigenic cartography (*140*) will allow comparison of antigenic vs. phylogenetic distance, identifying the transition at which SARSr-CoV strains escape SARS-CoV based vaccines, informing immunotherapeutic and vaccine design strategies (*141-143*). Similar approaches will be applied to novel MERS-related viruses, other CoV, FVs or henipaviruses. While most wildlife-origin viruses with unknown transmission potential or pathogenicity in people uncategorized, we use BSL-3 for isolation as standard, and cell culture for potentially BSL-4 pathogens will be undertaken at collaborating institute NEIDL. To facilitate this, duplicate samples in culture medium from animals PCR +ve for FVs and very close relatives of NiV or HeV will be shipped to NEIDL **(See letter of support)** for culture, characterization and sharing with other approved agencies including NIH Rocky Mountain Laboratories where PI Daszak and Co-Is Baric have ongoing collaborations. Similarly, some duplicate samples of animals PCR +ve for CoVs will be shipped to UNC for further characterization by Co-I Baric. Where feasible and appropriate, training opportunities at NEIDL and UNC for in-country staff will be given, thereby enriching hands-on collaboration across sites.

1.5.b. Host ACE2, DPPR, Niemann-Pick C1 (NPC1) and EphrinB2/EphrinB3 receptors: We will sequence relevant host receptors for select mammalian wildlife species we find positive for strains of high-priority CoV clades, and all new henipaviruses and FVs. Of particular interest is homology across 18 bat and human orthologue ACE2 contact interface residues that engage the SARS RBD as a potential indicator of SARSr-CoV cross species transmission potential and growth in human cells (*70*). Likewise, variation in the NPC1 receptor has been shown to alter EBOV ability to infect in humans and bats, so this can be structurally mapped for prediction and downselection of strains (*144*). Signatures of positive selection in bat NPC1 were concentrated at the virus-receptor interface, with the strongest signal at the same residue that controls EBOV infection in *Eidolon helvum* cells. Thus NPC1 is a genetic determinant of FV susceptibility in bats, and some NPC1 variations likely reflect host adaptations to reduce FV replication and virulence (*145*). NiV and HeV use ephrin B2 or ephrin B3 as a receptor, and variation affects its ability to use human vs bat receptor molecules (*103, 146*).

1.5.c. Host-virus evolution and predicting receptor binding: We will use Bayesian and Maximum Likelihood phylogenetic analyses of RNA-dependent RNA polymerase (RdRp) sequences from PCR screening, receptor binding glycoproteins, and/or full genome sequence data (when available) to reconstruct the evolutionary history of the novel bat SARSr-CoVs, bat MERSr-CoV, FVs and henipaviruses that we identify. We will run co-phylogenetic analyses to map out instances of host-switching (*147, 148*) and ancestral state reconstruction analyses **(Fig. 4)** to identify the most important species for shaping evolutionary diversity for these viruses (*51, 149*), allowing for more targeted future surveillance and spillover mitigation interventions. For receptor binding glycoprotein sequence data from characterized viruses, we will apply codon usage analyses and codon adaptation indices, as used recently on henipaviruses (*150*), to assess the relative contributions of natural selection and mutation pressure in shaping host adaptation and host range.

1.5.d. Viral strain prioritization: Of the expected 100-200 novel SARSr-CoV strains, and approximately 600 total CoV strains, we will down-select to prioritize for further characterization based on S genes that are: i) different from SHC014, WIV1, SARS-CoV with diversity ranges of 10-25%; ii) have virus S RBD that could use human/bat receptors; iii) have recombinant chimeric spikes indicative of gene flow between clade I and II strains; iv) have bat ACE2 or DPP4 receptors that might select for spike RBDs that can use human receptors for entry (15/18 conserved residues in human/bat ACE2 molecules that bind SARSs-CoV S RBD domains are likely more efficient receptors than 3/18 conserved sites). Using structural models based on the SARS S glycoprotein, the extent and location of antigenic variation will be annotated onto the structure, further resolving the locations of highly evolving and conserved structural motifs/epitopes that function in protective immunity (*135, 151-153*). For novel henipaviruses, we will examine the receptor-binding proteins (e.g. envelope attachment (G) proteins) for sequence and structural homology with particular attention to conversation of amino acids that mediate ephrin receptor binding. We will prioritize strains that are predicted to interact with ephrin-B2 and expression V/W proteins to determine whether these viruses represent zoonotic threats. Using the recombinant Cedar virus (rCedV) platform, we will be able to rescue rCedV that express heterotypic G proteins to explore receptor utilization and tissue dissemination in animal models. We will assess ability to infect human cells using a continuous lung epithelial cell line, Calu3, which our group has previously used to generate data on NiV and HeV growth curves and a variety of host expression changes after infection (*154*). We will also use primary human airway cultures and microvascular endothelial cells to assess human infection for FVs, henipaviruses and MERSr- and SARSr-CoVs (*155*) (*156*) . There is some evidence for NiV and HeV replication in mouse lung, low level titers in young animals, 10 fold higher titers in aged mice (*157, 158*). Thus it is likely these models could be improved in the collaborative cross mouse resource at UNC. We have identified CC lines that are highly vulnerable to wildtype SARS and others that develop hemorrhagic disease following mouse adapted Ebola infections (*86-88*).

1.5.e. Animal models: We will use transgenic mouse models and the UNC Collaborative Cross model to assess spillover potential of viruses. For work on CoVs, we will follow the protocols in the transgenic mouse model used by the Baric lab previously. Briefly, in the BSL3 lab, 10- to 20-week old hACE2 or hDPP4 transgenic mice will be intranasally inoculated with 5 x 10^4 PFU of full length wildtype rbat CoV, or chimeric SARSr-CoV or MERSr-CoV encoding different bat CoV spike proteins respectively, then monitored daily for weight loss, morbidity, and clinical signs of disease. Mice will be euthanized at 2, 4, and 6 dpi (endpoint of 14 dpi), organs harvested and virus quantified by plaque assay or genome (mRNA1) RT-PCR. After 7 days in formalin, tissues for histopathology will be removed from the BSL3 and stained with H&E, and for immunohistochemistry using polyclonal antibodies against the N protein. We will conduct limited evaluation of existing countermeasures using therapeutic monoclonal antibodies *in vitro*. Existing SARSr-CoV or MERS-CoV mAbs (*159, 160*) will be diluted in DMEM starting at 1:20, and serial dilutions mixed with an equal volume of 50 PFU of chimeric bat SARSr-CoVs encoding different spike proteins and RFP indicator genes, then incubated on Vero E6 cells at 37°C for 1 h. Antibody neutralization titers will be evaluated by plaque quantification at 4-5dpi. In select instances, hACE2 transgenic mice will be injected with SARS-CoV mAbs, and infected with chimeric bat SARSr-CoVs. Clinical signs and morbidity will be assessed and tissue pathology examined and compared with mice without treatment of mAbs to determine the therapeutic effect on SARSr-CoV infection, and protection of SARSr-CoV by wildtype SARS-S based vaccines assessed as described (*132, 161*). We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-

CoV cross neutralization, synthetically reconstruct a small subset (1-2), recover full length viruses and evaluate the ability of nucleoside analogues to inhibit growth in HAE cultures and/or *in vivo* (*63, 132*). For the Collaborative Cross model, we will infect six to eight mice from 6-8 Collaborative Cross mouse lines (10 weeks of age) with six test viruses/yr, intransasally or by subcutaneous infection with 1 x 10e4 virus. Animals will be followed for weight loss, morbidity, mortality and other clinical disease symptoms (e.g., hunching, hindleg paralysis). One half the animals will be sacrificed at day 3 and day 7 pi. to evaluate virus growth in various organs, and organ pathology. In highly vulnerable lines, additional experiments will be performed using more animals, evaluating virus growth, clinical disease symptoms, respiratory pathology, tropism and organ pathology through day 28 pi. We anticipate that some CC lines will prove highly vulnerable to select wildtype viruses, because of host susceptibility combinations that promote severe disease outcomes.

1.6. Potential problems/alternative approaches: Challenges with logistics or obtaining permission for wildlife sampling in sites we select. We have a >20-year track record of successful field work in Malaysia, and >10 years in Thailand, and have strong established partnerships with local wildlife authorities and communities to ensure access. We have existing permissions in place which will be renewed at the start of this project. Due to the abundance of wildlife habitats in the region, if access to a site is withdrawn, we will rapidly identify a suitable, complementary site with similar bat species composition and abundance. Finally, we have already collected tens of thousands of wildlife samples from the region and will focus on these while waiting for permission. **We may not identify novel viruses in our sample wildlife species due to seasonality of viral shedding or other factors.** We have calculated sample sizes based on realistic detection rates from our extensive preliminary data, and are confident that we will detect and identify large numbers of potentially zoonotic pathogens, particularly for CoVs and PMVs. We acknowledge that FV strain diversity may be harder to capture, given relatively low seroprevalence in bat populations – suggesting lower levels of viral circulation (*42, 43*). However, our team was the first and only group to sequence FV RNA from Asian wildlife species (*46, 47, 57*), and we are confident that with our targeted sampling and testing strategy offers the best chance globally to identify additional strains of Ebola and related viruses. The sampling regions we have selected are subtropical, and our previous data, and even published studies in temperate regions (*162*), do not suggest a strong pattern of seasonality in CoV shedding, and where seasonal patterns do occur, i.e. for henipaviruses in Thailand (*163*), or can be predicted from wildlife serology data, we will be sure to conduct sampling at different timepoints throughout the year to account for this.

Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays.

2.1 Rationale/Innovation: Rapid response requires early detection. Assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities that interface frequently with wildlife that harbor high risk emerging viral strains. To enhance the low statistical probability of identifying these rare events, In Aim 2 we will target rural human communities inhabiting regions identified in Aim 1 as having high viral diversity in wildlife, that also engage in practices that increase the risk of spillover (e.g. hunting and butchering wildlife). We will initially identify 4 subsites in each of Thailand, Peninsular Malaysia, Sarawak, and Sabah for sampling. We will design and deploy human risk factor questionnaires, and specific and sensitive serological assays to identify the baseline frequency, and causes of, spillover of known and related novel viral pathogens in these populations. **We will use the results to test a key hypothesis: that hunting wildlife increases the risk of exposure to novel viruses, compared to the rest of the community.** The alternative hypothesis is that seroprevalence in the general community is not statistically different to those who hunt and butcher wildlife, because exposure to wildlife pathogens is largely through inhabiting a biodiverse landscape where wildlife make indirect contact with people (e.g. via fomites). For participants who report symptoms related to 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever; or 6) unusual presentation of high fever with severe diarrhea; all within the last 10 days, an additional sample type will be collected, two nasal or oropharyngeal swabs. Samples will be marked for additional PCR-based assays to identify presence of known and novel CoVs, henipaviruses and FVs, and for isolation and biological characterization of potential pathogens, using the approach laid out in Aim 1.

2.2 Preliminary data on high-zoonotic risk human community surveillance: 2.2.a Biological sample collection in SE Asia: Our long-term collaboration in the region has included qualitative and quantitative survey data, and collecting large archives of banked human sera and other biological samples from high zoonotic-risk populations. For example, under the USAID-PREDICT project, EHA has collected 1,400 and 678 specimens in Malaysia and Thailand respectively from people living in zoonotic disease interfaces, all of which will be available for retrospective serological testing by EID-SEARCH which we have ensured is compliant with our existing IRB. Our core group has collected and tested many thousand additional specimens from other important community cohorts **(Table 2)**, and some of these will continue under EID-SEARCH **(Section 2.4)**.

Country, site	Lead	# enrolled, focus	Findings
Peninsular Malaysia	Hughes	9,800+ samples, Orang Asli indigenous pop., for PCR/serol.	25+ novel CoVs, influenza, Nipah ab+ve, FV ab+ve
Malaysia Sabah	Kamruddin	1,283 for serology	40% JE, 5% ZIKV ab+ve
Malaysia Sabah	William	10,800 for zoonotic malaria study	High burden of macaque-origin malaria
Malaysia Sabah	Hughes	150 bat cave workers	Ongoing
Malaysia Sarawak	Siang	500 Bidayuh and Iban people	8% PCR prevalence HPV in women
Malaysia PREDICT	Hughes	1,400 high zoonotic-risk communities for viral PCR, serology	Ongoing. Multiple known and novel CoVs, PMVs, others.
Thailand	Wacharaplu-esadee	100s of bat guano harvesters/villagers	Novel HKU1-CoV assoc. clinical findings
Thailand PREDICT	Wacharaplu-esadee	678 high zoonotic-risk communities for viral PCR, serology	Ongoing. Multiple known and novel CoVs, PMVs, others.
Singapore	Wang	856, for Melaka virus	7-11% MELV ab+ve

Table 2: Biological sample collection from healthy populations conducted by members of **EID-SEARCH** in our hub countries.

Of particular interest is the access to indigenous communities in Peninsular Malaysia (Orang Asli) and Sarawak (Bidayuh, Iban people), and the bat guano harvesters in Thailand. These communities hunt, butcher and eat wildlife daily, and work in supremely high-risk zoonotic interfaces (e.g. bat caves). One of our indigenous community sites is experiencing an ongoing outbreak of unknown etiology that we are investigating currently **(Section 3.2.a)**.

2.2.b Human Contact Risk Factors: EHA is the lead organization across all 27 USAID-PREDICT project countries for collecting data on, and analyzing the human aspects of, zoonotic spillover risk (*164, 165*). We conducted exploratory studies using standardized one-on-one semi-structured interviews and site observation data with people living in hotspot areas or engaged in high-zoonotic spillover risk activities, using these data to assess local social and cultural norms underlying wildlife contact (*165*). We then developed a risk factor survey to accompany human biological sampling to evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. We conducted cross-sectional studies in high risk populations in 27 countries, and study participants provided biological samples (oral/oropharyngeal swab, nasal/nasopharyngeal swab, urine sample/urogenital swab, fecal sample/rectal swab, whole blood, serum, plasma). For example, survey and biological samples were collected from: 1,585 participants from 7 sites in Yunnan, Guangxi, and Guangdong provinces, China; 1,400 participants from 4 sites in Malaysia; and 678 participants from 4 sites in Thailand. Samples were tested with novel serological assays and results analyzed against risk factors from survey data (*16*). In China, CoV-seropositive individuals were mostly farmers/peasants (8/9), living in Yunnan province (7/9), 41-60 yrs old (7/9), with domestic contact with rodents (6/9), that are male (6/9). Although these results are preliminary and don't provide detailed information on routes of exposure, they identify key subpopulations to target that would likely increase the opportunity for identifying evidence of spillover for other CoVs, PMVs and FVs. Data from PREDICT surveys have been used to help identify target populations in Aim 2, identify strategies to better target at-risk people, and conduct focused **human risk factor surveys and serosurveys to produce statistically significant findings for henipaviruses, CoVs and FVs.** In Aim 2, we will use combined biological sampling and risk factor surveillance in targeted populations within the community to 1) test the hypothesis that occupational (i.e. wildlife hunting) risk factors correlate with seropositivity to henipaviruses, FVs and CoVs; 2) assess possible health effects of infection in people; and 3) where recent

illness is reported, obtain biological samples for PCR testing and potential viral isolation and characterization. Obtaining this information could be a significant step in understanding the likelihood of recent 'hidden' spillover events and their public health impacts, as well as the risk of future emergence of viral disease.

2.2.c. Risk factors for illness: Our preliminary data in Thailand, Malaysia and other SE Asian countries revealed that frequent contact with wild (e.g. bats, rodents, NHPs) and domestic animals (e.g. poultry, swine) among local communities was associated with a high percentage of self-reported severe/acute respiratory illness (SARI/ARI) and Influenza-like illness (ILI) symptoms **(Section 3.2.b)**. In addition, people involved in crop production, hunting and butchering of wildlife reported statistically significantly higher SARI and ILI symptoms (Li *et al.*, in prep.). **These preliminary data suggest a target population and sites for current proposal with the correct targets for serological surveillance and risk factor surveys in Aim 2 to produce statistically significant findings**. Coupled with better serological tools from our team **(Section 2.2.d)**, we will be able to identify the likely routes of exposure to known or novel coronaviruses, henipaviruses, and FVs in the study region, as well as build data on the illnesses they cause in people.

2.2.d. Serological platform development: Most emerging viruses produced a short-lived viremia in people so that large samples sizes are required to provide enough PCR-positive individuals to analyze infection patterns. For Aim 2, we will focus on serological sampling in the community, because antibodies are typically long-lived and can be used to assess prior virus exposure or infection with much smaller samples sizes (*123*). Most serological assays target a single protein, and for emerging viruses, it's often unclear which viral antigen will be immunodominant during human infection, and therefore which to target in developing serological assays. Co-Is Wang, Anderson (Duke-NUS) and Broder, Laing (USUS) have developed a series of approaches to deal with this problem, and which will be used in testing human biological samples in Aims 2 and 3. Henipaviruses, FV and CoVs express envelope receptor-binding proteins that are the target of protective antibody responses. Co-I Broder has led efforts to express and purify henipavirus native-like virus surface proteins for immunoassay application (*166, 167*), developing monoclonal antibodies (*168, 169*) and as subunit vaccines (*170, 171*), has developed a novel, multiplex microsphere immunoassay (MMIA) allowing serum samples to be simultaneously tested for antibodies reactive with henipaviruses, FVs and CoVs. This MMIA uses a Luminex-based multiplex platform and custom designed virus receptor-binding proteins expressed in a mammalian cell-culture system and purified as native-like oligomeric proteins. These oligomeric antigens retain post-translational modifications and quaternary structures, allowing capture of conformational-dependent antibodies raised during a natural infection, which represent the majority of the protective humoral response. The known diversity of henipaviruses includes five presently described viruses, NiV, HeV, CedV, GhV and MojV and this assay has been expanded to include all described species **(Fig. 11)**. In the past, our collaborators have used NiV and HeV G protein antigens in a MMIA assay to identify a serological footprint of NiV-related African henipaviruses and exposure to virus(es) antigenically-related to NiV in livestock sampled in Bangladesh (*78, 79*). To strengthen serological data interpretation, the G protein from each virus is integrated into the MMIA so that virus-specific vs. henipavirus cross-reactive antibodies can be detected and address specificity of past virus exposure. Extensive cross-reactivity exists among ebolaviruses and between ebolaviruses and marburgviruses (*172-174*). We are validating the MMIA pan-FV assay for specificity, and as part of this, in collaboration with Duke-NUS, we have found serological evidence of past exposure to a filovirus(es) most antigenically-related to ebolaviruses (e.g. Ebola virus, Bundibugyo virus), but unreactive with Reston virus GP in three under-sampled fruit bat species, suggesting that novel FVs circulate within bat hosts in SE Asia (*43*). **This platform has now been set up for use in Malaysia and Thailand and will be available for use in this proposed work.** Co-Is Hughes, Broder, Laing have conducted initial screening of human, wildlife and livestock samples at high-risk interfaces in Malaysia, finding serological evidence of past exposure to viruses antigenically-related to NiV and EBOV in humans, bats and non-human primates (NHPs).

Fig. 11: Validation of multiplex microsphere immunoassay (MMIA) specificity and identification of immunologically cross-reactive viruses for A) NiV B) EBOV.

Co-Is Wang and Anderson (Duke-NUS) have developed a phage-display method to screen antibodies for all known and related bat-borne viruses, including henipaviruses, FVs and CoVs and have set up a similar Luminex-based assay. Additionally the Duke-NUS team has demonstrated capacity for rapid and cost-effective development of LIPS serological assays against novel viral agents **(Sections 2.6.a, 3.2.a)**. These will be used for verification and expanded surveillance. For molecular investigation (Aim 2 & 3), we will combine targeted PCR with a discovery-driven enrichment NGS strategy to not only survey currently known viruses, but also to discover potential novel viruses. Both the phage-display serological and enrichment platforms can be rapidly updated to include new viruses identified.

2.3 General Approach/Innovation: Our human sampling approach uses two approaches in Aims 1 & 2 (Fig. 12). In Aim 2 we will conduct community-based surveillance using a brief, focused questionnaire to identify the prevalence and frequency of risk-factors for viral spillover in tandem with collection of high quality biological samples to determine the seroprevalence of spillover viruses in at-risk human populations. We will use generalized linear models to analyze correlation between biological results and risk-factors for viral spillover. **In Aim 3**, we will conduct clinic-based syndromic surveillance and biological sampling at clinics/hospitals that are the catchment healthcare facilities for the communities sampled in Aim 2. Both community-based and clinic-based syndromic surveillance programs are cross sectional case-control studies designed with the sample sizes necessary to statistically quantify (with a power of 80%) risk factors and health impacts for viral spillover, linked to serological status and PCR prevalence in symptomatic patients. All human sampling conducted under Aims 2 and 3 will adhere to strict criteria for inclusion and recruitment and protection of human subjects from research risk **(see Human Subjects and Clinical Trials Information)**.

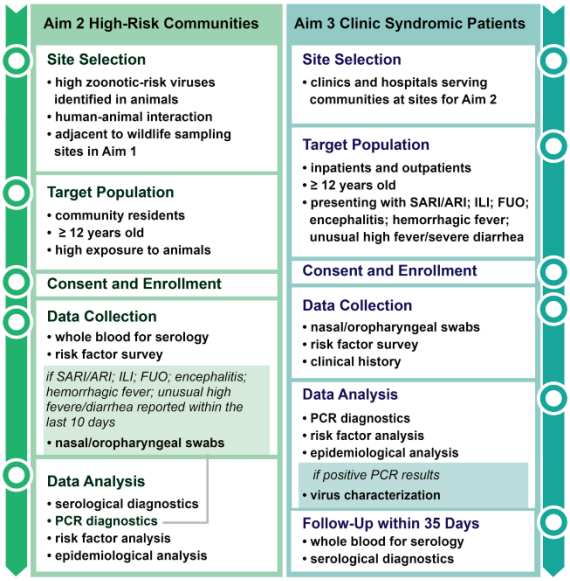


Fig. 12: Human sampling approach. Aim 2 **(left)** focuses on serology in high-risk communities to identify evidence of population exposure to novel zoonotic viruses. Aim 3 **(right)** sets up clinical cohorts at hospitals serving these high-risk communities to conduct syndromic surveillance and identify the etiology of new viral syndromes

2.4 Target population & sample sizes: We will target populations in the same geographic regions as those identified in Aim 1 that harbor wildlife with high potential zoonotic viral diversity, that are EID hotspots, and that are well-connected through regional travel and trade hubs to the global travel network **(Fig 13)**. Within each geographic region we will identify populations at four sub-sites, building on our preliminary data **(Table 2)**. We will target specific communities based on our analysis of previously collected behavioral questionnaire data, and other publicly available data (e.g. wildlife hunting licenses sold in a region, number of guano caves accessed for fertilizer etc.) to identify key at-risk populations and occupations with high exposure to wildlife. We will conduct concurrent sampling trips at visits to sub-sites during which members of the community will be enrolled and wildlife surveillance activities conducted. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock, or slaughtering wildlife will be considered for enrollment. Participants will complete a consent form and questionnaire in addition to having biological samples collected. During these community trips after data collection the field research staff will conduct community meetings to help inform the public health risk of zoonoses. **Target populations:** *Thailand (Co-I Wacharapluesadee):* 100 subjects of healthy high-risk community from 2 study sites, Chonburi (NiV, other PMVs and CoVs found by PCR in bats in our previous work), and Ratchaburi (bat guano harvester villages where we found a MERSr-CoV in bat guano (*50, 175*) and serological evidence of α-CoVs, HKU1-CoV in people (*175*). *Peninsular Malaysia (Co-I Hughes, CM Ltd.):* We will expand our sampling of the Orang Asli indigenous communities to include sub-sites in communities in Districts we have already sampled and

additional Districts in the States of Kelantan Perak, Pahang, and Kelantan all close to bat caves. Samples will be stored and tested at NPHL. *Sarawak (Co-I Tan, Fac. Med. Hlth. Sci. UNIMAS):* We will expand our sampling of the Bidayuh and Iban indigenous people to include the Dayak and Orang Ulu ("people of the interior") because this group has a more intimate connection to remote wildlife-rich areas than the other two, albeit all are hunters. Human sampling will be led by UniMAS in collaboration with CM Ltd / EHA. *Sabah:* We will conduct sampling at the Madai bat and bird cave that has 200 permanent residents who harvest bird's nests for soup, and >1,000 during the harvesting season and at Gomantong cave – one of the largest bat and cave swiftlet nesting sites in the world. *Sabah: (Co-I Hughes):* We will conduct sampling at the Madai bat and bird cave that has 200 permanent residents who harvest bird's nests for soup, and >1,000 during the harvesting season and at Gomantong cave – one of the largest bat and cave swiftlet nesting sites in the world. Protocols follow those for Peninsular Malaysia *Singapore: (Co-I Wang, Duke-NUS):* Active human surveillance will be not be conducted in Singapore, but archived human specimens e.g. from our Melaka virus serosurvey (*8*), will be made available to conduct retrospective serosurveys for specific pathogens that are identified as high interest to the EIDRC, NIH, and EID-CC.

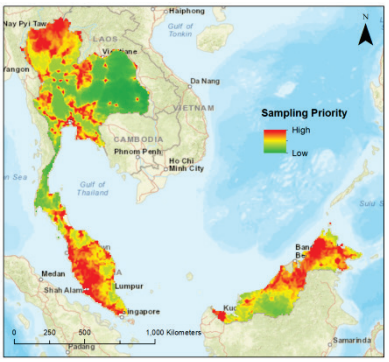


Fig. 13: Targeting high-risk sites for human zoonotic disease surveillance in Thailand and Malaysia. This map plots areas with high numbers of expected viruses in wildlife (*3*), greater spillover probability based on EID risk factors (*2*), and higher levels of connectivity (measured by road density data) that may facilitate person-person spread.

Sample sizes: From our previous work we conservatively anticipate that 10-30% of the community population will have had exposure to wildlife allowing us to capture highly exposed and non-exposure individuals at each site. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock farming, transportation, selling, or slaughtering wildlife in the surveyed areas will be targeted so that they make up ≥30% of the sampled population in each community. We will stratify sampling to ensure appropriate representation of sex, demographic, and socio-economic factors at each community site. In each of our regions, we will conduct sampling at four sub-sites in populations with high prevalence of high occupational and environmental risk of exposure to wildlife populations, sampling 175 persons at each sub-site. We will target populations with, on average, 30% or higher prevalence of occupational or environmental exposures to wildlife based on data from previous studies. This design will give us >80% power to detect 5 percentage point differences in seroprevalence against any of our viral groups between baseline and high-risk subgroups. We conservatively assume 5% base seroprevalence, as well as assuming high spatio-temporal variance amongst sub-sites, with baseline varying 0-25% amongst sub-sties and the fraction of the population at risk varying from 20-40%. (Power calculation conducted by 500 simulations of a generalized linear mixed model (GLMM)). For community-based surveillance, we will enroll 700 individuals per study region, allowing us to make county/province-level comparisons of differing effects; the total sample will be 3,500 participants over 5 years.

2.5 Data & sample collection: Following enrollment, biological specimens (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max 500 µL of whole blood and two 500 µL serum samples) will be collected by locally certified healthcare professional proficient in phlebotomy techniques and a short questionnaire will be administered by trained project staff that speak appropriate local language to all participants. Through the serological findings and the survey we will investigate the details of five risk factors related to high risk wildlife exposure, to maximize the power of the analyses, based on continuing analysis of our previous work, and will focus on: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. For participants who report the symptoms relating to 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever within the last 10 days, an additional sample type will be collected, nasal or oropharyngeal swab (2x). Blood of participants whose serum tests positive for emerging viral pathogens will be taken and Peripheral Blood Mononuclear Cells (PBMCs) frozen for future use.

These will be used for future harvesting of polyclonal and monoclonal antibodies as potential therapeutics/ diagnostics **(see Letter of Support NEIDL)**.

2.6: Laboratory analysis: 2.6.a Serological testing: We will screen all human specimens for henipaviruses, FVs and CoVs using our novel, multiplex microsphere immunoassay (MMIA) **(Section 2.2.d)**. We will use ELISA and serum neutralization tests (SNT) as confirmatory, where feasible and appropriate for the biocontainment level given sensitivity and specificity variation, and the need for live virus for SNTs **(See Select Agent Research)**. Serologic evidence of local spillover will be used to prioritize zoonotic strains for recombinant virus recovery from full length sequences. Preliminary data suggest ELISA will be effective for some viral groups. We previously developed a SARSr-CoV specific ELISA for serosurveillance using the purified NP of a bat SARSr-CoV (Rp3). The specificity of this assay was evaluated using polyclonal antibodies against HKU1, OC43, 229E, NL63, MERS-CoV and EBOV and no significant cross-activity detected (*16*). **This suggests that if we can expand our NP serology tests to cover other bat CoVs, and that doing so may identify many more seropositive individuals.** For CoVs, we will therefore use two serological testing approaches: 1) testing human sera collected from both community- and clinic- based sampling for a panel of bat CoVs that will include SARS-CoV, MERS-CoV, SADS-CoV and a range of bat SARSr-CoVs (*16*); 2) testing for antibodies to common human CoVs (HCoV NL63, OC43 – **Section 2.8**). We will test panels of NP and G recombinant proteins to assess if this approach will act as a comparison to MMIA for FVs and PMVs.

2.6.b RT-PCR testing. Specimens from individuals in the community who reported being symptomatic within the last 10 days **(Section 2.5)** will be screened using RT-PCR initially for CoVs, PMVs, and FVs following published protocols **(Section 1.4.c)**. Positive samples will be subjected to full genome sequencing and RT-PCR amplification of the glycoprotein genes. Samples from the clinic-based syndromic surveillance will also be tested using RT-PCR for Influenza A & B, HCoV NL63, OC43, HKU1, SARS-CoV & 229E, NiV, HeV, Ebola (WHO PCR protocol) based on the symptom as rule-outs.

2.6.c Biological characterization of viruses identified: Novel viruses identified in humans will be recovered by cultivation or viruses re-derived from full length genome sequences using reverse genetic approaches. Viruses will be characterized for growth in primary human cell lines and in mouse models, including the Collaborative Cross Mice or humanized mice expressing human receptors from related strains. The approaches are discussed in more detail under Aim 1.

2.7 Epidemiological analysis: We will conduct a cross sectional case-control study to identify risk factors for Henipavirus, FVs, and CoVs spillover. “Cases” are defined as participants whose samples tested positive for Henipavirus, FVs, or CoVs by serological tests. “Controls” will be selected from the pool of participants admitted to the studies and whose samples tested negative for Henipavirus, FVs, or CoVs. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models and least absolute shrinkage and selection operator (LASSO) regression analyses (*176*) to analyze correlation between biological results, serological/PCR status, and risk factors related to high risk wildlife exposure including: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) home or work proximity to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms lifetime and past 12mo. Additionally, we will use this procedure to determine how clinical presentation differs between Henipavirus, FVs, or CoVs in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms.

2.8 Potential problems/alternative approaches: Rarity of spillover events means it may be difficult to identify sufficient seropositives to statistically analyze human risk profiles. First, we will be targeting our community-based surveillance to subpopulations with high-levels of wildlife exposure, at sites selected for diverse and prevalent viruses to increase likelihood of finding positive individuals. Second, our serology testing will include a multi-plex panel of assays for a large diversity of viruses, increasing likelihood of positives. For example, in previous work our overall bat CoV PCR prevalence in the community was, 11.8%; β-CoV, 3.4%; α-CoV, 9.1%). Thus, using this broad serological panel to screen individuals prone to contact with species of higher zoonotic risk increases the potential for detecting spillover with enough power for statistical analyses,

and will shed light on behaviors that predispose to spillover. Third, we will include common human CoVs, PMVs, and influenza viruses in our panel, so that even if low prevalence of wildlife viruses is found, we will be able to conduct a valuable cross-sectional study of the seroprevalence of more common human viruses. Finally, we will be able to assess relative measures of human-wildlife contact from our survey work. We will analyze intensity of contact with high-risk taxa against other risk factors to provide useful proxy information for spillover risk. **Serological testing may be difficult to interpret in the presence of known and novel viruses.** We will use the three-tiered serological testing system outline in **2.6.a** to try to identify exposure to these 'novel' viruses. However, we know exposure to a range of related viruses generates antisera that are cross-reactive with known, related virus antigens - a serious challenge to serological surveillance and diagnostics. Our collaborations have already demonstrated that SE Asia bat sera samples screened with our pan-filovirus MMIA have been exposed to an Ebola-like virus, that is antigenically-distinct from known Asiatic filoviruses, Reston virus and Měnglà virus (*43*). Using these three serological platforms we will address inherent challenges of indirect virus surveillance through antibody capture by testing multiple binding assay platforms and antigens, using positive control samples to establish serological profiles against known viruses that will aid in our interpretations of cross-reactive antisera responses likely representative of 'novel' viruses.

Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts.

3.1 Rationale/Innovation: Our previous research **(Table 1)** and other data suggests that there are substantial numbers of outbreaks or clinical cases for which the etiologic agent is not diagnosed, e.g. (*177, 178*). To investigate this in SE. Asia, we have assembled a multi-disciplinary team that includes senior infectious disease doctors and clinicians and will work directly with local clinics and regional hospitals to identify the 'cryptic' outbreaks or cases that occur in the region. **In Aim 2, we will conduct serology in high zoonotic-risk communities to find evidence of prior exposure in people,** i.e. evidence of viral spillover into the community. In Aim 3 we identify members of these communities presenting at clinic with undiagnosed illness that may have high-impact zoonotic viral etiology. **Therefore, in Aim 3 we focus on PCR to identify the putative viruses that are replicating in these actively-infected patients (Fig. 12).** We will enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and who present at regional clinics or hospitals with symptoms typical of high impact zoonotic viruses. We will collect detailed survey data to assess their contact type and frequency with wildlife and livestock and conduct molecular and serological diagnostic assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. We will conduct molecular viral discovery assays on patients who present with symptoms indicative of a high-risk zoonotic virus. If novel pathogens are identified by PCR, we will attempt to isolate, obtain full genome sequence and/or biologically characterize the wildtype or molecularly re-derived pathogen, using the *in vitro* and *in vivo* strategy laid out in Aim 1.

3.2 Preliminary data clinical surveillance: 3.2.a. Clinical surveys and outbreak investigations: Our longterm collaboration in the region has included the following activities investigating clinical syndromes: *Peninsular Malaysia*: **At the time of writing, an outbreak of suspected undiagnosed viral illness in the indigenous Batek Orang Asli ("people of the forest") community living at Gua Musang ("civet cat cave") district, in Kelantan, Malaysia, close to Taman Negara – a National Park containing the oldest known rainforest**. This community is one where we have conducted prior routine surveillance and biological sampling, demonstrating proof-of-concept that our two-pronged high-risk community/clinic approach will work. Dozens of people are affected and the outbreak is currently being investigated by our group in collaboration with the Malaysian NPHL. The Orang Asli continue to practice traditional subsistence hunting of wild animals (bats, rodents, primates). These communities are remotely located, in heavily forested areas, with limited access to medical services. This outbreak, and others that will continue to emerge just like it, underscore the critical need for our EIDRC - to improve the technological tools, training, and human capacity for rapid EID surveillance, diagnosis, and response at the frontlines of disease emergence in SE Asia. **Investigating this outbreak is a key priority if EID-SEARCH is funded.** *Sabah*: Co-I Kamruddin (BMHRC) is leading an asymptomatic leptospirosis study on sanitation workers, an ILI study in a Kota Kinabalu clinic and diarrheal study in children under 5 yrs. Co-Is William, Tan and others have conducted clinical observational studies to determine the etiology of central nervous system infections and acute undifferentiated febrile illness in Sabah.

Co-Is William and consultant Yeo have collaborated with Co-I Wang to enroll 90 adult and over 100 pediatric participants with central nervous system infections, negative tests for malaria and dengue from 3 district hospitals in Sabah to determine the various etiologies. While a proportion tested positive for rickettsial agents, the majority had no clear diagnosis. Co-Is William, Rajahram, and Yeo have conducted clinical studies of over 200 inpatients with acute febrile illness with negative tests for malaria and dengue from 3 district hospitals in Sabah to determine the various etiologies. While a high proportion tested positive for rickettsial agents, the majority had no clear diagnosis. *Sarawak:* Co-I Siang (UNIMAS) is conducting a survey of high-risk female Kelabit indigenous people and has found 8% with HPV52 as the dominant strain. He is working on clinical cohorts of hand, foot and mouth disease and will recruit patients from that study in the current proposed work. Finally, Co-Is Siang, Kamruddin are conducting a long-term study of rotavirus and other viral etiologies of acute gastroenteritis in Kuching, Sarawak that will include swine herders and farmers. The Baric lab is a global leader in norovirus VLP diagnostic reagents to detect serologic evidence of norovirus gastroenteritis, as well as RT-PCR detection of novel noroviruses in humans, and has identified novel bat noroviruses, suggesting further opportunities for expansion of this work (*142, 179-181*). This work is particularly relevant in Malaysia because pig farms are shunned by the government and local villagers to they are placed far away from town centers, deep in the forest, and it is this scenario that led to the emergence of Nipah virus in 1998-9 (*6, 22*). *Thailand:* Co-Is Hemachudha, Wacharapluesedee (TRC-EID, Chulalongkorn Univ. Hosp.) work in coordination with the Thailand Ministry of Health to investigate outbreak etiology, including the first case of imported MERS-CoV in Thailand, **for which we produced sequence confirmation within 24 hours from acquiring the specimen**. Three imported MERS cases have been detected in Thailand since 2015, and all cases were confirmed at the TRC-EID. We worked during the Zika outbreak to identify > 500 PCR-positive Zika patients with sequence confirmation. We have also worked on returning and suspected Ebola travelers, syndromic surveillance for SARI, encephalitis, Hand Foot and Mouth disease in children and viral diarrhea. Consultant Hickey (US CDC, Thailand), screened specimens collected between 1998 and 2002 from a large cohort of children from Kamphaeng Phet Province to examine Dengue virus and JE epidemiology (*182, 183*). Among the 784/2,574 convalescent sera henipavirus-specific antibodies were detected in 13 serum samples (1.7% seroprevalence), suggesting prior exposure to endemic henipaviruses or a related PMV in rural Thailand. *Singapore:* Duke-NUS has worked with the Ministry of Health to investigate Zika cases (*184*), and other EIDs. With EHA and others, Duke-NUS has developed novel CoV assays for clinical surveillance, and for expedited analysis during outbreaks. Our pipeline of novel virus detection, reservoir host identification, serological assay development, and serological testing is as rapid as several months, with the development of novel serological assays from sequence data in 7 days (*17*). **For example, with the discovery of SADS-CoV, we identified and fully sequenced a new virus, screened archived wildlife specimens to identify the natural reservoir host species (*Rhinolophus* spp. bats), developed LIPS serological assays, and tested human and swine samples to assess seroprevelance in less than 3 months (*17*).**

3.2.b Analysis of self-reported illness: We have developed a standardized approach for analyzing data from study participants on self-reported symptoms related to target illnesses: fever with cough and shortness of breath or difficulty breathing (severe acute respiratory illness - SARI); fever with muscle aches (fevers of unknown origin (FUO); and, cough or sore throat (influenza-like illness - ILI). For all of our prior human clinical surveillance in 20+ countries, including in Malaysia and Thailand, we used a least absolute shrinkage and selection operator (LASSO) regression to identify associations between participants who reported ILI and/or SARI symptoms and demographics, occupation, and contact with animals in the last year. Results have clear biological relevance. For example, in Yunnan, China, factors strongly associated with prior disease involve animal hunting and consumption **(Fig. 14).** We will expand this approach for all clinical syndromes in Aim 3, and use targeted questions to assess patient's exposure to wildlife in terms that are relevant to each specific country.

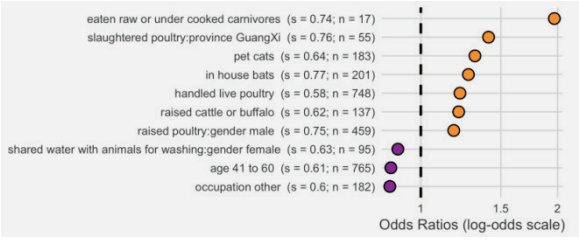


Fig. 14: Factors associated with self-reported ILI & SARI in prior 12 months (s = bootstrap support; n = #+ve out of 1,585 respondents). **Orange circles** = odds ratios > 1 (+ve association); **purple** = odds ratios <1 (-ve association).

3.3 General Approach: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent has not been identified. To capture these, we will expand on our current syndromic surveillance to enroll, and collect high quality biological samples from patients who present at regional clinics and live in rural communities linked to Aim 2. Case definitions and enrollment criteria include: 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever; or 6) high fever/diarrhea with unusual presentation. We will collect survey data to assess contact with wildlife, and occupational and environmental exposures, conduct molecular and serological assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. If novel pathogens are identified by PCR, we will attempt to isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work. We will then use behavioral survey data, ecological and phylogeographic analyses to assess the likely reservoir hosts to inform intervention programs.

3.4 Clinical cohorts. 3.4.a Cohort selection: We will initiate active clinic-based syndromic surveillance at 2 town-level level clinics and 2 provincial-level hospitals in each Thailand, Peninsular Malaysia, Sabah and Sarawak. These serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2 and are inclusive of wildlife sampling sites in Aim 1. Patients ≥ 12 years old presenting at the health facility who meet the syndromic and clinical case definitions above will be recruited into the study after completing a consent form. We will aim to enroll 300 participants per hospital, with that we will have a 95% probability of detecting live virus in patients in each hospital assuming a population prevalence of 1%. This sampling effort will total of 4,800 individuals for clinical syndromic surveillance. We assume up to 40% loss from follow-up, therefore yielding 2880 participants that will be available for follow-up blood sampling **(Section 3.4.b)**. While enrollment is limited by the number of patients that come to the healthcare facility presenting with relevant clinical symptoms, in our work in the PREDICT project we enrolled an average of 100 participants per year, so we are confident this is an achievable enrollment target. Study data will be pooled across country regions, as clinical patients are limited by the number of individuals presenting at hospitals. "Cases" will be determined as participants whose samples tested positive for either CoVs, henipavirus, or FVs, PCR tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. Sites: *Thailand:* We will work with two primary hospital, Phanat Nikhom Hosp., Chonburi district (Key Pers. Dr. P. Hemachudha) & Photharam Hosp., Ratchaburi Province, and a large tertiary hospital, King Chulalongkorn Memorial Hospital, Bangkok Thailand (Co-I Dr. T. Hemachudha). *Peninsular Malaysia:* **Focusing immediately on the current outbreak in Orang Asli, we will work closely with the Malaysian Ministry of Health to support their investigation, and with 6 Orang Asli clinics which focus solely on this indigenous community.** Co-I Sellaran (Lintang Clinic, Kuala Kangsar) refers patients to Sungai Siput Hospital and Gen. Hospital, Ipoh. Key Pers. l Lotfi (Pos Betau clinic, Kuala Lipis) will continue collaboration with Co-I Hughes in the communities at that site. *Sarawak:* Key Pers. Diyana (Director, Bario Clinic) refers patients to Miri Hospital (Co-I Utap) and serves people from the Kelabit, and nomadic tribes in the region, as well as populations within a number of large towns. *Sabah:* We will continue our collaboration with Queen Elizabeth Hospital, QEH (Key Pers. Lee, Rajahram) and Hospital University Malaysia Sabah, HUMS (Co-I Lasimbang, Director). Both clinics include our targeted sites for **Aim 2** in their catchment, and both have ongoing collaboration with the Borneo Medical Health Ctr (Co-I Kamruddin, Director). *Singapore:* Currently, there are no plans to conduct clinic surveillance based on budget constraints. However, in the event that that is an evolving need for clinic surveillance, enrollment, and sampling, we have close working relationships with all hospitals in the country, and the central infectious disease reference lab (key clinicians at which are aware of our proposal). This would allow us to rapidly on-board a hospital based site.

3.4.b Clinic enrollment and follow-up: We will recruit inpatients and outpatients to participate in the study after initial screening to determine if they meet our clinical case definitions. Once consented and enrolled, biological samples will be collected by trained and locally certified hospital staff and a questionnaire administered by trained hospital or research staff that speak appropriate local language. Every effort will be made to take samples concurrently when collecting samples for normative diagnostics. For both inpatients and outpatients, samples will be collected no more than 10 days of reported onset of illness to increase the chance

of PCR detection of viruses (*185*). Where possible, we will follow up 35 days after enrollment to collect an additional two 500 µL serum samples conduct a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. This gives adequate time for development of IgG, which occurs <28 days after onset of symptoms for SARS patients (*186-188*). Serum samples will be used to screen panels of high risk human and local zoonotic Filovirus, Henipavirus and Coronavirus strains as described in Aim 2. These samples may prove valuable to collaborators in that the PBMC could be used in production of therapeutic antibodies.

3.4.c Sampling and clinical interview: Biological specimens whole blood (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples) and two nasal or oropharyngeal swabs will be collected from all participants, and a questionnaire will be administered. We will investigate five risk factors, to maximize the power of the analyses, all related to high risk wildlife exposure, based on continuing analysis of our previous work, and will include: 11) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With additional consent considerations for participants enrolled from clinical settings, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology. Blood will be taken for future harvesting of Peripheral Blood Mononuclear Cells (PBMCs), as per **Section 2.5**.

3.5 Sample testing: The standard syndromic diagnostic PCR assays for common pathogens will be conducted and the results will be shared with the hospital with 48 hours. Specimens will be further tested for 3 viral families; CoV, PMV and filovirus by consensus PCR **(Section 1.4.c)**. Necropsy samples from deceased patients will be further characterized by NGS if previous PCRs are negative and unable to identify the cause of infection. Co-Is Wang, Anderson will conduct a VirScan serological assay using paired serum samples at Duke-NUS that identifies antibodies with a four-fold rise in titer in the convalescent sample compared with the acute sample, thereby indicating a possible etiological agent. A PCR test with the specific primer from each virus antibody positive case will be further tested from acute specimens to confirm infection.

3.6 Viral risk characterization and potential for pandemic spread: We will use statistical models built from collated biological, ecological, and genetic data to assess the likelihood of pathogenicity for the range of diverse viruses we will characterize. The aim is to quickly identify and assess which viruses pose the most significant risk to human health and thus prioritize additional resources to more fully characterize, better understand pathogenicity, and invest in our animal model pipeline and ultimately therapeutic development for those pathogens. We will combine two independent but related statistical models to predict pathogen spillover risk and pathogenicity. First, we will expand a simple generalized additive modeling approach (*3*) to assess spillover risk probability to a new virus that is currently not known to infect people, when we have only limited genetic data from initial PCR screening and sequencing. Additional data from receptor binding protein homology and cell line infection experiments will be incorporated into the model framework as available. Second, given high likelihood to spillover and infect humans, or direct evidence of human infection from our clinical surveillance, we will then assess the likelihood of pathogenicity based on traits for phylogenetically related nearest neighbor viruses as a first approximation and incorporating more detailed data from genomic characterization and animal model experiments (Aim 1). Epidemiological trait data for ~300 viral species known to infect people (e.g. case fatality rate, global estimates of number of human cases per year or decade, R_0, infectious period, and primary symptoms) have been collated by recent studies and will be used as predictor variables (*189*). Thirdly, we will apply tools already developed, and being refined by EHA under DTRA and DHS supported research, to predict pandemic spread for viruses using global flight models, as well as additional datasets on human movement and connectivity across Southeast Asia (*90, 91*) **(Fig. 15)**.

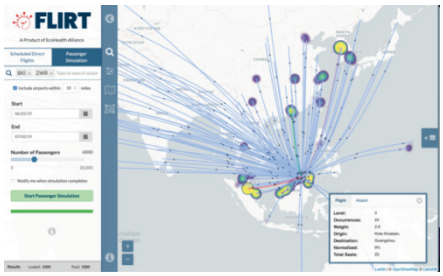


Fig. 15: Probability feed from EHA's Flight Risk Tracker tool (FLIRT). This image is of the likely pathways of spread and establishment for a Nipah-like virus emerging from Sabah, based on human movement and airline flight data (*90, 91*).

3.7 Potential problems/alternative approaches: Patients visiting clinics may have cleared virus, but not yet developed IgG antibodies, reducing seropositive cases. Our 35 day follow-up sampling should account for this because the maximum lag time between SARS infection and IgG development is ~28 days and Ebola virus infection and IgG approximately 20 days (*185-188, 190*). We also expect that patients in rural communities will only visit clinics when symptoms have progressed, likely coinciding with late illness and onset of IgG. We will also have data from our community study that can be analyzed in conjunction with, or in the absence of, clinical data from the hospital data to identify PCR- or seropositives. Finally, the risk of limited detection is outweighed by the potential public health importance of discovering active spillover of new henipaviruses, FVs or CoVs infection.

4. Administrative Plan

4.1. Project management: 4.1.a. Administrative core: The EID-SEARCH organizational partner roles are laid out by Specific Aim above **(see Fig. 3)**. EcoHealth Alliance (EHA) will lead the administrative core of the EID-SEARCH, including: coordination and management of all human and animal research; communication with consortium partners, NIH, other EIDRCs, and the EIDRC-CC; data management and quality assurance; modeling and analysis; and compliance with all US, international, and institutional rules and regulations. The administration will be led by PI Daszak, who has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5-20 yrs on NIAID- and USAID-funded research **(Fig. 16)**. He also has direct experience as one of two external reviewers of one of the CEIRS partners for NIAID (Program officer Diane Post). PI Daszak will be assisted by Deputy Lead, co-I Olival, who has overseen zoonotic disease research in Southeast Asia for >10 years and working directly with our partners in Thailand and Malaysia for 15 years. Senior program manager (Chmura) and full-time program assistant (Hamel) will provide administrative support. The EID-SEARCH data management, epidemiological analysis, and modeling activities will be led by co-Is Olival and Zambrana who have led all modeling and analytics on USAID USAID PREDICT for the last 10 years. They will overse a programmer/data scientist (Mendelsohn), evolutionary biologist/bioinformatician (Latinne), and human epidemiologists (Dawson, Li, Martinez). Our human epidemiology team will work directly with country teams in Malaysia and Thailand to manage human surveillance, IRB applications and approvals, and ensure compliance with all human subjects work. EHA's senior veterinarian (Field) and wildlife field biologist (Phelps) have extensive experience working that bats, rodents, and primates in SE Asia and will coordinate all training and field research in Malaysia and Thailand and ensure compliance with IACUCs.

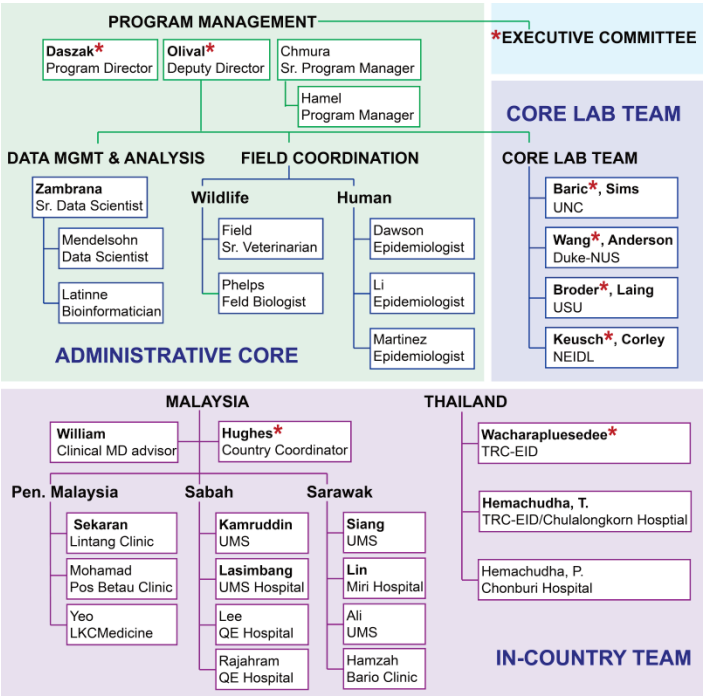


Fig. 16: Administration and program management for the **EID-SEARCH**

The EID-SEARCH core laboratory team includes global experts in virology, molecular pathogenesis, and the development of assays, reagents, and therapeutics for high consequence viral zoonoses (Co-Is Wang, Anderson at Duke-NUS; Baric, Sims at UNC; Broder, Laing at USU; Keusch, Corley at NEIDL). The core laboratory team will work with in-country diagnostic labs in Thailand (Wacharapluesedee, Hemachudha at the TRC-EID, Chulalongkorn University) and Malaysia (Hughes, Lee, CM Ltd.). Wacharapluesedee and Hughes will additionally serve as country coordinators for the EID-SEARCH, liaising directly with EHA on weekly conference calls, and working with in-country partners to ensure the smooth operation and coordination of clinical and community surveillance and wildlife research in Thailand and Malaysia, respectively. **A Core Executive Committee** comprising PI Daszak, Deputy Lead and Co-I Olival, and leads from each core partner and country: Co-Is Hughes, Wacharapleusadee, Baric, Wang, Broder, Keusch (or alternates), will conduct

regular conference calls and in-person meetings to facilitate rapid decision making within the EID-SEARCH. **This committee will also convene to manage EID-SEARCH response to outbreaks.**

4.1.b Project Management in Thailand and Malaysia: Wacharapluesedee and Hughes have collaborated directly with EHA for >15 years, including acting as country coordinators on the USAID PREDICT project for the last 10 years (project end date Sept. 2019). They maintain strong ties with Ministries of Health (MOH), Agriculture and Environment, multiple universities and research institutions, clinics, and hospitals, in their respective countries and across the region. The EID-SEARCH will use these connections to disseminate results, obtain permissions to conduct sampling, and also rapidly respond to and assist with outbreaks as they happen. Peninsular Malaysia, Sarawak, and Sabah are the three main Malaysian administrative regions, and effectively operate as three separate countries, with different regulations and government structures. We therefore provide specific details on the management of EID-SEARCH activities in each:

Coordination among Peninsular Malaysia, Sabah and Sarawak will be led by co-I Hughes (Conservation Medicine Ltd), and follow a successful model we implemented under USAID-PREDICT. **On Peninsular Malaysia** this project will be administered through the Zoonosis Technical Working Committee (ZTWC) established under the PREDICT project with a binding MOU among EHA, CM Ltd. and ZTWC, and including officers from MOH, Dept. of Veterinary Services, and PERHILITAN (the Govt. wildlife agency). EHA will communicate weekly with Co-I Hughes to coordinate and monitor implementation of research and reporting to ZTWC. Co-I Hughes will coordinate activities at all other Peninsular Malaysia institutions: NPHL, the National reference laboratory for diagnostic confirmation of pathogens, will manage molecular and serological screening (BioPlex) of Orang Asli samples, and serological screening of syndromic samples from Sabah and Sarawak; the PERHILITAN molecular zoonosis laboratory will store and conduct molecular and serological screening on wildlife samples; and Universiti Putra Malaysia (UPM) Faculty of Veterinary Medicine will conduct molecular and serological screening (BioPlex) of livestock samples, should these be required. **For Sabah & Sarawak**, work will be administered through the Sabah Zoonotic Diseases Committee (SZDC), a working technical committee comprising appointed and authorized officers from Sabah State Health Dept., Department of Veterinary Services, Sabah Wildlife Dept. (SWD), Universiti Malaysia Sabah (UMS) and EHA, all of which are also committed through a signed MOU. Co-I Hughes will oversee work at all other partners in Sabah, including: the Kota Kinabalu Public Health Lab (KKPHL) for molecular screening of syndromic samples from Sabah and Sarawak; the SWD Wildlife Health and Genetic and Forensics Lab for molecular screening of Sabah wildlife samples; The Borneo Medical Health Research Center (BMHRC) for screening some Sabah wildlife and livestock samples, if required, and human syndromic samples from Sabah and Sarawak. **In Thailand** all human community and wildlife research and testing will be coordinated by co-I Wacharapluesedee from the TRC-EID center. Clinical surveillance will be overseen by senior clinical physician and co-I T. Hemachudha.

4.1.c. Approval and release of results: In our experience, it is critical when working in resource-poor countries, on potentially important pathogens, to strictly adhere to protocols for release of results. EID-SEARCH will liaise with existing points of contact in the Ministries of Health, Environment, and Agriculture in each our administrative areas to approve and release project findings publicly. Results from human screening will be shared with participants when they become available, as per our IRB agreements ensuring no violations to anonymize data requirements (**see Protection of Human Subjects**).

4.2. Flexibility to extend the EID-SEARCH to new sites as needed: The EID-SEARCH consortium partners maintain extensive working relationships with leaders in EID outbreak control, clinical investigations and research at over 50 clinics, research institutes and public health laboratories across Southeast Asia. Due to space constraints, we haven't listed each of these, nor have we solicited >50 Letters of Support for this project. However, each core EID-SEARCH partner has contacted their networks and obtained permission for inclusion in the broader goals of the EIDRC. As examples of these contacts, our core partner, the Thai Red Cross Emerging Infectious Disease Health Science Centre (TRC-EID) at Chulalongkorn University, also serves as the WHO Collaborating Centre for Research and Training on Viral Zoonoses and has ongoing research collaborations across WHO SEARO countries including Bangladesh, Bhutan, Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, (Thailand), Timor-Leste; and has recently served as a training hub for scientists from Malaysia, Myanmar, Laos, the Philippines, and China to learn methods of wildlife sampling and diagnostic screening. Our Thai clinicians (Co-I T. Hemachudha and KP P.

Hemachudha) provide regular case consultations and clinical trainings for doctors across SEARO countries, including with Yangoon General Hospital and the National Health Lab in Myanmar, 2018. To maximize leverage of this broad network, EHA has budgeted for annual meetings in SE Asia, in addition to regular smaller network meetings, with our core team and key public health experts from network labs in each of the 10 SE Asian countries**.** Additionally, we will set up a listserv and an internal communication network to facilitate collaboration and information exchange, including on the first reports of new disease outbreaks. Our annual and smaller network meetings will critically allow face-to-face meetings of the EID-SEARCH that will foster greater sharing of information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks in the region, guided by the PI, Deputy and the Executive Committee.

4.3. Outbreak response: EHA collaboration with expert networks around the world allows us to mobilize and enhance effective One Health response to disease emergencies (*191*), ranging from real-time situation updates and risk analyses to on-the-ground investigations (*192-194*). We will adopt management tools from Emergency Operating Center (EOCs) (*195*) and Incident Management Systems (IMS) (*196*), to shift resources where necessary to help respond to novel zoonotic outbreak events and other public health emergencies. EHA has extensive experience working with governments in low and middle income countries (LMIC) applying these principles of epidemic preparedness during outbreak responses we've been involved with under the USAID-PREDICT project. For example, at the request of the government of Bangladesh, we provided technical field and laboratory support for Nipah virus and avian influenza outbreak investigations, assisting with wildlife sampling as part of the outbreak response alongside human and domestic animal sampling. In India, we provided technical assistance in response to the Nipah virus outbreak in Kerala in 2018. Last month in Indonesia were assisted the Ministry of Health's Center for Health Laboratory in Makassar to provide technical assistance in a mysterious outbreak in a small village in South Sulawesi that killed 4 villagers and infected 72. Our network partners include the key government and govt. approved laboratories that would be directly involved in public health emergency response in their respective countries. The serological and PCR platforms that EID-SEARCH develops will be made available to the main government outbreak investigation teams for clinical work and research during the outbreak. EID-SEARCH will also offer assistance training and conducting animal sampling during an outbreak, epidemiological analysis and modeling to help identify likely reservoirs or likely pathways to spread. Technical and material support for lab, field and analytical activities during an outbreak will be provided by EHA, UNC, USU, Duke-NUS, and NEIDL, as well as in-country partners. Any clinical samples, viral isolates and sequence data will be shared among partners to promote the rapid development of new diagnostic assays, reagents, and therapeutics that can be deployed to the region or other regions as part of the larger NIH EIDRC network.

Finally, while the initial pathogen focus of our group is on CoVs, PMVs and FVs, our broad collaborative group has multidisciplinary expertise on a number of virus-host systems. For example: PI Daszak was PI on a subaward from PI Laura Kramer's U01 on Poxviruses and Flaviviruses, managing a multidisciplinary research project on West Nile virus ecology. He was also co-I on a 5-year NSF-funded project to understand West Nile virus dynamics and risk in the USA (*197-201*); Co-I Baric is a global leader in Norovirus research leading to the development of vaccines and therapeutics (*202-205*); Co-I Wang has conducted significant work on bat immunology, therapeutic, and reagent development, as well as being involved in a range of outbreak investigations, viral discovery programs and other research on a wide diversity of viral groups (*206-215*). Additionally, the serological and PCR-based diagnostic platforms being developed by Co-Is Wang and Broder are adaptable to other viral targets. The modeling tools developed by Co-Is Olival and Zambrana-Torrelio can be used to predict the emergence and spread of diverse viral targets, including influenza, antimicrobial resistance, and vector-borne diseases (*216-221*). Our clinicians working in Thailand and Malaysia have a wide range of infectious disease investigations to adapt to any outbreak situation.

4.4. Communications: EHA will coordinate communication among all co-Is and key personnel, including:

- Multiple meetings per week with PI, Deputy Lead, Senior Program Manager (on project and task status)
- Weekly web/phone meetings between Program Manager and subawardee admin. staff
- Monthly web/phone conferences between EHA PIs and all subawardee PIs.

- Monthly web conferences between key personnel (research presentations/coordination)
- In-person Annual meetings with partner leads, key personnel at EHA and two in-person partner meetings annually between subawardees.
- Annual in-person meeting among all key personnel

4.5. Problem identification and resolution: Regular planning, monitoring, and evaluation meetings will be the primary mechanisms for problem identification. Minor problems (e.g. delays in sample availability or test results) will be dealt with internally through appropriate action and resolution monitored by PI Daszak and co-I Olival, and our Senior Program Manager. In the event of significant problems, such as prolonged poor productivity, inadequate scientific collaboration, or major disputes regarding research direction or resource allocation, EHA will assist with resolving the problem through negotiation with relevant co-Is and consultation with the Executive Committee. Should a resolution not be forthcoming, consultation with the EIDRC-CC, additional external technical advisors, and NIH staff may be warranted.

4.6. Adaptive management and risk mitigation: Maintaining a timeline and meeting milestones will require strict and continuous oversight of all project phases, frequent and regularly scheduled communication, and the ability to make decisions and implement strategies. To maintain our timeline on all projects, including the EID-SEARCH, we use an adaptive management approach to continually evaluate these trade-offs, to make decisions about when iteration is appropriate and when it is necessary to move forward with current information. Our ethos is that regular, scheduled communication among all staff, partners and collaborators will go a long way towards mitigating risks, especially if the process is collaborative and transparent.

5. Data Management Plan

EHA will house the Data Management and Analysis (DMA) team for EID-SEARCH, led by Co-PIs Olival and Zambrana-Torreilo and include Key Personnel Latinne and Mendelsohn. EHA has served as the data and analysis hub for numerous multi-institutional, multi-sectoral, international disease research groups, including acting as Modeling and Analytics lead for the PREDICT project (*122*), the Western Asia Bat Research Network (*222*) and EHA's Rift Valley Fever Consortium. We will leverage our experience and infrastructure from those projects to benefit the EID-SEARCH. **5.1. Project Database:** We will create a dedicated, centralized EID-SEARCH database to ingest, store, link, and provide for analysis all data associated with the proposed study and other expanded projects associated with the research network. The database will be SQL-based and use encrypted, secure cloud hosting services and enable export to archival and platform-independent formats. It will ensure data and metadata compatibility between project components, track data versioning and annotations. The system will be designed to work with both with both paper- and tablet-based field data entry and with the Lockbox laboratory information management systems **(Section 5.2)** in place in individual partner labs. The database will use existing metadata standards, including NCBI standards for genetic and molecular data and Ecological Metadata Language (EML) for field and wildlife data, as well as other standards and formats designated by the EIRDC CC. This will enable rapid publication and deposition of data. Granular security and privacy controls will be applied so that specific expansion projects undertaken in the network may be managed while maintaining data confidentiality as needed.

5.2. Biological Specimen Management: Project laboratories will use the Lockbox Laboratory Information Management System (LIMS), to manage the security, traceability, and quality of biological specimens. The LIMS will support sample barcoding at creation, tracking through transport, storage/inventory, and use via portable scanners. Lockbox supports CLIA and ISO 17025 as well as direct export to NCBI formats such as Sequence Read Archive. We will use the Lockbox LIMS application programming interface (API) to link to the central project database and associated samples with field and ecological data. We note that the project focuses on highly pathogenic viruses, including select agents; Lockbox LIMS supports sample tracking and movement compliant with US Select Agent Regulations and US Department of Commerce Pathogen Import and Export Control Regulations, and includes all necessary encryption, security, and backup protocols.

5.3. Training: Members of the DMA team will team will develop documentation and provide training for field and laboratory teams at all partner institutions in data management, metadata standards and data hygiene best practices. The DMA team will act as trainers and consultants for partner institutions in experimental

design, power analysis, data analysis, and computational and reproducibility issues, and visit each partner institution and/or field team base for training workshops and analysis consultations.

5.4. Data Identification and Privacy: For human clinical data and questionnaires, data will be identified by a unique identification code assigned to each individual and only this, de-identified code will be accessible in the project database, and destroyed at the end of the project - as per details provided in the Clinical Management Plan and Protection of Human Subjects forms.

5.5. Computing Resources: EHA operates a cluster of high-performance servers for data analysis activities, as well as infrastructure to launch cloud-based computing environments (**see EHA Facilities**). Our servers host all necessary software for statistical and bioninformatics work that is available to the DMA and partners anywhere in the world. We use a mixture of cloud services (AWS, Azure, Backblaze, GitHub) to provide redundancy, backup, version control, and rapid post-disaster recovery, and will be available to all project partners for analysis and training.

5.6. Data and Code Sharing: See details provided in the **Resource Sharing Plan.**

6. Clinical Management Plan

6.1. Clinical site selection: Our consortium partners have been conducting lab and human surveillance research, including during outbreaks, for >20 years and have developed strong relationships with local clinical facilities and processes in SE Asia and in LMIC globally. The plan for selection of clinical sites will begin in the same geographic regions as those identified in Aim 1 with high zoonotic viral diversity. Clinical sites will additionally serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2. We have already developed successful working relationships with the major healthcare facilities in Thailand and Malaysia and will use these established partners to rapidly gain appropriate permits and begin data collection quickly. Focusing on these EID hotspots in select biogeographic areas (see **Fig 13**) also reduces the number of additional sites needed to meet our goals within our geographic range. The capacity and capability requirements for clinical sites are fairly minimal, and include ability to enroll patients that meet the clinical case definitions of interest, collect and temporarily store biological samples, and follow standards for data management and subject protection with locked filing cabinets to store all paper records and an encrypted computer. We will be enrolling inpatient and outpatient participants within 10 days of the presentation of symptoms to increase the chance of PCR detection of viruses so we will not wait for advanced normative diagnostic tests, that are likely unavailable in the more remote sites, to be completed. We will work with clinical sites to determine the best process for project staff, in most cases supporting the time of currently hired staff at each site. We will recruit and train hospital staff in project-specific procedures including enrollment of patients, sample types to be collected, storage of samples, administration of the questionnaire, and data management plans. During the development and onboarding of new clinical sites we will assess the physical needs of the site and what supplies will be required for collection of data.

6.2. Standardized approach, oversight, and implementation: Management and oversite for all study sites will be undertaken by the local country coordinator with support from our Core Administrative team at EHA. Our research team has over 10 years of experience building capacity on human subjects' research and has developed training resources and materials for standardized implementation of community and clinical research and SOPs for screening, enrollment, and retention of participants. The country coordinator will conduct regular site visits to the clinical sites and annual visits to observe, monitor and evaluate the research process, and conduct follow-up training if required. Through our work with clinical sites under the USAID-PREDICT project we have developed culturally appropriate screening measures for clinal sites that do not disrupt the flow or quality of medical treatment received by the patient. This was done by working collectively with clinical staff to evaluate current procedures and patient flow at the site to determine the most efficient while minimal invasive inclusion of our study into the daily working of the clinal site. Most efficiently this was done by adding minimal basic screening questions to the current clinal intake forms, which allows the clinal research officer to quickly scan charts or logs for potential patients to enroll avoiding potential enrollees from being overlooked if staff are too busy or not on duty. Patients will be enrolled following established clinical criteria (**see Section 6.3**), samples collected and brief surveys conducted to assess the participants contact with wildlife through: 1) occupational exposures; 2) animal contact; and 3) the environment. With permission

from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology. Additionally, we will be following up with clinical participants to determine how clinical presentation differs between CoV, henipavirus, or FV in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. The country coordinator will be continually monitoring the project database to ensure we hit target sample sizes. While patient's enrollment is limited by the number of individuals presenting at hospitals, in previous research we enrolled an average of 105 patients per year, ranging from 77-244.

6.3. Clinical cohort setup, recruitment, enrollment: We will recruit inpatients and outpatients from clinical sites to participate in the study after initial screening to determine if they meet the clinical case definitions for 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever, of unknown etiology or severe diarrhea with unusual presentation for symptoms to increase the chance of PCR detection of viruses. Once enrolled, biological samples will be collected by trained hospital staff. When possible, samples will be taken concurrently with samples being collected for normative diagnostics. We will collect 5mL of blood for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples and two nasal or oropharyngeal swabs will be collected. Controls who test positive for CoVs, FVs, or Henipaviruses will be selected from the pool of participants admitted to the studies and whose samples tested negative for coronaviruses, henipavirus, or filoviruses. We will follow up 35 days after enrollment to collect an additional 5mL blood draw for collection of two 500 µL serum samples and a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. Following up 35 days post initial collection gives adequate time for 1) development of IgG and IgM, which occurs <28 days after onset of symptoms to evaluate the immunological progression of disease and 2) further the risk assessment of the participants to monitor contact with wildlife, people, and to assess the likely reservoir hosts to collect information to inform potential intervention programs.

6.4. Utilization of collected data: Sampling of wildlife and the enrollment of community surveillance participants and a clinical cohort of participants give us the ability to assess the viruses that are circulating in each of the three populations in a similar geographic region. We will use phylogenetic analysis to compare the relationship between wildlife viruses found and viruses fond in human participants. Additionally, the questionnaire data will allow us to assess relative measures of human-wildlife contact that we will analyze the intensity of contact with species known to be at a higher risk for spillover against other risk to provide useful proxy information for spillover risk. The clinical cohort will be split into cases and controls. "Cases" are defined as participants whose samples tested positive for either CoV, henipavirus, or FV via PCR tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. In clinical study, we will use this procedure to determine how clinical presentation differs between virus-exposed and -unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. We will model the outcomes to analyze correlation between biological results and risk factors related to high risk wildlife exposure. With this collective data set we aim to quantify and detect novel viruses likely to spillover regularly to people, are often unreported or misdiagnosed as their clinical manifestations, and are unknown to detect cryptic outbreaks causing previously 'hidden' clinical syndromes in people. Our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence.

6.5. Development of reagents of value to the community. Members of the EID-SEARCH consortium have substantial experience producing reagents, assays, and other products that are used widely by the clinical and research community, and some of which are on a pathway to commercialization. These include: PIs Daszak and Co-I Olival have produced software for analyzing the spread of novel viral agents through air travel networks; Co-I Baric has collaborated with a Norovirus surveillance collaboration with surveillance cohort at CDC and has developed therapeutics that have reached phase 2 and 3 clinical trials, He is currently working with Takeda Sanofi Pasteur on a Dengue therapeutic and with NIH on a tetravalent vaccine; Co-I Broder

developed a Hendra virus subunit vaccine that was commercially produced by Zoetis for horses and is labeled for human use under compassionate circumstances during outbreak situations.

6.6. Potential expansion: Expansion of the research project to other clinical sites or research areas could happen as new information is gathered, either through our research, the EID-SEARCH information network, or an outbreak being identified in the region by other organizations. If expansion is required we would rapidly shift research activities towards the clinical or community sites where the outbreak is active, using the same process we used to set up initial research locations. First, by working with local stakeholders to create a working relationship and on-boarding new clinical research staff through advanced trainings of project aims, SOPs on data collection and storage, and ethical guidelines. The network of infectious disease researchers in the community and hospital settings would allow for an accelerated response to any potential infectious disease outbreaks as staff are well trained on ethical guidelines for working with human subjects, data and specimen collection, storage, and transpiration, project staff would be on the front lines ready for the call to action. Ideally, our testing for viruses in priority viral families may lead to the discovery cryptic outbreaks before it spreads in the general public. Biological results coupled with survey data on key risk factors will provided necessary information for granular understand of disease spread.

7. Statistical Analysis Plan:

7.1. Framework: Statistical analyses across the project will be conducted under a common Bayesian framework. These models provide a unified, probabilistic approach best-suited for estimating effect sizes in heterogenous populations of human clinical and wildlife subjects in observational studies. Within this Bayesian framework, we will use generalized linear mixed models to estimate population prevalences and seroprevalences, and estimate the effects of demographic, occupational and environmental factors affecting these. We will use occupancy models (*223*) to estimate total viral species and strain diversity and completeness of sampling within the human and wildlife sub-populations, and discrete phylogeographic models to identify taxonomic and geographic centers of viral diversification. All statistical analyses will be performed reproducibly using scripted, programmatic workflows (e.g., the R and Stan languages) and maintained under source code version control (git). As with data management, the DMA team will act as trainers and consultants for exploratory data analysis, power analysis, and study design with project partners, and the EHA computing cluster will be available for partners undertaking additional or expansion studies. Power analyses, current and expansion, are performed via simulation approaches allowing planning for complex, hierarchical variation in study populations. Power analyses and specific analytical components of this study are detailed under each Specific Aim.

7.2. Data Quality Control and Data Harmonization: All data will be are examined at entry by field and lab teams upon data entry, followed by examination by DMA team members at upload and integration, for complete de-identification, completeness, accuracy, and logical consistency. The DMA will provide field and lab teams with reports, produced automatically, of data summaries, including aggregates, distribution, detected outliers and possible mis-entries. On a regular basis (quarterly or as-needed during data collection), DMA team members will review reports with field and lab teams to identify errors and update collection and entry procedures as necessary.

7.3. Statistical Considerations for Behavioral Questionnaires and Clinical Metadata: The data collected from the questionnaire will be analyzed to assess the reported measures of contact for each risk group under study, related to1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, rodents, and primates in/around house and other livestock animals (e.g. farming, butchering, slaughtering); 3) proximity of residence or workplace to environments of increased risks (e.g. nearby bat roosts); 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12 months and lifetime. Specific measures we are interested in are the proportion of respondents indicating they consume wildlife, where wildlife is obtained for consumption, have hunted wildlife, butchered or slaughtered live animals, seen wildlife in their homes, been bitten or scratched by animals, etc. Comparisons of measures of exposure contacts and types between men and women, children and adults, different study regions will be conducted in order to explore the occupational, environmental, and demographic factors (gender, age, socioeconomic status (SES)) that influence contact with animals and to determine who is

most at-risk. Statistical analysis will be employed to identify differences between groups with a 95% probably of detecting a difference. Measures of contact related to different activities within specific groups will be compared to determine the activities that put groups at most risk. As appropriate multivariate analysis (e.g. ordinary linear regression, logistic regression, non-normal distributions of outcome, least absolute shrinkage and selection operator (LASSO) regression, etc.) will be utilized to evaluate the relationship between the outcome variables, positive biological results (PCR or serology) key measures of contact and the factors that influence frequency and types of human-animal contact.

8. Project Milestones and Timelines

8.1 Milestones: <u>End of Year 1:</u> Aim 1: Sample targeting locations, species (for wildlife), sample size justifications completed for whole project and reported to in-country teams; Sample testing, viral isolation, NGS, glycoprotein sequencing begun for all archival and some newly-collected samples; *in vitro* work begun; host-pathogen dynamic analyses; animal model work begun. **Aim 2:** Target human community populations identified and sample sizes calculated for some sites in each country; Community data collection, serological testing and RT-PCR testing begun; first epidemiological analyses of data begin in last quarter. **Aim 3:** Clinical cohort selection underway; clinical enrollment, data collection and sample analysis begun. First Annual meeting in last quarter. First publications submitted by end of year, summary overview papers or reviews.

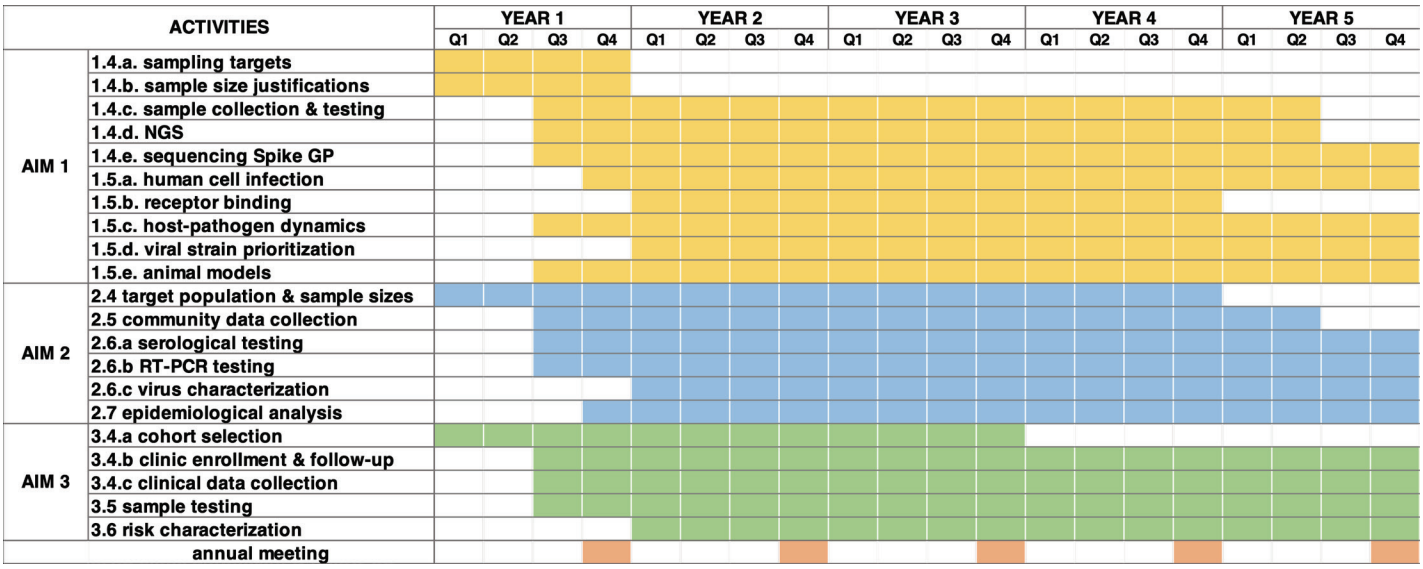
<u>End of Year 2:</u> Aim 1: No sample targeting or sample size justification analyses needed. All other aspects underway **Aim 2:** All aspects underway **Aim 3:** All sub-aims underway. Second Annual meeting in last quarter. Further 2 publications submitted by end of year, including first data papers.

<u>End of Year 3:</u> Aim 1: No sample targeting or sample size justification analyses needed. All other aspects underway **Aim 2:** All aspects underway **Aim 3:** All sub-aims underway. Third Annual meeting in last quarter. Further 3 publications submitted by end of year, largely data papers.

<u>End of Year 4:</u> Aim 1: No sample targeting or sample size justification analyses needed. All other aspects underway **Aim 2:** All aspects underway. Receptor binding work completed. **Aim 3:** No further cohort selection required; all other sub-aims underway. Fourth Annual meeting in last quarter. 3 further publications submitted, including first papers analyzing risk factors, pathogenic potential of novel viruses submitted.

<u>End of Year 5:</u> Aim 1: No sample targeting or sample size justification analyses needed. No receptor binding assays continuing. Serological and PCR testing completed end of 2nd quarter. Glycoprotein, *in vitro* and *in vivo* analyses, analysis of viral risk continue to end of project. **Aim 2:** No further community targeting or sample size work. Community data collection completed at end of 2nd quarter. All other aspects continue to end of project **Aim 3:** All sub-aims underway. Final Annual meeting in last quarter. Further 3 publications submitted.

8.2. Timeline:



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From:	Peter Daszak on behalf of Peter Daszak <daszak@ecohealthalliance.org>
To:	Laing, Eric; Thomas Hughes
Cc:	Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Supaporn Wacharapluesadee; Aleksei Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross; Kevin Olival
Subject:	EID-SEARCH Word version of proposal
Date:	Friday, June 28, 2019 9:00:08 PM
Attachments:	EIDRC Southeast Asia v7 FINAL_FINAL.docx
Importance:	High

..and here's the word version so you can use text for other purposes…

Cheers,

Peter

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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

From: Peter Daszak
Sent: Wednesday, June 26, 2019 5:19 PM
To: 'Laing, Eric'; Thomas Hughes
Cc: Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Supaporn Wacharapluesadee; Aleksei Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross; Kevin Olival
Subject: EID-SEARCH Comments

Thanks everyone for sending the comments back. Working on v5 now, while others in the office are cranking out sample size calculations, new figures, human and vert. subjects and all the additional information needed after the research plan.

As I go through all your comments, I'll send the occasional email with questions to clarify specific points, so please be on standby for a quick turnaround.

Cheers,

Peter

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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

From: Laing, Eric [mailto:eric.laing@usuhs.edu]
Sent: Wednesday, June 26, 2019 12:59 PM
To: Thomas Hughes
Cc: Peter Daszak; Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Supaporn Wacharapluesadee; Aleksei Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross; Kevin Olival
Subject: Re: EID-SEARCH v4

Hi Peter,

Built on top of Chris' suggestions. Included a prelimin data figure from Hughes et al, in prep. Double check that is ok with Tom?

- Eric

Eric D. Laing, Ph.D.
Research Assistant Professor
Department of Microbiology and Immunology
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eric.laing@usuhs.edu

On Wed, Jun 26, 2019 at 11:26 AM Tom Hughes <tom.hughes@ecohealthalliance.org> wrote:

> Hi Peter,
>
> I have added my edits and comments to Kevin's.
>
> Please let me know if you ave any questions or need more details.
>
> Thanks.
>
> Tom

From: Kevin Olival <olival@ecohealthalliance.org>
Sent: 26 June 2019 2:51 PM
To: Peter Daszak
Cc: Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Eric Laing; Thomas Hughes; Supaporn Wacharapluesadee; Aleksei Avery Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross
Subject: Re: EID-SEARCH v4

Peter, v4 with my edits, some revised figs, and additional comments.

Cheers,
Kevin

Kevin J. Olival, PhD
Vice President for Research

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460 West 34th Street, Suite 1701
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On Jun 25, 2019, at 9:26 AM, Peter Daszak <daszak@ecohealthalliance.org> wrote:

Dear All,

Here's version 4. Apologies for the delay. It's in better shape now, but there are still lots of areas that need your attention. Please put in time today, and tomorrow to edit and firm up the text. I've put your names in some of the comment boxes where I need specific help but I also need you to read this critically now and edit and tighten it up. We have 30 pages of space, and our research plan is now 21 pages, so we need to lose maybe 3 or 4 pages. There are also some weak points, specifically on Filoviruses, on the details of where we'll do our clinical work, and on what we'll do with the animal models for henipas and filos.

Please get comments back with me before Wednesday 26th 4pm New York time, so that I have Wendesday evening and all day Thursday to incorporate them. In the meantime, I'll be working with folks here on better figures, and all the extra sections that come after the research plan. I'll need you all to help with those on Thursday while I'm finishing off the draft.

Thanks to all of you in advance and look forward to the next round.

Cheers,

Peter

Peter Daszak
President

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Twitter: @PeterDaszak

EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

From: Peter Daszak
Sent: Thursday, June 20, 2019 9:41 PM
To: Wang Linfa; Danielle Anderson (danielle.anderson@duke-nus.edu.sg); Ralph Baric (rbaric@email.unc.edu); Baric, Toni C; Sims, Amy C; Christopher Broder (christopher.broder@usuhs.edu); Eric Laing; 'Tom Hughes'
Cc: Aleksei Chmura (chmura@ecohealthalliance.org); Alison Andre; Luke Hamel (hamel@ecohealthalliance.org); Kevin Olival, PhD (olival@ecohealthalliance.org)
Subject: EIDRC-SEA v.3
Importance: High

Dear all,

Thanks to everyone for comments and text for the earlier draft at the beginning of the week. Kevin's spent the last couple of days pulling all the comments together into one draft and inserting his changes, and I've now attached a v3 of the draft. It's still rough, and now too long, but it's getting there. I wanted you to look at this rough version because I'm stuck in Liberia in meetings Friday, then flying back home Saturday. I'll continue editing this draft on the plane and will be working solely on this grant from home on Sunday, Monday, Tues, Wed, Thurs.

Please work on this rapidly if possible. If you can all push your comments through, using track changes, and get them back to me **by Sunday evening New York Time**, I will turn round a polished draft by Tuesday, giving us time for significant last go-over on Wed, Thurs. It's tight, but unfortunately, it is what it is, so please commit the time if you can!! As well as general edits (using track changes, with references in comment boxes), please do the following…

Linfa/Danielle – We especially need your input in section 1.1, 1.3, 1.4 and 2.5

Ralph/Amy - Please work your magic on this draft, inserting text, editing and reducing text, and making sure the technical details of the characterization are correct and fit the proposed work on CoVs, PMVs and Henipaviruses

Chris/Eric – Eric – congratulations on your (b) (6) and thanks for working on it at this time! Chris – please help and edit/finesse the language in the relevant sections for you

Tom – Your edits crossed over with Kevin's changes, so please insert all the bits that were new in the last version you sent to me, into this version. I think these are mainly the sections and comments from the other Malaysian colleagues, but please check and re-insert all key text. We especially need to be specific about which partner in Malaysia is doing what cohorts, and where, and about who's doing the testing

Thanks again all, and I'm looking forward to non-stop work on this as soon as I get on my flight in 24 hours…

Cheers,

Peter

Peter Daszak
President

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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

<EIDRC Southeast Asia v4.docx>

II. Research Strategy:

1. Significance: Southeast Asia is a critical hotspot for emerging diseases due to its high biodiversity of wildlife and their viruses, and the presence of key ecological and socioeconomic drivers of emergence **(Fig. 1)** (*1*). These include dense human populations living in networks of rural and urban communities, with strong cultural and occupational connection to wildlife and livestock farming and trading, and intimate connection to global travel networks (*2*). The region is undergoing rapid environmental and demographic change, both of which increase the risk of disease emergence and spread. Pathogens that emerge in this region often spread and sometimes enter the USA (e.g. prior influenza pandemics, SARS) and threaten global health security.

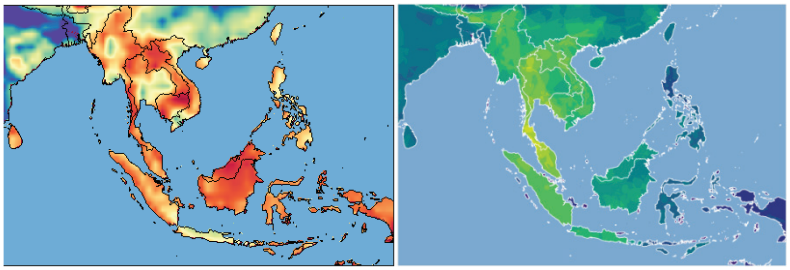


Fig. 1: Southeast Asia, and in particular, parts of Thailand and Malaysia are hotspots of risk for disease emergence **(left)**, and predicted high diversity of 'missing' or as-yet undiscovered viruses, yellow = highest diversity **(left)**. From (*1-3*).

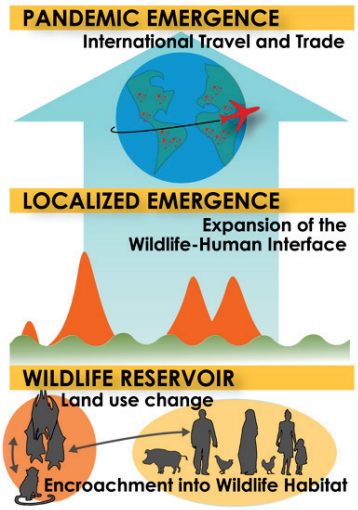
Not surprisingly novel viruses, including near-neighbors of known agents, have emerged in the region leading to often unusual clinical presentations **(Table 1)**.This adds to a significant background burden of repeated Dengue virus outbreaks in the region (*20*), and over 30 known *Flavivirus* species circulating throughout S. and SE Asia (*21*). The events in **Table 1** are dominated by three viral families: coronaviruses (CoVs), paramyxoviruses (PMVs - particularly henipaviruses) and filoviruses (FVs), which are initial examples of our team's research focus and capabilities. These viral groups have led to globally important emerging zoonoses (*4, 5, 22-31*), causing tens of thousands of deaths, and costing billions of dollars (*32-34*). Furthermore, near-neighbors of known pathogens in these families have been reported throughout the region, including by members of our consortium: henipaviruses in bats in Thailand, Cambodia, Philippines, Laos, Indonesia, China, Malaysia, Bangladesh and India (*35-41*); a novel henipavirus, Mòjiāng virus, in wild rats in Yunnan (*14*); serological evidence of FVs in bats in Bangladesh (*42*) and Singapore (*43*); Nipah- and Ebola-like viruses in *Hipposideros*, *Cynopterus* and *Rhinolophus* species in Malaysia (Hughes *et al.*, in prep.); evidence of novel FVs in bats in China (*44-46*), including Měnglà virus that appears capable of infecting human cells (*47*); novel PMVs in bats and rodents consumed as bushmeat in Vietnam (*48*); a lineage C β-CoV in bats in China using the same cell receptor as MERS-CoV to infect human cells *in vitro* (*49*); MERSr-CoVs in bat guano harvested as fertilizer in Thailand (*50*), and directly in bats (Wacharapluesadee *et al.*, in prep.); 172 related viruses.

Viral agent	Site, date	Impact	Novelty of event	Ref.
Nipah virus	Malaysia, Singapore 1998-9	~246 human cases, ~40% fatal	2nd emergence of a zoonotic henipavirus, 1st large outbreak	(*4-6*)
Melaka & Kampar virus	Malaysia 2006	SARI in family group, individual	1st disease due to bat-origin reoviruses, also Singapore, Vietnam etc.	(*7-10*)
Reston filovirus	Philippines 2008	Seropositive people killed pigs,	No prior FVs in pigs	(*11*)
Thrombocytopenia Syndrome virus	E. Asia 2009	100s of deaths in people	Novel tick-borne zoonosis with large caseload	(*12*)
Reston filovirus	Shanghai 2011	PCR positive pigs	Further evidence of pig RESTV infection	(*13*)
Mòjiāng virus	Yunnan 2012	Death of 3 mineworkers	1st evidence of rodent origin henipavirus in people	(*14*)
Nipah-like virus	Philippines 2015	Killed horses	No prior horse infection for NiV, known for HeV	(*15*)
SARSr-CoV & HKU10-CoV	S. China 2015	Seropositive people	1st evidence human infection HKU10 & SARSr-CoV	(*16*)
SADS-CoV	China 2017	>25,000 pig deaths seronegative people	Novel emergence of bat-origin CoV	(*17*)
Nipah virus	Kerala 2018, 2019	Killed 17/19 people 2018, 1 infected 2019	1st outbreak of NiV outside Bangladesh, W. Bengal focus	(*18, 19*)

Table 1: Recent emergence events in SE Asia indicating potential for novel pathways of emergence, or unusual presentations for known or

novel β-CoVs (52 novel SARSr-CoVs) and a new β-CoV clade ("lineage E") in bats in S. China that occur throughout the region (*24, 49, 51, 52*); 9 novel wildlife-origin CoVs and 27 PMVs in Thailand, and 9 novel CoVs in Malaysia reported by us from USAID-PREDICT funding (*51, 53*). These discoveries underscore the clear and present danger of zoonotic events in the region. The wide diversity of potentially pathogenic viral strains also has significant potential use in broadly active vaccine, immunotherapeutic and drug development (*49*).

Most high-impact zoonotic viruses originate in wildlife reservoirs, sometimes spilling over first into livestock 'amplifier' hosts, or directly into localized human populations with high levels of animal contact **(Fig. 2)**. Efforts to prevent emerging zoonoses have targeted these high-risk populations in regions prone to disease emergence (*31, 54*). However, these regions tend to be in the developing world, where nations often lack the resources to investigate novel zoonotic events, or wildlife reservoirs of zoonoses, and prioritize more immediate public health concerns such as Dengue virus or NTDs (*20*). Zoonotic disease surveillance and control is also hampered by inadequate information on basic disease ecology (e.g. range of wildlife reservoirs, risk factors for spillover) and pathogen biology (viral diversity, pathogenesis), and by logistical challenges sharing novel diagnostics, animal models, samples and reagents at sites where outbreaks begin (*55*). Countermeasure and vaccine development is challenged by the small number of isolates available, and the presence of closely-related strains, some with evidence of transmission to people but with unknown pathogenicity. For example, using NIH and other funding, we found serological evidence of exposure to African henipaviruses in 3-4% of a sample of people in Cameroon, with a significant risk factor being butchering of bats; and serological responses to bat SARSr-CoVs in 6/209 (2.87%) and bat HKU10-CoV in 2/412 (0.5%) people living close to a bat cave in Yunnan, all novel viruses with unknown clinical impact (*16, 56*). We discovered two novel bat-borne reoviruses, Melaka and Pulau viruses, and showed that 14/109 (13%) of people living in close proximity to bat roosts on Tioman Island, Malaysia were exposed to these viruses (*7*) as well as 12/856 (1.4%) of a random sample screened in Singapore (*8*). Previous studies in the Philippines found famers and butchers who had contact with sick pigs tested positive for Reston ebolavirus antibodies but had not experienced illness (*11*), and it was our team that discovered Philippine bats as natural reservoir hosts for Reston virus (*57*). In preliminary screening in Malaysia with funding from DTRA we found serological evidence of exposure to henipaviruses (Hendra, Nipah and Cedar) in 14 bats and 6 human samples, and Ebola (Zaire and Sudan)-related viruses in 11 bats, 2 non-human primates (NHP) and 3 human samples (Hughes, in prep.).



Even when spillover leads to outbreaks of illness and mortality, they are often unreported, undiagnosed or misdiagnosed. We initiated targeted syndromic surveillance of encephalitis patients in Bangladesh clinics and showed that Nipah virus (NiV) causes repeated annual outbreaks with an overall mortality rate of ~70% (*58*). NiV has been reported in people in West Bengal, India, which borders Bangladesh (*28*), in bats in Central North India (*59*), and now in people in Kerala, South India in 2018 and 2019, raising the specter of future spillover at other sites in the region (*18, 60*).

Fig. 2: Our Early Warning Approach: Most zoonotic viruses emerge from wildlife hosts, sometimes via livestock **(lower panel)**, to cause small scale outbreaks (green) or larger chains (red) of human-to-human transmission **(middle)**. In some cases, these spread more widely via air travel **(upper)**. Our EIDRC proposal targets each stage: **SA1** examines the diversity and spillover potential of viral threats in targeted SE Asian wildlife; **SA2** seeks evidence of their spillover into focused high-risk human populations; **SA3** identifies their capacity to cause illness and to spread more widely. Figure from (*54*).

Under a current NIAID R01, we have shown that we can use analytical approaches to target surveillance of wildlife and people and identify likely sites of spillover. We used phylogenetic analysis of bat hosts and their CoVs to identify a key cave complex in SW Yunnan Province that harbors all the genetic elements of SARS-CoV (*61*), as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics (*24, 49, 51, 52, 62-64*). We conducted risk factor surveys and biological sampling of human populations living close to this cave and found serological evidence of spillover in people highly exposed to wildlife (*16*). **This work provides proof-of-**

concept for an early warning approach that we will expand in this EIDRC to target other viral groups in one of the world's most high-risk EID hotspots.

The overall premise of this EIDRC proposal is that there is substantial evidence that: 1) diverse CoVs, henipaviruses and FVs related to known human pathogens are circulating in wildlife reservoirs in Southeast Asia; **2)** these viruses likely spillover regularly to people, are often unreported or misdiagnosed, and their clinical manifestations and potential to cause pandemics are unknown and underestimated; and **3)** our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence. To scale-up this approach, we propose to launch **EID-SEARCH** (the **E**merging **I**nfectious **D**iseases - **S**outh **E**ast **A**sia **R**esearch **C**ollaboration **H**ub), to better understand, and respond to, the risk of zoonotic viral emergence in Southeast Asia. We will initially focus on the diversity of known and novel CoVs, henipaviruses, and FVs in wildlife reservoirs. We will use this work to build our center's interactive network throughout the region, bridge barriers, provide early focal point for basic and translational studies, develop sample handling and storage routines, communication networks, cohort management, infrastructure development, and cross-training in surveillance and novel diagnostic platforms. We will develop and transfer technology to test these viruses' capacity to infect human cells and mouse models, develop new specific and sensitive serological and molecular diagnostic tools, conduct surveillance of human communities with high risk of exposure to wildlife, and clinical cohorts to identify evidence of spillover of viruses causing previously 'cryptic' clinical syndromes in people. We will test the capacity of novel viruses to infect human cells and/or mouse models, and thus asses potential for spillover and spread which will feed into our risk models. Our group also has extensive experience in other viruses, including vector-borne flaviviruses, and alphaviruses, and will use this experience to provide robust long-term surveillance, diagnostics, and basic science opportunities. While the current proposal focuses its basic and applied research agendas on this group of viral EID, we note that the EID-SEARCH is poised to rapidly respond to any outbreak. **Thus, FVs, henipaviruses and CoVs are examples of sentinel surveillance systems in place that illustrate EID-SEARCH's capacity to identify and initiate a rapid response targeting any novel microbial pathogen that emerges in this region.**

2. Innovation: Previous work by our consortium has developed strategies to target surveillance in wildlife and people to better anticipate and identify early spillover events and pre-empt outbreaks of emerging viruses. In the EID-SEARCH we scale up this approach in a central research "hub" made up of leading laboratories, clinics and surveillance sites in three critically high-risk EID hotspot countries in SE Asia. EID-SEARCH's reach is expanded through its regional network of collaborators that covers nearly all of the greater SE Asia region. The innovation of the EID-SEARCH is in: **1)** Its multidisciplinary approach that combines modeling to target geographic and taxonomic sampling targets with on-the-ground zoonotic disease surveillance in human and wildlife populations; **2)** the biological characterization approach that identifies how likely viruses are to be able to infect people, and enables evaluation of existing countermeasure technologies; an approach that is grounded in our successful work on SARSr-CoVs; and **3)** the strategic sampling of both high-risk communities with extensive animal contact at the front-lines of viral spillover *and* syndromic clinical cohorts that present with signs of novel viral emergence, to identify risk of viral spillover in an early warning system approach **(Fig. 2)**. **In Aim 1, we will target viruses in archived and newly collected wildlife samples, and characterize their risk of spillover to people**. We will use spatial and phylogeographic analyses to identify the geography and species of wildlife to test samples from, and to further characterize viruses we have recently discovered. We will use *in silico* methods (novel ecological-phylogenetic risk ranking and mapping of receptor binding domains), cell culture, and animal models to assess their potential for spillover into high-risk human populations. **In Aim 2, we will conduct targeted cross-sectional serological surveys of human communities with high levels of animal contact to find evidence of viral spillover, and identify occupational and other risk factors for zoonotic virus transmission.** We will use serological tests targeting viruses identified in Aim 1 to identify the baseline spillover of known or novel CoVs, henipaviruses and FVs in these populations. Serological findings will be analyzed together with subject metadata (including a simple animal-contact survey, location data, viral sequence/isolates etc.) to identify zoonotic risk factors and routes of exposure from specific wildlife or vector species. **In Aim 3, we will use syndromic surveillance of clinical human cohorts to identify evidence of viral etiology for otherwise 'cryptic' outbreaks.** We will enroll and

collect biological samples from patients in local clinics and hospitals who present with syndromes previously linked to known viral agents and live within communities at high risk for disease emergence. We will conduct molecular and follow-up serological diagnostic assays to identify likely viral etiology of syndromes that may represent otherwise cryptic events. Where viruses are identified, we will attempt to isolate or molecularly re-derive them, and use survey data, ecological and phylogenetic analysis to identify likely reservoir hosts and inform intervention programs. Our US partner BSL-4 laboratory, NEIDL, will attempt isolation and characterization of viruses requiring BSL-4 of containment (e.g. novel filoviruses, close relatives of Hev/NiV).

3. Approach: 3.1. Research team: The EID-SEARCH builds on long-term collaboration among world leaders in emerging virus research with proven experience collaborating internationally on field, lab and human surveillance research **(Fig. 3)**. Over the past two decades, our consortium partners have conducted high profile research on EIDs within the region and globally, including identifying the wildlife reservoirs for NiV and Hendra virus (HeV), MERS- and SARS-CoVs (*24, 27, 59, 61, 65-67*), discovering SADS-CoV (*17*), and developing an array of serological, molecular, *in vitro* and *in vivo* approaches to characterizing high-risk CoVs, henipaviruses, and FVs (*62-64, 68-81*). Our team has substantial experience conducting human surveillance in the community and during outbreaks **(Sections 2.2.a., 2.2.b., 2.2.c, 3.2.a, 3.2.b)**, including: USAID-PREDICT, and DTRA-funded viral surveillance of indigenous communities in Peninsular Malaysia and syndromic surveillance of hospital patients in Sabah (Co-I Hughes); collection of >2,000 serum samples from healthy blood donors and sanitation workers, febrile and influenza like illness (ILI), and diarrheal stool samples from children under 5 years of age across Borneo Malaysia (Co-I Kamruddin); collaboration on the MONKEYBAR project with the London School of Hygiene and Tropical Medicine to analyze risk and prevalence of the zoonotic malaria *Plasmodium knowlesi* through case control and cross-sectional studies in Sabah villagers (n=~800, 10,000 resp.) and clinical observational studies to determine the etiology of central nervous system infections and acute undifferentiated febrile illness in Sabah. (Co-Is William, Rajahram, Yeo); and community-based surveillance of hundreds of bat-exposed villagers in Thailand (Co-Is Wacharapluesedee, Hemachudha). As a proof-of-concept of how our team can rapidly scale up resources for diagnostics and surveillance in the face of a new EID, in 2016, we discovered a novel bat-origin HKU-2 CoV (*82, 83*) killing >20,000 pigs in S. China, designed PCR and serological assays, surveyed pig farmers for evidence of exposure, and conducted experimental infections, pathogenesis and molecular studies, **all in the span of 3 months** (*17, 84*).

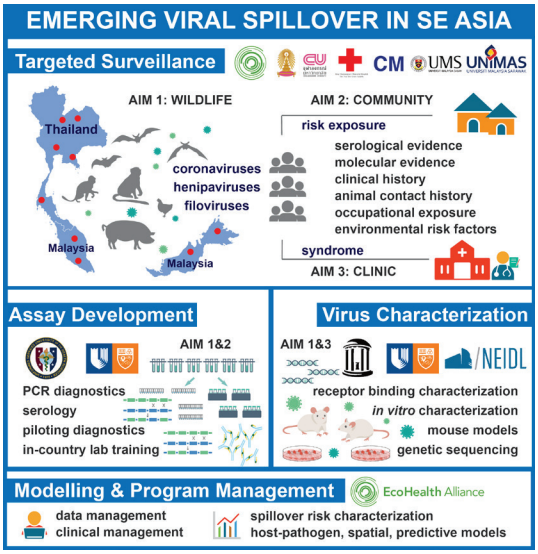


Fig. 3: EID-SEARCH scope, core institutions, and roles.

The administration of this center **(Section 4.1.)** will be centralized at EcoHealth Alliance (EHA) led by PI Daszak, who has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5-20 yrs on NIAID- and USAID-funded research. He will oversee all activities in this EIDRC, supported by Deputy lead, Dr. Olival – who has managed human and wildlife disease surveillance projects in SE Asia for >10 years, and a **Core Executive Committee (Section 4.1.a)**. Co-Is Olival and Zambrana are leaders infectious disease analytics, and head the modeling and analytics work for USAID-PREDICT. Co-Is Wang, Baric, Broder, Anderson, and Laing have developed a unique array of *in vitro* cell culture, serological and molecular reagents to identify and characterize emerging viruses in people and animals (henipaviruses and other PMVs, CoVs, FVs and many others). Co-Is Wacharapluesedee, Hemachudha, and Hughes and collaborators have coordinated clinical and community surveillance of people, and of wildlife and livestock within Thailand and Malaysia for the past 15 years, in collaboration with Ministries of Health, Agriculture and Environment. Finally, this consortium has extensive external funding from NIH, DoD, USAID, DHS and other USG agencies supporting laboratory, analytical and field studies directly related to the current proposal, and will bring substantial leverage to our EIDRC.

3.2. Geographical focus: The three core (hub) countries for this proposed EIDRC are Thailand, Malaysia and Singapore, three contiguous EID hotspot countries that stretch through one of the most significant foci of EID origins globally. Our proposed work includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sarawak and Sabah, and thus contains overlapping wildlife biogeography with India, Bangladesh, Myanmar, SW China, Laos, Cambodia, Vietnam, Philippines, and Indonesia. It encompasses diverse cultural and socioeconomic backgrounds which have correspondingly diverse and intensive interactions with wildlife reservoirs and the global travel network. We have already begun sampling of wildlife hunters, livestock workers, wildlife farmers and market workers, rural villagers living close to high risk wildlife species, people otherwise highly exposed to animals in these countries, and clinical cohorts, as part of current/prior NIH, DTRA, and USAID funding. Our core member's extensive network of collaborators in clinics, research institutes and public health laboratories in almost every other Southeast Asian country positions the EID-SEARCH within a much larger catchment area for emerging zoonoses and will allow us to rapidly scale up to include additional sites in response to an outbreak or shift in research priorities. We will conduct regular meetings with key research, public health and community leaders, including >50 leading laboratories/institutions in 10 countries **(Section 4.2)** to maintain these collaborative relationships with the core members of our consortium (**Fig. 4**). We will use these, and our annual meetings, to share information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks across the region.

Fig.4 : Map of Southeast Asia indicating three hub countries for this proposed EIDRC (**Red**: Thailand, Singapore, Malaysia) and countries in which Key Personnel have existing collaborating partners via other funded work (**Green**), indicating that EID-SEARCH provides access to key collaborators and sites throughout the region.

3.3. Capacity for linkage with other NIAID studies, and scaling up to respond to outbreaks: EID-SEARCH US-based partners include senior scientists with significant experience on NIAID-funded projects, extensive collaborations with other NIAID-funded scientists, and previous experience working with NIAID leadership through workshops, review of programs (e.g. PI Daszak's role in NIAID CEIRS external review), and work on study sections. These contacts ensure that the EID-SEARCH senior leadership will be ready and able to rapidly set up data, sample and reagent/assay sharing protocols with NIAID researchers, the other EIDRCs, the EIDRC CC and other NIAID research centers. EID-SEARCH in-country partners include senior medical doctors and infectious disease specialists at national and regional hospitals in each country and administrative state in this proposal. On news of potential outbreaks, **EID-SEARCH will rapidly mobilize its core staff and their extensive collaborative network across almost all SE Asian countries (Fig. 4) (Sections 4.1.a, 4.2. 4.3).** The core analytical approach can be used to rapidly identify sampling sites and targets to harvest vectors, vector borne arboviruses, birds and mammalian species that harbor most other viral threats. Through existing projects such as the USAID PREDICT Project and DTRA-funded biosurveillance projects, EHA is currently partnered with hospitals and researchers across South and Western Asia, West and Central Africa, and Latin America. Our partners already have capacity to conduct human and wildlife sampling and testing for novel viral agents, so expanding in geography or scale is readily achievable given sufficient resources.

Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife.

1.1 Rationale/Innovation: The vast majority of emerging zoonotic pathogens are linked directly or indirectly to wildlife host, particularly from important mammalian groups like bats, rodents and NHPs (*3*). Our previous work on wildlife origin viruses demonstrates that zoonotic viral emergence tends to occur in specific regions with high diversity of wildlife that harbor viruses of zoonotic potential, and human populations that have close contact to these wildlife **(Fig. 1)** (*2, 3*). In Aim 1 **(see Fig. 9 for overview)**, we will strategically conduct EID surveillance by applying risk analytics to prioritize the regions, wildlife species, and human populations that

have a particularly high risk of viral spillover. We will target high-risk zoonotic host reservoirs (bats, rodents and NHPs) in these regions and apply novel predictive models to estimate viral diversity and host range to identify wildlife species that have been ignored or under-sampled by previous zoonotic disease surveillance efforts. We will strategically collect specimens from these species and screen them, together with some of the tens of thousands of recently collected samples we have archived, for known and novel agents. We will begin screening using conserved PCR assays to identify known and novel viruses in high risk RNA viral families, *Coronaviridae*, *Paramyxoviridae*, and *Filoviridae*. We will expand to other groups of pathogens, including arboviruses, pending available resources and priorities established with the EIDRC coordinating center (EIDRC-CC) and other regional EIDRCs. Using risk ranking algorithms and a series of ecological and virological risk factors, we will triage the wildlife viruses we discover for further genomic characterization and viral isolation. On this subset of viruses, we will then use *in vitro* and *in vivo* approaches (viral rescue, cell lines, gene edited humanized transgenic mice and the collaborative cross genetic reference mouse population) previously developed and widely used by our team to predict capacity of novel viruses to infect people and spillover (*63, 85-88*). These high-risk viruses and their close relatives will be targets for human community serosurveys and clinical sampling in Aims 2 and 3, respectively.

1.2 Preliminary data: 1.2.a. Geographic targeting: EHA has led the field in analyzing where the highest risk of zoonotic spillover is across the globe, identifying SE Asia as a major EID hotspot (*1, 2*). These hotspots are sites where high wildlife biodiversity, human population density and rapid ecologic change collide **(Fig 1)**. Using host and ICTV viral data for all known mammalian viruses, and generalized additive models to correct for sampling bias, we are able to predict the relative number of yet-to-be-described viruses that a species harbors (*3*). This demonstrates substantial undiscovered viral diversity in mammals across Southeast Asia, and particularly in regions of Thailand, Singapore and Malaysia **(Fig. 1)**. Separately, we found that factors driving spillover (host diversity, climate) differ from those driving spread in human populations (population density, bushmeat hunting, livestock production) (*89*). We therefore analyze capacity for viral spread as a separate risk factor, using our unique demographic and air travel and flight data modeling software (*90, 91*). In the current proposed work, we will use all the above approaches to target wildlife sampling (archival and new field collections) in Aim 1, to inform sites for human sampling in Aim 2, and to assess risk of spread in Aim 3.

1.2.b. Reservoir host species targeting: Our analysis of all known mammalian host-viral associations demonstrates that bats, rodents and NHPs represent the three most important reservoirs for zoonoses, responsible for the highest proportion of known and predicted viral diversity of zoonotic potential (*3*). Thus, the bulk of our proposed wildlife sampling and testing will consist of these three taxa with a focus on bats, because this group is the known or hypothesized reservoir for the majority of high-impact zoonotic CoVs, PMVs and FVs (*50, 92-94*). Bats have the highest proportion of predicted zoonotic viruses in any mammalian group (*2*), and are exceedingly diverse in Southeast Asia (~500 species). To further prioritize taxonomic sampling targets within these diverse geographic hotspots, we used a novel Bayesian phylogeographic algorithm to identify host genera and species that are the most important centers of evolutionary diversity for a given viral group, e.g. bat genera for β-CoVs **(Fig. 5)** (*51*). This approach will be extended to other mammal taxa and viral families of interest, allowing us to identify the reservoir hosts of specific zoonotic viral groups, where viral diversity is likely highest.

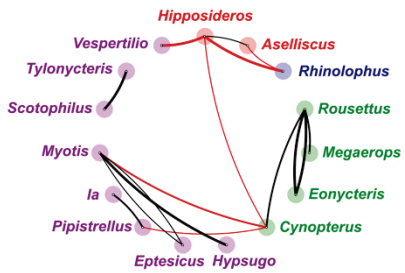


Fig 5: Preliminary analysis of sequence data collected under prior NIH funding, identifying SE Asian bat genera with the highest β-CoV evolutionary diversity. We will apply this Bayesian discrete phylogeographic model to identify taxonomic and geographic centers of viral diversification and sharing for relevant PMV, CoV, FV and other virus clades. Line thickness proportional to probability of virus sharing between two genera. Inter-family switches in red.

Finally, we have designed a strategy to estimate sampling gaps, using a viral 'mark-recapture' approach we previously published (*95, 96*) **(Fig. 6)**. Using prior data for bat, rodent and NHP viral discovery vs. sampling rates, we can identify the likely total viral diversity for specific host groups in a region, and use this to estimate sample sizes to discovering the majority of unknown viruses within a specific family or genus. **In the current**

proposal, we will use these analyses to estimate geographic and species-specific sampling targets **to more effectively identify new strains to support experimental infection studies and risk assessment.**

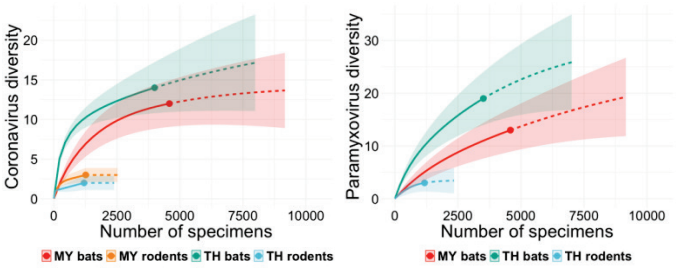


Fig. 6: Estimated CoV **(left)** and PMV **(right)** diversity in bats and rodents from Thailand and Malaysia, using data from PCR screening and RdRp sequences from >10,000 specimens in bats and 4,500 in rodents. Bats have 4X more viral species than rodents, controlling for sampling effort. We estimate that additional collection of 5k-9k bat specimens and testing of our archived bat and rodent specimens alone will identify >80% of remaining CoV and PMV viral species in these key reservoirs, yielding >800 unique viral strains.

1.2.c. Sample testing to identify known and novel viruses: We have conducted extensive wildlife surveillance for novel viruses across Southeast Asia in prior NIAID funded work and as part of the USAID PREDICT project. Under the 10-year PREDICT program globally, we identified 912 novel and 210 known viruses, more than the total number of viruses previously recognized in mammals by the ICTV (*97*). This work included collecting nearly 300,000 individual mammal samples from 14 EHA-led PREDICT countries, and PCR-screening of more than 60,000 individual specimens (*53*). In southern China alone, we identified 178 β-CoVs, of which 172 were novel, discovered a new β-CoV clade, "lineage E" (41), diverse HKU3r-CoVs (179 sequences) within a 'sister' clade to the SARS-CoV lineage, and a new bat-origin SADS-CoV, responsible for killing >25,000 pigs in Guangdong Province (*17*). We have collected 28,957 samples from bats, rodents and NHPs in Thailand and 47,178 in Malaysia, **but have only tested a minority of these using PCR.** We have identified 100 novel viruses in Thailand and 77 in Malaysia. **Furthermore, we have only sequenced a small segment of one gene for all novel viruses found. We have archived duplicate samples which are now available for use in this project**, including fecal, oral, urogenital, serum samples, and biopsies from bats, rodents, and NHPs. In the current proposed work, we will attempt to sequence, isolate and characterize those viruses that our analyses below suggest are most likely to be able to infect humans.

1.2.d. In vitro & in vivo *characterization of viral potential for human infection:* Using Coronaviridae as an example, we have conducted *in vitro* and *in vivo* experiments to characterize the pathogenic potential of novel SARSr-CoVs from bats. We isolated three SARSr-CoVs from bat feces: WIV1, WIV16 and Rs4874, with Spike (S) protein sequences that diverged from SARS-CoV by 3-7% (*24, 61, 98*). We conducted full-length genome sequencing of 12 other novel SARSr-CoVs, some highly similar to outbreak SARS-CoV in the most variable genes (*61*). Using our reverse genetics system we constructed chimeric viruses and rederived full length recombinant SARSr-CoV from in silco sequence. All 3 SARSr-CoV full length isolates and the two chimeric viruses replicated efficiently in Vero E6 cells and in HeLa cells expressing hACE2, civet and bat ACE2, but not in those without ACE2 (*24, 61, 98*). We used the SARS-CoV reverse genetics system (*72*) to generate a chimeric virus with a mouse-adapted SARS-CoV backbone expressing SHC014 S protein with 10% sequence divergence from SARS-CoV S. This and the other full length and chimera viruses replicated in primary human airway epithelium, using the human ACE2 receptor to enter into cells (*62*). Thus, **SARSr-CoVs with diverse variants of SL-CoV S protein without deletions in their RBD can use human ACE2 as receptor for cell entry.** The Baric lab has a well-established hACE2 and DPP4 transgenic mouse model that **we used to assess the capacity of novel SARSr-CoVs and MERS-CoV to infect humans and cause disease** (*85, 99*). Infection of bat SARSr-SHC014 or WIV1 caused SARS-like clinical signs in the transgenic hACE2 mouse model **that weren't reduced by immune-therapeutic monoclonals that attenuate SARS-CoV pathogenicity, nor after challenge following vaccination against SARS-CoV** (*62*) **(Fig. 7)**. We repeated this virus characterization approach with chimeras using HKU3r-CoV S proteins that are ~25% divergent from SARS-CoV S, **and found that they are unable to use the ACE2 receptor**. Additionally, we were unable to culture HKU3r-CoVs in Vero E6, or human cell lines.

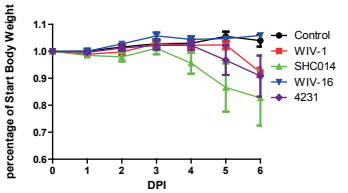


Fig. 7: Body weight change in hACE2 transgenic mice after SARSr-CoV infection, demonstrating that bat-origin SARSr-CoVs cause similar clinical signs to outbreak SARS-CoV strain (4231) (*62, 63*).

A similar approach to the above pipeline for CoVs will be applied to novel henipaviruses we discover during our research. The broad mammalian tropism of HeV and NiV (*68*) is likely mediated by their usage of highly conserved ephrin ligands for cell entry (*19, 100*). A third isolated henipavirus, Cedar virus does not cause pathogenesis in animal models. **Co-Is Broder and Laing** have developed a reverse genetics system and rescued a recombinant CedV to test ephrin receptor usage/tropism and pathogenicity during henipavirus infections (*77*). CedV is unable to use the Ephrin-B3 receptor (*77, 101*) which is found in spinal cord and may underlie NiV encephalitis (*102*) and that pathogenesis also involves virulence factors V and W proteins. The putative henipaviruses, Ghana virus and Mòjiāng virus, predict V and W protein expression, with GhV able to bind to ephrin-B2, but not -B2 (*103*), and the receptor for MoJV remains unknown (*104*) but is likely ephrin-B2 (*105*). Our group has also used similar methods to test the hypothesis that novel Filoviruses discovered in wildlife are able to infect people. NiV, EBOV and MERS-CoV all infect primary microvascular endothelial cells, which are available at Co-I Baric's lab for EID-SEARCH collaborators (*71, 106*). Primary human lung endothelial cells are highly susceptible to Ebolavirus infection (*107*), and Huh7 cells can be used to isolate virus from clinical samples (*108*) offering advantages for reverse genetic recovery of novel FVs (*109*). The three filovirus genera, *Ebolavirus*, *Marburgvirus* and *Cuevavirus*, use Niemann–Pick type C1 (NPC1) protein as cell entry receptor (*110*). For novel filoviruses, cell culture would be carried out under BSL-4, however they can readily be characterized following identification of their encoded GP sequences which can be pseudotyped into recombinant GP-bearing vesicular stomatitis virus (VSV) pseudovirions (*111*). These pseudovirions can be safely used to characterize the tropism and entry filoviruses mediated by GP without a need for BSL4 containment. **Co-Is Wang and Anderson** used cells originating from various mammalian species to determine spillover and/or zoonotic potential of MLAV, a newly discovered Asian filovirus (*47*). Despite the low amino acid sequence identity (22–39%) of the glycoprotein with other filoviruses, MLAV is capable of using NPC1 as entry receptor. Cells from humans, monkeys, dogs, hamsters and bats were able to be infected with a MLAV pseudovirus, implying a broad species cell tropism with a high risk of interspecies spillover transmission.

Mouse models. For novel viruses across all families, we will use the UNC collaborative cross mouse that captures 90% of the diversity within all mouse groups to assess the most suitable model for further experiments (*112*). We have used this model for CoV, FV (Ebola), Flaviviruses, and alphaviruses infections. Clinical signs such as weight loss, hemorrhage, encephalitis, and, acute or chronic arthritis were recapitulated in these mice following SARS, EBOV, West Nile virus and Chikungunya infections, respectively (*86-88, 113-116*). Note that CC OR3015 shows hemorrhagic disease phenotypes after EBOV infection **(Fig. 8)**. *Bat Models.* Duke-NUS has developed two models for bat *in vivo* studies: a colony of *Eonycteris spelaea* (cave dwelling small fruit bat) and a bat mouse model, which contains the bat immune system in an immunodeficient mouse (*117*). In a proof of concept study, **Co-Is Wang and Anderson** infected *E. spelaea* bats with MERS-CoV to demonstrate their susceptibility and suitability for infection with a bat borne virus under BSL3 containment. These bats and the bat mouse model will serve to complement and expand studies from the UNC collaborative cross mouse, pending further funding from EIDRC CC. Recombinant viruses, transgenic mouse models and experimental recombinant protein constructs described above will be made available to the EID-SEARCH consortium and other EIDRCs following standard procedures **(see Resource Sharing Plan)**.

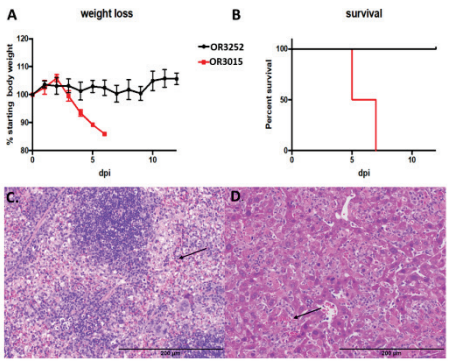


Fig. 8: EBOV Infection in Collaborative Cross Mouse. **Panel A/B:** Wt EBOV in two different CC lines demonstrate different disease patterns. **Panel C/D:** Hemorrhagic phenotypes on d. 6 (arrow) in spleen and liver, resp. From (*86*).

1.3. General Approach: We will conduct analyses to geographically- and taxonomically-target testing of samples from wild mammals that are most likely to harbor known high-profile zoonotic pathogens, or close relatives with potential to infect people. This includes selecting from tens of thousands of samples collected on recent projects and stored in freezers in our laboratories, and new sampling of wildlife in high risk locales. We will use viral family level PCR assays to identify and partially characterize viruses, then follow up sequencing of relevant receptor binding proteins to assess homology and predict binding affinity, then attempt to isolate and

biologically characterize viruses that phylogenetic and other data suggest have high spillover and pandemic potential. We will follow this with *in vitro* binding assays and cell culture experiments, and *in vivo* animal model infections to assess their potential to infect people and cause disease **(Fig. 9)**. EHA will lead the study design, targeted sampling, and data analysis; Co-Is Hughes and Wacharapluesadee will lead field sampling and in-country testing; Duke-NUS, UNC, USUS will lead the serological and PCR diagnostic development and the *in vitro* and *in vivo* analyses, as well as cross-training and tech transfer to the hub labs in Thailand and Malaysia. This will include via support of one Malaysian Ph.D student, Mei-Ho Lee (CM Ltd.) who has worked with the EID-SEARCH collaborators for 9 years. The focus of her study will be infection potential of novel CoVs and PMVs already found in bats in Malaysia under preliminary data for this proposal.

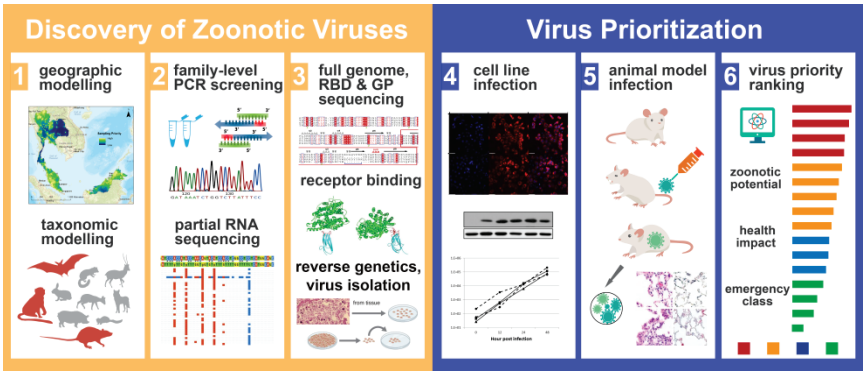


Fig. 9: Aim 1 will use analytical tools to target selection of archived and newly collected samples, conduct PCR and sequencing of novel viruses, recover by reverse genetics, and assess zoonotic potential using *in vitro* and *in vitro* models and analyses.

1.4 Wildlife samples: 1.4.a. Geographic and taxonomic targeting for newly collected wildlife samples: We will prioritize sites within each country that rank highest for both the risk of zoonotic disease emergence (*2*) and the predicted number of 'missing' zoonotic viruses (*3*). Our preliminary analysis **(Fig. 1)** suggests priority areas include: the Kra Isthmus (S. Thailand) and adjacent forests in N. Peninsular Malaysia, NW Thailand (near the Myanmar-Laos border), and lowland rainforests of Sarawak and Sabah. In each of these 'hottest of the hotspots' regions, we will identify 2-4 distinct sampling sites based on the geographic distribution of key hosts we prioritize for additional wildlife sampling. We will priority rank bat, rodent, and NHP species using host trait-based models (*3*). We will also identify species predicted to be centers of evolutionary diversity for CoVs, PMVs, and FVs using ancestral host reconstruction of available sequence data and Bayesian discrete phylogeographic models (*118-120*). We will leverage our strong collaborations with wildlife experts in Malaysia and Thailand, including many of whom are based at our core partner institutions, to ground-truth geographic and taxonomic model outputs with local expert knowledge to ensure the sampling plan is realistic based on current species distributions, population size estimates, seasonality, and accessibility to sites. Most zoonotic viruses are naturally carried by multiple wildlife host species, and there are strong, predictable relations between host phylogeny and viral taxonomy (*3*). We will use a combined network analysis and phylogeographic model to rapidly predict and prioritize new (unsampled) hosts for important, novel viruses we discover during our research. We have shown this relatively simple model (using just wildlife species range overlap and phylogenetic similarity between host species) is both generalizable to estimate host range for all mammalian and avian viruses *and* robust – accurately predicting hosts 98% of the time when cross-validated using data from known viruses (*121*).

1.4.b. Sample size justifications for testing new and archived wildlife specimens: We calculate initial sample sizes targets using our preliminary data on average PCR prevalence from screening tens of thousands of bat, rodent and NHP specimens for CoV and PMVs based on previous studies in Malaysia and Thailand. We will refine these sample size targets, in real-time, over the course of our project using viral discovery curve analyses **(Fig. 6)** to either scale-up or scale-back new sampling and testing of archived specimens for each species depending on viral capture rates. We will only collect additional specimens projected from each prioritized taxa to achieve statistically robust sample sizes and to complement our archived specimens. For example, *Rousettus* spp. bats, known FV reservoirs, were not adequately sampled under PREDICT research in Thailand. Preliminary analysis indicates that, for all viral target families, we will require ~14,000 new samples to identify 80% of remaining viral species diversity in our study region, ~5,000 samples from Thailand and ~9,000 samples from Malaysia **(Fig. 10).** Viral strain diversity from a sampling effort this size is expected to number in the hundreds of strains. For example, given ~6% prevalence of CoVs in bat species previously sampled under the PREDICT project, a target of 14,000 individuals would yield ~800 PCR positive individuals,

representing, an estimated ~600 novel sequences/strains. Similarly, for PMVs, which have an average PCR detection prevalence of ~1.5%, sampling of 14,000 individuals would yield ~200 positives and an estimated 150 unique strains. Filovirus estimates are not feasible yet due to the small number of positive samples in prior studies, but will be estimated as the project begins. From each mammal, we will collect serum, whole blood, throat swabs, urine and fecal samples or rectal swab using our previously-validated nondestructive methods. We will sample sites at different time-points throughout each year, and sample an equal number of males and females to account for seasonal or sex-specific differences viral shedding **(See Vertebrate Animals)** (*122, 123*). Dead or animals euthanized due to injury will be necropsied.

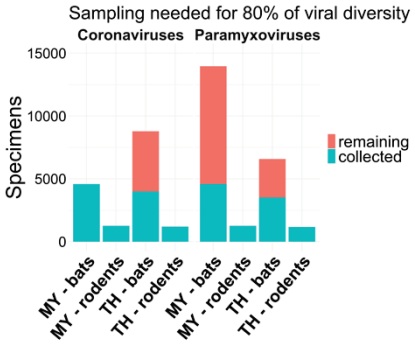


Fig. 10: Stacked bar plot showing number of currently-archived specimens **(blue)** and new specimens required **(orange)** to discover 80% of predicted number of CoVs and PMV species from high-risk bat and rodent taxa in Malaysia and Thailand.

1.4.c. Sample collection, testing, viral isolation: Wildlife will be captured and sampled using previously published techniques, by personnel wearing appropriate personal protective equipment (Tyvek suits or dedicated long external clothing, double nitrile gloves, leather gloves, an N95 or p100 respirator, and safety glasses) (*53, 93, 95, 96, 122, 124*). All samples will be placed in a vapor phase liquid nitrogen dry shipper immediately upon collection, and then transferred to a -80C freezer once back in the lab, until testing. Viral RNA will be extracted from bat fecal pellets/anal swabs. RNA will be aliquoted, and stored at -80C. PCR screening will be performed with pan-coronavirus (*125, 126*), filovirus (*127*) and paramyxovirus primers (*128*), in addition to virus specific primers where additional sensitivity is warranted in key viral reservoirs, i.e. Nipah virus or MERS virus specific primers (*129, 130*). PCR products will be gel purified and sequenced. We will attempt isolation on samples that contain viruses of interest (determined in **Aim 1.5 below**), using Vero cells and primary cell culture from the wildlife taxonomic order (bat, rodent and NHP cell lines). Over 70 bat cell lines are maintained at Duke-NUS from five different bat species. These include primary lung, brain, bone marrow, heart, kidney, intestine, liver, lymph node, muscle, spleen, PBMC and ovary/testes from *Eonycteris spelaea, Cynopterus bracyhotis, Rhinolophus Lepidus, Myotis muricola* and *Pteropus alecto* bats. In addition we have 8 immortalized cell lines, including those derived from lungs, therefore suitable for the isolation of respiratory viruses.

1.4.d. Moving beyond RNA viruses: To detect pathogens from other families, such as non-RNA viruses, we will combine conserved PCR assays with NGS. We will perform either unbiased NGS, or use a pan-viral enrichment library to sequence full-length genomes (*131*). We will conduct this work via EID-CC research projects or as part of outbreak detection/investigation in the region from DNA viruses or other agents.

1.4.e. Sequencing Spike Glycoproteins: Based on earlier studies, our working hypothesis is that zoonotic CoVs, FVs and henipaviruses encode receptor binding glycoproteins that elicit efficient infection of primary or continuous human cell lines (e.g., lung, liver, etc.) (*16, 61, 62*). Characterization these for SARSr-CoVs suggest that viruses up to 10%, but not 25%, different in the spike glycoprotein use human and bat ACE2 receptors for docking and entry. Uneven sampling gaps (e.g., no strains were found with 10-25% spike variation) prevent a thorough understanding of the transition point where the most divergent strains lose human ACE2 receptor usage. Our viral discovery curves **(Fig. 6)** suggest further sampling will reveal a rich diversity of as-yet-undiscovered SARSr-CoVs and strains of other viruses that fill in the phylogenetic tree between currently-described viruses. In this aim we will use phylogenetic and viral discovery analysis to specifically target bat species and regions that are under-sampled to allow sufficient power to identify and characterize missing strain diversity for potential zoonoses. We will sequence the S proteins of novel SARSr-CoVs to prioritize viruses for experimental work in Aim 3 to test this hypothesis. Likewise, we will test the hypothesis that phylogenetic related zoonotic receptor binding protein genes of MERS-related, Ebola-related and NiV/HeV-related viruses will also program efficient entry via human and ortholog receptors. For all novel virus strains, we will sequence the complete entry spike genes by amplifying overlapping fragments using degenerate primers as shown previously (*24, 61*). ***Reverse Genetics:*** Full-length genomes of selected CoVs, FVs or henipaviruses (representative across subclades) will be sequenced using NEBNext Ultra II DNA Library

Prep Kit for Illumina and sequenced on a MiSeq sequencer. PCR or MinIon sequencing will be performed to fill gaps in the genome. The full length spike gene sequences, including the amount of variation in the receptor binding residues that bind the appropriate human receptor will be used to select strains for Aim 3 experiments. We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-CoV cross neutralization, synthetically reconstruct a small subset (1-2) and evaluate growth in Vero, BHK and Huh7 cells (*63, 132*). From full length sequences, synthetic clones will be ordered to recover recombinant viruses using reverse genetics as described by our groups (*62, 63, 77, 109, 133, 134*). Recombinant virus growth will be accessed in Vero, BHK, Huh7 and select bat cells and recovered viruses used for downstream studies. Virus strain prioritization is described below.

1.5. Assessing risk for spillover. 1.5.a. Capacity to infect human cells: We will use a series of cell cultures to assess the capacity of target viruses to enter human cells. For CoVs, FVs and henipaviruses, we will use primary human ciliated airway epithelial cells (HAE) and lung endothelial cell cultures derived from the lungs of de-identified transplant recipients. HAE are highly differentiated and grown on an air-liquid interface for several weeks prior to use (*62, 63, 132*). We will prepare HAE and lung endothelial cultures from three different patient codes in triplicate in collaboration with the tissue procurement facility at the Cystic Fibrosis Center at UNC. Cultures will be inoculated with chimeric bat SARSr-CoVs or novel FVs or henipaviruses to assess efficient replication. At 72 hpi, cultures will be fixed for immunofluorescent staining using antisera to the SARS-CoV conserved nucleocapsid protein (N) or to structural genes of the other viruses (*64, 71*). SARSr-CoVs that differ significantly in S protein sequence (11-24%) from epidemic SARS-CoV yet replicate *in vitro*, will also be evaluated for sensitivity to neutralization in Vero cells by PRNT50 assays using broadly SARS-CoV cross reactive human mAbs S227.14, S230.15, S215.17, and S109.8 (*63, 135*) or monoclonal antibodies targeting the Ebola or Marburg E glycoprotein (Available through BEI), or NiV/HeV glycoproteins (*136*). As controls or if antisera is not available, the S genes of novel SARSr-CoV or spike entry genes of other novel viruses under investigation will be inserted into VEE 3526 replicon vectors (VRP3526-S), packaged and used to vaccinate mice (*137*). Polyclonal sera against test bat-SARS virus or other spike genes will be harvested and tested for ability to cross neutralize SARS-CoV, GD03, WIV-1, SHC014, WIV-16, other novel SARSr-CoV and HKU3-SRBD by PRNT50 assay (*63, 138, 139*). Using PRNT50 titers with sera (n=4 each) among these viruses, antigenic cartography (*140*) will allow comparison of antigenic vs. phylogenetic distance, identifying the transition at which SARSr-CoV strains escape SARS-CoV based vaccines, informing immunotherapeutic and vaccine design strategies (*141-143*). Similar approaches will be applied to novel MERS-related viruses, other CoV, FVs or henipaviruses. While most wildlife-origin viruses with unknown transmission potential or pathogenicity in people uncategorized, we use BSL-3 for isolation as standard, and cell culture for potentially BSL-4 pathogens will be undertaken at collaborating institute NEIDL. To facilitate this, duplicate samples in culture medium from animals PCR +ve for FVs and very close relatives of NiV or HeV will be shipped to NEIDL **(See letter of support)** for culture, characterization and sharing with other approved agencies including NIH Rocky Mountain Laboratories where PI Daszak and Co-Is Baric have ongoing collaborations. Similarly, some duplicate samples of animals PCR +ve for CoVs will be shipped to UNC for further characterization by Co-I Baric. Where feasible and appropriate, training opportunities at NEIDL and UNC for in-country staff will be given, thereby enriching hands-on collaboration across sites.

1.5.b. Host ACE2, DPPR, Niemann-Pick C1 (NPC1) and EphrinB2/EphrinB3 receptors: We will sequence relevant host receptors for select mammalian wildlife species we find positive for strains of high-priority CoV clades, and all new henipaviruses and FVs. Of particular interest is homology across 18 bat and human orthologue ACE2 contact interface residues that engage the SARS RBD as a potential indicator of SARSr-CoV cross species transmission potential and growth in human cells (*70*). Likewise, variation in the NPC1 receptor has been shown to alter EBOV ability to infect in humans and bats, so this can be structurally mapped for prediction and downselection of strains (*144*). Signatures of positive selection in bat NPC1 were concentrated at the virus-receptor interface, with the strongest signal at the same residue that controls EBOV infection in *Eidolon helvum* cells. Thus NPC1 is a genetic determinant of FV susceptibility in bats, and some NPC1 variations likely reflect host adaptations to reduce FV replication and virulence (*145*). NiV and HeV use ephrin B2 or ephrin B3 as a receptor, and variation affects its ability to use human vs bat receptor molecules (*103, 146*).

1.5.c. Host-virus evolution and predicting receptor binding: We will use Bayesian and Maximum Likelihood phylogenetic analyses of RNA-dependent RNA polymerase (RdRp) sequences from PCR screening, receptor binding glycoproteins, and/or full genome sequence data (when available) to reconstruct the evolutionary history of the novel bat SARSr-CoVs, bat MERSr-CoV, FVs and henipaviruses that we identify. We will run co-phylogenetic analyses to map out instances of host-switching (*147, 148*) and ancestral state reconstruction analyses **(Fig. 4)** to identify the most important species for shaping evolutionary diversity for these viruses (*51, 149*), allowing for more targeted future surveillance and spillover mitigation interventions. For receptor binding glycoprotein sequence data from characterized viruses, we will apply codon usage analyses and codon adaptation indices, as used recently on henipaviruses (*150*), to assess the relative contributions of natural selection and mutation pressure in shaping host adaptation and host range.

1.5.d. Viral strain prioritization: Of the expected 100-200 novel SARSr-CoV strains, and approximately 600 total CoV strains, we will down-select to prioritize for further characterization based on S genes that are: i) different from SHC014, WIV1, SARS-CoV with diversity ranges of 10-25%; ii) have virus S RBD that could use human/bat receptors; iii) have recombinant chimeric spikes indicative of gene flow between clade I and II strains; iv) have bat ACE2 or DPP4 receptors that might select for spike RBDs that can use human receptors for entry (15/18 conserved residues in human/bat ACE2 molecules that bind SARSs-CoV S RBD domains are likely more efficient receptors than 3/18 conserved sites). Using structural models based on the SARS S glycoprotein, the extent and location of antigenic variation will be annotated onto the structure, further resolving the locations of highly evolving and conserved structural motifs/epitopes that function in protective immunity (*135, 151-153*). For novel henipaviruses, we will examine the receptor-binding proteins (e.g. envelope attachment (G) proteins) for sequence and structural homology with particular attention to conversation of amino acids that mediate ephrin receptor binding. We will prioritize strains that are predicted to interact with ephrin-B2 and expression V/W proteins to determine whether these viruses represent zoonotic threats. Using the recombinant Cedar virus (rCedV) platform, we will be able to rescue rCedV that express heterotypic G proteins to explore receptor utilization and tissue dissemination in animal models. We will assess ability to infect human cells using a continuous lung epithelial cell line, Calu3, which our group has previously used to generate data on NiV and HeV growth curves and a variety of host expression changes after infection (*154*). We will also use primary human airway cultures and microvascular endothelial cells to assess human infection for FVs, henipaviruses and MERSr- and SARSr-CoVs (*155*) (*156*) . There is some evidence for NiV and HeV replication in mouse lung, low level titers in young animals, 10 fold higher titers in aged mice (*157, 158*). Thus it is likely these models could be improved in the collaborative cross mouse resource at UNC. We have identified CC lines that are highly vulnerable to wildtype SARS and others that develop hemorrhagic disease following mouse adapted Ebola infections (*86-88*).

1.5.e. Animal models: We will use transgenic mouse models and the UNC Collaborative Cross model to assess spillover potential of viruses. For work on CoVs, we will follow the protocols in the transgenic mouse model used by the Baric lab previously. Briefly, in the BSL3 lab, 10- to 20-week old hACE2 or hDPP4 transgenic mice will be intranasally inoculated with 5 x 10^4 PFU of full length wildtype rbat CoV, or chimeric SARSr-CoV or MERSr-CoV encoding different bat CoV spike proteins respectively, then monitored daily for weight loss, morbidity, and clinical signs of disease. Mice will be euthanized at 2, 4, and 6 dpi (endpoint of 14 dpi), organs harvested and virus quantified by plaque assay or genome (mRNA1) RT-PCR. After 7 days in formalin, tissues for histopathology will be removed from the BSL3 and stained with H&E, and for immunohistochemistry using polyclonal antibodies against the N protein. We will conduct limited evaluation of existing countermeasures using therapeutic monoclonal antibodies *in vitro*. Existing SARSr-CoV or MERS-CoV mAbs (*159, 160*) will be diluted in DMEM starting at 1:20, and serial dilutions mixed with an equal volume of 50 PFU of chimeric bat SARSr-CoVs encoding different spike proteins and RFP indicator genes, then incubated on Vero E6 cells at 37°C for 1 h. Antibody neutralization titers will be evaluated by plaque quantification at 4-5dpi. In select instances, hACE2 transgenic mice will be injected with SARS-CoV mAbs, and infected with chimeric bat SARSr-CoVs. Clinical signs and morbidity will be assessed and tissue pathology examined and compared with mice without treatment of mAbs to determine the therapeutic effect on SARSr-CoV infection, and protection of SARSr-CoV by wildtype SARS-S based vaccines assessed as described (*132, 161*). We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-

CoV cross neutralization, synthetically reconstruct a small subset (1-2), recover full length viruses and evaluate the ability of nucleoside analogues to inhibit growth in HAE cultures and/or *in vivo* (*63, 132*). For the Collaborative Cross model, we will infect six to eight mice from 6-8 Collaborative Cross mouse lines (10 weeks of age) with six test viruses/yr, intransasally or by subcutaneous infection with 1 x 10e4 virus. Animals will be followed for weight loss, morbidity, mortality and other clinical disease symptoms (e.g., hunching, hindleg paralysis). One half the animals will be sacrificed at day 3 and day 7 pi. to evaluate virus growth in various organs, and organ pathology. In highly vulnerable lines, additional experiments will be performed using more animals, evaluating virus growth, clinical disease symptoms, respiratory pathology, tropism and organ pathology through day 28 pi. We anticipate that some CC lines will prove highly vulnerable to select wildtype viruses, because of host susceptibility combinations that promote severe disease outcomes.

1.6. Potential problems/alternative approaches: Challenges with logistics or obtaining permission for wildlife sampling in sites we select. We have a >20-year track record of successful field work in Malaysia, and >10 years in Thailand, and have strong established partnerships with local wildlife authorities and communities to ensure access. We have existing permissions in place which will be renewed at the start of this project. Due to the abundance of wildlife habitats in the region, if access to a site is withdrawn, we will rapidly identify a suitable, complementary site with similar bat species composition and abundance. Finally, we have already collected tens of thousands of wildlife samples from the region and will focus on these while waiting for permission. **We may not identify novel viruses in our sample wildlife species due to seasonality of viral shedding or other factors.** We have calculated sample sizes based on realistic detection rates from our extensive preliminary data, and are confident that we will detect and identify large numbers of potentially zoonotic pathogens, particularly for CoVs and PMVs. We acknowledge that FV strain diversity may be harder to capture, given relatively low seroprevalence in bat populations – suggesting lower levels of viral circulation (*42, 43*). However, our team was the first and only group to sequence FV RNA from Asian wildlife species (*46, 47, 57*), and we are confident that with our targeted sampling and testing strategy offers the best chance globally to identify additional strains of Ebola and related viruses. The sampling regions we have selected are subtropical, and our previous data, and even published studies in temperate regions (*162*), do not suggest a strong pattern of seasonality in CoV shedding, and where seasonal patterns do occur, i.e. for henipaviruses in Thailand (*163*), or can be predicted from wildlife serology data, we will be sure to conduct sampling at different timepoints throughout the year to account for this.

Aim 2: Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays.

2.1 Rationale/Innovation: Rapid response requires early detection. Assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities that interface frequently with wildlife that harbor high risk emerging viral strains. To enhance the low statistical probability of identifying these rare events, In Aim 2 we will target rural human communities inhabiting regions identified in Aim 1 as having high viral diversity in wildlife, that also engage in practices that increase the risk of spillover (e.g. hunting and butchering wildlife). We will initially identify 4 subsites in each of Thailand, Peninsular Malaysia, Sarawak, and Sabah for sampling. We will design and deploy human risk factor questionnaires, and specific and sensitive serological assays to identify the baseline frequency, and causes of, spillover of known and related novel viral pathogens in these populations. **We will use the results to test a key hypothesis: that hunting wildlife increases the risk of exposure to novel viruses, compared to the rest of the community.** The alternative hypothesis is that seroprevalence in the general community is not statistically different to those who hunt and butcher wildlife, because exposure to wildlife pathogens is largely through inhabiting a biodiverse landscape where wildlife make indirect contact with people (e.g. via fomites). For participants who report symptoms related to 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever; or 6) unusual presentation of high fever with severe diarrhea; all within the last 10 days, an additional sample type will be collected, two nasal or oropharyngeal swabs. Samples will be marked for additional PCR-based assays to identify presence of known and novel CoVs, henipaviruses and FVs, and for isolation and biological characterization of potential pathogens, using the approach laid out in Aim 1.

2.2 Preliminary data on high-zoonotic risk human community surveillance: 2.2.a Biological sample collection in SE Asia: Our long-term collaboration in the region has included qualitative and quantitative survey data, and collecting large archives of banked human sera and other biological samples from high zoonotic-risk populations. For example, under the USAID-PREDICT project, EHA has collected 1,400 and 678 specimens in Malaysia and Thailand respectively from people living in zoonotic disease interfaces, all of which will be available for retrospective serological testing by EID-SEARCH which we have ensured is compliant with our existing IRB. Our core group has collected and tested many thousand additional specimens from other important community cohorts **(Table 2)**, and some of these will continue under EID-SEARCH **(Section 2.4)**.

Country, site	Lead	# enrolled, focus	Findings
Peninsular Malaysia	Hughes	9,800+ samples, Orang Asli indigenous pop., for PCR/serol.	25+ novel CoVs, influenza, Nipah ab+ve, FV ab+ve
Malaysia Sabah	Kamruddin	1,283 for serology	40% JE, 5% ZIKV ab+ve
Malaysia Sabah	William	10,800 for zoonotic malaria study	High burden of macaque-origin malaria
Malaysia Sabah	Hughes	150 bat cave workers	Ongoing
Malaysia Sarawak	Siang	500 Bidayuh and Iban people	8% PCR prevalence HPV in women
Malaysia PREDICT	Hughes	1,400 high zoonotic-risk communities for viral PCR, serology	Ongoing. Multiple known and novel CoVs, PMVs, others.
Thailand	Wacharaplu-esadee	100s of bat guano harvesters/villagers	Novel HKU1-CoV assoc. clinical findings
Thailand PREDICT	Wacharaplu-esadee	678 high zoonotic-risk communities for viral PCR, serology	Ongoing. Multiple known and novel CoVs, PMVs, others.
Singapore	Wang	856, for Melaka virus	7-11% MELV ab+ve

Table 2: Biological sample collection from healthy populations conducted by members of **EID-SEARCH** in our hub countries.

Of particular interest is the access to indigenous communities in Peninsular Malaysia (Orang Asli) and Sarawak (Bidayuh, Iban people), and the bat guano harvesters in Thailand. These communities hunt, butcher and eat wildlife daily, and work in supremely high-risk zoonotic interfaces (e.g. bat caves). One of our indigenous community sites is experiencing an ongoing outbreak of unknown etiology that we are investigating currently **(Section 3.2.a)**.

2.2.b Human Contact Risk Factors: EHA is the lead organization across all 27 USAID-PREDICT project countries for collecting data on, and analyzing the human aspects of, zoonotic spillover risk (*164, 165*). We conducted exploratory studies using standardized one-on-one semi-structured interviews and site observation data with people living in hotspot areas or engaged in high-zoonotic spillover risk activities, using these data to assess local social and cultural norms underlying wildlife contact (*165*). We then developed a risk factor survey to accompany human biological sampling to evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. We conducted cross-sectional studies in high risk populations in 27 countries, and study participants provided biological samples (oral/oropharyngeal swab, nasal/nasopharyngeal swab, urine sample/urogenital swab, fecal sample/rectal swab, whole blood, serum, plasma). For example, survey and biological samples were collected from: 1,585 participants from 7 sites in Yunnan, Guangxi, and Guangdong provinces, China; 1,400 participants from 4 sites in Malaysia; and 678 participants from 4 sites in Thailand. Samples were tested with novel serological assays and results analyzed against risk factors from survey data (*16*). In China, CoV-seropositive individuals were mostly farmers/peasants (8/9), living in Yunnan province (7/9), 41-60 yrs old (7/9), with domestic contact with rodents (6/9), that are male (6/9). Although these results are preliminary and don't provide detailed information on routes of exposure, they identify key subpopulations to target that would likely increase the opportunity for identifying evidence of spillover for other CoVs, PMVs and FVs. Data from PREDICT surveys have been used to help identify target populations in Aim 2, identify strategies to better target at-risk people, and conduct focused **human risk factor surveys and serosurveys to produce statistically significant findings for henipaviruses, CoVs and FVs.** In Aim 2, we will use combined biological sampling and risk factor surveillance in targeted populations within the community to 1) test the hypothesis that occupational (i.e. wildlife hunting) risk factors correlate with seropositivity to henipaviruses, FVs and CoVs; 2) assess possible health effects of infection in people; and 3) where recent

illness is reported, obtain biological samples for PCR testing and potential viral isolation and characterization. Obtaining this information could be a significant step in understanding the likelihood of recent 'hidden' spillover events and their public health impacts, as well as the risk of future emergence of viral disease.

2.2.c. Risk factors for illness: Our preliminary data in Thailand, Malaysia and other SE Asian countries revealed that frequent contact with wild (e.g. bats, rodents, NHPs) and domestic animals (e.g. poultry, swine) among local communities was associated with a high percentage of self-reported severe/acute respiratory illness (SARI/ARI) and Influenza-like illness (ILI) symptoms **(Section 3.2.b)**. In addition, people involved in crop production, hunting and butchering of wildlife reported statistically significantly higher SARI and ILI symptoms (Li *et al.*, in prep.). **These preliminary data suggest a target population and sites for current proposal with the correct targets for serological surveillance and risk factor surveys in Aim 2 to produce statistically significant findings**. Coupled with better serological tools from our team **(Section 2.2.d)**, we will be able to identify the likely routes of exposure to known or novel coronaviruses, henipaviruses, and FVs in the study region, as well as build data on the illnesses they cause in people.

2.2.d. Serological platform development: Most emerging viruses produced a short-lived viremia in people so that large samples sizes are required to provide enough PCR-positive individuals to analyze infection patterns. For Aim 2, we will focus on serological sampling in the community, because antibodies are typically long-lived and can be used to assess prior virus exposure or infection with much smaller samples sizes (*123*). Most serological assays target a single protein, and for emerging viruses, it's often unclear which viral antigen will be immunodominant during human infection, and therefore which to target in developing serological assays. Co-Is Wang, Anderson (Duke-NUS) and Broder, Laing (USUS) have developed a series of approaches to deal with this problem, and which will be used in testing human biological samples in Aims 2 and 3. Henipaviruses, FV and CoVs express envelope receptor-binding proteins that are the target of protective antibody responses. Co-I Broder has led efforts to express and purify henipavirus native-like virus surface proteins for immunoassay application (*166, 167*), developing monoclonal antibodies (*168, 169*) and as subunit vaccines (*170, 171*), has developed a novel, multiplex microsphere immunoassay (MMIA) allowing serum samples to be simultaneously tested for antibodies reactive with henipaviruses, FVs and CoVs. This MMIA uses a Luminex-based multiplex platform and custom designed virus receptor-binding proteins expressed in a mammalian cell-culture system and purified as native-like oligomeric proteins. These oligomeric antigens retain post-translational modifications and quaternary structures, allowing capture of conformational-dependent antibodies raised during a natural infection, which represent the majority of the protective humoral response. The known diversity of henipaviruses includes five presently described viruses, NiV, HeV, CedV, GhV and MojV and this assay has been expanded to include all described species **(Fig. 11)**. In the past, our collaborators have used NiV and HeV G protein antigens in a MMIA assay to identify a serological footprint of NiV-related African henipaviruses and exposure to virus(es) antigenically-related to NiV in livestock sampled in Bangladesh (*78, 79*). To strengthen serological data interpretation, the G protein from each virus is integrated into the MMIA so that virus-specific vs. henipavirus cross-reactive antibodies can be detected and address specificity of past virus exposure. Extensive cross-reactivity exists among ebolaviruses and between ebolaviruses and marburgviruses (*172-174*). We are validating the MMIA pan-FV assay for specificity, and as part of this, in collaboration with Duke-NUS, we have found serological evidence of past exposure to a filovirus(es) most antigenically-related to ebolaviruses (e.g. Ebola virus, Bundibugyo virus), but unreactive with Reston virus GP in three under-sampled fruit bat species, suggesting that novel FVs circulate within bat hosts in SE Asia (*43*). **This platform has now been set up for use in Malaysia and Thailand and will be available for use in this proposed work.** Co-Is Hughes, Broder, Laing have conducted initial screening of human, wildlife and livestock samples at high-risk interfaces in Malaysia, finding serological evidence of past exposure to viruses antigenically-related to NiV and EBOV in humans, bats and non-human primates (NHPs).

Fig. 11: Validation of multiplex microsphere immunoassay (MMIA) specificity and identification of immunologically cross-reactive viruses for A) NiV B) EBOV.

Co-Is Wang and Anderson (Duke-NUS) have developed a phage-display method to screen antibodies for all known and related bat-borne viruses, including henipaviruses, FVs and CoVs and have set up a similar Luminex-based assay. Additionally the Duke-NUS team has demonstrated capacity for rapid and cost-effective development of LIPS serological assays against novel viral agents **(Sections 2.6.a, 3.2.a)**. These will be used for verification and expanded surveillance. For molecular investigation (Aim 2 & 3), we will combine targeted PCR with a discovery-driven enrichment NGS strategy to not only survey currently known viruses, but also to discover potential novel viruses. Both the phage-display serological and enrichment platforms can be rapidly updated to include new viruses identified.

2.3 General Approach/Innovation: Our human sampling approach uses two approaches in Aims 1 & 2 (Fig. 12). In Aim 2 we will conduct community-based surveillance using a brief, focused questionnaire to identify the prevalence and frequency of risk-factors for viral spillover in tandem with collection of high quality biological samples to determine the seroprevalence of spillover viruses in at-risk human populations. We will use generalized linear models to analyze correlation between biological results and risk-factors for viral spillover. **In Aim 3**, we will conduct clinic-based syndromic surveillance and biological sampling at clinics/hospitals that are the catchment healthcare facilities for the communities sampled in Aim 2. Both community-based and clinic-based syndromic surveillance programs are cross sectional case-control studies designed with the sample sizes necessary to statistically quantify (with a power of 80%) risk factors and health impacts for viral spillover, linked to serological status and PCR prevalence in symptomatic patients. All human sampling conducted under Aims 2 and 3 will adhere to strict criteria for inclusion and recruitment and protection of human subjects from research risk **(see Human Subjects and Clinical Trials Information)**.

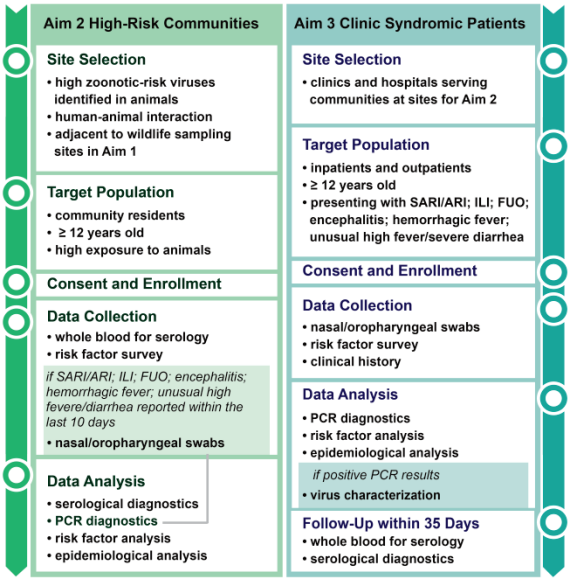


Fig. 12: Human sampling approach. Aim 2 **(left)** focuses on serology in high-risk communities to identify evidence of population exposure to novel zoonotic viruses. Aim 3 **(right)** sets up clinical cohorts at hospitals serving these high-risk communities to conduct syndromic surveillance and identify the etiology of new viral syndromes

2.4 Target population & sample sizes: We will target populations in the same geographic regions as those identified in Aim 1 that harbor wildlife with high potential zoonotic viral diversity, that are EID hotspots, and that are well-connected through regional travel and trade hubs to the global travel network **(Fig 13)**. Within each geographic region we will identify populations at four sub-sites, building on our preliminary data **(Table 2)**. We will target specific communities based on our analysis of previously collected behavioral questionnaire data, and other publicly available data (e.g. wildlife hunting licenses sold in a region, number of guano caves accessed for fertilizer etc.) to identify key at-risk populations and occupations with high exposure to wildlife. We will conduct concurrent sampling trips at visits to sub-sites during which members of the community will be enrolled and wildlife surveillance activities conducted. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock, or slaughtering wildlife will be considered for enrollment. Participants will complete a consent form and questionnaire in addition to having biological samples collected. During these community trips after data collection the field research staff will conduct community meetings to help inform the public health risk of zoonoses. **Target populations:** *Thailand (Co-I Wacharapluesadee):* 100 subjects of healthy high-risk community from 2 study sites, Chonburi (NiV, other PMVs and CoVs found by PCR in bats in our previous work), and Ratchaburi (bat guano harvester villages where we found a MERSr-CoV in bat guano (*50, 175*) and serological evidence of α-CoVs, HKU1-CoV in people (*175*). *Peninsular Malaysia (Co-I Hughes, CM Ltd.):* We will expand our sampling of the Orang Asli indigenous communities to include sub-sites in communities in Districts we have already sampled and

additional Districts in the States of Kelantan Perak, Pahang, and Kelantan all close to bat caves. Samples will be stored and tested at NPHL. *Sarawak (Co-I Tan, Fac. Med. Hlth. Sci. UNIMAS):* We will expand our sampling of the Bidayuh and Iban indigenous people to include the Dayak and Orang Ulu ("people of the interior") because this group has a more intimate connection to remote wildlife-rich areas than the other two, albeit all are hunters. Human sampling will be led by UniMAS in collaboration with CM Ltd / EHA. *Sabah:* We will conduct sampling at the Madai bat and bird cave that has 200 permanent residents who harvest bird's nests for soup, and >1,000 during the harvesting season and at Gomantong cave – one of the largest bat and cave swiftlet nesting sites in the world. *Sabah: (Co-I Hughes):* We will conduct sampling at the Madai bat and bird cave that has 200 permanent residents who harvest bird's nests for soup, and >1,000 during the harvesting season and at Gomantong cave – one of the largest bat and cave swiftlet nesting sites in the world. Protocols follow those for Peninsular Malaysia *Singapore: (Co-I Wang, Duke-NUS):* Active human surveillance will be not be conducted in Singapore, but archived human specimens e.g. from our Melaka virus serosurvey (*8*), will be made available to conduct retrospective serosurveys for specific pathogens that are identified as high interest to the EIDRC, NIH, and EID-CC.

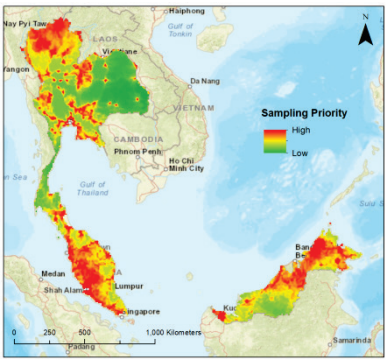


Fig. 13: Targeting high-risk sites for human zoonotic disease surveillance in Thailand and Malaysia. This map plots areas with high numbers of expected viruses in wildlife (*3*), greater spillover probability based on EID risk factors (*2*), and higher levels of connectivity (measured by road density data) that may facilitate person-person spread.

Sample sizes: From our previous work we conservatively anticipate that 10-30% of the community population will have had exposure to wildlife allowing us to capture highly exposed and non-exposure individuals at each site. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock farming, transportation, selling, or slaughtering wildlife in the surveyed areas will be targeted so that they make up ≥30% of the sampled population in each community. We will stratify sampling to ensure appropriate representation of sex, demographic, and socio-economic factors at each community site. In each of our regions, we will conduct sampling at four sub-sites in populations with high prevalence of high occupational and environmental risk of exposure to wildlife populations, sampling 175 persons at each sub-site. We will target populations with, on average, 30% or higher prevalence of occupational or environmental exposures to wildlife based on data from previous studies. This design will give us >80% power to detect 5 percentage point differences in seroprevalence against any of our viral groups between baseline and high-risk subgroups. We conservatively assume 5% base seroprevalence, as well as assuming high spatio-temporal variance amongst sub-sites, with baseline varying 0-25% amongst sub-sties and the fraction of the population at risk varying from 20-40%. (Power calculation conducted by 500 simulations of a generalized linear mixed model (GLMM)). For community-based surveillance, we will enroll 700 individuals per study region, allowing us to make county/province-level comparisons of differing effects; the total sample will be 3,500 participants over 5 years.

2.5 Data & sample collection: Following enrollment, biological specimens (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max 500 µL of whole blood and two 500 µL serum samples) will be collected by locally certified healthcare professional proficient in phlebotomy techniques and a short questionnaire will be administered by trained project staff that speak appropriate local language to all participants. Through the serological findings and the survey we will investigate the details of five risk factors related to high risk wildlife exposure, to maximize the power of the analyses, based on continuing analysis of our previous work, and will focus on: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. For participants who report the symptoms relating to 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever within the last 10 days, an additional sample type will be collected, nasal or oropharyngeal swab (2x). Blood of participants whose serum tests positive for emerging viral pathogens will be taken and Peripheral Blood Mononuclear Cells (PBMCs) frozen for future use.

These will be used for future harvesting of polyclonal and monoclonal antibodies as potential therapeutics/ diagnostics **(see Letter of Support NEIDL)**.

2.6: Laboratory analysis: 2.6.a Serological testing: We will screen all human specimens for henipaviruses, FVs and CoVs using our novel, multiplex microsphere immunoassay (MMIA) **(Section 2.2.d)**. We will use ELISA and serum neutralization tests (SNT) as confirmatory, where feasible and appropriate for the biocontainment level given sensitivity and specificity variation, and the need for live virus for SNTs **(See Select Agent Research)**. Serologic evidence of local spillover will be used to prioritize zoonotic strains for recombinant virus recovery from full length sequences. Preliminary data suggest ELISA will be effective for some viral groups. We previously developed a SARSr-CoV specific ELISA for serosurveillance using the purified NP of a bat SARSr-CoV (Rp3). The specificity of this assay was evaluated using polyclonal antibodies against HKU1, OC43, 229E, NL63, MERS-CoV and EBOV and no significant cross-activity detected (*16*). **This suggests that if we can expand our NP serology tests to cover other bat CoVs, and that doing so may identify many more seropositive individuals.** For CoVs, we will therefore use two serological testing approaches: 1) testing human sera collected from both community- and clinic- based sampling for a panel of bat CoVs that will include SARS-CoV, MERS-CoV, SADS-CoV and a range of bat SARSr-CoVs (*16*); 2) testing for antibodies to common human CoVs (HCoV NL63, OC43 – **Section 2.8**). We will test panels of NP and G recombinant proteins to assess if this approach will act as a comparison to MMIA for FVs and PMVs.

2.6.b RT-PCR testing. Specimens from individuals in the community who reported being symptomatic within the last 10 days **(Section 2.5)** will be screened using RT-PCR initially for CoVs, PMVs, and FVs following published protocols **(Section 1.4.c)**. Positive samples will be subjected to full genome sequencing and RT-PCR amplification of the glycoprotein genes. Samples from the clinic-based syndromic surveillance will also be tested using RT-PCR for Influenza A & B, HCoV NL63, OC43, HKU1, SARS-CoV & 229E, NiV, HeV, Ebola (WHO PCR protocol) based on the symptom as rule-outs.

2.6.c Biological characterization of viruses identified: Novel viruses identified in humans will be recovered by cultivation or viruses re-derived from full length genome sequences using reverse genetic approaches. Viruses will be characterized for growth in primary human cell lines and in mouse models, including the Collaborative Cross Mice or humanized mice expressing human receptors from related strains. The approaches are discussed in more detail under Aim 1.

2.7 Epidemiological analysis: We will conduct a cross sectional case-control study to identify risk factors for Henipavirus, FVs, and CoVs spillover. “Cases” are defined as participants whose samples tested positive for Henipavirus, FVs, or CoVs by serological tests. “Controls” will be selected from the pool of participants admitted to the studies and whose samples tested negative for Henipavirus, FVs, or CoVs. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models and least absolute shrinkage and selection operator (LASSO) regression analyses (*176*) to analyze correlation between biological results, serological/PCR status, and risk factors related to high risk wildlife exposure including: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) home or work proximity to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms lifetime and past 12mo. Additionally, we will use this procedure to determine how clinical presentation differs between Henipavirus, FVs, or CoVs in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms.

2.8 Potential problems/alternative approaches: Rarity of spillover events means it may be difficult to identify sufficient seropositives to statistically analyze human risk profiles. First, we will be targeting our community-based surveillance to subpopulations with high-levels of wildlife exposure, at sites selected for diverse and prevalent viruses to increase likelihood of finding positive individuals. Second, our serology testing will include a multi-plex panel of assays for a large diversity of viruses, increasing likelihood of positives. For example, in previous work our overall bat CoV PCR prevalence in the community was, 11.8%; β-CoV, 3.4%; α-CoV, 9.1%). Thus, using this broad serological panel to screen individuals prone to contact with species of higher zoonotic risk increases the potential for detecting spillover with enough power for statistical analyses,

and will shed light on behaviors that predispose to spillover. Third, we will include common human CoVs, PMVs, and influenza viruses in our panel, so that even if low prevalence of wildlife viruses is found, we will be able to conduct a valuable cross-sectional study of the seroprevalence of more common human viruses. Finally, we will be able to assess relative measures of human-wildlife contact from our survey work. We will analyze intensity of contact with high-risk taxa against other risk factors to provide useful proxy information for spillover risk. **Serological testing may be difficult to interpret in the presence of known and novel viruses.** We will use the three-tiered serological testing system outline in **2.6.a** to try to identify exposure to these 'novel' viruses. However, we know exposure to a range of related viruses generates antisera that are cross-reactive with known, related virus antigens - a serious challenge to serological surveillance and diagnostics. Our collaborations have already demonstrated that SE Asia bat sera samples screened with our pan-filovirus MMIA have been exposed to an Ebola-like virus, that is antigenically-distinct from known Asiatic filoviruses, Reston virus and Měnglà virus (*43*). Using these three serological platforms we will address inherent challenges of indirect virus surveillance through antibody capture by testing multiple binding assay platforms and antigens, using positive control samples to establish serological profiles against known viruses that will aid in our interpretations of cross-reactive antisera responses likely representative of 'novel' viruses.

Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts.

3.1 Rationale/Innovation: Our previous research **(Table 1)** and other data suggests that there are substantial numbers of outbreaks or clinical cases for which the etiologic agent is not diagnosed, e.g. (*177, 178*). To investigate this in SE. Asia, we have assembled a multi-disciplinary team that includes senior infectious disease doctors and clinicians and will work directly with local clinics and regional hospitals to identify the 'cryptic' outbreaks or cases that occur in the region. **In Aim 2, we will conduct serology in high zoonotic-risk communities to find evidence of prior exposure in people,** i.e. evidence of viral spillover into the community. In Aim 3 we identify members of these communities presenting at clinic with undiagnosed illness that may have high-impact zoonotic viral etiology. **Therefore, in Aim 3 we focus on PCR to identify the putative viruses that are replicating in these actively-infected patients (Fig. 12).** We will enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and who present at regional clinics or hospitals with symptoms typical of high impact zoonotic viruses. We will collect detailed survey data to assess their contact type and frequency with wildlife and livestock and conduct molecular and serological diagnostic assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. We will conduct molecular viral discovery assays on patients who present with symptoms indicative of a high-risk zoonotic virus. If novel pathogens are identified by PCR, we will attempt to isolate, obtain full genome sequence and/or biologically characterize the wildtype or molecularly re-derived pathogen, using the *in vitro* and *in vivo* strategy laid out in Aim 1.

3.2 Preliminary data clinical surveillance: 3.2.a. Clinical surveys and outbreak investigations: Our longterm collaboration in the region has included the following activities investigating clinical syndromes: *Peninsular Malaysia*: **At the time of writing, an outbreak of suspected undiagnosed viral illness in the indigenous Batek Orang Asli ("people of the forest") community living at Gua Musang ("civet cat cave") district, in Kelantan, Malaysia, close to Taman Negara – a National Park containing the oldest known rainforest**. This community is one where we have conducted prior routine surveillance and biological sampling, demonstrating proof-of-concept that our two-pronged high-risk community/clinic approach will work. Dozens of people are affected and the outbreak is currently being investigated by our group in collaboration with the Malaysian NPHL. The Orang Asli continue to practice traditional subsistence hunting of wild animals (bats, rodents, primates). These communities are remotely located, in heavily forested areas, with limited access to medical services. This outbreak, and others that will continue to emerge just like it, underscore the critical need for our EIDRC - to improve the technological tools, training, and human capacity for rapid EID surveillance, diagnosis, and response at the frontlines of disease emergence in SE Asia. **Investigating this outbreak is a key priority if EID-SEARCH is funded.** *Sabah*: Co-I Kamruddin (BMHRC) is leading an asymptomatic leptospirosis study on sanitation workers, an ILI study in a Kota Kinabalu clinic and diarrheal study in children under 5 yrs. Co-Is William, Tan and others have conducted clinical observational studies to determine the etiology of central nervous system infections and acute undifferentiated febrile illness in Sabah.

Co-Is William and consultant Yeo have collaborated with Co-I Wang to enroll 90 adult and over 100 pediatric participants with central nervous system infections, negative tests for malaria and dengue from 3 district hospitals in Sabah to determine the various etiologies. While a proportion tested positive for rickettsial agents, the majority had no clear diagnosis. Co-Is William, Rajahram, and Yeo have conducted clinical studies of over 200 inpatients with acute febrile illness with negative tests for malaria and dengue from 3 district hospitals in Sabah to determine the various etiologies. While a high proportion tested positive for rickettsial agents, the majority had no clear diagnosis. *Sarawak:* Co-I Siang (UNIMAS) is conducting a survey of high-risk female Kelabit indigenous people and has found 8% with HPV52 as the dominant strain. He is working on clinical cohorts of hand, foot and mouth disease and will recruit patients from that study in the current proposed work. Finally, Co-Is Siang, Kamruddin are conducting a long-term study of rotavirus and other viral etiologies of acute gastroenteritis in Kuching, Sarawak that will include swine herders and farmers. The Baric lab is a global leader in norovirus VLP diagnostic reagents to detect serologic evidence of norovirus gastroenteritis, as well as RT-PCR detection of novel noroviruses in humans, and has identified novel bat noroviruses, suggesting further opportunities for expansion of this work (*142, 179-181*). This work is particularly relevant in Malaysia because pig farms are shunned by the government and local villagers to they are placed far away from town centers, deep in the forest, and it is this scenario that led to the emergence of Nipah virus in 1998-9 (*6, 22*). *Thailand:* Co-Is Hemachudha, Wacharapluesedee (TRC-EID, Chulalongkorn Univ. Hosp.) work in coordination with the Thailand Ministry of Health to investigate outbreak etiology, including the first case of imported MERS-CoV in Thailand, **for which we produced sequence confirmation within 24 hours from acquiring the specimen**. Three imported MERS cases have been detected in Thailand since 2015, and all cases were confirmed at the TRC-EID. We worked during the Zika outbreak to identify > 500 PCR-positive Zika patients with sequence confirmation. We have also worked on returning and suspected Ebola travelers, syndromic surveillance for SARI, encephalitis, Hand Foot and Mouth disease in children and viral diarrhea. Consultant Hickey (US CDC, Thailand), screened specimens collected between 1998 and 2002 from a large cohort of children from Kamphaeng Phet Province to examine Dengue virus and JE epidemiology (*182, 183*). Among the 784/2,574 convalescent sera henipavirus-specific antibodies were detected in 13 serum samples (1.7% seroprevalence), suggesting prior exposure to endemic henipaviruses or a related PMV in rural Thailand. *Singapore:* Duke-NUS has worked with the Ministry of Health to investigate Zika cases (*184*), and other EIDs. With EHA and others, Duke-NUS has developed novel CoV assays for clinical surveillance, and for expedited analysis during outbreaks. Our pipeline of novel virus detection, reservoir host identification, serological assay development, and serological testing is as rapid as several months, with the development of novel serological assays from sequence data in 7 days (*17*). **For example, with the discovery of SADS-CoV, we identified and fully sequenced a new virus, screened archived wildlife specimens to identify the natural reservoir host species (*Rhinolophus* spp. bats), developed LIPS serological assays, and tested human and swine samples to assess seroprevelance in less than 3 months (*17*).**

3.2.b Analysis of self-reported illness: We have developed a standardized approach for analyzing data from study participants on self-reported symptoms related to target illnesses: fever with cough and shortness of breath or difficulty breathing (severe acute respiratory illness - SARI); fever with muscle aches (fevers of unknown origin (FUO); and, cough or sore throat (influenza-like illness - ILI). For all of our prior human clinical surveillance in 20+ countries, including in Malaysia and Thailand, we used a least absolute shrinkage and selection operator (LASSO) regression to identify associations between participants who reported ILI and/or SARI symptoms and demographics, occupation, and contact with animals in the last year. Results have clear biological relevance. For example, in Yunnan, China, factors strongly associated with prior disease involve animal hunting and consumption **(Fig. 14).** We will expand this approach for all clinical syndromes in Aim 3, and use targeted questions to assess patient's exposure to wildlife in terms that are relevant to each specific country.

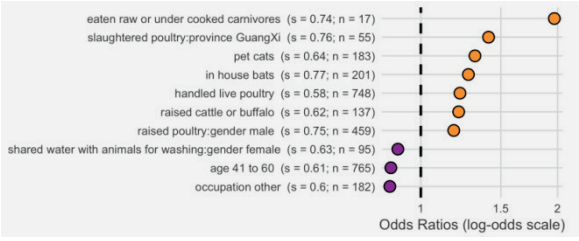


Fig. 14: Factors associated with self-reported ILI & SARI in prior 12 months (s = bootstrap support; n = #+ve out of 1,585 respondents). **Orange circles** = odds ratios > 1 (+ve association); **purple** = odds ratios <1 (-ve association).

3.3 General Approach: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent has not been identified. To capture these, we will expand on our current syndromic surveillance to enroll, and collect high quality biological samples from patients who present at regional clinics and live in rural communities linked to Aim 2. Case definitions and enrollment criteria include: 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever; or 6) high fever/diarrhea with unusual presentation. We will collect survey data to assess contact with wildlife, and occupational and environmental exposures, conduct molecular and serological assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. If novel pathogens are identified by PCR, we will attempt to isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work. We will then use behavioral survey data, ecological and phylogeographic analyses to assess the likely reservoir hosts to inform intervention programs.

3.4 Clinical cohorts. 3.4.a Cohort selection: We will initiate active clinic-based syndromic surveillance at 2 town-level level clinics and 2 provincial-level hospitals in each Thailand, Peninsular Malaysia, Sabah and Sarawak. These serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2 and are inclusive of wildlife sampling sites in Aim 1. Patients ≥ 12 years old presenting at the health facility who meet the syndromic and clinical case definitions above will be recruited into the study after completing a consent form. We will aim to enroll 300 participants per hospital, with that we will have a 95% probability of detecting live virus in patients in each hospital assuming a population prevalence of 1%. This sampling effort will total of 4,800 individuals for clinical syndromic surveillance. We assume up to 40% loss from follow-up, therefore yielding 2880 participants that will be available for follow-up blood sampling **(Section 3.4.b)**. While enrollment is limited by the number of patients that come to the healthcare facility presenting with relevant clinical symptoms, in our work in the PREDICT project we enrolled an average of 100 participants per year, so we are confident this is an achievable enrollment target. Study data will be pooled across country regions, as clinical patients are limited by the number of individuals presenting at hospitals. "Cases" will be determined as participants whose samples tested positive for either CoVs, henipavirus, or FVs, PCR tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. Sites: *Thailand:* We will work with two primary hospital, Phanat Nikhom Hosp., Chonburi district (Key Pers. Dr. P. Hemachudha) & Photharam Hosp., Ratchaburi Province, and a large tertiary hospital, King Chulalongkorn Memorial Hospital, Bangkok Thailand (Co-I Dr. T. Hemachudha). *Peninsular Malaysia:* **Focusing immediately on the current outbreak in Orang Asli, we will work closely with the Malaysian Ministry of Health to support their investigation, and with 6 Orang Asli clinics which focus solely on this indigenous community.** Co-I Sellaran (Lintang Clinic, Kuala Kangsar) refers patients to Sungai Siput Hospital and Gen. Hospital, Ipoh. Key Pers. l Lotfi (Pos Betau clinic, Kuala Lipis) will continue collaboration with Co-I Hughes in the communities at that site. *Sarawak:* Key Pers. Diyana (Director, Bario Clinic) refers patients to Miri Hospital (Co-I Utap) and serves people from the Kelabit, and nomadic tribes in the region, as well as populations within a number of large towns. *Sabah:* We will continue our collaboration with Queen Elizabeth Hospital, QEH (Key Pers. Lee, Rajahram) and Hospital University Malaysia Sabah, HUMS (Co-I Lasimbang, Director). Both clinics include our targeted sites for **Aim 2** in their catchment, and both have ongoing collaboration with the Borneo Medical Health Ctr (Co-I Kamruddin, Director). *Singapore:* Currently, there are no plans to conduct clinic surveillance based on budget constraints. However, in the event that that is an evolving need for clinic surveillance, enrollment, and sampling, we have close working relationships with all hospitals in the country, and the central infectious disease reference lab (key clinicians at which are aware of our proposal). This would allow us to rapidly on-board a hospital based site.

3.4.b Clinic enrollment and follow-up: We will recruit inpatients and outpatients to participate in the study after initial screening to determine if they meet our clinical case definitions. Once consented and enrolled, biological samples will be collected by trained and locally certified hospital staff and a questionnaire administered by trained hospital or research staff that speak appropriate local language. Every effort will be made to take samples concurrently when collecting samples for normative diagnostics. For both inpatients and outpatients, samples will be collected no more than 10 days of reported onset of illness to increase the chance

of PCR detection of viruses (*185*). Where possible, we will follow up 35 days after enrollment to collect an additional two 500 µL serum samples conduct a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. This gives adequate time for development of IgG, which occurs <28 days after onset of symptoms for SARS patients (*186-188*). Serum samples will be used to screen panels of high risk human and local zoonotic Filovirus, Henipavirus and Coronavirus strains as described in Aim 2. These samples may prove valuable to collaborators in that the PBMC could be used in production of therapeutic antibodies.

3.4.c Sampling and clinical interview: Biological specimens whole blood (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples) and two nasal or oropharyngeal swabs will be collected from all participants, and a questionnaire will be administered. We will investigate five risk factors, to maximize the power of the analyses, all related to high risk wildlife exposure, based on continuing analysis of our previous work, and will include: 11) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With additional consent considerations for participants enrolled from clinical settings, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology. Blood will be taken for future harvesting of Peripheral Blood Mononuclear Cells (PBMCs), as per **Section 2.5**.

3.5 Sample testing: The standard syndromic diagnostic PCR assays for common pathogens will be conducted and the results will be shared with the hospital with 48 hours. Specimens will be further tested for 3 viral families; CoV, PMV and filovirus by consensus PCR **(Section 1.4.c)**. Necropsy samples from deceased patients will be further characterized by NGS if previous PCRs are negative and unable to identify the cause of infection. Co-Is Wang, Anderson will conduct a VirScan serological assay using paired serum samples at Duke-NUS that identifies antibodies with a four-fold rise in titer in the convalescent sample compared with the acute sample, thereby indicating a possible etiological agent. A PCR test with the specific primer from each virus antibody positive case will be further tested from acute specimens to confirm infection.

3.6 Viral risk characterization and potential for pandemic spread: We will use statistical models built from collated biological, ecological, and genetic data to assess the likelihood of pathogenicity for the range of diverse viruses we will characterize. The aim is to quickly identify and assess which viruses pose the most significant risk to human health and thus prioritize additional resources to more fully characterize, better understand pathogenicity, and invest in our animal model pipeline and ultimately therapeutic development for those pathogens. We will combine two independent but related statistical models to predict pathogen spillover risk and pathogenicity. First, we will expand a simple generalized additive modeling approach (*3*) to assess spillover risk probability to a new virus that is currently not known to infect people, when we have only limited genetic data from initial PCR screening and sequencing. Additional data from receptor binding protein homology and cell line infection experiments will be incorporated into the model framework as available. Second, given high likelihood to spillover and infect humans, or direct evidence of human infection from our clinical surveillance, we will then assess the likelihood of pathogenicity based on traits for phylogenetically related nearest neighbor viruses as a first approximation and incorporating more detailed data from genomic characterization and animal model experiments (Aim 1). Epidemiological trait data for ~300 viral species known to infect people (e.g. case fatality rate, global estimates of number of human cases per year or decade, R_0, infectious period, and primary symptoms) have been collated by recent studies and will be used as predictor variables (*189*). Thirdly, we will apply tools already developed, and being refined by EHA under DTRA and DHS supported research, to predict pandemic spread for viruses using global flight models, as well as additional datasets on human movement and connectivity across Southeast Asia (*90, 91*) **(Fig. 15)**.

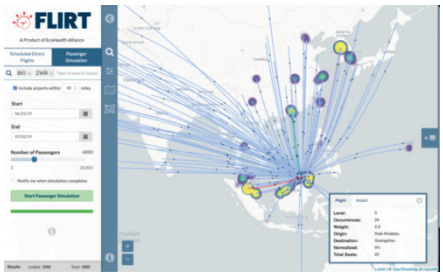


Fig. 15: Probability feed from EHA's Flight Risk Tracker tool (FLIRT). This image is of the likely pathways of spread and establishment for a Nipah-like virus emerging from Sabah, based on human movement and airline flight data (*90, 91*).

3.7 Potential problems/alternative approaches: Patients visiting clinics may have cleared virus, but not yet developed IgG antibodies, reducing seropositive cases. Our 35 day follow-up sampling should account for this because the maximum lag time between SARS infection and IgG development is ~28 days and Ebola virus infection and IgG approximately 20 days (*185-188, 190*). We also expect that patients in rural communities will only visit clinics when symptoms have progressed, likely coinciding with late illness and onset of IgG. We will also have data from our community study that can be analyzed in conjunction with, or in the absence of, clinical data from the hospital data to identify PCR- or seropositives. Finally, the risk of limited detection is outweighed by the potential public health importance of discovering active spillover of new henipaviruses, FVs or CoVs infection.

4. Administrative Plan

4.1. Project management: 4.1.a. Administrative core: The EID-SEARCH organizational partner roles are laid out by Specific Aim above **(see Fig. 3)**. EcoHealth Alliance (EHA) will lead the administrative core of the EID-SEARCH, including: coordination and management of all human and animal research; communication with consortium partners, NIH, other EIDRCs, and the EIDRC-CC; data management and quality assurance; modeling and analysis; and compliance with all US, international, and institutional rules and regulations. The administration will be led by PI Daszak, who has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5-20 yrs on NIAID- and USAID-funded research **(Fig. 16)**. He also has direct experience as one of two external reviewers of one of the CEIRS partners for NIAID (Program officer Diane Post). PI Daszak will be assisted by Deputy Lead, co-I Olival, who has overseen zoonotic disease research in Southeast Asia for >10 years and working directly with our partners in Thailand and Malaysia for 15 years. Senior program manager (Chmura) and full-time program assistant (Hamel) will provide administrative support. The EID-SEARCH data management, epidemiological analysis, and modeling activities will be led by co-Is Olival and Zambrana who have led all modeling and analytics on USAID USAID PREDICT for the last 10 years. They will overse a programmer/data scientist (Mendelsohn), evolutionary biologist/bioinformatician (Latinne), and human epidemiologists (Dawson, Li, Martinez). Our human epidemiology team will work directly with country teams in Malaysia and Thailand to manage human surveillance, IRB applications and approvals, and ensure compliance with all human subjects work. EHA's senior veterinarian (Field) and wildlife field biologist (Phelps) have extensive experience working that bats, rodents, and primates in SE Asia and will coordinate all training and field research in Malaysia and Thailand and ensure compliance with IACUCs.

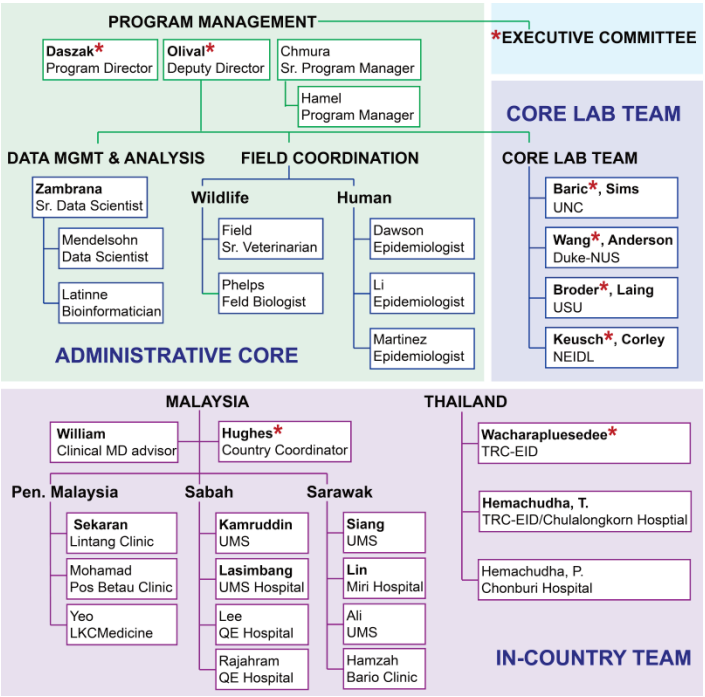


Fig. 16: Administration and program management for the **EID-SEARCH**

The EID-SEARCH core laboratory team includes global experts in virology, molecular pathogenesis, and the development of assays, reagents, and therapeutics for high consequence viral zoonoses (Co-Is Wang, Anderson at Duke-NUS; Baric, Sims at UNC; Broder, Laing at USU; Keusch, Corley at NEIDL). The core laboratory team will work with in-country diagnostic labs in Thailand (Wacharapluesedee, Hemachudha at the TRC-EID, Chulalongkorn University) and Malaysia (Hughes, Lee, CM Ltd.). Wacharapluesedee and Hughes will additionally serve as country coordinators for the EID-SEARCH, liaising directly with EHA on weekly conference calls, and working with in-country partners to ensure the smooth operation and coordination of clinical and community surveillance and wildlife research in Thailand and Malaysia, respectively. **A Core Executive Committee** comprising PI Daszak, Deputy Lead and Co-I Olival, and leads from each core partner and country: Co-Is Hughes, Wacharapleusadee, Baric, Wang, Broder, Keusch (or alternates), will conduct

regular conference calls and in-person meetings to facilitate rapid decision making within the EID-SEARCH. **This committee will also convene to manage EID-SEARCH response to outbreaks.**

4.1.b Project Management in Thailand and Malaysia: Wacharapluesedee and Hughes have collaborated directly with EHA for >15 years, including acting as country coordinators on the USAID PREDICT project for the last 10 years (project end date Sept. 2019). They maintain strong ties with Ministries of Health (MOH), Agriculture and Environment, multiple universities and research institutions, clinics, and hospitals, in their respective countries and across the region. The EID-SEARCH will use these connections to disseminate results, obtain permissions to conduct sampling, and also rapidly respond to and assist with outbreaks as they happen. Peninsular Malaysia, Sarawak, and Sabah are the three main Malaysian administrative regions, and effectively operate as three separate countries, with different regulations and government structures. We therefore provide specific details on the management of EID-SEARCH activities in each:

Coordination among Peninsular Malaysia, Sabah and Sarawak will be led by co-I Hughes (Conservation Medicine Ltd), and follow a successful model we implemented under USAID-PREDICT. **On Peninsular Malaysia** this project will be administered through the Zoonosis Technical Working Committee (ZTWC) established under the PREDICT project with a binding MOU among EHA, CM Ltd. and ZTWC, and including officers from MOH, Dept. of Veterinary Services, and PERHILITAN (the Govt. wildlife agency). EHA will communicate weekly with Co-I Hughes to coordinate and monitor implementation of research and reporting to ZTWC. Co-I Hughes will coordinate activities at all other Peninsular Malaysia institutions: NPHL, the National reference laboratory for diagnostic confirmation of pathogens, will manage molecular and serological screening (BioPlex) of Orang Asli samples, and serological screening of syndromic samples from Sabah and Sarawak; the PERHILITAN molecular zoonosis laboratory will store and conduct molecular and serological screening on wildlife samples; and Universiti Putra Malaysia (UPM) Faculty of Veterinary Medicine will conduct molecular and serological screening (BioPlex) of livestock samples, should these be required. **For Sabah & Sarawak**, work will be administered through the Sabah Zoonotic Diseases Committee (SZDC), a working technical committee comprising appointed and authorized officers from Sabah State Health Dept., Department of Veterinary Services, Sabah Wildlife Dept. (SWD), Universiti Malaysia Sabah (UMS) and EHA, all of which are also committed through a signed MOU. Co-I Hughes will oversee work at all other partners in Sabah, including: the Kota Kinabalu Public Health Lab (KKPHL) for molecular screening of syndromic samples from Sabah and Sarawak; the SWD Wildlife Health and Genetic and Forensics Lab for molecular screening of Sabah wildlife samples; The Borneo Medical Health Research Center (BMHRC) for screening some Sabah wildlife and livestock samples, if required, and human syndromic samples from Sabah and Sarawak. **In Thailand** all human community and wildlife research and testing will be coordinated by co-I Wacharapluesedee from the TRC-EID center. Clinical surveillance will be overseen by senior clinical physician and co-I T. Hemachudha.

4.1.c. Approval and release of results: In our experience, it is critical when working in resource-poor countries, on potentially important pathogens, to strictly adhere to protocols for release of results. EID-SEARCH will liaise with existing points of contact in the Ministries of Health, Environment, and Agriculture in each our administrative areas to approve and release project findings publicly. Results from human screening will be shared with participants when they become available, as per our IRB agreements ensuring no violations to anonymize data requirements (**see Protection of Human Subjects**).

4.2. Flexibility to extend the EID-SEARCH to new sites as needed: The EID-SEARCH consortium partners maintain extensive working relationships with leaders in EID outbreak control, clinical investigations and research at over 50 clinics, research institutes and public health laboratories across Southeast Asia. Due to space constraints, we haven't listed each of these, nor have we solicited >50 Letters of Support for this project. However, each core EID-SEARCH partner has contacted their networks and obtained permission for inclusion in the broader goals of the EIDRC. As examples of these contacts, our core partner, the Thai Red Cross Emerging Infectious Disease Health Science Centre (TRC-EID) at Chulalongkorn University, also serves as the WHO Collaborating Centre for Research and Training on Viral Zoonoses and has ongoing research collaborations across WHO SEARO countries including Bangladesh, Bhutan, Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, (Thailand), Timor-Leste; and has recently served as a training hub for scientists from Malaysia, Myanmar, Laos, the Philippines, and China to learn methods of wildlife sampling and diagnostic screening. Our Thai clinicians (Co-I T. Hemachudha and KP P.

Hemachudha) provide regular case consultations and clinical trainings for doctors across SEARO countries, including with Yangoon General Hospital and the National Health Lab in Myanmar, 2018. To maximize leverage of this broad network, EHA has budgeted for annual meetings in SE Asia, in addition to regular smaller network meetings, with our core team and key public health experts from network labs in each of the 10 SE Asian countries**.** Additionally, we will set up a listserv and an internal communication network to facilitate collaboration and information exchange, including on the first reports of new disease outbreaks. Our annual and smaller network meetings will critically allow face-to-face meetings of the EID-SEARCH that will foster greater sharing of information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks in the region, guided by the PI, Deputy and the Executive Committee.

4.3. Outbreak response: EHA collaboration with expert networks around the world allows us to mobilize and enhance effective One Health response to disease emergencies (*191*), ranging from real-time situation updates and risk analyses to on-the-ground investigations (*192-194*). We will adopt management tools from Emergency Operating Center (EOCs) (*195*) and Incident Management Systems (IMS) (*196*), to shift resources where necessary to help respond to novel zoonotic outbreak events and other public health emergencies. EHA has extensive experience working with governments in low and middle income countries (LMIC) applying these principles of epidemic preparedness during outbreak responses we've been involved with under the USAID-PREDICT project. For example, at the request of the government of Bangladesh, we provided technical field and laboratory support for Nipah virus and avian influenza outbreak investigations, assisting with wildlife sampling as part of the outbreak response alongside human and domestic animal sampling. In India, we provided technical assistance in response to the Nipah virus outbreak in Kerala in 2018. Last month in Indonesia were assisted the Ministry of Health's Center for Health Laboratory in Makassar to provide technical assistance in a mysterious outbreak in a small village in South Sulawesi that killed 4 villagers and infected 72. Our network partners include the key government and govt. approved laboratories that would be directly involved in public health emergency response in their respective countries. The serological and PCR platforms that EID-SEARCH develops will be made available to the main government outbreak investigation teams for clinical work and research during the outbreak. EID-SEARCH will also offer assistance training and conducting animal sampling during an outbreak, epidemiological analysis and modeling to help identify likely reservoirs or likely pathways to spread. Technical and material support for lab, field and analytical activities during an outbreak will be provided by EHA, UNC, USU, Duke-NUS, and NEIDL, as well as in-country partners. Any clinical samples, viral isolates and sequence data will be shared among partners to promote the rapid development of new diagnostic assays, reagents, and therapeutics that can be deployed to the region or other regions as part of the larger NIH EIDRC network.

Finally, while the initial pathogen focus of our group is on CoVs, PMVs and FVs, our broad collaborative group has multidisciplinary expertise on a number of virus-host systems. For example: PI Daszak was PI on a subaward from PI Laura Kramer's U01 on Poxviruses and Flaviviruses, managing a multidisciplinary research project on West Nile virus ecology. He was also co-I on a 5-year NSF-funded project to understand West Nile virus dynamics and risk in the USA (*197-201*); Co-I Baric is a global leader in Norovirus research leading to the development of vaccines and therapeutics (*202-205*); Co-I Wang has conducted significant work on bat immunology, therapeutic, and reagent development, as well as being involved in a range of outbreak investigations, viral discovery programs and other research on a wide diversity of viral groups (*206-215*). Additionally, the serological and PCR-based diagnostic platforms being developed by Co-Is Wang and Broder are adaptable to other viral targets. The modeling tools developed by Co-Is Olival and Zambrana-Torrelio can be used to predict the emergence and spread of diverse viral targets, including influenza, antimicrobial resistance, and vector-borne diseases (*216-221*). Our clinicians working in Thailand and Malaysia have a wide range of infectious disease investigations to adapt to any outbreak situation.

4.4. Communications: EHA will coordinate communication among all co-Is and key personnel, including:

- Multiple meetings per week with PI, Deputy Lead, Senior Program Manager (on project and task status)
- Weekly web/phone meetings between Program Manager and subawardee admin. staff
- Monthly web/phone conferences between EHA PIs and all subawardee PIs.

- Monthly web conferences between key personnel (research presentations/coordination)
- In-person Annual meetings with partner leads, key personnel at EHA and two in-person partner meetings annually between subawardees.
- Annual in-person meeting among all key personnel

4.5. Problem identification and resolution: Regular planning, monitoring, and evaluation meetings will be the primary mechanisms for problem identification. Minor problems (e.g. delays in sample availability or test results) will be dealt with internally through appropriate action and resolution monitored by PI Daszak and co-I Olival, and our Senior Program Manager. In the event of significant problems, such as prolonged poor productivity, inadequate scientific collaboration, or major disputes regarding research direction or resource allocation, EHA will assist with resolving the problem through negotiation with relevant co-Is and consultation with the Executive Committee. Should a resolution not be forthcoming, consultation with the EIDRC-CC, additional external technical advisors, and NIH staff may be warranted.

4.6. Adaptive management and risk mitigation: Maintaining a timeline and meeting milestones will require strict and continuous oversight of all project phases, frequent and regularly scheduled communication, and the ability to make decisions and implement strategies. To maintain our timeline on all projects, including the EID-SEARCH, we use an adaptive management approach to continually evaluate these trade-offs, to make decisions about when iteration is appropriate and when it is necessary to move forward with current information. Our ethos is that regular, scheduled communication among all staff, partners and collaborators will go a long way towards mitigating risks, especially if the process is collaborative and transparent.

5. Data Management Plan

EHA will house the Data Management and Analysis (DMA) team for EID-SEARCH, led by Co-PIs Olival and Zambrana-Torreilo and include Key Personnel Latinne and Mendelsohn. EHA has served as the data and analysis hub for numerous multi-institutional, multi-sectoral, international disease research groups, including acting as Modeling and Analytics lead for the PREDICT project (*122*), the Western Asia Bat Research Network (*222*) and EHA's Rift Valley Fever Consortium. We will leverage our experience and infrastructure from those projects to benefit the EID-SEARCH. **5.1. Project Database:** We will create a dedicated, centralized EID-SEARCH database to ingest, store, link, and provide for analysis all data associated with the proposed study and other expanded projects associated with the research network. The database will be SQL-based and use encrypted, secure cloud hosting services and enable export to archival and platform-independent formats. It will ensure data and metadata compatibility between project components, track data versioning and annotations. The system will be designed to work with both with both paper- and tablet-based field data entry and with the Lockbox laboratory information management systems **(Section 5.2)** in place in individual partner labs. The database will use existing metadata standards, including NCBI standards for genetic and molecular data and Ecological Metadata Language (EML) for field and wildlife data, as well as other standards and formats designated by the EIRDC CC. This will enable rapid publication and deposition of data. Granular security and privacy controls will be applied so that specific expansion projects undertaken in the network may be managed while maintaining data confidentiality as needed.

5.2. Biological Specimen Management: Project laboratories will use the Lockbox Laboratory Information Management System (LIMS), to manage the security, traceability, and quality of biological specimens. The LIMS will support sample barcoding at creation, tracking through transport, storage/inventory, and use via portable scanners. Lockbox supports CLIA and ISO 17025 as well as direct export to NCBI formats such as Sequence Read Archive. We will use the Lockbox LIMS application programming interface (API) to link to the central project database and associated samples with field and ecological data. We note that the project focuses on highly pathogenic viruses, including select agents; Lockbox LIMS supports sample tracking and movement compliant with US Select Agent Regulations and US Department of Commerce Pathogen Import and Export Control Regulations, and includes all necessary encryption, security, and backup protocols.

5.3. Training: Members of the DMA team will team will develop documentation and provide training for field and laboratory teams at all partner institutions in data management, metadata standards and data hygiene best practices. The DMA team will act as trainers and consultants for partner institutions in experimental

design, power analysis, data analysis, and computational and reproducibility issues, and visit each partner institution and/or field team base for training workshops and analysis consultations.

5.4. Data Identification and Privacy: For human clinical data and questionnaires, data will be identified by a unique identification code assigned to each individual and only this, de-identified code will be accessible in the project database, and destroyed at the end of the project - as per details provided in the Clinical Management Plan and Protection of Human Subjects forms.

5.5. Computing Resources: EHA operates a cluster of high-performance servers for data analysis activities, as well as infrastructure to launch cloud-based computing environments (**see EHA Facilities**). Our servers host all necessary software for statistical and bioninformatics work that is available to the DMA and partners anywhere in the world. We use a mixture of cloud services (AWS, Azure, Backblaze, GitHub) to provide redundancy, backup, version control, and rapid post-disaster recovery, and will be available to all project partners for analysis and training.

5.6. Data and Code Sharing: See details provided in the **Resource Sharing Plan.**

6. Clinical Management Plan

6.1. Clinical site selection: Our consortium partners have been conducting lab and human surveillance research, including during outbreaks, for >20 years and have developed strong relationships with local clinical facilities and processes in SE Asia and in LMIC globally. The plan for selection of clinical sites will begin in the same geographic regions as those identified in Aim 1 with high zoonotic viral diversity. Clinical sites will additionally serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2. We have already developed successful working relationships with the major healthcare facilities in Thailand and Malaysia and will use these established partners to rapidly gain appropriate permits and begin data collection quickly. Focusing on these EID hotspots in select biogeographic areas (see **Fig 13**) also reduces the number of additional sites needed to meet our goals within our geographic range. The capacity and capability requirements for clinical sites are fairly minimal, and include ability to enroll patients that meet the clinical case definitions of interest, collect and temporarily store biological samples, and follow standards for data management and subject protection with locked filing cabinets to store all paper records and an encrypted computer. We will be enrolling inpatient and outpatient participants within 10 days of the presentation of symptoms to increase the chance of PCR detection of viruses so we will not wait for advanced normative diagnostic tests, that are likely unavailable in the more remote sites, to be completed. We will work with clinical sites to determine the best process for project staff, in most cases supporting the time of currently hired staff at each site. We will recruit and train hospital staff in project-specific procedures including enrollment of patients, sample types to be collected, storage of samples, administration of the questionnaire, and data management plans. During the development and onboarding of new clinical sites we will assess the physical needs of the site and what supplies will be required for collection of data.

6.2. Standardized approach, oversight, and implementation: Management and oversite for all study sites will be undertaken by the local country coordinator with support from our Core Administrative team at EHA. Our research team has over 10 years of experience building capacity on human subjects' research and has developed training resources and materials for standardized implementation of community and clinical research and SOPs for screening, enrollment, and retention of participants. The country coordinator will conduct regular site visits to the clinical sites and annual visits to observe, monitor and evaluate the research process, and conduct follow-up training if required. Through our work with clinical sites under the USAID-PREDICT project we have developed culturally appropriate screening measures for clinal sites that do not disrupt the flow or quality of medical treatment received by the patient. This was done by working collectively with clinical staff to evaluate current procedures and patient flow at the site to determine the most efficient while minimal invasive inclusion of our study into the daily working of the clinal site. Most efficiently this was done by adding minimal basic screening questions to the current clinal intake forms, which allows the clinal research officer to quickly scan charts or logs for potential patients to enroll avoiding potential enrollees from being overlooked if staff are too busy or not on duty. Patients will be enrolled following established clinical criteria (**see Section 6.3**), samples collected and brief surveys conducted to assess the participants contact with wildlife through: 1) occupational exposures; 2) animal contact; and 3) the environment. With permission

from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology. Additionally, we will be following up with clinical participants to determine how clinical presentation differs between CoV, henipavirus, or FV in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. The country coordinator will be continually monitoring the project database to ensure we hit target sample sizes. While patient's enrollment is limited by the number of individuals presenting at hospitals, in previous research we enrolled an average of 105 patients per year, ranging from 77-244.

6.3. Clinical cohort setup, recruitment, enrollment: We will recruit inpatients and outpatients from clinical sites to participate in the study after initial screening to determine if they meet the clinical case definitions for 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever, of unknown etiology or severe diarrhea with unusual presentation for symptoms to increase the chance of PCR detection of viruses. Once enrolled, biological samples will be collected by trained hospital staff. When possible, samples will be taken concurrently with samples being collected for normative diagnostics. We will collect 5mL of blood for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples and two nasal or oropharyngeal swabs will be collected. Controls who test positive for CoVs, FVs, or Henipaviruses will be selected from the pool of participants admitted to the studies and whose samples tested negative for coronaviruses, henipavirus, or filoviruses. We will follow up 35 days after enrollment to collect an additional 5mL blood draw for collection of two 500 µL serum samples and a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. Following up 35 days post initial collection gives adequate time for 1) development of IgG and IgM, which occurs <28 days after onset of symptoms to evaluate the immunological progression of disease and 2) further the risk assessment of the participants to monitor contact with wildlife, people, and to assess the likely reservoir hosts to collect information to inform potential intervention programs.

6.4. Utilization of collected data: Sampling of wildlife and the enrollment of community surveillance participants and a clinical cohort of participants give us the ability to assess the viruses that are circulating in each of the three populations in a similar geographic region. We will use phylogenetic analysis to compare the relationship between wildlife viruses found and viruses fond in human participants. Additionally, the questionnaire data will allow us to assess relative measures of human-wildlife contact that we will analyze the intensity of contact with species known to be at a higher risk for spillover against other risk to provide useful proxy information for spillover risk. The clinical cohort will be split into cases and controls. "Cases" are defined as participants whose samples tested positive for either CoV, henipavirus, or FV via PCR tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. In clinical study, we will use this procedure to determine how clinical presentation differs between virus-exposed and -unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. We will model the outcomes to analyze correlation between biological results and risk factors related to high risk wildlife exposure. With this collective data set we aim to quantify and detect novel viruses likely to spillover regularly to people, are often unreported or misdiagnosed as their clinical manifestations, and are unknown to detect cryptic outbreaks causing previously 'hidden' clinical syndromes in people. Our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence.

6.5. Development of reagents of value to the community. Members of the EID-SEARCH consortium have substantial experience producing reagents, assays, and other products that are used widely by the clinical and research community, and some of which are on a pathway to commercialization. These include: PIs Daszak and Co-I Olival have produced software for analyzing the spread of novel viral agents through air travel networks; Co-I Baric has collaborated with a Norovirus surveillance collaboration with surveillance cohort at CDC and has developed therapeutics that have reached phase 2 and 3 clinical trials, He is currently working with Takeda Sanofi Pasteur on a Dengue therapeutic and with NIH on a tetravalent vaccine; Co-I Broder

developed a Hendra virus subunit vaccine that was commercially produced by Zoetis for horses and is labeled for human use under compassionate circumstances during outbreak situations.

6.6. Potential expansion: Expansion of the research project to other clinical sites or research areas could happen as new information is gathered, either through our research, the EID-SEARCH information network, or an outbreak being identified in the region by other organizations. If expansion is required we would rapidly shift research activities towards the clinical or community sites where the outbreak is active, using the same process we used to set up initial research locations. First, by working with local stakeholders to create a working relationship and on-boarding new clinical research staff through advanced trainings of project aims, SOPs on data collection and storage, and ethical guidelines. The network of infectious disease researchers in the community and hospital settings would allow for an accelerated response to any potential infectious disease outbreaks as staff are well trained on ethical guidelines for working with human subjects, data and specimen collection, storage, and transpiration, project staff would be on the front lines ready for the call to action. Ideally, our testing for viruses in priority viral families may lead to the discovery cryptic outbreaks before it spreads in the general public. Biological results coupled with survey data on key risk factors will provided necessary information for granular understand of disease spread.

7. Statistical Analysis Plan:

7.1. Framework: Statistical analyses across the project will be conducted under a common Bayesian framework. These models provide a unified, probabilistic approach best-suited for estimating effect sizes in heterogenous populations of human clinical and wildlife subjects in observational studies. Within this Bayesian framework, we will use generalized linear mixed models to estimate population prevalences and seroprevalences, and estimate the effects of demographic, occupational and environmental factors affecting these. We will use occupancy models (*223*) to estimate total viral species and strain diversity and completeness of sampling within the human and wildlife sub-populations, and discrete phylogeographic models to identify taxonomic and geographic centers of viral diversification. All statistical analyses will be performed reproducibly using scripted, programmatic workflows (e.g., the R and Stan languages) and maintained under source code version control (git). As with data management, the DMA team will act as trainers and consultants for exploratory data analysis, power analysis, and study design with project partners, and the EHA computing cluster will be available for partners undertaking additional or expansion studies. Power analyses, current and expansion, are performed via simulation approaches allowing planning for complex, hierarchical variation in study populations. Power analyses and specific analytical components of this study are detailed under each Specific Aim.

7.2. Data Quality Control and Data Harmonization: All data will be are examined at entry by field and lab teams upon data entry, followed by examination by DMA team members at upload and integration, for complete de-identification, completeness, accuracy, and logical consistency. The DMA will provide field and lab teams with reports, produced automatically, of data summaries, including aggregates, distribution, detected outliers and possible mis-entries. On a regular basis (quarterly or as-needed during data collection), DMA team members will review reports with field and lab teams to identify errors and update collection and entry procedures as necessary.

7.3. Statistical Considerations for Behavioral Questionnaires and Clinical Metadata: The data collected from the questionnaire will be analyzed to assess the reported measures of contact for each risk group under study, related to1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, rodents, and primates in/around house and other livestock animals (e.g. farming, butchering, slaughtering); 3) proximity of residence or workplace to environments of increased risks (e.g. nearby bat roosts); 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12 months and lifetime. Specific measures we are interested in are the proportion of respondents indicating they consume wildlife, where wildlife is obtained for consumption, have hunted wildlife, butchered or slaughtered live animals, seen wildlife in their homes, been bitten or scratched by animals, etc. Comparisons of measures of exposure contacts and types between men and women, children and adults, different study regions will be conducted in order to explore the occupational, environmental, and demographic factors (gender, age, socioeconomic status (SES)) that influence contact with animals and to determine who is

most at-risk. Statistical analysis will be employed to identify differences between groups with a 95% probably of detecting a difference. Measures of contact related to different activities within specific groups will be compared to determine the activities that put groups at most risk. As appropriate multivariate analysis (e.g. ordinary linear regression, logistic regression, non-normal distributions of outcome, least absolute shrinkage and selection operator (LASSO) regression, etc.) will be utilized to evaluate the relationship between the outcome variables, positive biological results (PCR or serology) key measures of contact and the factors that influence frequency and types of human-animal contact.

8. Project Milestones and Timelines

8.1 Milestones: <u>End of Year 1:</u> Aim 1: Sample targeting locations, species (for wildlife), sample size justifications completed for whole project and reported to in-country teams; Sample testing, viral isolation, NGS, glycoprotein sequencing begun for all archival and some newly-collected samples; *in vitro* work begun; host-pathogen dynamic analyses; animal model work begun. **Aim 2:** Target human community populations identified and sample sizes calculated for some sites in each country; Community data collection, serological testing and RT-PCR testing begun; first epidemiological analyses of data begin in last quarter. **Aim 3:** Clinical cohort selection underway; clinical enrollment, data collection and sample analysis begun. First Annual meeting in last quarter. First publications submitted by end of year, summary overview papers or reviews.

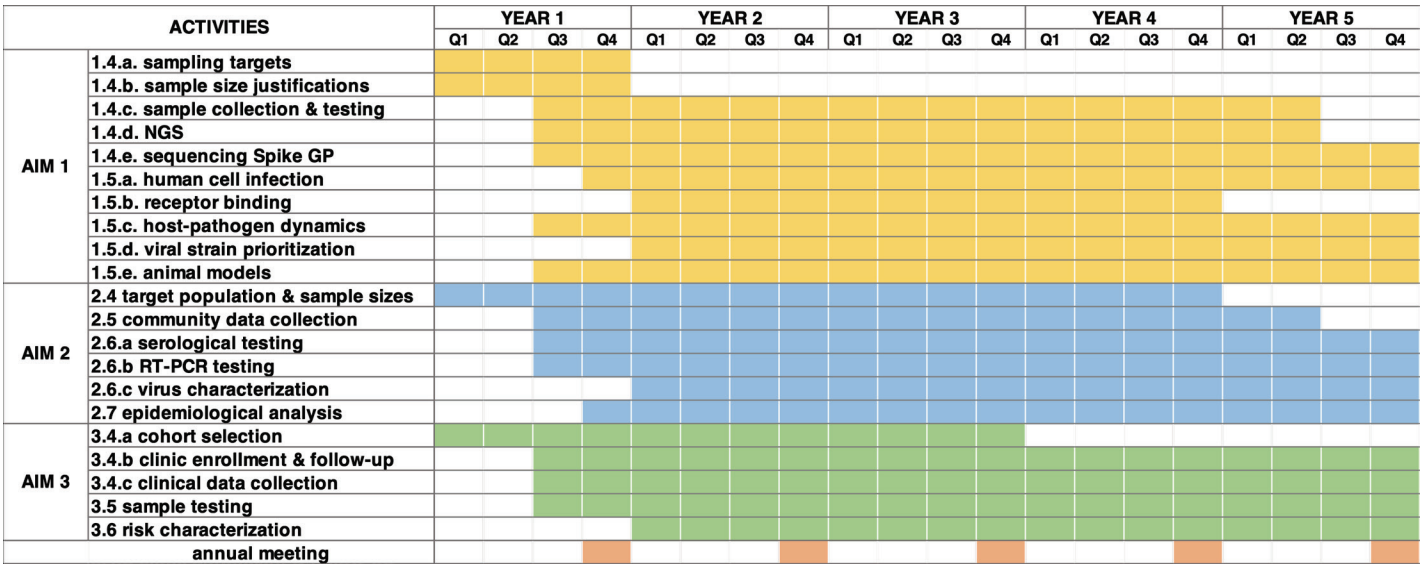
<u>End of Year 2:</u> Aim 1: No sample targeting or sample size justification analyses needed. All other aspects underway **Aim 2:** All aspects underway **Aim 3:** All sub-aims underway. Second Annual meeting in last quarter. Further 2 publications submitted by end of year, including first data papers.

<u>End of Year 3:</u> Aim 1: No sample targeting or sample size justification analyses needed. All other aspects underway **Aim 2:** All aspects underway **Aim 3:** All sub-aims underway. Third Annual meeting in last quarter. Further 3 publications submitted by end of year, largely data papers.

<u>End of Year 4:</u> Aim 1: No sample targeting or sample size justification analyses needed. All other aspects underway **Aim 2:** All aspects underway. Receptor binding work completed. **Aim 3:** No further cohort selection required; all other sub-aims underway. Fourth Annual meeting in last quarter. 3 further publications submitted, including first papers analyzing risk factors, pathogenic potential of novel viruses submitted.

<u>End of Year 5:</u> Aim 1: No sample targeting or sample size justification analyses needed. No receptor binding assays continuing. Serological and PCR testing completed end of 2nd quarter. Glycoprotein, *in vitro* and *in vivo* analyses, analysis of viral risk continue to end of project. **Aim 2:** No further community targeting or sample size work. Community data collection completed at end of 2nd quarter. All other aspects continue to end of project **Aim 3:** All sub-aims underway. Final Annual meeting in last quarter. Further 3 publications submitted.

8.2. Timeline:



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From:	Peter Daszak on behalf of Peter Daszak <daszak@ecohealthalliance.org>
To:	Laing, Eric; Thomas Hughes
Cc:	Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Supaporn Wacharapluesadee; Aleksei Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross; Kevin Olival
Subject:	EIDRC grant submitted
Date:	Friday, June 28, 2019 8:59:44 PM
Attachments:	EIDRC Southeast Asia v7 FINAL_FINAL.pdf
Importance:	High

Dear All,

Just to let you all know that the grant was successfully submitted today with time to spare, and no errors. It's in the system, and it's all down to the reviewers now! Thanks to all of you for your help and support in getting this finalized and completed. Please pass on my personal thanks to the Co-Investigators, Key Personnel and Consultants who you also brought into the team.

I've attached a pdf of the final proposal text and will send the Word version in a few minutes. It's actually a good read, and I'm especially grateful to Hongying who generated all the cool graphics, based largely on Ralph's previous NIH grant proposals.

As you read the text, please remember that the wording is very carefully targeted to a typical US-based NIH reviewer, and to the Program Officers, with the sole purpose of trying to win the grant. If I've exaggerated or made mistakes, or used language that isn't quite right, I apologize, but I did it for the key goal of getting funded.

In the meantime, please don't share this proposal beyond our group on this email chain, so we don't give our competitors an edge!

Good luck, and I'll be keeping my fingers crossed all summer in the hope that we win this…

Cheers,

Peter

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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

From: Peter Daszak
Sent: Wednesday, June 26, 2019 5:19 PM
To: 'Laing, Eric'; Thomas Hughes
Cc: Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Supaporn Wacharapluesadee; Aleksei Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross; Kevin Olival
Subject: EID-SEARCH Comments

Thanks everyone for sending the comments back. Working on v5 now, while others in the office are cranking out sample size calculations, new figures, human and vert. subjects and all the additional information needed after the research plan.

As I go through all your comments, I'll send the occasional email with questions to clarify specific points, so please be on standby for a quick turnaround.

Cheers,

Peter

Peter Daszak
President

EcoHealth Alliance
460 West 34th Street – 17th Floor
New York, NY 10001

Tel. +1 212-380-4474
Website: www.ecohealthalliance.org
Twitter: @PeterDaszak

EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

From: Laing, Eric [mailto:eric.laing@usuhs.edu]
Sent: Wednesday, June 26, 2019 12:59 PM
To: Thomas Hughes
Cc: Peter Daszak; Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C;

Chris Broder; Supaporn Wacharapluesadee; Aleksei Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross; Kevin Olival
Subject: Re: EID-SEARCH v4

Hi Peter,

Built on top of Chris' suggestions. Included a prelimin data figure from Hughes et al, in prep. Double check that is ok with Tom?

- Eric

Eric D. Laing, Ph.D.
Research Assistant Professor
Department of Microbiology and Immunology
Uniformed Services University
4301 Jones Bridge Road
Bethesda, MD 20814
cell: (301) 980-8192
office: (301) 295-9884
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eric.laing@usuhs.edu

On Wed, Jun 26, 2019 at 11:26 AM Tom Hughes <tom.hughes@ecohealthalliance.org> wrote:

> Hi Peter,
>
> I have added my edits and comments to Kevin's.
>
> Please let me know if you ave any questions or need more details.
>
> Thanks.
>
> Tom

From: Kevin Olival <olival@ecohealthalliance.org>
Sent: 26 June 2019 2:51 PM
To: Peter Daszak
Cc: Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Eric Laing; Thomas Hughes; Supaporn Wacharapluesadee; Aleksei Avery Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross
Subject: Re: EID-SEARCH v4

Peter, v4 with my edits, some revised figs, and additional comments.

Cheers,
Kevin

Kevin J. Olival, PhD
Vice President for Research

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On Jun 25, 2019, at 9:26 AM, Peter Daszak <daszak@ecohealthalliance.org> wrote:

Dear All,

Here's version 4. Apologies for the delay. It's in better shape now, but there are still lots of areas that need your attention. Please put in time today, and tomorrow to edit and firm up the text. I've put your names in some of the comment boxes where I need specific help but I also need you to read this critically now and edit and tighten it up. We have 30 pages of space, and our research plan is now 21 pages, so we need to lose maybe 3 or 4 pages. There are also some weak points, specifically on Filoviruses, on the details of where we'll do our clinical work, and on what we'll do with the animal models for henipas and filos.

Please get comments back with me before Wednesday 26th 4pm New York time, so that I have Wendesday evening and all day Thursday to incorporate them. In the meantime, I'll be working with folks here on better figures, and all the extra sections that come after the research plan. I'll need you all to help with those on Thursday while I'm finishing off the draft.

Thanks to all of you in advance and look forward to the next round.

Cheers,

Peter

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From: Peter Daszak
Sent: Thursday, June 20, 2019 9:41 PM
To: Wang Linfa; Danielle Anderson (danielle.anderson@duke-nus.edu.sg); Ralph Baric (rbaric@email.unc.edu); Baric, Toni C; Sims, Amy C; Christopher Broder (christopher.broder@usuhs.edu); Eric Laing; 'Tom Hughes'
Cc: Aleksei Chmura (chmura@ecohealthalliance.org); Alison Andre; Luke Hamel (hamel@ecohealthalliance.org); Kevin Olival, PhD (olival@ecohealthalliance.org)
Subject: EIDRC-SEA v.3
Importance: High

Dear all,

Thanks to everyone for comments and text for the earlier draft at the beginning of the week. Kevin's spent the last couple of days pulling all the comments together into one draft and inserting his changes, and I've now attached a v3 of the draft. It's still rough, and now too long, but it's getting there. I wanted you to look at this rough version because I'm stuck in Liberia in meetings Friday, then flying back home Saturday. I'll continue editing this draft on the plane and will be working solely on this grant from home on Sunday, Monday, Tues, Wed, Thurs.

Please work on this rapidly if possible. If you can all push your comments through, using track changes, and get them back to me **by Sunday evening New York Time**, I will turn round a polished draft by Tuesday, giving us time for significant last go-over on Wed, Thurs. It's tight, but unfortunately, it is what it is, so please commit the time if you can!! As well as general edits (using track changes, with references in comment boxes), please do the following…

Linfa/Danielle – We especially need your input in section 1.1, 1.3, 1.4 and 2.5

Ralph/Amy - Please work your magic on this draft, inserting text, editing and reducing text, and

making sure the technical details of the characterization are correct and fit the proposed work on CoVs, PMVs and Henipaviruses

Chris/Eric – Eric – congratulations on your (b) (6) and thanks for working on it at this time! Chris – please help and edit/finesse the language in the relevant sections for you

Tom – Your edits crossed over with Kevin's changes, so please insert all the bits that were new in the last version you sent to me, into this version. I think these are mainly the sections and comments from the other Malaysian colleagues, but please check and re-insert all key text. We especially need to be specific about which partner in Malaysia is doing what cohorts, and where, and about who's doing the testing

Thanks again all, and I'm looking forward to non-stop work on this as soon as I get on my flight in 24 hours…

Cheers,

Peter

Peter Daszak
President

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Website: www.ecohealthalliance.org
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<EIDRC Southeast Asia v4.docx>

II. Research Strategy:

1. Significance:

Southeast Asia is a critical hotspot for emerging diseases due to its high biodiversity of wildlife and their viruses, and the presence of key ecological and socioeconomic drivers of emergence **(Fig. 1)** (*1*). These include dense human populations living in networks of rural and urban communities, with strong cultural and occupational connection to wildlife and livestock farming and trading, and intimate connection to global travel networks (*2*). The region is also undergoing rapid environmental and demographic change, both of which increase the risk of disease emergence and spread.

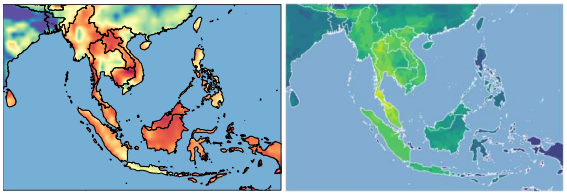

Fig. 1: Southeast Asia, and in particular, parts of Thailand and Malaysia are hotspots of risk for disease emergence **(left)**, and predicted high diversity of 'missing' or as-yet undiscovered viruses, yellow = highest diversity **(left)**. From (*1, 2*).

Not surprisingly a number of novel viruses, including near-neighbors of known agents, have emerged in the region leading to sometimes unusual clinical presentations **(Table 1)**.These factors are also behind a significant background burden of repeated Dengue virus outbreaks in the region (*16*), and over 30 known *Flavivirus* species circulating throughout S. and SE Asia (*17*). The events in **Table 1** are dominated by three viral families: coronaviruses, henipaviruses and filoviruses, which serve as initial examples of team research focus and capabilities. These viral groups have led to globally important emerging zoonoses (*18-29*). Their outbreaks have caused tens of thousands of deaths, and billions of dollars in economic damages (*30-32*). Furthermore, relatives and near-neighbors of known pathogens in these families have been reported throughout the region, including by members of our consortium: henipaviruses in bats in Thailand, Cambodia, Philippines, Laos, Indonesia, China, Malaysia, Bangladesh and India (*33-39*); a novel henipavirus, MòjiāngMojiang virus, in wild rats in Yunnan (*10*); serological evidence of filoviruses in bats in Bangladesh (*40*) and Singapore (*41*); Nipah- and Ebola-like viruses in *Hipposideros*, *Cynopterus* and *Rhinolophus* species in Malaysia (Hughes *et al.*, in prep.); evidence of novel filoviruses in bats in China (*42-44*), including Měnglà virus that appears capable of infecting human cells (*45*); novel paramyxoviruses in bats and rodents consumed as bushmeat in Vietnam (*46*); a lineage C β-CoV in bats in China that uses the same cell receptor as MERS-CoV and can infect human cells *in vitro* (*47*); MERSr-CoVs in dry bat guano being harvested as fertilizer in Thailand (*48*), and directly in bats (Wacharapluesadee *et al.*, in prep.);

Commented [EL1]: Did you want preliminary data?

Viral agent	Site, date	Impact	Novelty of event	Ref.
Melaka & Kampar virus	Malaysia 2006	SARI in family group, individual	1st disease due to bat-origin reoviruses, also Singapore, Vietnam etc.	(*3-6*)
Reston filovirus	Philippines 2008	Seropositive people killed pigs,	No prior findings of filoviruses in pigs	(*7*)
Thrombocytopenia Syndrome virus	E. Asia 2009	100s of deaths in people	Novel tick-borne zoonosis with large caseload	(*8*)
Reston filovirus	Shanghai 2011	PCR positive pigs	Further evidence of pig RESTV infection	(*9*)
~~Mojiang~~Mòjiāng virus	Yunnan 2012	Death of 3 mineworkers	1st evidence of rodent origin henipavirus in people	(*10*)
Nipah-like virus	Philippines 2015	Killed horses	No prior horse infection for NiV, known for HeV	(*11*)
SARSr-CoV & HKU10-CoV	S. China 2015	Seropositive people	1st evidence human infection HKU10 & SARSr-CoV	(*12*)
SADS-CoV	China 2017	>25,000 pig deaths seronegative people	Novel emergence of bat-origin CoV	(*13*)
Nipah virus	Kerala 2018, 2019	Killed 17/19 people 2018, 1 infected 2019	1st outbreak of NiV outside Bangladesh, W. Bengal focus	(*14, 15*)

Table 1: Recent emergence events in SE Asia indicating potential for novel pathways of emergence, or unusual presentations for known or close relatives of known viruses.

172 novel β-CoVs (52 novel SARSr-CoVs) and a new β-CoV clade ("lineage E") in bat species in S. China that are also found throughout the region (*20, 47, 49, 50*); and 9 and 27 novel wildlife-origin CoVs and

paramyxoviruses in Thailand (respectively) and 9 novel CoVs in Malaysia identified as part our work under USAID-PREDICT funding (*49, 51*). These discoveries underscore the clear and present danger of future zoonotic events in the region. Furthermore, the wide diversity of potentially pathogenic viral strains has significant potential to help in the development of broadly active vaccines, immunotherapeutics and drugs (*47*).

Most high-impact zoonotic viruses originate in wildlife reservoirs, sometimes spilling over first into livestock 'amplifier' hosts, or directly into localized human populations with high levels of animal contact **(Fig. 2)**. Efforts to prevent emerging zoonoses have targeted these high-risk populations in regions prone to disease emergence (*29, 52*). However, these regions tend to be in the developing world, where nations often lack the resources to investigate novel zoonotic events, or wildlife reservoirs of zoonoses, and prioritize more immediate public health concerns such as Dengue virus or NTDs (*16*). Zoonotic disease surveillance and control is also hampered by inadequate information on basic disease ecology (e.g. range of wildlife reservoirs, risk factors for spillover) and pathogen biology (viral diversity, pathogenesis), and by logistical challenges sharing novel diagnostics, animal models, samples and reagents at sites where outbreaks begin (*53*). Countermeasure and vaccine development is challenged by the small number of isolates available, and the presence of closely-related strains, some with evidence of transmission to people but with unknown pathogenicity. For example, using NIH and other funding, we found serological evidence of exposure to African henipaviruses in 3-4% of a sample of people in Cameroon, with a significant risk factor being butchering of bats; and serological responses to bat SARSr-CoVs in 6/209 (2.87%) and bat HKU10-CoV in x/xx (x%) people living close to a bat cave in Yunnan, all novel viruses with unknown clinical impact (*12, 54*). We discovered two novel bat-borne reoviruses, Melaka and Pulau viruses, and showed that 14/109 (13%) of people living in close proximity to bat roosts on Tioman Island, Malaysia were exposed to these viruses (*3*) as well as 12/856 (1.4%) people screened in Singapore (*4*). Previous studies in the Philippines found famers and butchers who had contact with sick pigs tested positive for Reston ebolavirus antibodies but had not experienced illness (*7*), and it was our team that discovered Philippine bats as natural reservoir hosts for Reston virus (*55*). Preliminary screening in Malaysia with funding from DTRA for serological surveillance of Henipaviruses and Filoviruses spillover found serological evidence of past exposure to Nipah and Ebola antigenically-related viruses in non-human primates (NHPs) and people (Hughes, in prep.). Even when spillover leads to outbreaks of illness and mortality, they are often unreported, undiagnosed or misdiagnosed. Under a prior NIH R01, we initiated targeted syndromic surveillance of encephalitis patients in Bangladesh clinics and showed that NiV causes repeated annual outbreaks with an overall mortality rate of ~70% (*56*). Nipah virus has been reported in people in West Bengal, India, which borders Bangladesh (*26*), in bats in Central North India (*57*), and now in people in Kerala, South India in 2018 and 2019, raising the specter of future spillover at other sites in the region (*14, 58*).

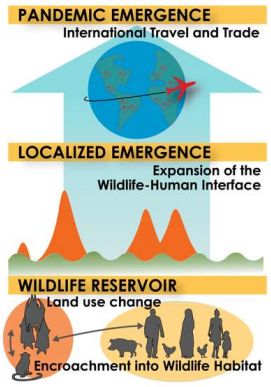

Fig. 2: Our Early Warning Approach: Most zoonotic viruses emerge from wildlife hosts, sometimes via livestock (Stage 1, lower panel), to cause small scale outbreaks (green) or larger chains (red) of human-to-human transmission (Stage 2, middle). In some cases, these spread more widely via air travel (Stage 3, upper). Our EIDRC proposal targets each stage: SA1 examines the diversity and spillover potential of viral threats in targeted SE Asian wildlife; SA2 seeks evidence of their spillover into focused high-risk human populations (stage 2); SA3 identifies their capacity to cause illness and to spread more widely (stage 3). Figure from (*52*).

Under a current NIAID R01, we have shown that we can use analytical approaches to target surveillance of wildlife and people and identify likely sites of spillover. We used phylogenetic analysis of bat hosts and their CoVs to identify a key cave complex in SW Yunnan Province that harbors all the genetic elements of SARS-CoV (*59*), as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics (*20, 47, 49, 50, 60-62*). We conducted behavioral risk surveys and biological sampling of human populations living close to this cave and found evidence of spillover (xx serology) in people highly exposed to wildlife. **This work provides proof-of-concept for an early warning approach that we will expand in this EIDRC to target key viral groups in**

Commented [PD2]: Peter will correct

Commented [PD3]: Peter to do

one of the world's most high-risk EID hotspots.

Commented [BRS4]: Statement should be in the aims. …Our center covers the worlds most high risk EID hostspot?

The overall premise for this EIDRC proposal is that there is substantial evidence that: 1) diverse CoVs, henipaviruses and filoviruses related to known zoonotic pathogens are circulating in wildlife reservoirs in Southeast Asia; **2)** these viruses likely spillover regularly to people, are often unreported or misdiagnosed, and their clinical manifestations and potential to cause pandemics are unknown and underestimated; and **3)** our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence. To scale-up this approach, we propose to launch **EID-SEARCH** (the **E**merging **I**nfectious **D**iseases - **S**outh **E**ast **A**sia **R**esearch **C**ollaboration **H**ub), to better understand the risk of zoonotic viral emergence in Southeast Asia. We will initially focus on the diversity of known and novel CoVs, henipaviruses, and filoviruses in wildlife reservoirs. We will use this work to build our center's interactive network throughout the region, bridge barriers, provide early focal point for basic and translational studies, develop sample handling and storage routines, communication networks, cohort management, infrastructure development, and cross-training in surveillance and novel diagnostic platforms. We will develop and transfer technology to test these viruses' capacity to infect human cells and mouse models, develop new specific and sensitive serological and molecular diagnostic tools, conduct surveillance of human communities with high risk of exposure to wildlife, and clinical cohorts to identify evidence of spillover of viruses causing previously 'cryptic' clinical syndromes in people. We will test the capacity of novel viruses we isolate, or genetically characterize, to infect human cells and/or mouse models, and thus asses potential for spillover and spread which will feed into our risk models. Our group also has extensive experience in other viruses, including vector-borne flaviviruses, and alphaviruses, and will use this experience to provide robust long-term surveillance, diagnostics, human and basic science opportunities. While the current proposal focuses its basic and applied research agendas on this group of viral EID, we note that the EID-SEARCH is poised to rapidly respond to any new emerging outbreak. Thus, filoviruses, henipaviruses and coronaviruses are examples of sentinel surveillance systems in place that can capture, identify and initiate a rapid response targeting any novel microbial pathogen that emerges in this region.

2. Innovation: Previous work by our consortium has developed strategies to target surveillance in wildlife and people to better anticipate and identify early spillover events and pre-empt outbreaks of emerging viruses. In the EID-SEARCH we scale up this approach in a central research "hub" made up of leading laboratories, clinics and surveillance sites in three critically high-risk EID hotspot countries in SE Asia. EID-SEARCH's reach is expanded through its regional network of collaborators that covers nearly all of the greater SE Asia region. The innovation of the EID-SEARCH is in: **1)** Its multidisciplinary approach that combines modeling to target geographic and taxonomic sampling targets with on-the-ground zoonotic disease surveillance in human and wildlife populations; **2)** the biological characterization approach that identifies how likely viruses are to be able to spillover into people, and enables evaluation of existing countermeasure technologies; an approach that is grounded in our successful work on SARSr-CoVs; and **3)** the strategic sampling of both high-risk communities with extensive animal contact at the front-lines of viral spillover *and* syndromic clinical cohorts that present with signs of novel viral emergence, to identify risk of viral spillover in an early warning system approach **(Fig. 2)**. **In Aim 1, we will target viruses in archived and newly collected wildlife samples, and characterize their risk of spillover to people**. We will use spatial and phylogeographic analyses to identify the geography and species of wildlife to test samples from, and to further characterize viruses we have recently discovered. We will use *in silico* methods (inovel ecological-phylogenetic risk ranking and antigenic mapping of receptor binding domains), cell culture, and animal models to assess their potential for spillover into

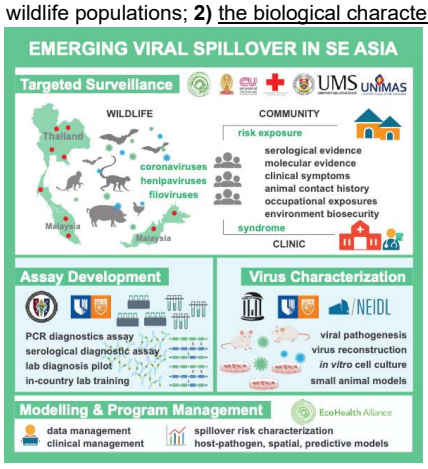

Fig. 2: EID-SEARCH approach, core members, and roles.

Commented [PD5]: Hongying – Figure needs edits to text: top panel, 'wildlife' should read "Aim 1: WILDLIFE", "AIM 2: COMMUNITY", "AIM 3: CLINIC". Right hand side: "Clinical symptoms" should be "Clinical history", "Occupational exposures" should be "Occupational exposure", "environment biosecurity" should be "environmental risk factors"

Middle panel: "Assay Development" and "Virus Characterization" should have "(Aim 1)" after them. Left hand side: should read "PCR diagnostics", "serology", "piloting diagnostics" and the last one is ok. Right hand side bullets should be "receptor binding characterization" "in vitro characterization" and "mouse models"

Bottom panel: all good

high-risk human populations. **In Aim 2, we will conduct focused, targeted surveys and sampling of human communities with high exposure to wildlife and other animals.** We will use serological tests targeting viruses identified in Aim 1 to identify the baseline spillover of known or novel CoVs, henipaviruses and filoviruses in these populations. Serological findings will be analyzed together with clinical metadata (including a simple animal-contact survey, location data, viral sequence/isolates etc.) to identify zoonotic risk factors and routes of exposure from specific wildlife or vector species. **In Aim 3, we will use syndromic surveillance of clinical cohorts to identify evidence of viral etiology for otherwise 'cryptic' outbreaks.** We will enroll and collect biological samples from patients in local clinics and hospitals who present with syndromes previously linked to known viral agents and live within communities at high risk for disease emergence. We will conduct molecular and serological diagnostic assays to identify likely viral etiology of syndromes that may represent otherwise cryptic events. Where viruses are identified, we will attempt to isolate and characterize them, and use survey data, ecological and phylogeographic analysis to identify likely reservoir hosts and inform intervention programs. Our US partner BSL-4 laboratory, NEIDL, ~~will attempt isolation and characterize~~ any viruses requiring high levels of containment (e.g. any novel filoviruses discovered).

Commented [PD6]: PD will get stronger letter from Jerry Keusch: opportunity for visiting scholars to be certified for BSL-4 research, strong Ebola person at NEIDL to conduct characterization if we find filos.

3. Approach

Research team: The EID-SEARCH builds on long-term collaboration among world leaders in emerging virus research with proven experience collaborating internationally on field, lab and human surveillance research **(Fig. 2)**. Over the past two decades, our consortium partners have conducted high profile research on EIDs within the region and globally, including identifying the wildlife reservoirs for Nipah and Hendra virus, MERS- and SARS-CoVs (*20, 25, 59, 63-66*), discovering SADS-CoV (*13*), and developing an array of serological, molecular, *in vitro* and *in vivo* approaches to characterizing high-risk CoVs, henipaviruses, and filoviruses (*60-62, 67-80*). Our team has substantial experience conducting human surveillance in the community and during outbreaks **(See sections 2.x.x, 3.x.x)**, including: USAID-PREDICT, and DTRA-funded viral surveillance of indigenous communities in Peninsular Malaysia and syndromic surveillance of hospital patients in Sabah (Co-I Hughes); collection of >2,000 serum samples from healthy blood donors and sanitation workers, febrile and influenza like illness (ILI), and diarrheal stool samples from children under 5 years of age (Co-I Kamruddin); collaboration on the MONKEYBAR project with the London School of Hygiene and Tropical Medicine to analyze risk and prevalence of the zoonotic malaria *Plasmodium knowlesi* (Co-Is William, Tock Hing); case control and cross-sectional studies in Sabah villages (~800, 10,000 participants resp.) (Co-Is GR, TYW). As a proof-of-concept of how our team can rapidly scale up resources for diagnostics and surveillance in the face of a new EID, in 2016, we discovered a novel bat-origin HKU-2 CoV (*81, 82*) killing >20,000 pigs in S. China, designed PCR and LIPS serology tests, then surveyed pig farmers for evidence of exposure, and conducted experimental infections, pathogenesis and molecular studies, **all in the span of 3 months** (*13, 83*).

The administration of this center **(See section X)** will be led by PI Daszak, who has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5- 20 yrs on NIAID- and USAID-funded research. He will oversee all activities in this EIDRC. Drs. Olival and Zambrana are leaders in analytical approaches to targeting surveillance and head the modeling and analytics work for USAID-PREDICT. Drs. Wang, Baric, Broder, Anderson, and Laing have developed a unique array of *in vitro* cell culture, serological and molecular reagents to identify and characterize emerging viruses in people and animals (henipaviruses and other paramyxoviruses, CoVs, filoviruses and many others). Dr. Wacharapluesedee, Hughes and collaborators have coordinated surveillance of people, wildlife, and livestock within Thailand and Malaysia for the past 10 years, in collaboration with Ministries of Health, Agriculture and Environment. Finally, this consortium has extensive external funding from NIH, DoD, USAID, DHS and other USG agencies that supports a range of laboratory, analytical and field studies directly related to the current proposal, and will bring substantial leverage to our EIDRC.

Geographical focus: The three core (hub) countries for this proposed EIDRC are Thailand, Malaysia and Singapore, three contiguous EID hotspot countries that stretch through one of the most significant foci of EID origins globally. Our proposed work includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sarawak and Sabah, and thus contains overlapping wildlife biogeography with India, Bangladesh, Myanmar, SW China, Laos, Cambodia, Vietnam, Philippines, and Indonesia. It encompasses diverse cultural and

socioeconomic backgrounds which have correspondingly diverse and intensive interactions with wildlife reservoirs and the global travel network. We have already begun sampling of wildlife hunters, livestock workers, wildlife farmers and market workers, rural villagers living close to high risk wildlife species, people otherwise highly exposed to animals in these countries, and clinical cohorts, as part of current/prior NIH, DTRA, and USAID funding. To develop a larger catchment area for emerging zoonoses, we will deploy our core member's extensive network of collaborators in clinics, research institutes and public health laboratories in almost every other Southeast Asian country. We will conduct regular meetings with key research, public health and community leaders, including >50 leading laboratories/institutions in XX countries that are currently collaborating with core members of our consortium via other funded work (**Fig. 3**). We will use these, and our annual meetings, to share information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks across the region.

Fig.3 : Map of Southeast Asia indicating three hub countries for this proposed EIDRC (Red: Thailand, Singapore, Malaysia) and countries in which Key Personnel have collaborating partners (Green), indicating that EID-SEARCH provides access to key collaborators throughout the region.

Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife

1.1 Rationale/Innovation: The vast majority of emerging zoonotic pathogens are linked directly or indirectly to wildlife host, particularly from important mammalian groups like bats, rodents and non-human primates (*84*). Our previous work on wildlife origin viruses demonstrates that zoonotic viral emergence tends to occur in specific regions with high diversity of wildlife that harbor viruses of zoonotic potential, and human populations that have close contact to these wildlife **(Fig. 1)** (*2*). In Aim 1, we will strategically conduct EID surveillance by applying risk analytics to prioritize the regions, wildlife species, and human populations that have a particularly high risk of viral spillover. We will target high-risk zoonotic host reservoirs (bats, rodents and primates) in these regions and apply novel predictive models to estimate viral diversity and host range to identify wildlife species that have been ignored or under-sampled by previous zoonotic disease surveillance efforts. We will strategically collect specimens from these species and screen them, together with some of the tens of thousands of recently collected samples we have archived in freezers in our labs, for known and novel agents. We will begin screening using conserved PCR assays to identify known and novel virus in high risk RNA viral families, *Coronaviridae*, *Paramyxoviridae*, and *Filoviridae*. We will expand to other groups of pathogens, pending available resources and priorities established with the EIDRC coordinating center (EIDRC-CC) and other regional EIDRCs. Using risk ranking algorithms and a series of ecological and virological risk factors, we will triage the wildlife viruses we discover for further genomic characterization and viral isolation. On this subset of viruses, we will then use *in vitro* and *in vivo* approaches (viral rescue, cell lines, gene edited humanized transgenic mice and the collaborative cross genetic reference mouse population) previously developed and widely used by our team to predict capacity of novel viruses to infect people and spillover (*61, 85-88*). These high-risk viruses and their close relatives will be targets for human community and clinical sampling in Aim 2 and 3, respectively.

1.2 Preliminary data: 1.2.a. Geographic targeting: EcoHealth Alliance has led the field in conducting analyses to indicate where the highest risk of zoonotic spillover is across the globe, identifying SE Asia as a major high risk location for pathogen emergence (*1, 2*). For Southeast Asia, these EID hotspots are sites where high wildlife biodiversity, human population density and rapid ecologic change collide **(Fig 1)**. Using host and ICTV viral data for all known mammalian viruses, and generalized additive models to correct for sampling bias, we are able to predict the relative number of yet-to-be-described viruses that a species harbors (*2*). Using these data to map unknown viral diversity demonstrates substantial undiscovered viral diversity in mammals across Southeast Asia, and particularly in regions of Thailand, Singapore and Malaysia **(Fig. 1)**. In a separate paper on risk of viruses emerging from bat hosts, we found that factors driving spillover (host diversity, climate)

Commented [PD7]: Hongying – "EIDRC Program Countries" should be "EID-SEARCH core countries" and "External Partner Countries" should be "Collaborating Partners"

Commented [PD8]: PD to Remember to correct specific aims with same titles

Commented [KJO9]: Will we? If so, make sure we mention primates more and include in vert animal sections.

Commented [PD10]: PD will discuss both of these comments with Kevin

Commented [KJO11]: Do you think we need to add in or mention vectors (arthropods)? We don't have big banks of these as far as I know, so maybe best not too?

Commented [CB12]: I would not mention arthropods. The scope here so far is well laid out, and expansive enough as it is, and most of our focus are viruses known to originate in mammalian hosts.

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differ from those driving spread in human populations (population density, bushmeat hunting, livestock production) (*89*). We therefore analyze capacity for viral spread as a separate risk factor, using demographic and air travel data and flight model software we have produced with Dept Homeland Security funds (*90*). In the current proposed work, we will use all the above approaches to target wildlife sampling (archive and new field collections), and to inform sites for human sampling in Aim 2, to areas of high risk for spillover and spread.

1.2.b. Reservoir host species targeting: Our analysis of all known mammalian host-viral associations demonstrates that bats, rodents and primates represent the three most important reservoirs for zoonoses, responsible for the highest proportion of known and predicted viral diversity of zoonotic potential(*91*). Thus, the bulk of our proposed wildlife sampling and testing will consist of these three taxa with a focus on bats, because this group is the known or hypothesized reservoir for the majority of high-impact zoonotic CoVs, henipaviruses and filoviruses (*48, 92-94*). Bats also have the highest proportion of predicted zoonotic viruses in any mammalian group (*2*), and are exceedingly diverse in Southeast Asia (~500 species). To further prioritize taxonomic sampling targets within these diverse geographic hotspots, we have used a novel, Bayesian phylogeographic analysis to identify host taxa that are the most important centers of evolutionary diversity for a given viral group, e.g. bat genera for β-CoVs using an extensive CoV sequence dataset from our NIH-funded research **(Fig. 5)** (*49*). This approach will be extended to other mammal taxa and viral families of interest, allowing us to identify the ancestral wildlife hosts of specific zoonotic viral groups, where their diversity is likely highest.

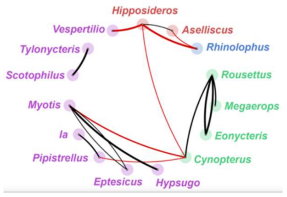

Fig 5: Preliminary analysis to identify the most important bat genera in Southeast Asia as hosts for beta-CoVs using our previously-collected CoV sequence data. Line thickness is proportional to the probability of virus sharing between two genera. Inter-family switches are highlighted in red. We will apply this Bayesian discrete phylogeographic model to identify taxonomic and geographic centers of viral diversification and sharing for relevant paramyxovirus, coronavirus, and other virus clades.

Finally, we have designed a strategy to estimate sampling gaps, using a viral 'mark-recapture' approach we previously published (*95, 96*) **(Fig. 6)**. Using prior data for bat, rodent and primate viral discovery vs. sampling rates, we can identify the likely total viral diversity for specific host groups in a region, and use this to estimate sample sizes to discovering the majority of unknown viruses within a specific family or genus. **In the current proposal,** we will use these analyses to estimate geographic and species-specific sampling targets **to more effectively identify new strains to support experimental infection studies and risk assessment.**

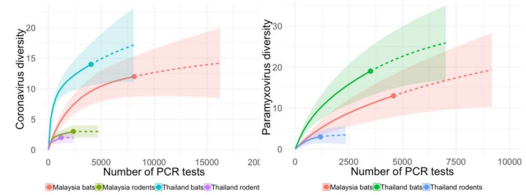

Fig. 6: Estimated coronavirus **(left)** and paramyxovirus **(right)** putative viral 'species' diversity in bats and rodents for Thailand and Malaysia, using RdRp gene sequence preliminary data from >13,000 PCR tests in bats and 4,500 in rodents. Bats in Thailand and Malaysia have 4X the number of viruses than rodent species, controlling for sampling effort. CoV and paramyxovirus diversity estimates are comparable, but discovery has not yet saturated in any taxonomic group or location. We estimate that additional sampling of ~5,000 bats and testing of our archived rodents specimens alone will identify >80% of remaining CoV and paramyxovirus viral species in these key reservoirs. We will use this approach to define sampling targets for other wildlife taxa, and for viral *strain* diversity.

1.2.c. Sample testing to identify known and novel viruses: We have conducted extensive wildlife surveillance for novel viruses across Southeast Asia in prior NIAID funded work and as part of the USAID PREDICT project. Under the 10-year PREDICT program globally, we identified 912 novel and 210 known viruses, more than the total number of viruses previously recognized in mammals by the International Committee on Taxonomy of Viruses. This work included collecting nearly 300,000 individual mammal samples from 14 EHA-led PREDICT countries and PCR-screening more than 60,000 individual specimens(*51*). In southern China alone, this sampling lead to the identification of 178 β-CoVs, of which 172 were novel (52 novel SARSr-CoVs). Included were members of a new β-CoV clade, "lineage E" (41), diverse HKU3-related CoVs (179 sequences) within a 'sister' clade to the SARS-CoV lineage, and the wildlife origin of a new virus, SADS-CoV, responsible for killing >20,000 pigs in Guangdong Province (*13*). Many of the identified bat species are

Commented [BRS14]: I would say we focus on these three species (plus vectors?). To demonstrate the capability of the team, this proposal will focus on bat surveillance (for a variety of reasons), noting that this approach will also be applied to other zoonotic vectors during the 5 year program throughout the region.

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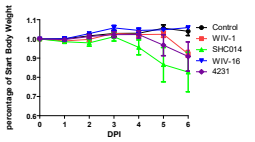
found across the region. We have collected 28,957 samples from bats, rodents and primates in Thailand and 51,373 in Malaysia, conducting 146,527 PCR tests on a large proportion of these specimens. We have **archived duplicate samples which are now available for use in this project**, including fecal, oral, urogenital and serum samples from bats, rodents, and non-human primates, and primate biopsies. In Thailand this screening has identified 100 novel viruses and 37 known viruses in wildlife reservoirs. In Malaysia this screening has identified 77 novel viruses and 23 known viruses in wildlife reservoirs (66 novel and 19 known in Sabah and 11 novel and 9 known from Peninsular Malaysia). We used family level primers for henipaviruses, filoviruses and CoVs and have already discovered 9 novel and 7 known CoVs and 26 novel and 2 known paramyxoviruses in Thailand. This is in addition to 13 novel and 5 known CoVs and 15 novel and 1 known paramyxovirus in Malaysia from wildlife. In the current proposed work, we will attempt to sequence, isolate and characterize those viruses that our analyses below suggest are most likely to be able to infect humans.

Our collaborative group has worked together on these projects, as well as other DTRA funded projects in Singapore/Thailand (Co-Is Broder, Wacharapleusadee, Wang) to design, cross-train, and use novel serological and molecular platforms to investigate virus infection dynamics in bats and examine potential spillover events with or without human disease. For serology, Co-Is Wang and Anderson (Duke-NUS) will develop a phage-display method to screen antibodies for all known and related bat-borne viruses, focusing on henipaviruses, filoviruses and coronaviruses. Once various "trends" are discovered, additional platforms (including Luminex and LIPS) will be used for verification and expanded surveillance. For molecular investigation, we will combine targeted PCR approach with a discovery-driven enrichment NGS strategy to not only survey currently known viruses, but also to discover potential novel viruses. Both the phage-display serological and enrichment platforms can be rapidly updated to include new viruses identified. In Malaysia (Co-Is Hughes, Broder, Laing) ~~we~~ have transferred the Luminex-based serology platform to partner laboratories for screening of wildlife, livestock and human samples to investigate the spillover of henipaviruses and filoviruses at high risk interfaces. These interfaces include farms near the forest and indigenous communities with lots of subsistence hunting in Peninsular Malaysia, and preliminary screening has found serological evidence of past exposure to viruses antigenically-related to Nipah and Ebola in humans, bats and non-human primates (NHPs). In Thailand, Co-Is Broder, Laing and Wacharapluesadee have transferred a Luminex-based serology platform for detecting exposure to novel Asiatic filoviruses and known henipaviruses in wildlife and human samples, with testing underway and currently being validated in our Chulalongkorn University laboratory **(See also section 2.2.d)**.

1.2.d. In vitro & in vivo *characterization of viral potential for human infection:* Using Coronaviridae as an example, we have conducted *in vitro* and *in vivo* experiments to characterize the pathogenic potential of novel SARSr-CoVs from bats. We isolated three SARSr-CoVs from bat feces: WIV1, WIV16 and Rs4874, with S protein sequences that diverged from SARS-CoV by 3-7% (*20, 59, 97*). We conducted full-length genome sequencing of 12 other novel SARSr-CoVs, some highly similar to outbreak SARS-CoV in the most variable genes: N-terminal domain and receptor binding domain (RBD) of the S gene, ORF8 and ORF3 (*59*). Using our reverse genetics system, we constructed chimeric viruses with SARSr-CoV WIV1 backbone and the S gene of two different variants. All 3 SARSr-CoV isolates and the two chimeric viruses replicated efficiently in Vero E6 cells and in HeLa cells expressing hACE2, but not in those without ACE2 (*20, 59, 97*). We used the SARS-CoV reverse genetics system (*71*) to generate a chimeric virus with a mouse-adapted SARS-CoV backbone expressing SHC014 S protein with 10% sequence divergence from SARS-CoV S. This chimera replicated in primary human airway epithelium, using the human ACE2 receptor to enter into cells (*60*). Thus, **SARSr-CoVs with diverse variants of SL-CoV S protein without deletions in their RBD can use human ACE2 as receptor for cell entry.** The Baric lab has a well-established hACE2 and DPP4 transgenic mouse model that we will use this to characterize the capacity of specific SARSr-CoVs to infect (*85, 98*). We used this chimera approach to demonstrate that pathogenicity of bat SARSr-CoVs in humanized mice differs with divergent S proteins, **confirming the value of this model in assessing novel SARSr-CoV pathogenicity**. Infection of rWIV1-SHC014S caused mild SARS-like clinical signs in the transgenic hACE2 mouse model **that weren't reduced by immune-therapeutic monoclonals that attenuate SARS-CoV pathogenicity, nor after challenge following vaccination against SARS-CoV** (*60*). We repeated this virus characterization approach with chimeras using HKU3r-CoV S proteins that are ~25% divergent from SARS-CoV S, **and found that they**

are unable to use the ACE2 receptor. Additionally, we were unable to culture HKU3r-CoVs in Vero E6 cells, or human cell lines.

A similar approach to the above pipeline for CoVs will be applied to novel henipaviruses and filoviruses we discover during our research. Thus far, paramyxovirus attachment glycoproteins have been divided into three major groups: hemagglutinin (H), hemagglutinin-neuraminidase (HN), and attachment glycoproteins (G), where all known species within the paramyxovirus genus, *Henipavirus*, encode a receptor-binding (G) ~~G~~ glycoprotein.~~.~~ The broad mammalian tropism of Hendra virus and Nipah virus (*67*) is likely mediated by their G glycoprotien's usage of highly conserved ephrin ligands for cell entry (*15, 99*). A third isolated henipavirus, Cedar virus (CedV) does not cause pathogenesis in animal models. Recently, **Co-Is Broder and Laing** have developed a reverse genetics system and rescued a recombinant CedV to test ephrin receptor usage/tropism and pathogenicity during henipavirus infections (*76*). CedV is unable to use the e~~E~~phrin-B3 receptor (*76, 100*) which is found in spinal cord and may underlie NiV encephalitis (*101*) and that pathogenesis also involves virulence factors V and W proteins. CedV is also capable of utilizing ephrin-B1 and two additional A-class ephrins (Broder and Laing, submitted).~~.~~ The putative henipaviruses, Ghana virus (GhV) and ~~Mojiang~~Mòjiāng virus (MojV), predict V and W protein expression, with GhV able to bind to ephrin-B2, but not -B2 (*102*), ~~and~~ but the receptor for MoJV ~~remaining~~ remains unknown (*103*) ~~but is likely ephrin-B2~~ (*104*). Novel henipaviruses can be rapidly characterized by an analysis of their encoded G glycoprotein and structural modeling and comparison to several known x-ray structures, to predict ephrin ligand binding competence, while at the same time, either infectious virus isolates (if available) can be assayed for host cell tropism using a panel of B and A class ephrin ligand expressing cells. If other novel henipaviruses are discovered that also encode the know interferon antagonist gene products (V or W) which would preclude work in low level containment or reverse genetics experiments, the receptor tropism and characteristics of these henipaviruses can be analyzed by through the reverse genetics and generation of chimeric viruses using the recombinant CedV platform and glycoprotein genes of other henipaviruses, which the Broder lab is currently doing with Nipah and Hendra to create a BSL-2 based neutralization system.



The host cell binding step of known filoviruses is less well understood, but filovirus tropism is determined by the glycoprotein spike (GP) that facilitates binding with target cells. Ebolavirus is the best well-characterized filovirus, and it productively infects a broad range of cell types such as monocytes, macrophages, dendritic cells, endothelial cells, fibroblasts, hepatocytes, and adrenal cortical cells (Feldmann H, Geisbert TW Lancet. 2011 Mar 5; 377(9768):849-62). Indeed, primary human lung endothelial cells are highly susceptible to Ebolavirus infection (105). Huh7 cells are good candidates for ebolavirus in the liver, oftentimes used to isolate virus from clinical samples (106) Huh7 cells also offer advantages for reverse genetic recovery of novel filoviruses (107). Following host cell attachment, the virus is internalized by macropinocytosis, a non-selective process of engulfment (Davey RA, Shtanko O, Anantpadma M, Sakurai Y, Chandran K, Maury W. Curr Top Microbiol Immunol. 2017;411:323-352). Binding to target cells is mediated by a variety of different attaching factors, and no single class of molecules serves as a classical entry receptor. However, the surface GP is a class I fusion protein with a well-defined mechanism of mediating membrane fusion and virion entry following the endocytosis of the virus particle. In its native state, GP is a triplet of heterodimers, each composed of a receptor binding subunit (GP1) and a fusion subunit (GP2). The GP1 and GP2 subunits are derived by the cleavage within the Golgi complex of a single precursor protein, and remain associated as a timer. After initial internalization, virus particles traffic into the endo-lysosomal pathway, where the low-pH-dependent cysteine proteases cathepsins B and L process GP1 into a 19 kDa fusogenic form, leading to a putative receptor binding domain exposure and allowing for the interaction between the processed GP1 and the late endosomal/lysosomal protein Neimann-Pick C1 (NPC-1) triggering GP2-dependent fusion of the viral envelope with the endosomal membrane (Carette J.E., et al. Nature. 2011;477:340–343; Côté M., et al. Nature. 2011;477:344–348). Here, novel filoviruses however can readily be characterized following the identification of their encoded GP sequences which can be easily pseudotyped into recombinant GP-bearing vesicular stomatitis virus (VSV) pseudovirions (Brouillette RB, Maury W.Methods Mol Biol. 2017;1628:53-63). These pseudovirions can be safely used to characterize the tropism and entry filoviruses mediated by GP without a

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need for BSL4 containment. Primary human lung endothelial cells are highly susceptible to Ebolavirus infection (*105*). Huh7 cells are good candidates for ebola in the liver, oftentimes used to isolate virus from clinical samples (*106*) Huh7 cells also offer advantages for reverse genetic recovery of novel filoviruses (*107*). Thus far, paramyxovirus attachment glycoproteins have been divided into three major groups: hemagglutinin (H), hemagglutinin-neuraminidase (HN), and attachment glycoproteins (G).

Fig. 7: Body weight change in hACE2 transgenic mice after SARSr-CoV infection, demonstrating that bat-origin SARSr-CoVs cause similar clinical presentation to outbreak strain (4231). From (*60, 61*).

Models of Outbred Populations. For novel viruses across all families, we will use the UNC collaborative cross mouse that captures 90% of the diversity within all mouse groups to assess the most suitable model for further experiments (*108*). We have used this model for CoV, filo (Ebola), Flaviviruses, and alphaviruses infections. In subpopulations it reproduces SARS weight loss, hemorrhage in EBOV, enceph in WNV, acute or chronic arthiritis in Chikungunya infection (*86-88, 109-112*). *Bat Models.* Duke-NUS has developed two models for bat *in vivo* studies: a colony of *Eonycteris spelaea* (cave dwelling small fruit bat) and a bat mouse model, which contains the bat immune system in an immunodeficient mouse (*113*).

1.3. General Approach: We will conduct analyses to geographically- and taxonomically-target testing of samples from wild mammals that are most likely to harbor known high-profile zoonotic pathogens, or close relatives with potential to infect people. This includes selecting from tens of thousands of samples collected on recent projects and stored in freezers in our laboratories, and new sampling of wildlife in high risk locales. We will use viral family level PCR screening to identify and partially characterize viruses, then follow up sequencing of relevant receptor binding proteins to assess homology and predict binding affinity, then attempt to isolate and biologically characterize viruses that phylogenetic and other data suggest have high spillover and pandemic potential. We will follow this with *in vitro* binding assays and cell culture experiments, and *in vivo* animal model infections to assess their potential to infect people and cause disease **(Fig. 8)**. EHA will lead the study design, targeted sampling, and data analysis; Co-Is Hughes and Wacharapluesadee will lead field sampling and in-country testing; Duke-NUS, UNC, USUS will lead the serological and PCR diagnostic development and the *in vitro* and *in vivo* analyses, as well as cross-training and tech transfer to the hub labs in Thailand and Malaysia. This will include via support of one Malaysian Ph.D student, Mei-Ho Lee (CM Ltd.) who has worked with the EID-SEARCH collaborators for 9 years. The focus of her study will be infection potential of novel CoVs and paramyxoviruses already found in bats in Malaysia under preliminary data for this proposal.

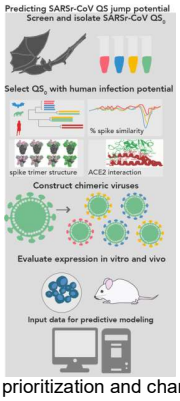

Fig. 8: Schematic showing EID-SEARCH approach to selection of sampling, viral testing, prioritization and characterization to identify novel, high zoonotic-risk viruses in wildlife.

1.4 Wildlife samples: 1.4.a. Geographic and taxonomic targeting for newly collected wildlife samples: We will prioritize sites within each country that rank highest for both the risk of zoonotic disease emergence (*114*) and the predicted number of 'missing' zoonotic viruses (*91*). Our preliminary analysis **(Fig. 1)** suggests priority areas include: the Isthmus of Kra (S. Thailand and N. Peninsular Malaysia), NW Thailand (near the Myanmar-Laos border), and the lowland rainforests of Sarawak and Sabah. In each of these 'hottest of the hotspots' regions, we will identify 2-4 distinct sampling sites based on the geographic distribution of key hosts we prioritize for intensive wildlife sampling. We will priority rank bat, rodent, and primate species using analysis of host trait data for the highest predicted number of viruses based (*91*). We will also identify species predicted to be centers of evolutionary diversity for Corona-, Paramyxo-, and Filoviridae using ancestral host reconstruction of available sequence data and Bayesian discrete phylogeographic models (*115-117*). We will ensure wildlife sampling is complimentary to specimens archived in our extensive freezer banks. We will collect only the additional specimens projected from each prioritized taxa to achieve statistically robust sample sizes given predicted levels of viral richness and rates of discovery **(see 1.4.b)**. We will leverage our strong collaborations with wildlife experts in Malaysia and Thailand to ground-truth geographic and taxonomic model

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outputs with local expert knowledge to ensure the sampling plan is realistic based on current species distributions, population size estimates, seasonality, and accessibility to sites.

Recognizing that most zoonotic viruses are multi-host, i.e. naturally harbored by multiple wildlife species, and that the phylogenetic relatedness of those hosts is predictable and varies across viral families (*91*) – we will also apply a generalizable phylogenetic and spatial modeling approach to rapidly predict new (unsampled) hosts for novel viruses we discover during our research. We will use a combined network analysis and a phylogeographic model to estimate a viruses' host range and to prioritize additional wildlife species for sampling and testing for viruses we discover. Our recently developed model of viral sharing uses just two phylogeographic traits (wildlife species range overlap and phylogenetic similarity between host species) to successfully predict host species in the top 2% of all 4,200 possible mammal species (*118*).

1.4.b. Sample size justifications for testing new and archived wildlife specimens: We will use our preliminary data on average PCR prevalence from screening tens of thousands of bat, rodent and primate specimens for CoV and Paramyxoviruses under the USAID-PREDICT project in Malaysia and Thailand. We will refine these sample size targets, in real-time, over the course of our project using viral discovery curve analyses **(Fig. 6)** to either scale-up or scale-back sampling and testing of archived specimens for each species depending on their viral diversity. For example, given 5-12% prevalence of CoVs in the most common bat species we previously-sampled in Thailand and Malaysia, a target of XXX individuals per species would yield XX (± x) PCR positive individual bats, and an estimated ~xx novel strains. Similarly, for paramyxoviruses with an average PCR detection rate of X%, we would need XX individuals per species to detect 80% of predicted viral strain diversity with 80% power. Preliminary analysis indicates we will require XX,000 samples to identify 80% of remaining viral diversity **(Fig. 9)**. All wildlife sampling will be nondestructive. In the event that an animal is found dead or needs to be euthanized due to injury we will collect biopsy samples for screening.

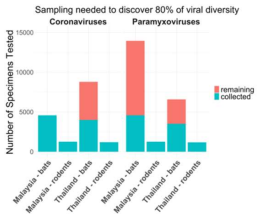

Fig. 9. Stacked bar plot showing number of currently-archived specimens **(blue)** and new specimens required **(orange)** to discover 80% of predicted number of CoVs and PMVs from high-risk bat and rodent taxa in Malaysia and Thailand

We estimate the following sampling effort, broken down for each region: *Peninsular Malaysia:* Wildlife sampling will be led by CM Ltd / EHA in collaboration with PERHILITAN. Archive bat, rodent and NHP samples will be screened serologically using the BioPlex and other serology techniques identified through this project. New wildlife sampling will focus on bats but it is expected that rodents and NHPs will be sample as well. Wildlife sampling will be conducted around Orang Asli communities sampled as part of aim 2. Three new communities will be identified in the districts we have already sampled in. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife mainly bats will be sampled. Wildlife and domestic animal samples will include serum, whole blood, throat swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and tested at PERHILITAN's NWFL. *Sarawak, Malaysia:* Wildlife sampling will be led by CM Ltd / EHA in collaboration with UniMAS. Wildlife sampling will be conducted around Dyak communities sampled as part of aim 2. One community will be identified. Concurrent sampling trip will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife mainly bats will be sampled. Wildlife and domestic animal samples will include serum, whole blood, throat swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and tested at UniMAS or BMHRC. *Sabah, Malaysia:* Bat sampling will be led by CM Ltd / EHA in collaboration with SWD and WHU. Bat sampling will initially focus on 3 caves. Batu Supu Sabah low disturbance cave and surrounding area, Madai medium disturbance cave and surrounding area, Gomantong cave high disturbance cave and surrounding area. Each cave will be sampled twice once in wet season and once in dry season targeting 500 bats at each cave on each trip for a total of 3000 bats. Wildlife samples will include serum, whole blood, throat swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored at WHGFL, molecular testing will be conducted at WHGFL and serology screening at BMHRC. In addition at Madi cave the community that lives in the mouth of the cave will also be sampled targeting 150 people, while at

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Gomantong cave 25 workers involved in the bird next farming will be enrolled into the study. *Thailand:* XXXXXXXXXX

1.4.c. Sample testing, viral isolation: Viral RNA will be extracted from bat fecal pellets/anal swabs with High Pure Viral RNA Kit (Roche). RNA will be aliquoted, and stored at -80C. PCR will be performed with pan-coronavirus, filovirus and paramyxovirus primers. PCR products will be gel purified and sequenced. We will attempt isolation on samples that contain viruses of interest (determined in **1.4, below**), using Vero cells and primary cell culture from the wildlife taxonomic order (bat, rodent and primate cell lines). This includes the over 30 bat cell lines maintained at Duke-NUS from four different bat species.

1.4.d. Moving beyond RNA viruses: To detect pathogens from other families, such as non-RNA viruses we will combine conserved PCR assays with NGS. We will perform either unbiased NGS, or use a pan-viral enrichment library to sequence full-length genomes. We will conduct this work via EID-CC research projects or as part of outbreak detection/investigation in the region from DNA viruses or other agents.

1.4.e. Sequencing Spike Glycoproteins: Based on earlier studies, our working hypothesis is that zoonotic coronaviruses, filoviruses and henipaviruses encode receptor binding glycoproteins that elicit efficient infection of primary human cells (e.g., lung, liver, etc.) (*12, 59, 60*). Characterization of these suggests SARSr-CoVs that are up to 10%, but not 25% different in the spike glycoprotein use human and bat ACE2 receptors for docking and entry. Uneven sampling gaps (e.g., no strains were found with 10-25% spike variation) prevent a thorough understanding of the transition point where the most divergent strains lose human ACE2 receptor usage. Our viral discovery curves **(Fig. 4)** suggest further sampling will reveal a rich diversity of as-yet-undiscovered SARSr-CoVs. In this aim we will use phylogenetic and viral discovery analysis to specifically target bat species and regions that are undersampled to allow sufficient power to identify and characterize missing SARSr-CoV strain diversity. Our previous work also suggests that SARSr-CoVs with S proteins that are ~10% divergent from SARS-CoV resist neutralization by therapeutic mAbs and escape SARS-CoV vaccines (*20, 60, 119*), suggesting that existing vaccines and immunotherapeutics could easily fail against future emerging SARS-like CoV. Moreover, viruses with 10-25% divergence in S proteins may bind to human cell receptors, but completely evade therapeutic and vaccine effects, and could therefore be a higher risk for public health. We will sequence the S proteins of novel SARSr-CoVs to prioritize viruses for experimental work in Aim 3 to test this hypothesis. Likewise, we will test the hypothesis that phylogenetic related zoonotic receptor binding protein genes of MERS-related, Ebola-related and Nipah/Hendra-related viruses will also program efficient entry via human ortholog receptors. For all novel virus strains, we will sequence the complete entry spike genes by amplifying overlapping fragments using degenerate primers as shown previously (*20, 59*). Full-length genomes of selected CoV strains (representative across subclades) will be sequenced using NEBNext Ultra II DNA Library Prep Kit for Illumina and sequenced on a MiSeq sequencer. PCR or MinIon sequencing will be performed to fill gaps in the genome. The full length spike gene sequences, including the amount of variation in the receptor binding residues that bind the appropriate human receptor will be used to select strains for Aim 3 experiments.

1.5. Assessing risk for spillover. 1.5.a. Capacity to infect human cells: We will use a series of cell cultures to assess the capacity of target viruses to enter human cells. For CoVs, filoviruses and henipaviruses, we will use primary human ciliated airway epithelial cells (HAE) and lung endothelial cell cultures from the lungs of transplant recipients represent highly differentiated human airway epithelium containing ciliated and non-ciliated epithelial and goblet cells, grown on an air-liquid interface for several weeks prior to use (*60, 61, 120*). We will prepare HAE and lung endothelial cultures from three different patient codes in triplicate in collaboration with the tissue procurement facility at the Cystic Fibrosis Center at UNC. Cultures will be inoculated with chimeric bat SARSr-CoVs or other test filoviruses or henipaviruses to assess efficient replication. At 72 hpi, cultures will be fixed for immunofluorescent staining using antisera to the SARS-CoV conserved nucleocapsid protein (N) or to structural genes of the other viruses (*62, 70*). SARSr-CoVs that differ significantly in S protein sequence (11-24%) from epidemic SARS-CoV yet replicate *in vitro*, will also be evaluated for sensitivity to neutralization in Vero cells by PRNT50 assays using broadly SARS-CoV cross reactive human mAbs S227.14, S230.15, S215.17, and S109.8 (*61, 121*) or monoclonal antibodies targeting the Ebola or Marburg E glycoprotein (Available through BEI), or Nipah or Hendra viruses (*122*). As controls or

Commented [PD33]: Kevin – who can write this bits?

Commented [PD34]: Linfa – can you give a v. brief description of these please, e.g.: "This includes 13 primary lung, 5 primary gut epithelial, and 1 primary liver cell line. In addition we have 12 immortalized cell lines, 7 of which are intestinal, and therefore suitable for CoVs. Cell lines are derived from insectivorous and frugivorous bats (genera……)"

Commented [BRS35]: Any antibodies available? If not we can synthesize them from published co-crystals.

Commented [EL36R35]: Yes, Broder has NiV and HeV G binding, characterized mAbs. Have been used in crystal structural/ephrin analyses

Commented [PD37]: Can someone answer Ralph's question please?

if antisera is not available, the S genes of novel SARSr-CoV or spike entry genes of other novel viruses under investigation will be inserted into VEE 3526 replicon vectors (VRP3526-S), packaged and used to vaccinate mice (*123*). Polyclonal sera against SARS-related viruses will be harvested and tested for ability to cross neutralize SARS-CoV, GD03, WIV-1, SHC014, WIV-16, other novel SARSr-CoV and HKU3-SRBD by PRNT50 assay (*61, 124, 125*). Using PRNT50 titers with sera (n=4 each) among these viruses, antigenic cartography (*126*) will allow comparison of antigenic vs. phylogenetic distance, identifying the transition at which SARSr-CoV strains escape SARS-CoV based vaccines, informing immunotherapeutic and vaccine design strategies (*127-129*). Similar approaches will be applied to novel MERS-related viruses, other CoV, filoviruses or henipaviruses. While most wildlife-origin viruses with unknown transmission potential or pathogenicity in people are BSL-2 or -3, cell culture for potentially BSL-4 pathogens will be undertaken at collaborating institute NEIDL. To facilitate this, duplicate samples in culture medium from animals PCR +ve for filoviruses will be shipped to NEIDL **(See letter of support)** for culture, characterization and sharing with other approved agencies including NIH RML. When appropriate of feasible, training opportunities at NEIDL for in-country staff will be given.

1.5.b. Host ACE2, DPPR, Niemann-Pick C1 (NPC1) and EphrinB2/EphrinB3 receptors: We will sequence host ACE2 receptors of different bat species or different bat populations from a single species (e.g. *Rhinolophus sinicus*) to identify relative importance of different hosts of high risk SARSr-CoVs and the *intra*specific scale of host-CoV coevolution. Of particular interest is homology across 18 bat and human orthologue ACE2 contact interface residues that engage the SARS RBD as a potential indicator of SARSr-CoV cross species transmission potential and growth in human cells (*69*). A lot of variation in the NPC1 receptor alters EBOV ability to infect in humans and bats, so this can be structurally mapped for prediction and downselection of strains (*130*). Signatures of positive selection in bat NPC1 were concentrated at the virus-receptor interface, with the strongest signal at the same residue that controls EBOV infection in *Eidolon helvum* cells. Thus NPC1 is a genetic determinant of filovirus susceptibility in bats, and some NPC1 variations likelyreflect host adaptations to reduce filovirus replication and virulence (*131*). Nipah and Hendra use ephrin B2 or ephrin B3 as a receptor, and variation affects its ability to use human vs bat receptor molecules (*102, 132*).

1.5.c. Host-virus evolution and distribution: We will use Bayesian and Maximum Likelihood phylogenetic analyses of RdRp (or L genes), receptor binding glycoproteins, and full genome sequence (when available) data to reconstruct the evolutionary history of the novel bat SARSr-CoVs, bat MERSr-CoV, filoviruses and henipaviruses that we identify. We will rerun MCC analyses **(Fig. 3)** to reconstruct β-CoV evolutionary origins using our expanded dataset. Ecological niche models will be used to predict spatial distribution of PCR-positive species, identify target sites for additional bat sampling, and analyze overlap with people. We will use our viral discovery/accumulation curve approach **(Fig. 4)** to monitor progress towards discovery of >80% of predicted diversity of SARSr-CoVs (lineage 1 & 2) or other virus families within species and sites, halting sampling when this target is reached, and freeing up resources for work other work (*96, 133*).

Commented [PD38]: Kevin/Alice – please edit this section to include relevance for filos and henipas as well (and other hosts)

1.5.d. Viral strain prioritization: Of the expected 100-200 novel SARSr-CoV strains, we will down-select to prioritize for further characterization based on S genes that are: i) different from SHC014, WIV1, SARS-CoV with diversity ranges of 10-25%; ii) have virus S RBD that could use human/bat receptors; iii) have recombinant chimeric spikes indicative of gene flow between clade I and II strains; iv) have bat ACE2 receptors that might select for spike RBDs that can use human receptors for entry (15/18 conserved residues in human/bat ACE2 molecules that bind SARSs-CoV S RBD domains are likely more efficient receptors than 3/18 conserved sites). Using structural models based on the SARS S glycoprotein, the extent and location of antigenic variation will be annotated onto the structure, further resolving the locations of highly evolving and conserved structural motifs/epitopes that function in protective immunity (*121, 134-136*). For novel henipaviruses, we will examine the receptor-binding proteins (e.g. envelope attachment (G) proteins) for sequence and structural homology with particular attention to conversation of amino acids that mediate ephrin receptor binding. We will prioritize strains that are predicted to interact with ephrin-B2 and expression V/W proteins to determine whether these viruses represent zoonotic threats. Using the recombinant Cedar virus (rCedV) platform, we will be able to rescue rCedV that express heterotypic G proteins to explore receptor utilization and tissue dissemination in animal models. We will assess ability to infect human cells using a

continuous lung epithelial cell line, Calu3, that our group has previously used to generate data on NiV and HeV growth curves and a variety of host expression changes after infection (*137*). We will also use primary human airway cultures to assess human infection for Henipavirus and MERS- and SARS-related CoV (*138*) (*139*) . There is some evidence for Nipah and Hendra virus replication in mouse lung, low level titers in young animals, 10 fold higher titers in aged mice (*140, 141*). Thus it is likely these models could be improved in the collaborative cross mouse resource at UNC. We have identified CC lines that are highly vulnerable to wildtype SARS and others that develop hemorrhagic disease following mouse adapted Ebola infections (*86-88*).

1.5.e. Animal models: We will use transgenic mouse models and the UNC Collaborative Cross model to assess spillover potential of viruses. For work on CoVs, we will follow the protocols in the transgenic mouse model used by the Baric lab previously. Briefly, in the BSL3 lab, n=8 10- to 20-week old hACE2 or hDPP4 transgenic mice will be intranasally inoculated with 5 x 10^4 PFU of wildtype WIV-1 or chimeric bat SARSr-CoVs with different spike proteins or MERS-CoV and MERS-related CoV, respectively, then monitored daily for weight loss, morbidity, and clinical signs of disease. Mice will be euthanized at 2, 4, and 6 dpi (endpoint of 14 dpi), organs harvested and virus quantified by NP RT-PCR. After 7 days in formalin, tissues for histopathology will be removed from the BSL3 and stained with H&E, and for immunohistochemistry using polyclonal antibodies against the N protein. We will conduct limited evaluation of existing countermeasures using therapeutic monoclonal antibodies *in vitro* and *in vivo*. Existing SARSr-CoV or MERS-CoV mAbs (*142, 143*) will be diluted in DMEM starting at 1:20, and serial 2-fold dilutions mixed with an equal volume of 50 PFU of chimeric bat SARSr-CoVs with different spike proteins, then incubated on Vero E6 cells at 37°C for 1 h, then changed to a semi-solid medium (DMEM containing 1% methylcellulose and 2% FBS). Antibody neutralization titers will be evaluated by plaque quantification at 4-5dpi. hACE2 transgenic mice will be injected with SARS-CoV mAbs, and infected with chimeric bat SARSr-CoVs. Clinical signs and morbidity will be assessed and tissue pathology examined and compared with mice without treatment of mAbs to determine the therapeutic effect on SARSr-CoV infection, and protection of SARSr-CoV by wildtype SARS-S based vaccines assessed as described (*120, 144*). We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-CoV cross neutralization, synthetically reconstruct a small subset (1-2) and evaluate the ability of nucleoside analogues to inhibit growth in HAE cultures and/or *in vivo* (*61, 120*). For the Collaborative Cross model, we will….

Finally, for more detailed pathogenesis studies in the natural reservoir, we will use the bat models (natural and transgenic mouse model) at Duke-NUS. Both animal systems can be used to test susceptibility, pathogenesis and immune responses of any newly discovered viruses.

1.6. Potential problems/alternative approaches: to sample bats in sites or provinces we select. We have a >20-year track record of successful field work in Malaysia, Thailand and >10 years in Singapore, and have worked with local authorities and communities to ensure access. We have existing permissions in place which will be renewed at the start of this project. Due to the abundance of wildlife habitats in the region, if access to a site is withdrawn, we will rapidly identify a suitable, complementary site with similar bat species composition and abundance. Finally, we have already collected tens of thousands of wildlife samples from the region and will focus on these while waiting for permission. **We may not identify novel viruses in our sample wildlife species due to seasonality of viral shedding or other factors**. We have calculated sample sizes based on a range of PCR data and are fairly conservative in our approach. However, as a back-up, we already have identified dozens of novel viruses that are near-neighbors to known high-impact agents, and will continue characterizing these should discovery efforts yield few novel viruses. The sampling regions we have selected are subtropical, and our previous data, and even published studies in temperate regions (*145*), do not suggest a strong pattern of seasonality in CoV shedding, although there are studies that suggest this occurs in henipavirus and filovirus (MARV?) shedding (REFS?). Nonetheless to account for this we will conduct sampling evenly on quarterly basis within each province.

Aim 2: Test evidence of viral spillover in high-risk communities using novel serological assays.

Commented [PD39]: Ralph – please insert a couple of sentences on how we'll use the cc mouse and what we'd expect

Commented [PD40]: Linfa/Danielle – could you put a couple of sentences in on what we might do with the models. Remember – this could be an additional short project from the EIDCC extra funding.

Commented [PD41]: Kevin – please edit/check

Commented [PD42]: Kevin – please check the veracity of these comments

2.1 Rationale/Innovation: Rapid response requires early detection. Thus, assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities that interface frequently with wildlife that harbor high risk emerging viral strains. To enhance the low statistical probability of identifying these rare events, we will target rural human communities inhabiting regions of high wildlife biodiversity, that also engage in practices that increase the risk of spillover (e.g. hunting and butchering wildlife). In Aim 2 we will build and expand on our preliminary biological sampling and enroll large cross-sectional samples in human populations with high behavioral risk of exposure to wildlife-origin viruses and that live close to sites identified in Aim 1 as having high viral diversity in wildlife. We will initially identify 2 sites in each of Thailand, Peninsular Malaysia, Sarawak, and Sabah for repeated biological sampling. We will design and deploy behavioral risk factor questionnaires, and specific and sensitive serological assays to identify the baseline frequency, and causes of, spillover of known and related novel viral pathogens in these populations. In participants where symptoms are reported in the last 10 days, paired serum specimens (acute and 21 days after fever) will be collected to determine titer of positive antibody. Samples will be marked for additional PCR-based assays to identify presence of known and novel CoVs, henipaviruses and filoviruses, and for isolation and biological characterization of potential pathogens, using the approach laid out in Aim 1.

Commented [KJO43]: Emily: Will we be sampling participants more than once?

Commented [PD44]: I think that would be good, depending on the sample size

Commented [KJO45]: Need to decide if we do PCR on community samples, we can add in the questionnaire if you've been sick in the last 10 days and if so, then maybe test only this subset.

Commented [PD46]: Kevin's comment makes sense to me

Country, site	Lead	# enrolled, focus	Findings
Peninsular Malaysia	Hughes	1,390 Orang Asli indigenous population, PCR/serol.	25+ novel CoVs, influenza, Nipah ab+ve, filovirus ab+ve
Malaysia Sabah	Kamruddin	1,283 serology	40% JE, 5% ZIKV ab+ve
Malaysia Sabah	William	10,800 zoonotic malaria	High burden of macaque-origin malaria
Malaysia Sabah	Hughes	150 bat cave workers	Ongoing
Malaysia Sarawak	Tan	500 Bidayuh and Iban people	8% PCR prevalence HPV in women
Malaysia PREDICT	Hughes	9,933 high zoonotic-risk communities, viral PCR, serology	Ongoing. Multiple known and novel CoVs, paramyxoviruses, others.
Thailand	Wacharaplu-esadee	1,000 bat guano harvesters/villagers	Novel HKU1-CoV assoc. clinical findings
Thailand PREDICT	Wacharaplu-esadee	9,269 high zoonotic-risk communities, viral PCR, serology	Ongoing. Multiple known and novel CoVs, paramyxoviruses, others.
Singapore	Wang	856, Melaka virus	7-11% ab+ve

2.2 Preliminary data on high-zoonotic risk human community surveillance: 2.2.a Biological sample collection in SE Asia: Our longterm collaboration in the region has included qualitative and quantitative survey data, and collecting large archives of banked human sera and other biological samples from high zoonotic-risk populations. For example, under the USAID-PREDICT project, EHA has collected 9,933 and 9,269 specimens in Malaysia and Thailand respectively from people living in zoonotic disease interfaces, all of which will be available for retrospective serological testing by EID-SEARCH. Our core group has collected and tested many thousand additional specimens from other important community cohorts **(Table 2)**, and some of these will continue under EID-SEARCH **(See section 2.4)**. Of particular interest is the access to indigenous communities in Peninsular Malaysia (Orang Asli) and Sarawak (Bidayuh, Iban people), and the bat guano harvesters in Thailand. These communities hunt, butcher and eat wildlife daily, and work in supremely high-risk zoonotic interfaces (e.g. bat caves). One of our indigenous community sites is experiencing an ongoing outbreak of unknown etiology that we are investigating currently **(See section 3.2.a)**.

Table 2: Current or recent biological sample collection from healthy populations conducted by members of our consortium in EID-SEARCH hub countries

2.2.b Human Contact Risk Factors: EHA is the lead organization across all 27 USAID-PREDICT project countries for collecting data on, and analyzing the human aspects of, zoonotic spillover risk (*146, 147*). We conducted exploratory studies using standardized one-on-one semi-structured interviews and site observation data with people living in hotspot areas or engaged in high-zoonotic spillover risk activities, using these data to assess local social and cultural norms underlying wildlife contact (*147*). We then developed a risk factor survey to accompany human biological sampling to evaluate the type and frequency of animal contact, wildlife

exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. We conducted cross-sectional studies in high risk populations in 27 countries, and study participants provided biological samples (oral/oropharyngeal swab, nasal/nasopharyngeal swab, urine sample/urogenital swab, fecal sample/rectal swab, whole blood, serum, plasma). For example, survey and biological samples were collected from: 1,585 participants from 7 sites in Yunnan, Guangxi, and Guangdong provinces, China; 1,400 participants from 4 sites in Malaysia; and 678 participants from 4 sites in Thailand. Samples were tested with novel serological assays and results analyzed against risk factors from survey data (*12*). In China, CoV-seropositive individuals were mostly farmers/peasants (8/9), living in Yunnan province (7/9), 41-60 yrs old (7/9), with domestic contact with rodents (6/9), that are male (6/9). Although these results are preliminary and don't provide detailed information on routes of exposure, they identify key subpopulations to target that would likely increase the opportunity for identifying evidence of spillover for other CoVs, paramyxoviruses and filoviruses. Data from PREDICT surveys will be used to help identify target populations in Aim 2, identify strategies to better target at-risk people, and conduct focused **human animal contact surveys and serosurveys to produce statistically significant findings for henipaviruses, CoVs and filoviruses.** In Aim 2, we will use combined biological sampling and risk factor surveillance in targeted populations within the community to 1) identify risk factors correlated with seropositivity to henipaviruses, filoviruses and CoVs; 2) assess possible health effects of infection in people; and 3) where recent illness is reported, obtain clinical samples for PCR testing and potential viral isolation and characterization. Obtaining this information could be a significant step in understanding the likelihood of recent 'hidden' spillover events and their public health impacts, as well as the risk of future emergence of viral disease.

2.2.c. Risk factors for illness: Our preliminary data from PREDICT in Thailand, Malaysia and China revealed that frequent contact with wild (e.g. bats, rodents, non-human primates) and domestic animals (e.g. poultry, swine) among local communities was associated with a high percentage of self-reported severe/acute respiratory illness (SARI/ARI) and Influenza-like illness (ILI) symptoms. In addition, people involved in crop production, hunting and butchering of wildlife reported statistically significantly higher SARI and ILI symptoms (Li *et al.*, in prep.). **These preliminary data suggest a target population and sites for current proposal with the correct targets for serological surveillance and risk factor surveys in Aim 2 to produce statistically significant findings**. Coupled with further refining serological tools, we will be able to identify the likely routes of exposure to known or novel coronaviruses, henipaviruses, and filoviruses in the study region, as well as build data on the illnesses they cause in people.

2.2.d. Serological platform for community surveillance: One of the present challenges in emerging disease surveillance is that viremia can be short-lived, complicating viral-RNA detection (i.e. traditional PCR-based approaches), especially in zoonotic reservoir hosts, and requiring large samples sizes to understand infection patterns or host range. By contrast, antibodies are typically long-lived and can be used to assess prior virus exposure or infection with much smaller samples sizes (*148*). However, most serological assays only target a single protein, and it's not always clear which antigen is the most immunodominant during an adaptive humoral immune response – i.e. which is the best target for a serological assay. Henipaviruses, filovirus and CoVs express envelope receptor-binding proteins (e.g. CoV=spike (S) protein, filoviruses/henipaviruses=glycoprotein (GP/G)), which are the target of protective antibody responses. The Broder lab at USU, a leader in the expression and purification of native-like virus surface proteins for immunoassay application (*149*)?, developing monoclonal antibodies (*150, 151*) and as subunit vaccines (*152, 153*), is presently developing a novel, multiplex microsphere immunoassay (MMIA) allowing serum samples to be simultaneously tested for antibodies reactive with henipaviruses, filoviruses and coronaviruses. This MMIA uses a Luminex-based multiplex platform and custom designed virus receptor-binding proteins expressed in a mammalian cell-culture system and purified as native-like oligomeric proteins ~~(149).~~ These oligomeric antigens retain post-translational modifications and quaternary structures. This structural advantage over peptide antigens, allows the capture of conformational-dependent antibodies raised during a natural infection, which represent the majority of the protective humoral response.

Commented [CB47]: #149 is OK, Yes. Add:

Chowdhury S, et al., PLoS Negl Trop Dis. 2014 Nov 20;8(11):e3302.

McNabb L, et al., J Virol Methods. 2014 May;200:22-8.

Baker KS, et al., J Anim Ecol. 2014 Mar;83(2):415-28.

Peel AJ, et al., PLoS One. 2012;7(1):e30346.

Brook CE, et al., J Anim Ecol. 2019 Mar 25. doi: 10.1111/1365-2656.12985.

Peel AJ, et al., Sci Rep. 2018 Mar 1;8(1):3859. doi: 10.1038/s41598-018-22236-6.

Laing ED, et al. Emerg Infect Dis. 2018 Jan;24(1):114-117.

Commented [PD48]: Chris/Eric Is this the right ref?

Commented [EL49R48]: Yes (until I finish a JVM manuscript) and add this reference: 15890907

Serological platforms have been historically limited by the starting antigen, and cross-reactivity between known and unknown related viruses not included in the assay. The known diversity of henipaviruses includes five presently described viruses, NiV, HeV, CedV, GhV and MojV and this assay has been expanded to include all described species **(Fig. XXX)**. In the past, our collaborators have used NiV and HeV G protein antigens in a MMIA assay to identify a serological footprint of NiV-related African henipaviruses and exposure to virus(es) antigenically-related to NiV in livestock sampled in Bangladesh (*77, 78*). To strengthen serological data interpretation, the G protein from each virus is integrated into the MMIA so that virus-specific versus henipavirus cross-reactive antibodies can be detected and address specificity of past virus exposure. Extensive cross-reactivity exists within ebolaviruses and between ebolaviruses and marburgviruses, highlighted by studies examining antibody binding between heterologous viruses (*154-156*). Ongoing work is aimed at validating the MMIA pan-filovirus assay for specificity. The majority of ebolaviruses are endemic to Africa, however the discovery of Měnglà virus in Yunnan, China increased the further demonstrated that a diversity of Asiatic filoviruses with unknown pathogenicity and zoonotic potential exists. Our past work in collaboration with Duke-NUS demonstrated that three under-sampled fruit bat species had serological evidence of past exposure to a filovirus(es) most antigenically-related to ebolaviruses (e.g. Ebola virus, Bundibugyo virus), but unreactive with Reston virus GP, suggesting that novel filoviruses circulate within bat hosts (*41*).

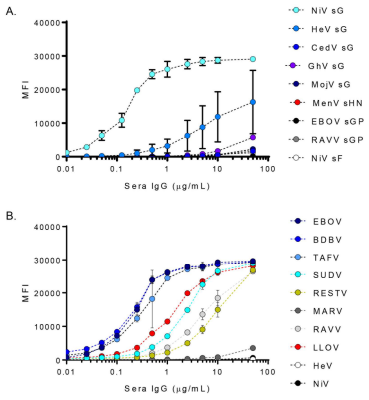

Fig. XXX: Validation of multiplex microsphere immunoassay (MMIA) specificity and identification of immunologically cross-reactive viruses for Nipah (A) and Ebola (B).

2.3 General Approach/Innovation: We will use a dual study design to gain in depth understanding of exposure and risk factors for viral spillover **(Fig. XX)**. In Aim 2 we will conduct community-based surveillance using a brief, focused questionnaire to identify the prevalence and frequency of risk-factors for viral spillover in tandem with collection of high quality biological samples to determine the seroprevalence of spillover viruses in at-risk human populations. We will use generalized linear models to analyze correlation between biological results and risk-factors for viral spillover. In Aim 3, we will conduct clinic-based syndromic surveillance and biological sampling at clinics/hospitals that are the catchment healthcare facilities for the communities sampled in Aim 2. Both community-based and clinic-based syndromic surveillance programs are cross sectional case-control studies designed with the sample sizes necessary to statistically quantify (with a power of 80%) risk factors and health impacts for viral spillover, linked to serological status and PCR prevalence in symptomatic patients.

2.4 Target population & sample sizes: We will target populations in the same geographic regions as those identified in Aim 1 that harbor wildlife with high potential zoonotic viral diversity, that are EID hotspots, and that are well-connected through regional travel and trade hubs to the global travel network **(Fig X)**. We will target specific communities based on our analysis of previously-collected PREDICT behavioral questionnaire data, to assess key at-risk populations and occupations with high exposure to wildlife, as well as using other publicly available data (e.g. wildlife hunting licenses sold in a region, number of guano caves accessed for fertilizer etc.). We will identify populations at two sites in each of the following regions, building on our preliminary data **(Table 2)**: *Thailand (Co-I Wacharapluesadee):* We will continue surveillance in bat guano harvesters and their villages in Ratchaburi province where we have identified MERS-related CoV in bat guano and rectal swabs (*48, 157*) and serological evidence of α-CoVs, HKU1-CoV in people (Wacharapluesadee in prep.). Our second site will be in Chonburi province where we have found Nipah virus (99% identity of whole genome sequence to NiV from a Bangladesh patient) in flying foxes but no outbreaks have yet been reported (REF), and have found several novel paramyxoviruses and CoVs have been found in bat feces roosting there (Wacharapluesadee in prep.).

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Commented [PD51]: Supaporn/Kevin/ -Need sample sizes for both populations and power calculations for serology for a typical virus from one of the three families we're targeting

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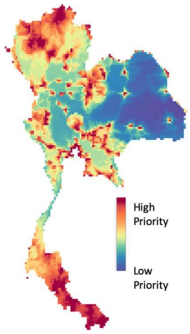

Fig. X: . Geographic analysis to target high-risk sites for human zoonotic disease surveillance in Thailand. Our spatial analysis from Aim 1 will be extended to optimize sites for human community surveillance in Thailand and Malaysia by ranking areas with high numbers of expected viruses in wildlife, greater spillover probability based on EID risk factors, and higher levels of connectivity (measured by road density data) that may facilitate person-person spread.

Peninsular Malaysia (Co-I Hughes, CM Ltd.): We will expand our sampling of the Orang Asli indigenous communities to include additional communities in the 3 Districts we have already sampled, and additional Districts in the States of Kelantan Perak, Pahang, and Kelantan. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife (mainly bats) will be sampled. Human samples will include serum, whole blood, throat swabs, nasal swabs, urine, and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and tested at NPHL. Participants who hunt, butcher or consume wildlife, or who rear animals will be considered suitable for enrolment. Participants will complete a consent form and questionnaire in addition to having samples collected. During these community trips the field team will conduct community meetings to help inform the Orang Asli about the public health risk of zoonoses. *Sarawak (Co-I Tan, UNIMAS):* We will expand our sampling of the Bidayuh and Iban indigenous people to include the Dayak and Orang Ulu ("people of the interior") because this group has a more intimate connection to remote wildlife-rich areas than the other two, albeit all are hunters. Human sampling will be led by UniMAS in collaboration with CM Ltd / EHA. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife (mainly bats) will be sampled, following the same protocols as for Peninsular Malaysia. *Sabah:* We will conduct sampling at the Madai bat and bird cave that has 200 permanent residents who harvest bird's nests for soup, and >1,000 during the harvesting season and at Gomantong cave – one of the largest bat and cave swiftlet nesting sites in the world. Protocols follow those for Peninsular Malaysia. *Singapore:* Active human surveillance will be not be conducted in Singapore, but archived human specimens e.g. from our Melaka virus serosurvey (*4*), will be made available to conduct retrospective serosurveys for specific pathogens that are identified as high interest to the EIDRC, NIH, and EID-CC.

Sample sizes: From our previous work we anticipate that 10-30% of the community population will have had exposure to wildlife allowing us to capture highly exposed and non-exposure individuals at each site. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock farming, transportation, selling, or slaughtering wildlife in the surveyed areas will be targeted so that they make up ≥30% of the sampled population in each community. We will stratify sampling to ensure appropriate representation of sex, demographic, and socio-economic factors at each community site. Estimating 5% overall seroprevalence in these high-exposure populations, these sample sizes are sufficient to estimate effect sizes of risk factors of 2X or greater with 80% power. For community-based surveillance, we will enroll xxxx individuals per county/province, pooled across two sites for each, allowing us to make county/province-level comparisons of differing effects.

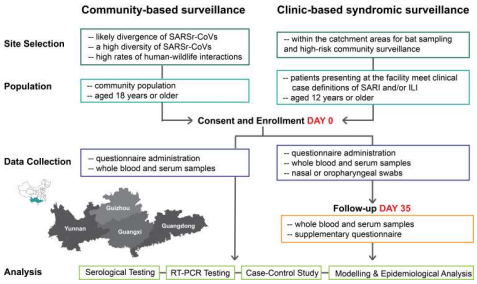

Fig. XX: Human survey and sampling study design overview (Aim 2 and 3)

2.5 Data & sample collection: Following enrollment of eligible participants and completion of the consent procedure, biological specimens (5ml will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 2.5ml whole blood; two 500 µL serum samples) will be collected and a questionnaire will be administered by trained project staff that speak appropriate local language to all participants. Through the serological findings and the survey we will investigate the details of five risk factors related to high risk wildlife exposure, to maximize the power of the analyses, based on continuing analysis of our previous work, and will focus on: 1) occupation and occupational exposures; 2) observed or reported

Commented [PD53]: Need this figure for Singapore, Pen. Malaysia, Sarawak, Sabah as well – all together is best

Commented [PD54]: Hongying– this figure is from our CoV R01 renewal. Please modify to make relevant to SE Asia and henipas, filos and CoVs.

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interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With additional consent considerations for participants enrolled from clinical settings we will review clinical records to collect recent data on medical history, clinical syndromes, and patient etiology.

2.6: Laboratory analysis: 2.6.a Serological testing:

In a previous R01, we expressed his-tagged nucleocapsid protein (NP) of SARSr-CoV Rp3 in *E.coli* and developed a SARSr-CoV specific ELISA for serosurveillance using the purified Rp3 NP. The specificity of this assay was evaluated using polyclonal antibodies against HKU1, OC43, 229E, NL63, MERS-CoV and EBOV and no significant cross-activity detected (*12*). **This suggests that if we can expand our serology tests to cover other bat CoVs, and that doing so may identify many more seropositive individuals.** We will therefore use two serological testing approaches. First, we will expand test all human sera collected from both community- and clinic- based sampling for a panel of bat CoVs that will include SARS-CoV (outbreak strain), a range of lineage 2 bat SARSr-CoVs (including WIV1 and SHC014), lineage 1 HKU3r-CoVs, MERS-CoV, and the α-CoVs SADS-CoV and HKU10. We previously found serological evidence of human exposure to HKU10 (*12*), and HKU10 is known to jump from one host bat species to another (*158*) so is likely to have infected people more widely. It is possible that non-neutralizing cross reactive epitopes exist that afford an accurate measure of cross reactivity between clade1 and clade 2 strains which would allow us to target exposure to strains of 15-25% divergence from SARS-CoV, at a phylogenetic distance where therapeutic or vaccine efficacy may falter. Additionally, we will test samples for antibodies to common human CoVs (HCoV NL63, OC43 – see potential pitfalls/solutions below). Secondly, CoVs have a high propensity to recombine. To serologically target 'novel' recombinant virus exposure, we will conduct 1) ELISA screening with SARSr-CoV S or S1 (n-terminal domain); 2) confirm these results by Western blot; then 3) use NP based ELISA and LIPS assays with a diversity of SARSr-CoV NP. For NP ELISA, micro-titer plates will be coated with 100 ng/well of recombinant batCoV NP and incubated with human sera in duplicates followed by detection with HRP labeled goat anti-human IgG antibodies. For confirmation, all ELISA positive samples will be subjected to Western blot at a dilution of 1:100 by using batCoV-NP as antigen. We will use an S protein-based ELISA to distinguish the lineage of SARSr-CoV. As new SARSr-CoVs are discovered, we will rapidly design specific Luciferase immunoprecipitation system (LIPS) assays targeting the S1 genes of bat-CoV strains for follow-up serological surveillance, as per our previous work (*13*). Additionally, in Thailand, as an initial project on our EIDRC, we will develop serology assays to the novel CoVs found in bats at the bat guano site using LIPS assays and viral genomic data, and will screen archived specimens from 230 participants (2 years' collection in 2017-2018, a subset of the subjects have been sampled at multiple timepoints to allow us to assess seroconversion). To complement ELISA and LIPS assays we will also simultaneously screen sera for the presence of antibodies specific to henipaviruses, filoviruses and select coronaviruses using a Luminex-based platform with a multiplex microsphere immunoassay (MMIA). This proposed serological approach for biosurveillance is based on the detection, or capture, of serum antibodies specific to or cross-reactive with virus envelope receptor-binding glycoproteins from all presently described species of filoviruses (e.g. Ebola virus), henipaviruses (e.g. Nipah virus) and bat-borne SARS-CoV. The Broder lab produces soluble native-like, oligomeric virus glycoproteins that retain quaternary structures and retain conformational epitopes that can be used to detect conformational-dependent antibodies generated during a humoral immune response to viral infections. These virus glycoproteins are coupled to microspheres and combined into a multiplex immunoassay utilizing Luminex-based machine technology so that sera can be simultaneously tested for antibodies reactive with all 20 virus antigens included in this pan-filovirus/henipavirus/coronoavirus immunoassay. Oligomeric antigens in combination with a MMIA have advantages in specificity and sensitivity compared to linear or monomeric antigens and traditional enzyme-linked immunosorbent assays (ELISAs), and can enhance and complement nucleic-acid based biosurveillance programs. Control sera samples for each virus antigen will be run in parallel to field sample screening to establish the expected positive and negative serological cutoffs and virus-specific antibody reactivity. Statistical approaches will be utilized to determine population level seroprevalence.

Commented [PD56]: Chris, Eric, Linfa, Dani etc. please draft some language here…

Commented [EL57]: Not sure if this is redundant from before

2.6.b RT-PCR testing. Specimens will be screened using RT-PCR for the RdRp gene (See section 1.3.b for details). Positive samples will be subjected to full genome sequencing and RT-PCR amplification of the S glycoprotein gene. Samples from the clinic-based syndromic surveillance will also be tested using RT-PCR for Influenza A & B, HCoV NL63, OC43, HKU1, SARS-CoV & 229E as rule-outs, and SADS-CoV and HKU10 as an opportunistic survey for potential spillover these CoVs as proposed above.

2.6.c Biological characterization of viruses identified: Novel viruses identified in humans will be recovered by cultivation or from full length genome sequences using reverse genetic approaches. Viruses will be characterized for growth in primary human cell lines and in mouse models, including the Collaborative Cross Mice or humanized mice expressing human receptors from related strains. The approaches are discussed in more detail under Aim 1.

2.7 Epidemiological analysis: We will conduct a cross sectional case-control study to identify risk factors for Henipavirus, Filoviruses, and CoVs spillover. "Cases" are defined as participants whose samples tested positive for Henipavirus, Filoviruses, or CoVs by serological tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative for Henipavirus, Filoviruses, or CoVs. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between biological results, serological/PCR status, and risk factors related to high risk wildlife exposure including: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) home or work proximity to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms lifetime and past 12mo. Additionally, we will use this procedure to determine how clinical presentation differs between Henipavirus, Filoviruses, or CoVs in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms.

2.8 Potential problems/alternative approaches: Rarity of spillover events means it may be difficult to identify sufficient seropositives to statistically analyze human risk profiles. First, we will be targeting our community-based surveillance to subpopulations with high-levels of wildlife exposure, at sites selected for diverse and prevalent viruses to increase likelihood of finding positive individuals. Second, our serology testing will include a panel of assays for a large diversity of viruses, increasing likelihood of positives. For example, in previous work our overall bat CoV PCR prevalence in the community was, 11.8%; β-CoV, 3.4%; α-CoV, 9.1%). Thus, using this broad serological panel to screen individuals prone to contact with species of higher zoonotic risk increases the potential for detecting spillover with enough power for statistical analyses, and will shed light on behaviors that predispose to spillover. Third, we will include common human CoVs, paramyxoviruses, and influenza viruses in our panel, so that even if low prevalence of wildlife viruses is found, we will be able to conduct a valuable cross-sectional study of the seroprevalence of more common human viruses. Finally, we will be able to assess relative measures of human-wildlife contact from our survey work. We will analyze intensity of contact with high-risk taxa against other risk factors to provide useful proxy information for spillover risk. **Serological testing may not match known viruses due to recombination events, particularly for CoVs.** We will use the three-tiered serological testing system outline in **2.6.a** to try to improve specificity of detecting and interpreting the presence of antibodies that bind to known viruses targeted in this project. Furthermore, we must also consider that ~~dentify these~~ 'novel' viruses likely exist and that exposure to these viruses generates antisera that is cross-reactive with known, related virus antigens, which is a serious challenge of serological surveillance and diagnostics. Our collaborations have already demonstrated that SE Asia bat sera samples screened with our pan-filovirus MMIA have been exposed to an Ebola-like virus, that is antigenically-distinct from known Asiatic filoviruses, Reston virus and Mengla virus (**Figure XXX**) (Laing, Mendnehall 2018).

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Commented [EL60]: Why highlight a very specific challenge for only CoVs?

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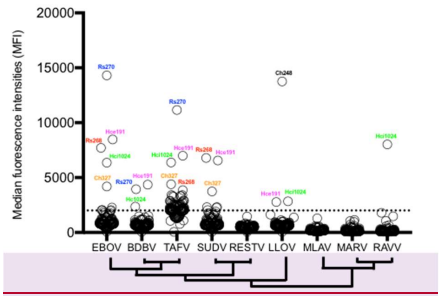

Figure XXX. Serolgoical evidence of 'novel' Ebola-like virus in three bat genera sampled in Peninsular Malaysia. Colors indicate likely cross-reactivity across related filovirus antigens within the same bat serum samples (Hughes et. al. in prep)

Using these three serological platforms we will be able to address inherent challenges of indirect virus surveillance through antibody capture by testing multiple binding assay platforms and antigens and using positive control samples to establish serological profiles against known viruses that will aid in our interpretations of cross-reactive antisera responses likely representative of 'novel' viruses.

, however, we will also remain flexible on interpretation of data to ensure we account for recombination.

Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts.

3.1 Rationale/Innovation: Our previous research **(Table 1)** and other data suggests that there are substantial numbers of outbreaks or clinical cases for which the etiologic agent is not diagnosed, e.g. (*159, 160*). To investigate this in SE. Asia, we will work directly with clinics and outbreak control organizations to identify the causes of some 'cryptic' outbreaks or cases in the region. Specifically, we will expand on current syndromic surveillance activities to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and who present at regional clinics or hospitals with symptoms typical of high impact zoonotic viruses. We will collect detailed survey data to assess their contact type and frequency with wildlife and livestock and conduct molecular and serological diagnostic assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. We conduct novel viral discovery assays on patients who present with symptoms indicative of a high-risk zoonotic virus. If novel pathogens are identified by PCR, we will attempt to isolate, obtain full genome sequence and/or biologically characterize the pathogen, using the *in vitro* and *in vivo* strategy laid out in Aim 1.

3.2 Preliminary data clinical surveillance: 3.2.a. Clinical surveys and outbreak investigations: Our longterm collaboration in the region has included the following activities investigating clinical syndromes: *Peninsular Malaysia*: **At the time of writing, there is an outbreak of suspected viral undiagnosed illness in the indigenous Batek Orang Asli ("people of the forest") community living at Gua Musang ("civet cat cave") district, in Kelantan, Malaysia, close to Taman Negara – a National Park containing the oldest known rainforest**. This community is one where we have conducted prior routine surveillance and biological sampling, demonstrating proof-of-concept that our two-pronged high-risk community/clinic approach will work. Dozens of people are affected and the outbreak is currently being investigated by our collaborators at the Malaysian NPHL. The Orang Asli live in remote villages in the rainforest of Malaysia, with limited access to modern health care and extensive contact hunting, butchering, and eating wildlife as their primary source of protein. This outbreak, and others that will continue to emerge just like it, underscore the critical need for our EIDRC - to improve the technological tools, training, and human capacity for rapid EID surveillance, diagnosis, and response at the frontlines of disease emergence in SE Asia. **Investigating this outbreak be a key priority if EID-SEARCH is funded.** *Sabah*: Co-I Kamruddin (BMHRC) is leading an asymptomatic leptospirosis study on sanitation workers, an ILI study in a Kota Kinabalu clinic and diarrheal study in children under 5 yrs. Co-Is William, Tan and others have conducted clinical observational studies to determine the etiology of central nervous system infections and acute undifferentiated febrile illness in Sabah. Co-Is Tan,

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Commented [KJO61]: Make this more general. There's a greater challenge of interpretation of serological data, esp. if we're using many platforms (each will have its own problems that our lab colleagues are probably more familiar with). Noam may be able to generally comment on a solution here from a statistical perspective.

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William have collaborated with Co-I Wang to enroll 90 adult and over 100 pediatric participants with central nervous system infections, negative tests for malaria and dengue from 3 district hospitals in Sabah to determine the various etiologies. While a high proportion tested positive for rickettsial agents, the majority had no clear diagnosis. *Sarawak:* Dr Tan (UNIMAS) is conducting a survey of high-risk female Kelabit indigenous people and has found 8% with HPV52 as the dominant strain. He is working on clinical cohorts of hand, foot and mouth disease and will recruit patients from that study in the current proposed work. Finally, he is conducting a longterm study of rotavirus and other viral etiologies of acute gastroenteritis in Kuching, Sarawak that will include swine herders and farmers. These are an important group in Malaysia because pig farms are shunned by the government and local villagers to they are placed far away from town centers, deep in the forest, and it is this scenario that led to the emergence of Nipah virus in 1998-9 (*18, 161*). *Thailand:* The Chulalongkorn Univ. TRC-EID laboratory works in coordination with the Thailand Ministry of Health to investigate outbreak etiology, including the first case of imported MERS-CoV in Thailand, **for which we produced sequence confirmation within 24 hours from acquiring the specimen (Fig. X)**. Three imported MERS cases have been detected in Thailand since 2015, and all cases were confirmed at the TRC-EID. We worked during the Zika outbreak to identify > 500 PCR-positive Zika patients with sequence confirmation. We have also worked on returning and suspected Ebola travelers, syndromic surveillance for SARI, Encephalitis, Hand Foot and Mouth disease in children and Viral diarrhea. Working with the US CDC (Thailand), between 1998 and 2002 we enrolled a large cohort of children from Kamphaeng Phet Province to examine Dengue virus and JE epidemiology (*162, 163*). Among the 784/2,574 convalescent sera Henipavirus-specific antibodies were detected in 13 serum samples (1.7% seroprevalence), suggesting prior exposure to endemic henipaviruses or a related paramyxovirus in rural Thailand. *Singapore:* Duke-NUS has worked with the Ministry of Health to investigate Zika cases (*164*), and with EcoHealth and others, Duke-NUS has developed novel CoV assays for clinical surveillance, and for expedited analysis during outbreaks. Our pipeline of novel virus detection, reservoir host identification, serological assay development, and serological testing is as rapid as several months, with the development of novel serological assays from sequence data in 7 days (*13*). **For example, with the discovery of SADS-CoV, we identified and fully sequenced a new virus, screened archived wildlife specimens to identify the natural reservoir host species (*Rhinolophus* spp. bats), developed LIPS serological assays, and tested human and swine samples to assess seroprevelance in less than 3 months (*13*).**

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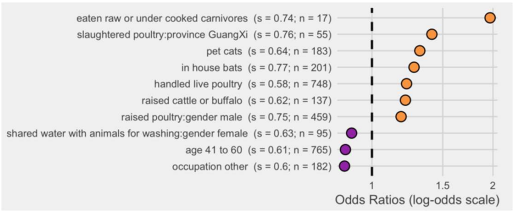
3.2.b Analysis of self-reported illness: In addition to biological outcomes we have developed a standardized approach in PREDICT for analyzing data on self-reported symptoms related to targeted illnesses; of fever with cough and shortness of breath or difficulty breathing (severe acute respiratory illness - SARI), fever with muscle aches (fevers of unknown origin (FUO) and, cough or sore throat (influenza-like illness - ILI) from study participants. We used a least absolute shrinkage and selection operator (LASSO) regression to identify associations between participants who reported ILI and/or SARI symptoms and demographics, occupation, and contact with animals in the last year. Results have clear biological relevance. In Yunnan, China, salient associated variables or combination of variables were, in descending order of odds ratio: 1) having eaten raw or undercooked carnivores; 2) having slaughtered poultry and being a resident of Guangxi province; 3) having had contact with cats or bats in the house; and 4) being a male who has raised poultry **(Fig. 11).** We will expand this approach for all syndromes in Aim 3, and use more granular targeted questions designed to assess patient's exposure to wildlife in terms that are relevant to the specific country.

Fig. 11: Associated variables of self-reported ILI and/or SARI in prior 12 months (s = bootstrap support; n = number +ve out of 1,585 respondents). **Orange circles** = odds ratios > 1 (positively associated with the outcome); **purple** = odds ratios <1 (negatively associated with the outcome).



3.3 General Approach: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent is not identified. To capture these, we will expand on our current syndromic surveillance to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and present at regional clinics with influenza-like illness, severe respiratory illness, encephalitis, and other symptoms typical of high-impact emerging viruses. We will collect survey data tailored to the region to assess contact with wildlife, conduct molecular and serological assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. If novel pathogens are identified by PCR, we will isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work. We will then use behavioral survey data, ecological and phylogeographic analyses to assess the likely reservoir hosts to inform intervention programs.

3.4 Clinical cohorts. 3.4.a Cohort selection: We will initiate active clinic-based syndromic surveillance at 2 town-level level clinics and 2 provincial-level hospitals in Thailand, Peninsular Malaysia, Sabah and Sarawak. These all serve as the catchment healthcare facility for people in our community-based surveillance of Aim 2 and are inclusive of wildlife sampling sites in Aim 1. Patients ≥ 12 years old presenting at the health facility who meet the syndromic and clinical case definitions for SARI, ILI, encephalitis, fevers of unknown origin, or hemorrhagic fever will be recruited into the study. We will enroll a total of xxxx individuals for clinical syndromic surveillance, which accounts for up to 40% loss from follow-up. Study data will be pooled across country sites, as clinical patients are limited by the number of individuals presenting at hospitals. Sites: *Thailand:* XXXXX *Peninsular Malaysia:* Focusing immediately on the current outbreak in Orang Asli, we will work closely with the Malaysian Ministry of Health to support their investigation. We will also work with 6 Orang Asli clinics which focus solely on this indigenous community. *Sarawak:* XXXXXX *Sabah:* XXXXXXXXX. *Singapore:* XXXXX

3.4.b Behavioral and clinical interviews: 2.5 Clinic enrollment and follow-up: We will recruit inpatients and outpatients to participate in the study after initial screening to determine if they meet the clinical case definition of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever of unknown etiology; or 6) severe diarrhea. Once enrolled, biological samples will be collected and a questionnaire administered by trained hospital staff that speak appropriate local language. Samples will be taken concurrently when collecting samples for normative diagnostics. For inpatients, samples will be collected no more than 10 days of reported onset of illness to increase the chance of PCR detection of viruses (*165*). We will follow up 35 days after enrollment to collect an additional two 500 µL serum samples conduct a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. 35 days gives adequate time for development of IgG, which occurs <28 days after onset of symptoms for SARS patients (*166*). These samples may prove valuable to collaborators in that the PBMC could be used in production of therapeutic antibodies.

3.4.c Sampling:

Following enrollment with signed consent form, biological specimens (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples) including two nasal or oropharyngeal swabs will be collected from all eligible participants, and a questionnaire will be administered. We will investigate five risk factors, to maximize the power of the analyses, all related to high risk wildlife exposure, based on continuing analysis of our previous work, and will include: 11) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With permission from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology.

3.5 Sample testing: PCR, Serol to link symptoms to etiologic agents

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The standard syndromic diagnostic PCR assay for the common pathogens will be conducted and the results will be shared to the hospital with 48 hours. Specimens will be further tested for 3 viral families; CoV, PmV and filovirus by consensus PCR. Appropriate specimen from dead case will be further conducted by NGS if the previous PCR tests are negative to identified cause of infection. The serology panel assay will be conducted from paired serum.

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3.5 Assessing potential for pandemic spread: We will use statistical models built from collated biological, ecological, and genetic data to predict the likelihood of human infection (or spillover) and pathogenicity for a the range of diverse viruses we will characterize. The aim is to quickly identify and assess which viruses pose the most significant risk to human health and thus prioritize additional resources to more fully characterize, better understand pathogenicity, and invest in therapeutic development for those pathogens. We will combine two independent but related statistical models to predict pathogen spillover risk and pathogenicity. First, we will expand a simple generalized additive modeling approach (*91*) to assess spillover risk probability to a new virus that is currently not known to infect people, when we have only limited genetic data from initial PCR screening and sequencing. Additional data from receptor binding protein homology and cell line infection experiments can be incorporated into the model framework as available. Second, given high likelihood to spillover and infect humans, or direct evidence of human infection from our clinical surveillance, we will then predict pathogenicity using nearest neighbor phylogenetic analyses as a first approximation and by incorporating more detailed data from genomic characterization and animal model experiments. For the pathogenicity model, human epidemiological data for ~300 viral species known to infect people (case fatality rate, global estimates of number of human cases per year or decade, R_0, infectious period, and primary symptoms) will be aggregated including from previous reviews [Woolhouse ref] to use as predictor variables. Thirdly, we will build off EHA's previous DTRA and DHS supported research to predict pandemic spread for viurses we identify by integrating surveillance site data, global flight models (*167*), as well as additional datasets (road networks, shipping routes, cell phone data) to measure human movement and connectivity across Southeast Asia.

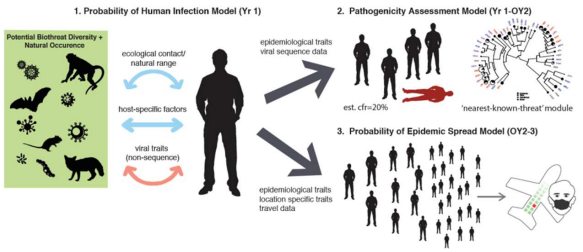

Fig. XXXXX: Conceptual framework highlighting 3 underlying models to estimate: **1)** probability of human infection, or spillover, the first necessary step to understand pathogenicity when status of human infection is unknown; **2)** pathogenicity, i.e. probability of causing clinical signs, case fatality rate estimates, and other metrics relevant to humans; and 3) probability of pathogen spread based on ecological, epidemiological, and airline connectivity and human migration data.

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3.7 Potential problems/alternative approaches: Patients visiting clinics may have cleared virus, but not yet developed IgG antibodies, reducing seropositive cases. Our 35 day follow-up sampling should avoid this because the maximum lag time between SARS infection and IgG development was ~28 days (*165*). We also expect that patients in rural communities will only visit clinics when symptoms have progressed, likely coinciding with late illness and onset of IgG. We will also have data from our community study, so won't be completely reliant on hospital data to identify PCR- or seropositives. Finally, the risk is outweighed by the potential public health importance of discovering active spillover of a new henipavirus, filovirus or CoV infection.

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Additional Instructions – Specific to the EIDRC FOA:

4. Administrative Plan

In a clearly labeled section entitled **"Administrative Plan":**

- Describe the administrative and organizational structure of the EIDCRC Administrative and Leadership Team. Include the unique features of the organizational structure that serve to facilitate accomplishment of the long range goals and objectives.

Chula TRC-EID is National reference laboratory for diagnostic and confirmation of EIDs pathogen along with the National public health laboratory at the Department of Medical Science and the WHO Collaborating Centre for Research and Training on Viral Zoonoses. We have built a EID laboratory network in-country among three Ministry including Ministry of Public Health (Department of Medical Science and Department of Disease Control), Department of Livestock and Development, and Department of National Parks, Wildlife and Plant Conservation and Universities and among SEARO countries. Our clinicians provide case consultation for Thailand and SEARO countries. The laboratory and clinic training are regularly conducted.

For Malaysia –
NPHL – Molecular and serological screening (BioPlex) of PM Orang Asli samples, serological screening of syndromic samples from Sabah (already approved) and Sarawak (would need approval)

PERHILITAN molecular zoonosis laboratory, at the National Wildlife Forensic Laboratory - Molecular and serological screening (BioPlex) of PM wildlife samples

UPM Faculty of Veterinary Medicine - Molecular and serological screening (BioPlex) of PM livestock samples

KKPHL - Molecular of syndromic samples from Sabah (already done) and Sarawak (would need approval)

SWD WHGFL - Molecular screening of Sabah wildlife samples

BMHRC - Molecular and serological screening (BioPlex) of Sabah livestock samples, serological screening (BioPlex) of Sabah wildlife samples, Molecular and serological screening of syndromic samples from Sabah (already approved) and Sarawak (would need approval). BMHRC will need renovations and BioPlex to do this work – Menzies team might have funds for BioPlex that they would base here as part of DTRA project.

- Describe how communications will be planned, implemented, and provided to collaborators, teams, or sites. In addition, describe plans to achieve synergy and interaction within the EIDRC to ensure efficient cooperation, communication and coordination.
- Specifically address how the EIDRC Team will communicate with other EIDRCs, the EIDRC CC and NIAID and how the EIDRC will implement plans and guidance from the EIDRC CC to ensure coordination and collaboration across the Emerging Infectious Disease Program as a whole. Describe how the study site will provide information, if any, back to the local health authorities and the study subjects as needed. Note any adjustments or changes needed for rapid and flexible responses to outbreaks in emerging infectious diseases.

List out all of our involvement with outbreak investigations, ministries of health surveillance etc.
If possible the clean or summarized answers from the questionnaire should be shared to the hospital Infectious Control team and Bureau of Epidemiology for their information for controlling the outbreak.

- Describe the overall management approach, including how resources will be managed, organized and prioritized, including pilot research projects.
- Highlight how the proposed staffing plan incorporates scientific and administrative expertise to fulfill the goal to develop the flexibility and capacity to respond rapidly and effectively to outbreaks in emerging

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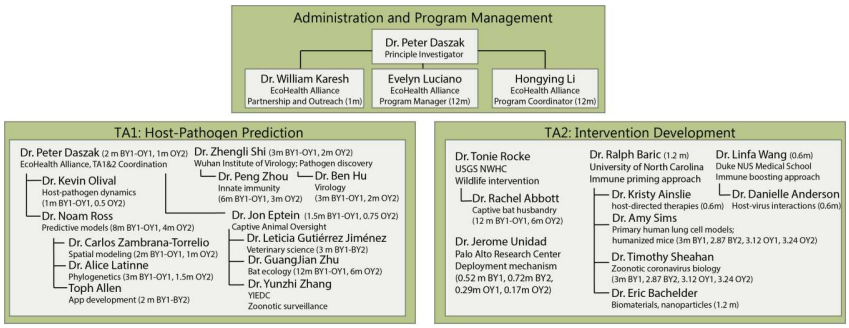
infectious diseases in an international setting.

CM/EHA helped establish and manages with SWD the Wildlife Health, Genetic and Forensic Laboratory (certified as a BSL- 2 laboratory according to the US standard for laboratory specifications) that has all the equipment necessary to store samples, run extractions, PCR and analysis on biological samples for wildlife disease surveillance. The lab is used to conduct health checks on rescued and relocated wildlife before being released into new areas or sanctuaries, to screen samples for the PREDICT and Deep Forest Project and for genetic research and forensic investigations. CM/EHA also helped establish the new molecular zoonosis laboratories (certified as a BSL- 2 laboratory according to the US standard for laboratory specifications), at DWNP's National Wildlife Forensic Laboratory. The lab is used to screen samples for the PREDICT & DTRA projects. In addition CM/EHA also has access to NPHL and KKPHL for screening human samples and the Virology lab at FVMUPM for screening Livestock samples.

The Borneo Medical and Health Research Centre (BMHRC) is a centre of excellence that conducts teaching and scientific researches on communicable diseases, outbreak investigations, and ethnomedicine. BMHRC has offices space including a meeting room and seven laboratories which including preparation lab, parasitology lab, cell culture lab, bacteriology lab, natural product lab, virology lab, and molecular biology lab. BMHRC is supported by an administration staff, medical lab technologist, and research assistants who are available to work on projects. Through this project we will help to establish a certified BSL-2 lab at the BMHRC. This lab can then be used for human testing to elevate pressure at KKPHL and QEH labs. It would also be an ideal location for a Luminex platform as human, wildlife and livestock samples could all be screened here. Co-Is Hughes and Kamruddin will be starting outbreak response training in August 2019 with a team from Sabah CDC, KKPHL and BMHRC. (Dr. Maria Suleiman now in PM But still collaborating with us, Dr. Muhammad Jikal current Director CDC working with us and Dato' Dr Cristina Rundi Director Sabah state health Dept is supportive) this collaborative group is working to develop a Sabah outbreak response team that will help support SSHD/ CDC outbreak team and also conduct sampling for disease surveillance to be based at BMHRC and we will develop this as part of this proposal. In addition the UMS hospital will come in line in 2021 providing additional opportunities for cohort studies and capacity building.

FROM DARPA grant:

Section 1.03 MANAGEMENT PLAN



Project DEFUSE lead institution is EcoHealth Alliance, New York, an international research organization focused on emerging zoonotic diseases. The PI, Dr. Daszak, has 25+ years' experience managing lab, field and modeling research projects on emerging zoonoses. Dr. Daszak will commit 2 months annually (one funded by DEFUSE, one funded by core EHA funds) to oversee and coordinate all project activities, with emphasis on modeling and fieldwork in TA1. Dr. Karesh has 40+ years' experience leading zoonotic and wildlife disease projects, and will commit 1 month annually to manage partnership activities and outreach. Dr. Epstein, with 20 years' experience working emerging bat zoonoses will coordinate animal trials across partners. Drs. Olival and Ross will manage modeling approaches for this project. Support staff includes

Commented [KJO75]: Figure like this would be good, but instead of breaking down by TA, maybe by country or Specific Aim. Per FOA, Need to also show how we'll link in with the Coordinating Center and other EIDRCs.

a field surveillance team, modeling analysts, developers, data managers, and field consultants in Yunnan Province, China. The EHA team has worked extensively with other collaborators: Prof. Wang (15+ yrs); Dr. Shi (15+ yrs); Prof. Baric (5+ yrs) and Dr. Rocke (15+ yrs).

Subcontracts: #1 to Prof. Baric, UNC, to oversee reverse engineering of SARSr-CoVs, BSL-3 humanized mouse experimental infections, design and testing of targeted immune boosting treatments; **#2** to Prof. Wang, Duke-NUS, to oversee broadscale immune boosting, captive bat experiments, and analyze immunological and virological responses to immune modulators; **#3** to Dr. Shi, Wuhan Inst. Virol., to conduct PCR testing, viral discovery and isolation from bat samples collected in China, spike protein binding assays, humanized mouse work, and experimental trials on *Rhinolophus* bats; **#4** to Dr. Rocke, USGS NWHC, to refine delivery mechanisms for immune boosting treatments. Dr. Rocke will use a captive colony of bats at NWHC for initial trials, and oversee cave experiments in the US and China. **#5** to Dr. Unidad, PARC, to develop innovative aerosal platform into a field-deployable device for large-scale inoculation of the bats. Dr. Unidad will collaborate closely with Dr. Rocke in developing a field deployable prototype for both initial trails and cave experiments in China.

Collaborator Coordination: All key personnel will join regularly-scheduled web conferencing and conference calls in addition to frequent ad hoc email and telephone interactions between individuals and groups of investigators. Regular calls will include:

- Weekly meetings between the PI and Program Manager (on project and task status)
- Weekly web/phone meetings between Program Manager and subawardee admin. staff
- Monthly web/phone conferences between EHA PIs and all subawardee PIs.
- Monthly web conferences between key personnel (research presentations/coordination)
- Four in-person partner meetings annually with key personnel at EHA and two in-person partner meetings annually between subawardees.
- Annual in-person meeting among all key personnel

Evaluation metrics will include the generation of high-quality data, successful achievement of milestones and timelines, scientific interaction and collaboration, the generation of high quality publications, and effective budget management. The PI and subawardee PIs will attend a kickoff meeting and the PI will meet regularly with DAPRA at headquarters and at site visits.

Problem identification and resolution: Regular planning, monitoring, and evaluation meetings will be the primary mechanisms for problem identification. Minor problems (e.g. delays in sample availability or test results) will be dealt with internally through appropriate action and resolution monitored by Dr. Daszak and the Project Manager. In the event of significant problems, such as prolonged poor productivity, inadequate scientific collaboration, or major disputes regarding research direction or resource allocation, EHA will assist with resolving the problem through negotiation. Should a resolution not be forthcoming, consultation with our technical advisors and DARPA Program staff may be warranted.

Risk management: Maintaining a timeline and meeting milestones will require strict and continuous oversight of all project phases, frequent and regularly scheduled communication, and the ability to make decisions and implement strategies. A project of this nature requires a different mindset from that typically associated with basic research activities that move at an incremental pace with investigators gradually optimizing experimental systems, refining data, or waiting for additional data before moving ahead with an analysis approach. To maintain our timeline, we will continually evaluate these trade-offs to make decisions about when iteration is appropriate and when necessary to move forward with current information.

Biographies:

Commented [PD76]: Some of references in this section are duplicates

Dr. Peter Daszak is President and Chief Scientist of EcoHealth Alliance, Chair of the NASEM Forum on Microbial Threats, member of the Executive Committee and the EHA institutional lead for the $130 million USAID-EPT-PREDICT. His >300 scientific papers include the first global map of EID hotspots, estimates of unknown viral diversity, predictive models of virus-host relationships, and evidence of the bat origin of SARS-CoV and other emerging viruses.

Prof. Ralph Baric is a Professor in the UNC-Chapel Hill Dept. of Epidemiology and Dept. of Microbiology & Immunology. His work focuses on coronaviruses as models to study RNA virus transcription, replication,

persistence, cross species transmission and pathogenesis. His group has developed a platform strategy to assess and evaluate countermeasures of the potential “pre-epidemic” risk associated with zoonotic virus cross species transmission.
Prof. Linfa Wang is the Emerging Infectious Diseases Programme Director at Duke-NUS Medical School. His research focuses on emerging bat viruses, including SARS-CoV, SADS-CoV, henipaviruses, and others and genetic work linking bat immunology, flight, and viral tolerance. A 2014 recipient of the Eureka Prize for Research in Infectious Diseases, he currently heads a Singapore National Research Foundation grant “Learning from bats” ($9.7M SGD).

Current list of contacts at key human (especially) and wildlife orgs in every SE Asian country from map above – please add to this if we’ve missed any

Indonesia – EHA: Eijkman (Dodi Safari), IPB (Imung Joko Pamungkas); **CM Ltd:** IPB (Imung Joko Pamungkas), Bogor Agricultural University (Diah Iskandriati)

SE China – EHA: Yunnan CDC (XX), Wuhan Inst. Virol. (Zhengli Shi);

Myanmar – Chulalongkorn (Thiravat and Supaporn) have collaborated extensively with Win Min Thit, MD (Yangoon General Hospital, Myanmar); Khin Yi Oo (National Health Laboratory, Myanmar) and also in training PREDICT Myanmar veterinarians in bat sampling under PREDICT.

Japan – **BMHRC**: Oita University (Hidekatsu Iha, Kiyoshi Aoyagi), Nagaskai University (Koichi Morita, Kazuhiko Moji)

Cambodia -
Laos - **CM Ltd:** Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit (Matthew Robinson)
Bangladesh – EHA: IEDCR (Meerjady Sabrina Flora, Ariful Islam), icddr,b (Mohammed Ziaur)

India – EHA: Jon’s collaborators;

Vietnam – Wellcome CRU ? Liaison with Zhengli Shi for Serol assays etc.?

Philippines – EHA, CM Ltd: Biodiversity Management Bureau, Department of Environment and Natural Resources (Anson Tagtag), Bureau of Animal Industry (Davinio Catbagan), Research Institute of Tropical Medicine (Catalino Demetria).

Malaysia – **CM Ltd:** Borneo Medical and Health Research Center (Ahmed Kamruddin), Department of Wildlife and National Parks Peninsular Malaysia (Frankie Sitam, Jeffrine Japning, Rahmat Topani, Kadir Hashim, Hatta Musa), Hospital Universiti Malaysia Sabah (Helen Lasimbang), Faculty of Medicine, Universiti of Malaya (Sazaly Abu Bakar), Kota Kinabalu Public Health Laboratory (Jiloris Dony, Joel Jaimin), Ministry of Health (Chong Chee Kheong, Khebir Verasahib, Maria Suleiman), National Public Health Laboratory (Hani Mat Hussin, Noorliza Noordin, Yu Kie Chem), Queen Elizabeth Hospital (Giri Rajahram, Heng Gee Lee), Sabah State Health Department (Christina Rundi, Mohammad Jikal), Sabah Wildlife Department (Rosa Sipangkui, Senthilvel Nathan, Augustine Tuuga, Jumrafiah Sukor), Gleneagles Hospital Kota Kinabalu (Timothy Wiliam); **BMHRC:** CM Ltd (Tom Hughes), HQEII (Shahnaz Sabri), Kota Kinabalu Public Health Laboratory (Jiloris Dony), Sabah State Health Department (Mohammad Jikal)

Thailand – **CM Ltd:** Department of Disease Control, Ministry of Public Health (Rome Buathong, Pawinee Doungngern, Pongtorn Chartpituck), Department of National Park, Wildlife and Plant Conservation (Pattarapol Manee-On), Faculty of Forestry, Kasetsart University (Prateep Duengkae), Mahidol-Oxford Tropical Medicine Research Unit, (Stuart Blacksell, Richard Maude),Chulalongkorn University (Supaporn Wacharapluesadee).

Chulalongkorn lab is a WHO Collaborating Centre for Research and Training on Viral Zoonoses, we work closely with WHO SEARO and SEARO countries including Bangladesh, Bhutan, Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, (Thailand), Timor-Leste.
We had conducted the PREDICT lab training for Veterinary lab in the Southeast Asia region for FAO.
We conducted (transferred?) the bat sampling technique for scientists from Malaysia, Myanmar, the Philippines, and China.

5. Data Management Plan

In a clearly labeled section entitled **"Data Management Plan":**

- Describe internal and external data acquisition strategies to achieve harmonization of systems and procedures for data management, data quality, data analyses, and dissemination for all data and data-related materials generated by the research study with the EIDRC CC.
- Within the plan, indicate the extent to which dedicated systems or procedures will be utilized to harmonize the acquisition, curation, management, inventory and storage of data and samples. Describe how training for the data and sample collection, in terms of the use of electronic data capture systems, will be provided to all staff including those at enrollment sites.
- Describe the quality control procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens.

This includes sample labeling etc. Get a bar-coding system, everyone has a scanner (cf. PREDICT) – Ralph has this for select agents and MERS. Also need a software system

Language from NIH CoV grant:

Data Sharing Plan: Data will be available to the public and researchers without cost or registration, and be released under a CC0 license, as soon as related publications are in press. Data will be deposited for in long-term public scientific repositories – all sequence data will be made publicly available via GenBank, species location data via the Knowledge Network for Biodiversity, and other data will be deposited in appropriate topic-specific or general repositories. Computer code for modeling and statistical analysis will be made available on a code-hosting site (GitHub), and archived in the Zenodo repository under an open-source, unrestrictive MIT license. Limited human survey and clinical data will be released following anonymization and aggregation per IRB requirements. Publications will be released as open-access via deposition to PubMed commons.

Viral isolates will remain at the Wuhan Institute of Virology initially. Isolates, reagents and any other products, should they be developed, will be made available to other NIH-funded researchers via applicable Wuhan Institute of Virology and EcoHealth Alliance Material Transfer Agreements and/or licensing agreements.

Sharing Model Organisms: We do not anticipate the development of any model organisms from this study. Should any be developed, they will be made available to other NIH-funded researchers via applicable Wuhan Institute of Virology and EcoHealth Alliance Material Transfer Agreements and/or licensing agreements.

Genomic Data Sharing: We anticipate obtaining genetic sequence data for 100s of novel coronavirus genotypes, including RNA-dependent RNA polymerase (RdRp) and Spike genes for all strains/genotypes. In addition, we will generate full viral genomes for a subset of the bat SARSr-CoVs that we identify. All sequence data will be deposited in the NIH genetic sequence database, GenBank. We will ensure that all meta-data

associated with these sequences, including collection locality lat/long, species-level host identification, date of collection, and sequencing protocols will also submitted. The genotype data will be made publicly available no later than the date of initial publication or six months after the receipt of final sequencing data, whichever comes first. We anticipate sequence generation will occur over the 5 year proposed project period.

Genome Wide Association Studies (GWAS): Not applicable.

Commented [KJO77]: From CoV NIH grant

Thailand TRC-EID Chulalongkorn lab:Specimen management and BioBank: The software called PACS (Pathogen Asset Control System) supported by BTRP DTRA, is now used for managing the specimen from specimen registration (barcoding, and may move to use QR code system soon), aliquoting, tracking, short patient history record, data summary, searching, test reporting and etc. Our biobank system contains 28 freezers (-80C) and liquid N2 system. Its size is around 120 sq meters. There is a separate electric generator for our lab and biobank. There are CC TV cameras at the biobank and laboratory.

Chula TRC-EID has our own server for data management and analysis. We have the necessary software including Bioinformatic tools (Blast Standalone, SAMTOOL, GATK, AliVIEW, RaxML, FigTREE, MAFFT, Guppy, Megan, PoreChop, MEGA), Database (MySQL, MongoDB) Web, jQuery, Node.js, Express.js React, API, PHP, Ruby, Python, Java, C#, HTML5, Bootstrap, CSS, Responsive, Cloud, Linux Command, PACS (sample management system), Virtualbox virtual machines, R programming languages, Perl scripts, Bash shell scripts, Open source software, Microsoft Office running on both Apple Mac OS X, Ubuntu, Linux, and Windows Operating Systems. Chula TRC-EID has a dedicated 16-core Ram 64GB Linux server with 4TB hard drives, dual quad-core Mac Pro Server with 2TB hard drives and another dedicated iMac Pro (Retina 4K, 21.5-inch, 2017) Processor 3.6GHz quad-core Intel Core i7 (Turbo Boost up to 4.2GHz) Ra, 16GB with 512GB hard drives. TRC-EID also has HPC Linux server CPU: 4 x 26cores Intel Xeon = 104 cores 2.1GHz with 52 TB HHD and 2x3.84TB SSD with GPU 2x Tesla v100 (CUDA core = 5120 cores/GPU, Tenser core = 640 cores/GPU). TRC-EID has a dedicated 10/100/1000 of LAN (Local Area Network), 2.4G / 5G Gigabit Wi-Fi 802.11ac.

Commented [KJO78]: From Supaporn for their lab, some may go in Facilities doc?

From DARPA:

Data Management and Sharing: EcoHealth Alliance will maintain a central database of data collected and generated via all project field, laboratory, and modeling work. The database will use secure cloud hosting services and enable export to archival and platform-independent formats. It will ensure data and metadata compatibility between project components, track data versioning and annotations, and enable compliance with DARPA data requests and disclosure requirements. All archived human sample data will be de-identified. Partners will provide raw and processed data to the central database throughout the course of the project. Project partners will have access to the data they generate in the database at all times, and maintain control over local copies. Release of any data from the database to non-DARPA outside parties or for public release or publication will occur only after consultations with all project partners. EHA has extensive experience in managing data for multi-partner partner projects (PREDICT, USAID IDEEAL, the Global Ranavirus Reporting System).

Commented [KJO79]: Text from DARPA Preempt

6. Clinical Management Plan

Clinical site selection

Our consortium partners have been conducting lab and human surveillance research, inclusive of human surveillance during outbreaks, for over the past two decades and in that time have developed strong relationships with local clinical facilities and processes. The plan for selection of clinical sites will begin in the same geographic regions as those identified in Aim 1. These are regions where we will sample wildlife due to the high potential of zoonotic viral diversity. Additionally, these will be clinical sites that serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2. In PREDICT we developed successful working relationships with the major healthcare facilities the hotspot areas in 6 different countries, including Thailand and Malaysia and we will continue to work with these sites going forward. Continuing to

Commented [EH80]: Is that true

work with these hospitals means we will begin this project with successfully established partners and we can begin data collection quickly. This also reduces the number of additional sites needed to meet our goals within our geographic range. The capacity and capability requirements for clinical sites for this study is limited. All that is required of sites will be the ability to enroll patients that meet the clinical case definitions of interest, capacity to collect and temporarily store biological samples, and follow standards for data management and protection. We will be enrolling inpatient and outpatient participants within 10 days of the presentation of symptoms to increase the chance of PCR detection of viruses so we will not wait for advanced normative diagnostic tests, that are likely unavailable in the more remote sites, to be completed. We will work with clinical sites to determine the best process for project staff, in most cases supporting the time of currently site staff. These will be persons who are locally trained and certified to collect biological specimen from participants including blood draw technique, e.g. doctor or nurse. Last, the clinical site will need the capacity to securely store all personal health information and personally identifiable research data, this will include locked offices with locked filing cabinets to store all paper records and an encrypted computer. Training on project procedures will be provided for all local project staff by local country project management staff and partners from headquarters. The study at clinical sites will begin by developing relationships with local stakeholders at the hospitals and clinics in our geographic areas of interest that serve as catchment facilities for people of within the community surveillance study. We will recruit hospital staff that will be extensively trained for project procedures including enrollment of patients, sample types to be collected, storage of samples, administration of the questionnaire, and data management plans. During the Development and onboarding of new clinical sites we will assess the physical needs of the site and what supplies will be required for collection of data (e.g. locked filing cabinet, dry shipper, secure laptop). [Did not specifically address CONDUCT]. Oversight for clinical sites will be managed by the country coordinator for each country. This will be done with regular site visits to the clinical sites and annual visits by headquarter research staff. Site visits will include monitoring and evaluating the process for patient enrollment, collecting and storage of samples, administration of the questionnaire, and secure and data management procedures for secure personal health information and research data. During these visits any additional training will also be provided.

Standardized approach oversight and implementation

Management and oversite for all study sites will be managed by the local country coordinator who is supported by staff at headquarters. Implementation of a standardized approach across all study sites, community and clinical, will begin by extensive training of staff on procedures on requirements of the project, this includes: enrollment, data collection, and data management. Our research team has over 5 years of experience building capacity on human subjects' research and has developed training resources and materials for standardized implementation of community and clinical research. This includes SOPs for screening, enrollment, and retention of participants. In addition to yearly monitory reports the country coordinator will regularly visit each clinical site to ensure quality standards and compliance of procedures are being met. Through our work with clinical sites in PREDICT we have developed culturally appropriate screening measures for clinal sites that do not disrupt the flow or quality of medical treatment received by the patient. This was done by working collectively with clinical research staff to evaluate current procedures and patient flow at the site to determine the most efficient while minimal invasive inclusion of our study into the daily working of the clinal site. Most efficiently this was done by adding minimal basic screening questions to the current clinal intake forms, which allows the clinal research officer to quickly scan charts or logs for potential patients to enroll, allowing for minimal disruption and prevents potential enrollees from being overlooked if the research staff is not on duty. At clinical sites that do scheduled outpatient days we may have a trained member of the research staff with the intake nurse to monitor the patients coming to the clinic more rapidly to no delay screening or enrollment in such a fast pace environment. Using the screening tools, we will recruit and enroll patients who present with symptoms that meet the clinical case definitions of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever, of unknown etiology within the past 10 days. These patients will be enrolled, samples collected and survey conducted to assess the participants contact with wildlife through: 1) occupational exposures; 2) animal contact; 3) environment biosecurity. With permission from each clinic, and consent from participants, we will review clinical records to

collect data on medical history, clinical syndromes, and patient etiology. The clinical cohort will be split into cases and controls. “Cases” are defined as participants whose samples tested positive for either coronaviruses, henipavirus, or filoviruses, serological tests. “Controls” will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between serological/PCR status and risk factors. In clinical study, we will use this procedure to determine how clinical presentation differs between virus-exposed and -unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. We will model the outcomes to analyze correlation between biological results and risk factors related to high risk wildlife exposure. Additionally, we will be following up with clinical participants to determine how clinical presentation differs between coronaviruses, henipavirus, or filoviruses in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. [How do these cohorts be leveraged for new emerging pathogens] Achieving the adequate numbers will be attained through duration of the as clinical participant enrollment. While patient’s enrollment is limited by the number of individuals presenting at hospitals, during the PREDICT project we had an average of 105 patients enrolled per year, ranging from 77-244. The country coordinator will be continually monitoring the project database to be sure that we are on track to reach our target.

Clinical cohort setup, recruitment, enrollment

We will recruit inpatients and outpatients from clinical sites to participate in the study after initial screening to determine if they meet the clinical case definitions for 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever, of unknown etiology for symptoms reported onset of illness to increase the chance of PCR detection of viruses. Once enrolled, biological samples will be collected by trained hospital staff. When possible, samples will be taken concurrently with samples being collected for normative diagnostics. We will collect 2* 5mL of blood for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples and two nasal or oropharyngeal swabs will be collected. The cases are defined as participants whose samples tested positive for Henipaviruses, Filoviruses, or CoVs by PCR or serological tests. The controls will be selected from the pool of participants admitted to the studies and whose samples tested negative for coronaviruses, henipavirus, or filoviruses. We will follow up 35 days after enrollment to collect an additional 5mL blood draw for collection of two 500 µL serum samples and a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. Following up 35 days post initial collection gives adequate time for 1) development of IgG and IgM, which occurs <28 days after onset of symptoms for coronaviruses, henipavirus, or filoviruses infected patients to evaluate the immunological progression of disease and 2) further the risk assessment of the participants to monitor contact with wildlife, people, and to assess the likely reservoir hosts to collect information to inform potential intervention programs.

Utilization of collected data

Sampling of wildlife and the enrollment of community surveillance participants and a clinical cohort of participants give us the ability to assess the viruses the are circulating in each of the three populations in a similar geographic region. We will use phylogenetic analysis to compare the relationship between wildlife viruses found and viruses fond in human participants. Additionally, the questionnaire allows us to assess relative measures of human-wildlife contact from our survey work that we will analyze the intensity of contact with species known to be at a higher risk for spillover against other risk to provide useful proxy information for spillover risk.

The biological specimens to be collected (2*5mL whole blood for whole blood and serum analysis and nasal or oropharyngeal swabs) from all eligible participants and follow up specimen 35 days (3mL of whole blood for serum) after enrollment with the standardized questionnaire. Capacity for specimen is collection is not more than standard phlebotomy skills and we will collected by locally trained and certified personnel that are part of the research team in country. Staff we will trainline on ethical guidelines for conducing human subjects

Commented [EH81]: Will these cohort fulfill the goals of the EIDRC.

Commented [EH82]: PREDICT we also make the stipulation that we will collective relatvive samples if avialble from treatmean collection, eg CSF if the MD is doing a spinal tap we will request an aliqot if possible

Commented [EH83]: Please add any information that might address: types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.

Commented [EH84]: Please add any information that might address: Describe methods that might be applied to elucidate a zoonotic source

research and survey data collection methods. Collection of whole blood, serum, and nasal or oropharyngeal swabs allows us to test the samples for PCR to detect viruses in our priority viral families, serologically test for immunological response and historical exposure to zoonotic diseases of relevance, and the questionnaire will assess individuals evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. With this collective data set we aim to quantify and detect novel viruses likely to spillover regularly to people, are often unreported or misdiagnosed as their clinical manifestations are unknown to detect cryptic outbreaks causing previously 'hidden' clinical syndromes in people. Our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence.

Potential expansion
Expansion of the research project to other clinical sites or research areas could happen as new information is gathered, either through our research or an outbreak in the region. If expansion is required we could rapidly implement research activities in additional clinical or community sites through the same process we on-boarded the initial locations. First, by working with local stakeholders to create a working relationship and on-boarding new clinical research staff through advanced trainings of project aims, SOPs on data collection and storage, and ethical guidelines. The network of infectious disease researchers in the community and hospital settings would allow for an accelerated response to any potential infectious disease outbreaks as staff are well trained on ethical guidelines for working with human subjects, data and specimen collection, storage, and transpiration, project staff would be on the front lines ready for the call to action. Ideally, our testing for viruses in priority viral families may lead to the discovery cryptic outbreaks before it spreads in the general public. Biological results coupled with survey data on key risk factors will provided necessary information for granular understand of disease spread.

In a clearly labeled section entitled **"Clinical Management Plan":**

- Describe plans, processes, and procedures for the following activities: clinical site selection including feasibility, capacity, and capabilities, and study development, conduct, and oversight.
- Describe strategies for oversight and implementation of standardized approaches in the recruitment and clinical characterization of adequate numbers of relevant patient populations to ensure the prompt screening, enrollment, retention and completion of studies. Describe novel approaches and solutions to study enrollment. including the clinical meta-data that will be captured and describe how the staff at each enrollment/collection site will be trained and comply with the quality standards for data and sample collection and compliance with the standardized procedures. Provide a general description of the methods for establishing clinical cohorts and how cohorts may be leveraged for new emerging pathogens.
- Provide general approaches to identification of the types of clinical cohorts needed to fulfill the goals of the EIDRC. Discuss types of clinical cohorts and clinical assessments that will be utilized to study natural history and pathophysiology of disease, including characteristics of the cohort, site for recruitment, types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.
- Describe methods that might be applied to either elucidate a zoonotic source or a vector of transmission.

Our research team incredibly diverse and capable of working with a group of other viral families: Lay out all our additional vector experience incl. my WNV work, our work on Chik etc.
Baric: Flavi, Noro,
Linfa: …
EHA: …

- Describe capacity for and approaches to clinical, immunologic and biologic data and specimen

collection, and how these data and specimens will address and accomplish the overall goals and objectives of the research study.

Ralph

1. Norovirus: collaboration with surveillance cohort CDC; first one to publish antigenic characterization of each new pandemic wave virus; and phase 2 and 3 trial of therapeutics
2. Tekada Sanofi Pasteur dengue
3. Nih tetravalent vaccine

- Provide plans for the potential addition of new sites and expanding scientific areas of research when needed for an accelerated response to an outbreak or newly emerging infectious disease.

Linfa – will launch VirCap platform on outbreak samples.

7. Statistical Analysis Plan

In a clearly labeled section within the Research Strategy entitled **"Statistical Analysis Plan":**

- Describe the overall plan for statistical analysis of preliminary research data, including observational cohort data.
- Describe the overall staffing plan for biostatistical support and systems available for following functions: 1) preliminary data analyses, 2) estimates of power and sample size, 3) research study design and protocol development.
- Describe the quality control and security procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens. Describe any unique or innovative approaches to statistical analysis that may be utilized.

8. Project Milestones and Timelines

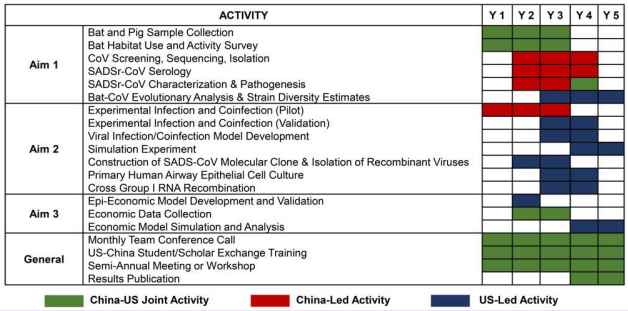
In a clearly labeled section entitled **"Project Milestones and Timelines":**

- Describe specific quantifiable milestones by annum and include annual timelines for the overall research study and for tracking progress from individual sites, collaborators, and Teams. Milestones must specify the outcome(s) for each activity. Milestones should be quantifiable and scientifically justified, and include the completion of major research study activities, including, for example, protocol development, case report forms, scheduled clinical visits, obtaining clearances study completion, and analysis of final data. Milestone criteria should not simply be a restatement of the specific aims. Using a Gantt chart or equivalent tool, describe the associated timelines and identified outcomes for the research study.

Show that for this proposal, we won't be doing the Specific Aims sequentially. We'll begin the human work (SA 2, 3) at the beginning of the project, along with SA1. We'll do the diagnostic assay development initially in Ralph's, Linfa's, Chris's lab, but then begin tech transfer in Y1….

Project Management & Timeline

PI Daszak will oversee all aspects of the project. Dr. Daszak and has two decades of experience managing international, multidisciplinary research in disease ecology, including successful multi-year projects funded by NSF, NIH and USAID. He will conduct monthly conference calls with all co-PIs, including the China team **(see Activity schedule below)**. He will be assisted by EHA Sr. Personnel Li, who is a US-based Chinese national with fluent Mandarin. Co-PI Olival will oversee Aim 1 working closely with China PI Tong, who will test samples. Co-PI Ma (China team) will coordinate and lead all Chinese experimental infections, with guidance from co-PI Saif, who will coordinate and lead all US experiments with co-PI Qiuhong Wang. Sr. Personnel Ross will conduct all modeling activities in Aim 2, working closely with co-PI Saif to parameterize the models. Co-PI Baric and Sr. Personnel Sims will lead infectious clone, recombinant viral and culture experiments. Consultant Linfa Wang will advise on serology, PCR and other assays. China team co-PIs Shi and Zhou will oversee viral characterization in Aim 1. Sr. Personnel Feferholtz will oversee the economic modeling in close collaboration with Ross.



Commented [KJO85]: Text and figure From NSF-NIH SADS grant. Peter will adapt

Another Example of Milestones and specific tasks from a prev DHS (unfunded) proposal:

Expand existing databases to include predictor variables for pathogenicity risk model

Task 1: Collate available human clinical information from the literature for ~300 viral pathogens known to infect humans (Contract Award Date to 6 month)

Task 2: Update EHA host-viral association database to include new data for mammalian and avian species from literature 2015-present (Contract Award Date to 3 month)

Task 3: Extract and curate full and partial genomic data from Genbank for 5-6 viral families with a high proportion of human-infectious pathogens for phylogenetic analyses – e.g. 'nearest-known-threat module'. (Month 2 to 6 month)

Develop underlying pathogenicity and spillover risk assessment models

Task 4: Fit generalized linear models in Stan for multiple response variables to measure pathogen impact (Month 7 to 10 month)

Task 5: Fit generalized linear models to estimate risk of human infection (spillover) based on host, ecological, and location specific traits (Month 4 to 8 month)

Task 6: Validate model effectiveness by simulating early-outbreak, limited-information scenarios from known pandemics to confirm model behavior (Month 8 to 10 month)

Commented [KJO86]: Just example, but probably just want to use a Gantt chart format per most of our grants, depending on space.

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II. Research Strategy:

1. Significance:

Southeast Asia is a critical hotspot for emerging diseases due to its high biodiversity of wildlife and their viruses, and the presence of key ecological and socioeconomic drivers of emergence **(Fig. 1)** (*1*). These include dense human populations living in networks of rural and urban communities, with strong cultural and occupational connection to wildlife and livestock farming and trading, and intimate connection to global travel networks (*2*). The region is also undergoing rapid environmental and demographic change, both of which increase the risk of disease emergence and spread.

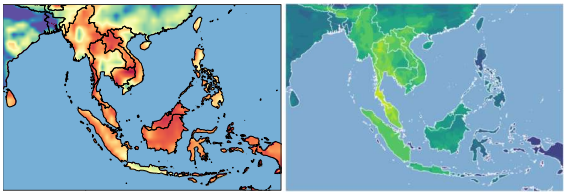

Fig. 1: Southeast Asia, and in particular, parts of Thailand and Malaysia are hotspots of risk for disease emergence **(left)**, and predicted high diversity of 'missing' or as-yet undiscovered viruses, yellow = highest diversity **(left)**. From (*1, 2*).

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Not surprisingly a number of novel viruses, including near-neighbors of known agents, have emerged in the region leading to sometimes unusual clinical presentations **(Table 1)**.These factors are also behind a significant background burden of repeated Dengue virus outbreaks in the region (*16*), and over 30 known *Flavivirus* species circulating throughout S. and SE Asia (*17*). The events in **Table 1** are dominated by three viral families: coronaviruses, henipaviruses and filoviruses, which serve as initial examples of our team's research focus and capabilities. These viral groups have led to globally important emerging zoonoses (*18-29*). Their outbreaks have caused tens of thousands of deaths, and billions of dollars in economic damages (*30-32*). Furthermore, relatives and near-neighbors of known pathogens in these families have been reported throughout the region, including by members of our consortium: henipaviruses in bats in Thailand, Cambodia, Philippines, Laos, Indonesia, China, Malaysia, Bangladesh and India (*33-39*); a novel henipavirus, Mojiang virus, in wild rats in Yunnan (*10*); serological evidence of filoviruses in bats in Bangladesh (*40*) and Singapore (*41*); Nipah- and Ebola-like viruses in *Hipposideros*, *Cynopterus* and *Rhinolophus* species in Malaysia (Hughes *et al.*, in prep.); evidence of novel filoviruses in bats in China (*42-44*), including Měnglà virus that appears capable of infecting human cells (*45*); novel paramyxoviruses in bats and rodents consumed as bushmeat in Vietnam (*46*); a lineage C β-CoV in bats in China that uses the same cell receptor as MERS-CoV and can infect human cells *in vitro* (*47*); MERSr-CoVs in dry bat guano being harvested as fertilizer in Thailand (*48*), and directly in bats (Wacharapluesadee *et al.*, in prep.);

Viral agent	Site, date	Impact	Novelty of event	Ref.
Melaka & Kampar virus	Malaysia 2006	SARI in family group, individual	1st disease due to bat-origin reoviruses, also Singapore, Vietnam etc.	(*3-6*)
Reston filovirus	Philippines 2008	Seropositive people killed pigs,	No prior findings of filoviruses in pigs	(*7*)
Thrombocytopenia Syndrome virus	E. Asia 2009	100s of deaths in people	Novel tick-borne zoonosis with large caseload	(*8*)
Reston filovirus	Shanghai 2011	PCR positive pigs	Further evidence of pig RESTV infection	(*9*)
Mojiang virus	Yunnan 2012	Death of 3 mineworkers	1st evidence of rodent origin henipavirus in people	(*10*)
Nipah-like virus	Philippines 2015	Killed horses	No prior horse infection for NiV, known for HeV	(*11*)
SARSr-CoV & HKU10-CoV	S. China 2015	Seropositive people	1st evidence human infection HKU10 & SARSr-CoV	(*12*)
SADS-CoV	China 2017	>25,000 pig deaths seronegative people	Novel emergence of bat-origin CoV	(*13*)
Nipah virus	Kerala 2018, 2019	Killed 17/19 people 2018, 1 infected 2019	1st outbreak of NiV outside Bangladesh, W. Bengal focus	(*14, 15*)

Table 1: Recent emergence events in SE Asia indicating potential for novel pathways of emergence, or unusual presentations for known or close relatives of known viruses.

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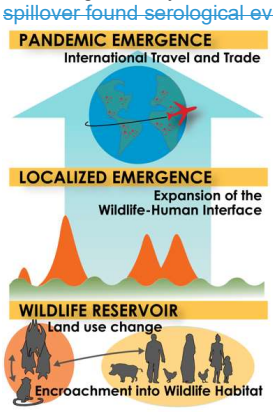
172 novel β-CoVs (52 novel SARSr-CoVs) and a new β-CoV clade ("lineage E") in bat species in S. China that are also found throughout the region (*20, 47, 49, 50*); and 9 and 27 novel wildlife-origin CoVs and

paramyxoviruses in Thailand (respectively) and 9 novel CoVs in Malaysia identified as part our work under USAID-PREDICT funding (*49, 51*). These discoveries underscore the clear and present danger of future zoonotic events in the region. Furthermore, the wide diversity of potentially pathogenic viral strains has significant potential to help in the development of broadly active vaccines, immunotherapeutics and drugs (*47*). Most high-impact zoonotic viruses originate in wildlife reservoirs, sometimes spilling over first into livestock 'amplifier' hosts, or directly into localized human populations with high levels of animal contact **(Fig. 2)**. Efforts to prevent emerging zoonoses have targeted these high-risk populations in regions prone to disease emergence (*29, 52*). However, these regions tend to be in the developing world, where nations often lack the resources to investigate novel zoonotic events, or wildlife reservoirs of zoonoses, and prioritize more immediate public health concerns such as Dengue virus or NTDs (*16*). Zoonotic disease surveillance and control is also hampered by inadequate information on basic disease ecology (e.g. range of wildlife reservoirs, risk factors for spillover) and pathogen biology (viral diversity, pathogenesis), and by logistical challenges sharing novel diagnostics, animal models, samples and reagents at sites where outbreaks begin (*53*). Countermeasure and vaccine development is challenged by the small number of isolates available, and the presence of closely-related strains, some with evidence of transmission to people but with unknown pathogenicity. For example, using NIH and other funding, we found serological evidence of exposure to African henipaviruses in 3-4% of a sample of people in Cameroon, with a significant risk factor being butchering of bats; and serological responses to bat SARSr-CoVs in 6/209 (2.87%) and bat HKU10-CoV in x/xx (x%) people living close to a bat cave in Yunnan, all novel viruses with unknown clinical impact (*12, 54*). We discovered two novel bat-borne reoviruses, Melaka and Pulau viruses, and showed that 14/109 (13%) of people living in close proximity to bat roosts on Tioman Island, Malaysia were exposed to these viruses (*3*) as well as 12/856 (1.4%) people screened in Singapore (*4*). Previous studies in the Philippines found famers and butchers who had contact with sick pigs tested positive for Reston ebolavirus antibodies but had not experienced illness (*7*), and it was our team that discovered Philippine bats as natural reservoir hosts for Reston virus (*55*). In pPreliminary screening in Malaysia with funding from DTRA ~~for serological surveillance of Henipaviruses and Filoviruses spillover found serological evidence of past exposure to Nipah and Ebola antigenically-related viruses in non-human primates (NHPs) and people (Hughes, in prep.).~~ we found serological evidence of exposure to Henipaviruses (Hendra, Nipah and Cedar) in 14 bats and 6 human samples, and Ebola (Zaire and Sudan) antigenically-related viruses in 11 bats, 2 non-human primates (NHP) and 3 human samples (Hughes, in prep.).



Even when spillover leads to outbreaks of illness and mortality, they are often unreported, undiagnosed or misdiagnosed. Under a prior NIH R01, we initiated targeted syndromic surveillance of encephalitis patients in Bangladesh clinics and showed that NiV causes repeated annual outbreaks with an overall mortality rate of ~70% (*56*). Nipah virus has been reported in people in West Bengal, India, which borders Bangladesh (*26*), in bats in Central North India (*57*), and now in people in Kerala, South India in 2018 and 2019, raising the specter of future spillover at other sites in the region (*14, 58*).

Fig. 2: Our Early Warning Approach: Most zoonotic viruses emerge from wildlife hosts, sometimes via livestock (Stage 1, lower panel), to cause small scale outbreaks (green) or larger chains (red) of human-to-human transmission (Stage 2, middle). In some cases, these spread more widely via air travel (Stage 3, upper). Our EIDRC proposal targets each stage: SA1 examines the diversity and spillover potential of viral threats in targeted SE Asian wildlife; SA2 seeks evidence of their spillover into focused high-risk human populations (stage 2); SA3 identifies their capacity to cause illness and to spread more widely (stage 3). Figure from (*52*).

Under a current NIAID R01, we have shown that we can use analytical approaches to target surveillance of wildlife and people and identify likely sites of spillover. We used phylogenetic analysis of bat hosts and their CoVs to identify a key cave complex in SW Yunnan Province that harbors all the genetic elements of SARS-CoV (*59*), as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics (*20, 47, 49, 50, 60-62*). We conducted behavioral risk surveys and biological sampling of human populations living close to this cave and found evidence of spillover (xx serology) in people highly exposed to wildlife. **This work provides proof-of-**

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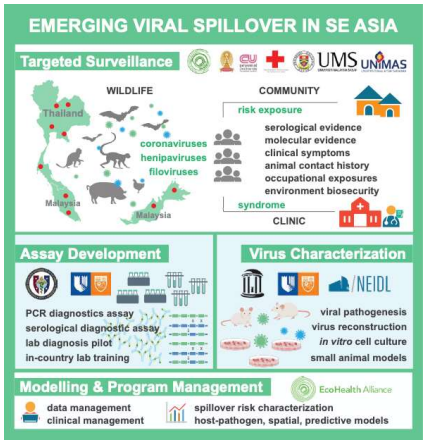
concept for an early warning approach that we will expand in this EIDRC to target key viral groups in one of the world's most high-risk EID hotspots.

The overall premise for this EIDRC proposal is that there is substantial evidence that: 1) diverse CoVs, henipaviruses and filoviruses related to known zoonotic pathogens are circulating in wildlife reservoirs in Southeast Asia; **2)** these viruses likely spillover regularly to people, are often unreported or misdiagnosed, and their clinical manifestations and potential to cause pandemics are unknown and underestimated; and **3)** our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence. To scale-up this approach, we propose to launch **EID-SEARCH** (the **E**merging **I**nfectious **D**iseases - **S**outh **E**ast **A**sia **R**esearch **C**ollaboration **H**ub), to better understand the risk of zoonotic viral emergence in Southeast Asia. We will initially focus on the diversity of known and novel CoVs, henipaviruses, and filoviruses in wildlife reservoirs. We will use this work to build our center's interactive network throughout the region, bridge barriers, provide early focal point for basic and translational studies, develop sample handling and storage routines, communication networks, cohort management, infrastructure development, and cross-training in surveillance and novel diagnostic platforms. We will develop and transfer technology to test these viruses' capacity to infect human cells and mouse models, develop new specific and sensitive serological and molecular diagnostic tools, conduct surveillance of human communities with high risk of exposure to wildlife, and clinical cohorts to identify evidence of spillover of viruses causing previously 'cryptic' clinical syndromes in people. We will test the capacity of novel viruses we isolate, or genetically characterize, to infect human cells and/or mouse models, and thus asses potential for spillover and spread which will feed into our risk models. Our group also has extensive experience in other viruses, including vector-borne flaviviruses, and alphaviruses, and will use this experience to provide robust long-term surveillance, diagnostics, human capacity building and basic science opportunities. While the current proposal focuses its basic and applied research agendas on this group of viral EID, we note that the EID-SEARCH is poised to rapidly respond to any new emerging outbreak. Thus, filoviruses, henipaviruses and coronaviruses are examples of sentinel surveillance systems in place that can capture, identify and initiate a rapid response targeting any novel microbial pathogen that emerges in this region.

2. Innovation: Previous work by our consortium has developed strategies to target surveillance in wildlife and people to better anticipate and identify early spillover events and pre-empt outbreaks of emerging viruses. In the EID-SEARCH we scale up this approach in a central research "hub" made up of leading laboratories, clinics and surveillance sites in three critically high-risk EID hotspot countries in SE Asia. EID-SEARCH's reach is expanded through its regional network of collaborators that covers nearly all of the greater SE Asia region. The innovation of the EID-SEARCH is in: **1)** Its multidisciplinary approach that combines modeling to target geographic and taxonomic sampling targets with on-the-ground zoonotic disease surveillance in human and wildlife populations; **2)** the biological characterization approach that identifies how likely viruses are to be able to spillover into people, and enables evaluation of existing countermeasure technologies; an approach that is grounded in our successful work on SARSr-CoVs; and **3)** the strategic sampling of both high-risk communities with extensive animal contact at the front-lines of viral spillover *and* syndromic clinical cohorts that present with signs of novel viral emergence, to identify risk of viral spillover in an early warning system approach **(Fig. 2)**. **In Aim 1, we will target viruses in archived and newly collected wildlife samples, and characterize their risk of spillover to people**. We will use spatial and phylogeographic analyses to identify the geography and species of wildlife to test samples from, and to further characterize viruses we have recently discovered. We will use *in silico* methods (novel ecological-phylogenetic risk ranking and antigenic mapping of receptor binding domains),



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cell culture, and animal models to assess their potential for spillover into

Fig. 2: EID-SEARCH approach, core members, and roles.

high-risk human populations. **In Aim 2, we will conduct ~~focused,~~ targeted cross-sectional surveys of ~~and sampling of~~ human communities with high levels of animal contact ~~exposure to wildlife and other animals~~to identify occupational and other risk factors for zoonotic virus exposure.** We will use serological tests targeting viruses identified in Aim 1 to identify the baseline spillover of known or novel CoVs, henipaviruses and filoviruses in these populations. Serological findings will be analyzed together with clinical metadata (including a simple animal-contact survey, location data, viral sequence/isolates etc.) to identify zoonotic risk factors and routes of exposure from specific wildlife or vector species. **In Aim 3, we will use syndromic surveillance of clinical cohorts to identify evidence of viral etiology for otherwise 'cryptic' outbreaks.** We will enroll and collect biological samples from patients in local clinics and hospitals who present with syndromes previously linked to known viral agents and live within communities at high risk for disease emergence. We will conduct molecular and follow-up serological diagnostic assays to identify likely viral etiology of syndromes that may represent otherwise cryptic events. Where viruses are identified, we will attempt to isolate and characterize them, and use survey data, ecological and phylogeographic analysis to identify likely reservoir hosts and inform intervention programs. Our US partner BSL-4 laboratory, NEIDL, will attempt isolation and characterize any viruses requiring high levels of containment (e.g. any novel filoviruses discovered).

3. Approach

Research team: The EID-SEARCH builds on long-term collaboration among world leaders in emerging virus research with proven experience collaborating internationally on field, lab and human surveillance research **(Fig. 2)**. Over the past two decades, our consortium partners have conducted high profile research on EIDs within the region and globally, including identifying the wildlife reservoirs for Nipah and Hendra virus, MERS- and SARS-CoVs (*20, 25, 57, 59, 63-65*), discovering SADS-CoV (*13*), and developing an array of serological, molecular, *in vitro* and *in vivo* approaches to characterizing high-risk CoVs, henipaviruses, and filoviruses (*60-62, 66-79*). Our team has substantial experience conducting human surveillance in the community and during outbreaks **(See sections 2.x.x, 3.x.x)**, including: USAID-PREDICT, and DTRA-funded viral surveillance of indigenous communities in Peninsular Malaysia and syndromic surveillance of hospital patients in Sabah (Co-I Hughes); collection of >2,000 serum samples from healthy blood donors and sanitation workers, febrile and influenza like illness (ILI), and diarrheal stool samples from children under 5 years of age (Co-I Kamruddin); collaboration on the MONKEYBAR project with the London School of Hygiene and Tropical Medicine to analyze risk and prevalence of the zoonotic malaria *Plasmodium knowlesi* through case control and cross-sectional studies in Sabah villages (~800, 10,000 participants resp.) and clinical observational studies to determine the etiology of central nervous system infections and acute undifferentiated febrile illness in Sabah. (Co-Is William, Rajahram, Yeo);~~(Co-Is William, Tock Hing); case control and cross-sectional studies in Sabah villages (~800, 10,000 participants resp.) (Co-Is GR, TYW);~~ and community-based surveillance of hundreds of bat-exposed villagers in Thailand (Co-Is Wacharapluesedee and Hemachudha). As a proof-of-concept of how our team can rapidly scale up resources for diagnostics and surveillance in the face of a new EID, in 2016, we discovered a novel bat-origin HKU-2 CoV (*80, 81*) killing >20,000 pigs in S. China, designed PCR and LIPS serology tests, then surveyed pig farmers for evidence of exposure, and conducted experimental infections, pathogenesis and molecular studies, **all in the span of 3 months** (*13, 82*).

The administration of this center **(See section X)** will be led by PI Daszak, who has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5-20 yrs on NIAID- and USAID-funded research. He will oversee all activities in this EIDRC. Drs. Olival and Zambrana are leaders in analytical approaches to targeting surveillance and head the modeling and analytics work for USAID-PREDICT. Drs. Wang, Baric, Broder, Anderson, and Laing have developed a unique array of *in vitro* cell culture, serological and molecular reagents to identify and characterize emerging viruses in people and animals (henipaviruses and other paramyxoviruses, CoVs, filoviruses and many others). Dr. Wacharapluesedee, Hughes and collaborators have coordinated surveillance of people,

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Commented [PD9]: Hongying – Figure needs edits to text: top panel, 'wildlife' should read "Aim 1: WILDLIFE", "AIM 2: COMMUNITY", "AIM 3: CLINIC". Right hand side: "Clinical symptoms" should be "Clinical history", "Occupational exposures" should be "Occupational exposure", "environment biosecurity" should be "environmental risk factors"

Middle panel: "Assay Development" and "Virus Characterization" should have "(Aim 1)" after them. Left hand side: should read "PCR diagnostics", "serology", "piloting diagnostics" and the last one is ok. Right hand side bullets should be "receptor binding characterization" "in vitro characterization" and "mouse models"

Bottom panel: all good

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wildlife, and livestock within Thailand and Malaysia for the past 150 years, in collaboration with Ministries of Health, Agriculture and Environment. Finally, this consortium has extensive external funding from NIH, DoD, USAID, DHS and other USG agencies that supports a range of laboratory, analytical and field studies directly related to the current proposal, and will bring substantial leverage to our EIDRC.

Geographical focus: The three core (hub) countries for this proposed EIDRC are Thailand, Malaysia and Singapore, three contiguous EID hotspot countries that stretch through one of the most significant foci of EID origins globally. Our proposed work includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sarawak and Sabah, and thus contains overlapping wildlife biogeography with India, Bangladesh, Myanmar, SW China, Laos, Cambodia, Vietnam, Philippines, and Indonesia. It encompasses diverse cultural and socioeconomic backgrounds which have correspondingly diverse and intensive interactions with wildlife reservoirs and the global travel network. We have already begun sampling of wildlife hunters, livestock workers, wildlife farmers and market workers, rural villagers living close to high risk wildlife species, people otherwise highly exposed to animals in these countries, and clinical cohorts, as part of current/prior NIH, DTRA, and USAID funding. To develop a larger catchment area for emerging zoonoses, we will deploy our core member's extensive network of collaborators in clinics, research institutes and public health laboratories in almost every other Southeast Asian country. We will conduct regular meetings with key research, public health and community leaders, including >50 leading laboratories/institutions in XX 10 countries that are currently collaborating with core members of our consortium via other funded work (**Fig. 3**). We will use these, and our annual meetings, to share information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks across the region.

Fig.3 : Map of Southeast Asia indicating three hub countries for this proposed EIDRC (Red: Thailand, Singapore, Malaysia) and countries in which Key Personnel have collaborating partners (Green), indicating that EID-SEARCH provides access to key collaborators throughout the region.

Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife

1.1 Rationale/Innovation: The vast majority of emerging zoonotic pathogens are linked directly or indirectly to wildlife host, particularly from important mammalian groups like bats, rodents and ~~non-human primates~~NHP (*83*). Our previous work on wildlife origin viruses demonstrates that zoonotic viral emergence tends to occur in specific regions with high diversity of wildlife that harbor viruses of zoonotic potential, and human populations that have close contact to these wildlife **(Fig. 1)** (*2*). In Aim 1, we will strategically conduct EID surveillance by applying risk analytics to prioritize the regions, wildlife species, and human populations that have a particularly high risk of viral spillover. We will target high-risk zoonotic host reservoirs (bats, rodents and ~~primates~~NHP) in these regions and apply novel predictive models to estimate viral diversity and host range to identify wildlife species that have been ignored or under-sampled by previous zoonotic disease surveillance efforts. We will strategically collect specimens from these species and screen them, together with some of the tens of thousands of recently collected samples we have archived in freezers in our labs, for known and novel agents. We will begin screening using conserved PCR assays to identify known and novel virus in high risk RNA viral families, *Coronaviridae (CoV)*, *Paramyxoviridae (PMV)*, and *Filoviridae (FV)*. We will expand to other groups of pathogens, including arboviruses, pending available resources and priorities established with the EIDRC coordinating center (EIDRC-CC) and other regional EIDRCs. Using risk ranking algorithms and a series of ecological and virological risk factors, we will triage the wildlife viruses we discover for further genomic characterization and viral isolation. On this subset of viruses, we will then use *in vitro* and *in vivo* approaches (viral rescue, cell lines, gene edited humanized transgenic mice and the collaborative cross genetic reference mouse population) previously developed and widely used by our team to predict capacity of novel viruses to

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infect people and spillover (*61, 84-87*). These high-risk viruses and their close relatives will be targets for human community serosurveys and clinical sampling in Aim 2 and 3, respectively.

1.2 Preliminary data: 1.2.a. Geographic targeting: EcoHealth Alliance has led the field in conducting analyses to indicate where the highest risk of zoonotic spillover is across the globe, identifying SE Asia as a major high risk location for pathogen emergence (*1, 2*). For Southeast Asia, these EID hotspots are sites where high wildlife biodiversity, human population density and rapid ecologic change collide **(Fig 1)**. Using host and ICTV viral data for all known mammalian viruses, and generalized additive models to correct for sampling bias, we are able to predict the relative number of yet-to-be-described viruses that a species harbors (*2*). Using these data to map unknown viral diversity demonstrates substantial undiscovered viral diversity in mammals across Southeast Asia, and particularly in regions of Thailand, Singapore and Malaysia **(Fig. 1)**. In a separate paper on risk of viruses emerging from bat hosts, we found that factors driving spillover (host diversity, climate) differ from those driving spread in human populations (population density, bushmeat hunting, livestock production) (*88*). We therefore analyze capacity for viral spread as a separate risk factor, using demographic and air travel data and flight model software we have produced with Dept Homeland Security funds (*89*). In the current proposed work, we will use all the above approaches to target wildlife sampling (archive and new field collections), and to inform sites for human sampling in Aim 2, to areas of high risk for spillover and spread.

1.2.b. Reservoir host species targeting: Our analysis of all known mammalian host-viral associations demonstrates that bats, rodents and ~~primates~~ NHP represent the three most important reservoirs for zoonoses, responsible for the highest proportion of known and predicted viral diversity of zoonotic potential(*90*). Thus, the bulk of our proposed wildlife sampling and testing will consist of these three taxa with a focus on bats, because this group is the known or hypothesized reservoir for the majority of high-impact zoonotic CoVs, ~~henipaviruses and filoviruses~~PMVs and FVs (*48, 91-93*). Bats also have the highest proportion of predicted zoonotic viruses in any mammalian group (*2*), and are exceedingly diverse in Southeast Asia (~500 species). To further prioritize taxonomic sampling targets within these diverse geographic hotspots, we have used a novel, Bayesian phylogeographic analysis to identify host taxa that are the most important centers of evolutionary diversity for a given viral group, e.g. bat genera for β-CoVs ~~using an extensive CoV sequence dataset from our NIH-funded research~~ **(Fig. 5)** (*49*). This approach will be extended to other mammal taxa and viral families of interest, allowing us to identify the ancestral wildlife hosts of specific zoonotic viral groups, where their diversity is likely highest.

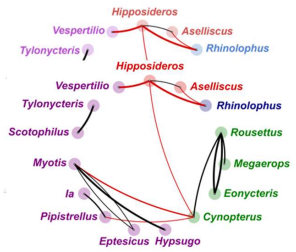

Fig 5: Preliminary analysis to identify the most important bat genera in Southeast Asia ~~as hosts~~ for beta-CoV evolutionary ~~diversity~~s using ~~our previously-collected CoV~~ sequence data collected under our NIH-funded research. Line thickness is proportional to the probability of virus sharing between two genera, with~~.~~ in ~~In~~ter-family switches ~~are highlighted~~ in red. We will apply this Bayesian discrete phylogeographic model to identify taxonomic and geographic centers of viral diversification and sharing for relevant ~~paramyxovirus~~PMV, CoV ~~, coronavirus,~~ and other virus clades.

Finally, we have designed a strategy to estimate sampling gaps, using a viral 'mark-recapture' approach we previously published (*94, 95*) **(Fig. 6)**. Using prior data for bat, rodent and ~~primate~~ NHP viral discovery vs. sampling rates, we can identify the likely total viral diversity for specific host groups in a region, and use this to estimate sample sizes to discover~~ing~~ the majority of unknown viruses within a specific family or genus. **In the current proposal,** we will use these analyses to estimate geographic and species-specific sampling targets **to more effectively identify new strains to support experimental infection studies and risk assessment.**

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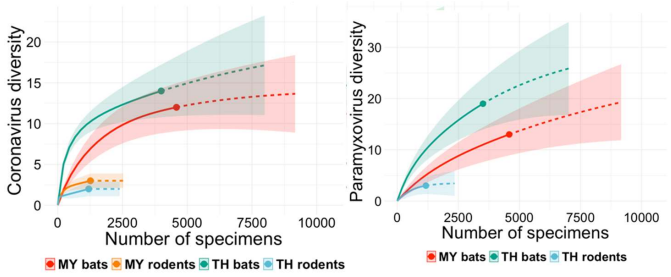

Fig. 6: Estimated ~~coronavirus~~ CoV **(left)** and ~~paramyxovirus~~ PMV **(right)** ~~putative~~ viral ~~'~~species~~'~~ diversity in bats and rodents ~~for~~ from Thailand and Malaysia, using data from PCR screening and RdRp ~~gene~~ sequence~~s~~ ~~preliminary~~ data from >1~~0~~3,000 ~~PCR tests~~specimens in bats and 4,500 ~~tests~~in ~~in~~ rodents. Bats in Thailand and Malaysia have 4X the number of viruses than rodent species, controlling for sampling effort. ~~CoV and paramyxovirus diversity estimates are comparable, but discovery has not yet saturated in any taxonomic group or location.~~ We estimate that additional collection of 5k-9k bat specimens ~~sampling of ~5,000 bats~~ and testing of our archived rodents specimens alone will identify >80% of remaining CoV and ~~paramyxovirus~~ PMV viral species in these key reservoirs, yielding >800 unique viral strains. ~~We will use this approach to define sampling targets for other wildlife taxa, and for viral *strain* diversity.~~

1.2.c. Sample testing to identify known and novel viruses: We have conducted extensive wildlife surveillance for novel viruses across Southeast Asia in prior NIAID funded work and as part of the USAID PREDICT project. Under the 10-year PREDICT program globally, we identified 912 novel and 210 known viruses, more than the total number of viruses previously recognized in mammals by the International Committee on Taxonomy of Viruses. This work included collecting nearly 300,000 individual mammal samples from 14 EHA-led PREDICT countries and PCR-screening more than 60,000 individual specimens(*51*). In southern China alone, this sampling lead to the identification of 178 β-CoVs, of which 172 were novel (52 novel SARSr-CoVs). Included were members of a new β-CoV clade, "lineage E" (41), diverse HKU3-related CoVs (179 sequences) within a 'sister' clade to the SARS-CoV lineage, and the wildlife origin of a new virus, SADS-CoV, responsible for killing >20,000 pigs in Guangdong Province (*13*). ~~Many of the identified bat species are found across the region.~~ We have collected 28,957 samples from bats, rodents and primates in Thailand and 47,178~~51,373~~ in Malaysia, conducting 146,527 PCR tests on a large proportion of these specimens. We have **archived duplicate samples which are now available for use in this project**, including fecal, oral, urogenital, ~~and~~ serum samples and biopsies from bats, rodents, and ~~non-human primates~~NHP, ~~and primate biopsies~~. In Thailand this screening has identified 100 novel viruses and 37 known viruses in wildlife reservoirs. In Malaysia this screening has identified 77 novel viruses and 23 known viruses in wildlife reservoirs (66 novel and 19 known in Sabah and 11 novel and 9 known from Peninsular Malaysia). ~~We used~~Using family level primers for ~~henipaviruses~~PMV, ~~filoviruses~~ FV, and CoVs, we ~~and~~ have already discovered 9 novel and 7 known CoVs and 26 novel and 2 known ~~paramyxoviruses~~ PMV in Thailand; and . ~~This is in addition to~~ 13 novel and 5 known CoVs and 15 novel and 1 known ~~paramyxovirus~~ PMV in Malaysia from wildlife. In the current proposed work, we will attempt to sequence, isolate and characterize those viruses that our analyses below suggest are most likely to be able to infect humans.

Our collaborative group has worked together on these projects, as well as other DTRA funded projects in Singapore/Thailand (Co-Is Broder, Wacharapleusadee, Wang) to design, cross-train, and use novel serological and molecular platforms to investigate virus infection dynamics in bats and examine potential spillover events with or without human disease (see Aim 2). For serology, Co-Is Wang and Anderson (Duke-NUS) will develop a phage-display method to screen antibodies for all known and related bat-borne viruses, focusing on henipaviruses, ~~filoviruses~~ FV and ~~coronaviruses~~CoV. Once various "trends" are discovered, additional platforms (including Luminex and LIPS) will be used for verification and expanded surveillance. For molecular investigation, we will combine targeted PCR approach with a discovery-driven enrichment NGS strategy to not only survey currently known viruses, but also to discover potential novel viruses. Both the phage-display serological and enrichment platforms can be rapidly updated to include new viruses identified. In Malaysia (Co-Is Hughes, Broder, Laing) ~~we~~ have transferred the Luminex serology platform to partner laboratories for screening of wildlife, livestock and human samples to investigate the spillover of henipaviruses and FV~~filoviruses~~ at high risk interfaces. These interfaces include farms near the forest and indigenous communities with lots of subsistence hunting in Peninsular Malaysia, and preliminary screening has found

Commented [PD26]: Kevin – please get someone to check EIDITH – I think I just guestimated these numbers…

Commented [MOU27R26]: These are correct, based on EIDITH P1 + P2 for EHA countries.

Commented [T28]: This is right number based on our records.

Commented [KJO29]: Leave this out as it may look like we've already done a bulk of the work?

Commented [MOU30]: Think we need to abbreviate these viral families throughout, should Find/Replace. Also, henipa is not a viral family, so changing back to PMVs.

serological evidence of past exposure to viruses antigenically-related to Nipah and Ebola in humans, bats and ~~non-human primates~~NHP ~~(NHPs)~~. In Thailand, Co-Is Broder, Laing and Wacharapluesadee have transferred a Luminex-based serology platform for detecting exposure to novel Asiatic ~~filoviruses~~ FV and known henipaviruses in wildlife and human samples, with testing underway and currently being validated in our Chulalongkorn University laboratory **(See also section 2.2.d)**.

1.2.d. In vitro & in vivo *characterization of viral potential for human infection:* Using Coronaviridae as an example, we have conducted *in vitro* and *in vivo* experiments to characterize the pathogenic potential of novel SARSr-CoVs from bats. We isolated three SARSr-CoVs from bat feces: WIV1, WIV16 and Rs4874, with S protein sequences that diverged from SARS-CoV by 3-7% (*20, 59, 96*). We conducted full-length genome sequencing of 12 other novel SARSr-CoVs, some highly similar to outbreak SARS-CoV in the most variable genes: N-terminal domain and receptor binding domain (RBD) of the S gene, ORF8 and ORF3 (*59*). Using our reverse genetics system, we constructed chimeric viruses with SARSr-CoV WIV1 backbone and the S gene of two different variants. All 3 SARSr-CoV isolates and the two chimeric viruses replicated efficiently in Vero E6 cells and in HeLa cells expressing hACE2, but not in those without ACE2 (*20, 59, 96*). We used the SARS-CoV reverse genetics system (*70*) to generate a chimeric virus with a mouse-adapted SARS-CoV backbone expressing SHC014 S protein with 10% sequence divergence from SARS-CoV S. This chimera replicated in primary human airway epithelium, using the human ACE2 receptor to enter into cells (*60*). Thus, **SARSr-CoVs with diverse variants of SL-CoV S protein without deletions in their RBD can use human ACE2 as receptor for cell entry.** The Baric lab has a well-established hACE2 and DPP4 transgenic mouse model that we will use this to characterize the capacity of specific SARSr-CoVs to infect (*84, 97*). We used this chimera approach to demonstrate that pathogenicity of bat SARSr-CoVs in humanized mice differs with divergent S proteins, **confirming the value of this model in assessing novel SARSr-CoV pathogenicity**. Infection of rWIV1-SHC014S caused mild SARS-like clinical signs in the transgenic hACE2 mouse model **that weren't reduced by immune-therapeutic monoclonals that attenuate SARS-CoV pathogenicity, nor after challenge following vaccination against SARS-CoV** (*60*). We repeated this virus characterization approach with chimeras using HKU3r-CoV S proteins that are ~25% divergent from SARS-CoV S, **and found that they are unable to use the ACE2 receptor**. Additionally, we were unable to culture HKU3r-CoVs in Vero E6 cells, or human cell lines.

A similar approach to the above pipeline for CoVs will be applied to novel henipaviruses and ~~filoviruses~~ FV we discover during our research. The broad mammalian tropism of Hendra virus and Nipah virus (*66*) is likely mediated by their usage of highly conserved ephrin ligands for cell entry (*15, 98*). A third isolated henipavirus, Cedar virus does not cause pathogenesis in animal models. Recently, **Co-Is Broder and Laing** have developed a reverse genetics system and rescued a recombinant CedV to test ephrin receptor usage/tropism and pathogenicity during henipavirus infections (*75*). CedV is unable to use the Ephrin-B3 receptor (*75, 99*) which is found in spinal cord and may underlie NiV encephalitis (*100*) and that pathogenesis also involves virulence factors V and W proteins. The putative henipaviruses, Ghana virus and Mojiang virus, predict V and W protein expression, with GhV able to bind to ephrin-B2, but not -B2 (*101*), and the receptor for MoJV remaining unknown (*102*) but is likely ephrin-B2 (*103*). Primary human lung endothelial cells are highly susceptible to Ebolavirus infection (*104*). Huh7 cells are good candidates for ebola in the liver, oftentimes used to isolate virus from clinical samples (*105*) Huh7 cells also offer advantages for reverse genetic recovery of novel filoviruses (*106*). Thus far, paramyxovirus attachment glycoproteins have been divided into three major groups: hemagglutinin (H), hemagglutinin-neuraminidase (HN), and attachment glycoproteins (G).

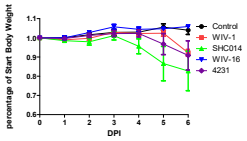

Fig. 7: Body weight change in hACE2 transgenic mice after SARSr-CoV infection, demonstrating that bat-origin SARSr-CoVs cause similar clinical presentation to outbreak strain (4231). From (*60, 61*).

Models of Outbred Populations. For novel viruses across all families, we will use the UNC collaborative cross mouse that captures 90% of the diversity within all mouse groups to assess the most suitable model for further experiments (*107*). We have used this model for CoV, ~~filo~~ FV (Ebola), Flaviviruses, and alphaviruses

Commented [KJO31]: Move to Aim 2? I think it's good to have some flexibility to test wildlife sera using these assays, but not readily clear how this fits in with our PCR screening? We could do a broader survey of a smaller number of wildlife samples from more species, and those with high seroprev, we can follow up w molecular assays – though not sure this is necessary. All the serology in this section can prob go to Aim 2.

Commented [T32R31]: All samples from concurrent Orang Asli sampling will be screened this year using Luminex under our current DTRA project as well as most of our concurrent farm samples. Doing PCR and serology on wildlife and livestock sample sets allows us to see what viruses where circulating at time of sampling while serology will show what has been circulating in past and provide indication of what novel viruses might be out there that we have yet to find in viremic animal or person.

Commented [PD33]: Ralph/Danielle/Eric/Chris – need something substantial here on filovirus binding that shows we have a valid approach for novel filos

Commented [PD34]: Ralph – I think I mis-spoke in this fig. description – please correct.

infections. In subpopulations it reproduces SARS weight loss, hemorrhage in EBOV, enceph in WNV, acute or chronic arthiritis in Chikungunya infection (*85-87, 108-111*). *Bat Models.* Duke-NUS has developed two models for bat *in vivo* studies: a colony of *Eonycteris spelaea* (cave dwelling small fruit bat) and a bat mouse model, which contains the bat immune system in an immunodeficient mouse (*112*).

1.3. General Approach: We will conduct analyses to geographically- and taxonomically-target testing of samples from wild mammals that are most likely to harbor known high-profile zoonotic pathogens, or close relatives with potential to infect people. This includes selecting from tens of thousands of samples collected on recent projects and stored in freezers in our laboratories, and new sampling of wildlife in high risk locales. We will use viral family level PCR ~~screening~~ assays to identify and partially characterize viruses, then follow up sequencing of relevant receptor binding proteins to assess homology and predict binding affinity, then attempt to isolate and biologically characterize viruses that phylogenetic and other data suggest have high spillover and pandemic potential. We will follow this with *in vitro* binding assays and cell culture experiments, and *in vivo* animal model infections to assess their potential to infect people and cause disease **(Fig. 8)**. EHA will lead the study design, targeted sampling, and data analysis; Co-Is Hughes and Wacharapluesadee will lead field sampling and in-country testing; Duke-NUS, UNC, USUS will lead the serological and PCR diagnostic development and the *in vitro* and *in vivo* analyses, as well as cross-training and tech transfer to the hub labs in Thailand and Malaysia. This will include via support of one Malaysian Ph.D student, Mei-Ho Lee (CM Ltd.) who has worked with the EID-SEARCH collaborators for 9 years. The focus of her study will be infection potential of novel CoVs and ~~paramyxoviruses~~ PMV already found in bats in Malaysia under preliminary data for this proposal.

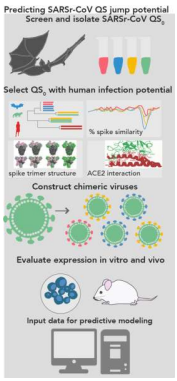

Fig. 8: Schematic showing EID-SEARCH approach to selection of sampling, viral testing, prioritization and characterization to identify novel, high zoonotic-risk viruses in wildlife.

1.4 Wildlife samples: 1.4.a. Geographic and taxonomic targeting for newly collected wildlife samples: We will prioritize sites within each country that rank highest for both the risk of zoonotic disease emergence (*113*) and the predicted number of 'missing' zoonotic viruses (*90*). Our preliminary analysis **(Fig. 1)** suggests priority areas include: the Kra Isthmus and the forests of northern Peninsular Malaysia, ~~of Kra (S. Thailand and N. Peninsular Malaysia),~~ NW Thailand (near the Myanmar-Laos border), and the lowland rainforests of Sarawak and Sabah. In each of these 'hottest of the hotspots' regions, we will identify 2-4 distinct sampling sites based on the geographic distribution of key hosts we prioritize for ~~intensive~~ additional wildlife sampling. We will priority rank bat, rodent, and ~~primate~~ NHP species using ~~analysis of host trait data for the highest predicted number of viruses based~~host trait-based models (*90*). We will also identify species predicted to be centers of evolutionary diversity for Corona-, Paramyxo-, and Filoviridae using ancestral host reconstruction of available sequence data and Bayesian discrete phylogeographic models (*114-116*). ~~We will ensure wildlife sampling is complimentary to specimens archived in our extensive freezer banks. We will collect only the additional specimens projected from each prioritized taxa to achieve statistically robust sample sizes given predicted levels of viral richness and rates of discovery **(see 1.4.b)**.~~ We will leverage our strong collaborations with wildlife experts in Malaysia and Thailand to ground-truth geographic and taxonomic model outputs with local expert knowledge to ensure the sampling plan is realistic based on current species distributions, population size estimates, seasonality, and accessibility to sites.

Recognizing that most zoonotic viruses are multi-host, i.e. naturally harbored by multiple wildlife species, and that the phylogenetic relatedness of those hosts is predictable and varies across viral families (*90*) – we will use a combined network analysis and a phylogeographic model ~~also apply a generalizable phylogenetic and spatial modeling approach~~ to rapidly predict and prioritize new (unsampled) hosts for important, novel viruses we discover during our research. ~~We will use a combined network analysis and a phylogeographic model to estimate a viruses' host range and to~~We have shown this relatively simple model ~~prioritize additional wildlife species for sampling and testing for viruses we discover. Our recently developed model of viral sharing uses just two phylogeographic traits~~ (using just wildlife species range overlap and phylogenetic similarity between host species) is both generalizable to estimate host range for all mammalian and avian viruses *and* robust –

Commented [PD35]: Ralph – please add data from the CC mouse re. filoviruses, to beef up the image of us as a filovirus group

Commented [PD36]: Linfa/Danielle – please draft a brief para explaining how we'll use these two models

Commented [PD37]: Hongying – this is a placeholder from DARPA proposal. We need a new one with Stages: 1) Geographic and taxonomic modeling to maximize discovery of potential zoonotic viruses; 2) PCR screening and partial RNA sequencing; 3) Full genome or RBD and Glycoprotien sequencing to model receptor binding based on sequence similarity; 4) Cell line infections (for prioritized viruses); 5) animal model infections (for prioritized); 6) High priority viruses ranked based on data (red, yellow, green) schematic.

Commented [T38]: Kra Isthmus is in Thailand

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accurately predicting hosts 98% of the time when cross-validated using data from known viruses ~~to successfully predict host species in the top 2% of all 4,200 possible mammal species~~ (*117*).

1.4.b. Sample size justifications for testing new and archived wildlife specimens: We calculate initial ~~We will~~sample sizes targets using ~~e~~ our preliminary data on average PCR prevalence from screening tens of thousands of bat, rodent and primate specimens for CoV and ~~Paramyxoviruses~~ PMVs under the USAID-PREDICT project in Malaysia and Thailand. We will refine these sample size targets, in real-time, over the course of our project using viral discovery curve analyses **(Fig. 6)** to either scale-up or scale-back new sampling and testing of archived specimens for each species depending on ~~their~~ viral ~~diversity~~capture rates. We will only collect additional specimens projected from each prioritized taxa to achieve statistically robust sample sizes and to complement our archived specimens. For example, *Rousettus* spp. bats, known filovirus reservoirs, were not adequately sampled under PREDICT research in Thailand. Preliminary analysis indicates we will require ~14,000 new samples to identify 80% of remaining viral species diversity in our study region, ~5,000 samples from Thailand and ~9,000 samples from Malaysia **(Fig. 9).** Viral strain di~~scovery~~versity from a sampling effort this size is expected to number in the hundreds of strains. For example, given ~6% prevalence of CoVs in bat species previously sampled under the PREDICT project, a target of 14,000 individuals would yield ~800 PCR positive individuals, representing, ~~conservatively,~~ an estimated ~~200~~600 novel ~~CoV strains~~sequences/strains. Similarly, for paramyxoviruses, which have an average PCR detection prevalence of ~1.5%, sampling of 14,000 individuals would yield ~200 positives and an estimated ~~50~~150 novel ~~paramyxovirus~~ strains. From each mammal, we will collect serum, whole blood, throat swabs, urine and fecal samples or rectal swab using our previously-validated ~~For example, given 5-12% prevalence of CoVs in the most common bat species we previously sampled in Thailand and Malaysia, a target of XXX individuals per species would yield XX (± x) PCR positive individual bats, and an estimated ~xx novel strains. Similarly, for paramyxoviruses with an average PCR detection rate of X%, we would need XX individuals per species to detect 80% of predicted viral strain diversity with 80% power. Preliminary analysis indicates we will require XX,000 samples to identify 80% of remaining viral diversity **(Fig. 9).** All wildlife sampling will be~~ nondestructive methods (see Vertebrate Animals section) [REFS]. In the event that an animal is found dead or needs to be euthanized due to injury we will collect ~~biopsy~~ necropsy samples for screening.

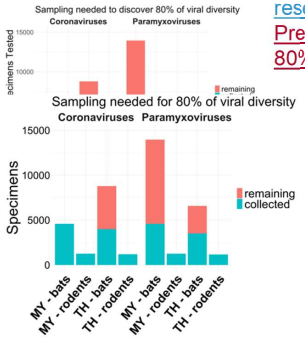

Fig. 9. Stacked bar plot showing number of currently-archived specimens **(blue)** and new specimens required **(orange)** to discover 80% of predicted number of CoVs and PMV species ~~s~~ from high-risk bat and rodent taxa in Malaysia and Thailand

We estimate the following sampling effort, broken down for each region: *Peninsular Malaysia:* Wildlife sampling will be led by CM Ltd / EHA in collaboration with PERHILITAN. Archive bat, rodent and NHP samples will be screened serologically using the BioPlex and other serology techniques identified through this project. New wildlife sampling will focus on bats but it is expected that rodents and NHPs will be sample as well. Wildlife sampling will be conducted around Orang Asli communities sampled as part of aim 2. Three new communities will be identified in the districts we have already sampled in. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife mainly bats will be sampled. Wildlife and domestic animal samples will include serum, whole blood, throat swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and tested at PERHILITAN's NWFL and screened using molecular and serological techniques. *Sarawak, Malaysia:* Archive bat, rodent and NHP samples will be screened serologically using the BioPlex and other serology techniques identified through this project. Wildlife sampling will be led by CM Ltd / EHA in collaboration with UniMAS (Co-Is Faisal Faculty of Resource Science and Technology). Wildlife sampling will be conducted around ~~Dyak~~ indigenous communities sampled as part of aim 2. One community will be identified. Concurrent sampling trip will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife mainly bats will be sampled. Wildlife and domestic animal samples will include serum, whole blood, throat swabs, urine sample of urogenital swab

Commented [PD39]: Kevin - That doesn't make clear sense straight away – please re-word better without increasing length

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Commented [KJO40]: Just assumed that 75% of detections would = unique sequences/strains. We can lower this to be more conservative.

Commented [MOU41]: Kevin will work with Noam and Evan to fill in these numbers.

Commented [KJO42]: PREDICT sampling protocols? Jon's FAO bat book manual, and other published for rodents and primates (from Bangladesh?)

Commented [PD43]: Hongying/KEvin Need less text on the figure, but larger font size, or crisper text to make it all visible at this size.

and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and initially tested at ~~UniMAS or~~ BMHRC using molecular and serological techniques. If over the course of the project we are able to develop the laboratory at the Faculty of Medicine and Health Sciences or Faculty of Resource Science and Technology molecular screening will be carried at UniMAS. *Sabah, Malaysia:* Bat sampling will be led by CM Ltd / EHA in collaboration with SWD and WHU. Bat sampling will initially focus on 3 caves. Batu Supu Sabah low disturbance cave and surrounding area, Madai medium disturbance cave and surrounding area, Gomantong cave high disturbance cave and surrounding area. Each cave will be sampled twice once in wet season and once in dry season targeting 500 bats at each cave on each trip for a total of 3000 bats. Wildlife samples will include serum, whole blood, throat swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored at WHGFL, molecular testing will be conducted at WHGFL and serology screening at BMHRC. In addition at Madi cave the community that lives in the mouth of the cave will also be sampled targeting 150 people, while at Gomantong cave 25 workers involved in the bird next farming will be enrolled into the study.

Thailand: XXXXXXXXXX New bat specimens will be collected from bat species that was not collected during PREDICT2 study for example *Rousettus* bats (targeted species for Filovirus study), *Taphozous* bat (targeted species for MERS-CoV), *Rhinolophus* (targeted species for SARS-CoV) for serology and PCR assays (n=100 for each species).

~~*Thailand: XXXXXXXXXX*~~

1.4.c. Sample testing, viral isolation: Viral RNA will be extracted from bat fecal pellets/anal swabs ~~with High Pure Viral RNA Kit (Roche)~~. RNA will be aliquoted, and stored at -80C. PCR screening will be performed with pan-coronavirus, filovirus and paramyxovirus primers, in addition to virus specific primers where additional sensitivity is warranted in key viral reservoirs, i.e. Nipah virus specific primers [REF]. PCR products will be gel purified and sequenced. We will attempt isolation on samples that contain viruses of interest (determined in **1.4, below**), using Vero cells and primary cell culture from the wildlife taxonomic order (bat, rodent and primate cell lines). This includes the over 30 bat cell lines maintained at Duke-NUS from four different bat species.

1.4.d. Moving beyond RNA viruses: To detect pathogens from other families, such as non-RNA viruses we will combine conserved PCR assays with NGS. We will perform either unbiased NGS, or use a pan-viral enrichment library to sequence full-length genomes. We will conduct this work via EID-CC research projects or as part of outbreak detection/investigation in the region from DNA viruses or other agents.

1.4.e. Sequencing Spike Glycoproteins: Based on earlier studies, our working hypothesis is that zoonotic ~~coronaviruses~~CoV, ~~filoviruses~~ FV and ~~henipaviruses~~ PMV encode receptor binding glycoproteins that elicit efficient infection of primary human cells (e.g., lung, liver, etc.) (*12, 59, 60*). Characterization of these suggests SARSr-CoVs that are up to 10%, but not 25% different in the spike glycoprotein use human and bat ACE2 receptors for docking and entry. Uneven sampling gaps (e.g., no strains were found with 10-25% spike variation) prevent a thorough understanding of the transition point where the most divergent strains lose human ACE2 receptor usage. Our viral discovery curves **(Fig. 4)** suggest further sampling will reveal a rich diversity of as-yet-undiscovered SARSr-CoVs. In this aim we will use phylogenetic and viral discovery analysis to specifically target bat species and regions that are under sampled to allow sufficient power to identify and characterize missing SARSr-CoV strain diversity. Our previous work also suggests that SARSr-CoVs with S proteins that are ~10% divergent from SARS-CoV resist neutralization by therapeutic mAbs and escape SARS-CoV vaccines (*20, 60, 118*), suggesting that existing vaccines and immunotherapeutics could easily fail against future emerging SARS-like CoV. Moreover, viruses with 10-25% divergence in S proteins may bind to human cell receptors, but completely evade therapeutic and vaccine effects, and could therefore be a higher risk for public health. We will sequence the S proteins of novel SARSr-CoVs to prioritize viruses for experimental work in Aim 3 to test this hypothesis. Likewise, we will test the hypothesis that phylogenetic related zoonotic receptor binding protein genes of MERS-related, Ebola-related and Nipah/Hendra-related viruses will also program efficient entry via human ortholog receptors. For all novel virus strains, we will sequence the complete entry spike genes by amplifying overlapping fragments using degenerate primers as shown previously (*20, 59*). Full-length genomes of selected CoV strains (representative across subclades) will be sequenced using NEBNext Ultra II DNA Library Prep Kit for Illumina and sequenced on a MiSeq sequencer.

Commented [T44]: I have just seen Dr Tan's lab – no -80 freezer, no biosafety cabinets.

Commented [PD45]: Kevin – who can write this bits?

Commented [KJO46]: I think all this could be deleted. Doesn't fit with the overall plan, or is redundant. We may want to call out a few specific sites Tom mentions (but won't that depend on the modeling!?), and you may want to keep the partner info Tom provided and call that out - maybe more in Aim 2 since its about people too?

Commented [T47R46]: These sites fit into geographic areas described above. If we want to sample a large number of bats the Sabah caves are our best bet. We could target caves in PM as well as in Sarawak. But based on earlier discussions I thought this was plan for PM and Sarawak?

In PM we could target –
1. A cave we found near OA GM but only around 300 bats and we sampled 171 already.
2. Gua payong, kelantan
3. Gua tempurug, perak
4. Gua kandu, perak
5. Bewah cave, terengganu
6. Cave kelam, perlis

In Sarawak we could target Niah, Mulu, Fairy, Deer, Wind caves. These are tourist attractions with many native settlements around the areas and hunting bushmeat for protein source is still a norm.

Commented [T48]: Will we not use throat swabs as well – thought this was where we had found the most viruses then in fecal samples?

Commented [MOU49]: Supaporn: From my DTRA study, specific Nipah primers and WHO 2C CoV primers is more sensitive than consensus PCR if we want to find the specific virus.

Commented [PD50]: Linfa – can you give a v. brief description of these please, e.g.: "This includes 13 primary lung, 5 primary gut epithelial, and 1 primary liver cell line. In addition we have 12 immortalized cell lines, 7 of which are intestinal, and therefore suitable for CoVs. Cell lines are derived from insectivorous and frugivorous bats (genera……)"

PCR or MinIon sequencing will be performed to fill gaps in the genome. The full length spike gene sequences, including the amount of variation in the receptor binding residues that bind the appropriate human receptor will be used to select strains for Aim 3 experiments.

1.5. Assessing risk for spillover. 1.5.a. Capacity to infect human cells: We will use a series of cell cultures to assess the capacity of target viruses to enter human cells. For CoVs, ~~filoviruses~~ FV and ~~henipaviruses~~PMV, we will use primary human ciliated airway epithelial cells (HAE) and lung endothelial cell cultures from the lungs of transplant recipients represent highly differentiated human airway epithelium containing ciliated and non-ciliated epithelial and goblet cells, grown on an air-liquid interface for several weeks prior to use (*60, 61, 119*). We will prepare HAE and lung endothelial cultures from three different patient codes in triplicate in collaboration with the tissue procurement facility at the Cystic Fibrosis Center at UNC. Cultures will be inoculated with chimeric bat SARSr-CoVs or other test ~~filoviruses~~ FV or ~~henipaviruses~~ PMV to assess efficient replication. At 72 hpi, cultures will be fixed for immunofluorescent staining using antisera to the SARS-CoV conserved nucleocapsid protein (N) or to structural genes of the other viruses (*62, 69*). SARSr-CoVs that differ significantly in S protein sequence (11-24%) from epidemic SARS-CoV yet replicate *in vitro*, will also be evaluated for sensitivity to neutralization in Vero cells by PRNT50 assays using broadly SARS-CoV cross reactive human mAbs S227.14, S230.15, S215.17, and S109.8 (*61, 120*) or monoclonal antibodies targeting the Ebola or Marburg E glycoprotein (Available through BEI), or Nipah or Hendra viruses (*121*). As controls or if antisera is not available, the S genes of novel SARSr-CoV or spike entry genes of other novel viruses under investigation will be inserted into VEE 3526 replicon vectors (VRP3526-S), packaged and used to vaccinate mice (*122*). Polyclonal sera against SARS-related viruses will be harvested and tested for ability to cross neutralize SARS-CoV, GD03, WIV-1, SHC014, WIV-16, other novel SARSr-CoV and HKU3-SRBD by PRNT50 assay (*61, 123, 124*). Using PRNT50 titers with sera (n=4 each) among these viruses, antigenic cartography (*125*) will allow comparison of antigenic vs. phylogenetic distance, identifying the transition at which SARSr-CoV strains escape SARS-CoV based vaccines, informing immunotherapeutic and vaccine design strategies (*126-128*). Similar approaches will be applied to novel MERS-related viruses, other CoV, ~~filoviruses~~ FV or ~~henipaviruses~~PMV. While most wildlife-origin viruses with unknown transmission potential or pathogenicity in people are BSL-2 or -3, cell culture for potentially BSL-4 pathogens will be undertaken at collaborating institute NEIDL. To facilitate this, duplicate samples in culture medium from animals PCR +ve for filoviruses will be shipped to NEIDL **(See letter of support)** for culture, characterization and sharing with other approved agencies including NIH Rocky Mountain Laboratories ~~RML~~where our EID-SEARCH team has ongoing collaborations. When appropriate o~~f~~r feasible, training opportunities at NEIDL for in-country staff will be given.

1.5.b. Host ACE2, DPPR, Niemann-Pick C1 (NPC1) and EphrinB2/EphrinB3 receptors: We will sequence host ACE2 receptors of different bat species or different bat populations from a single species (e.g. *Rhinolophus sinicus*) to identify relative importance of different hosts of high risk SARSr-CoVs and the *intra*specific scale of host-CoV coevolution. Of particular interest is homology across 18 bat and human orthologue ACE2 contact interface residues that engage the SARS RBD as a potential indicator of SARSr-CoV cross species transmission potential and growth in human cells (*68*). ~~A lot of v~~Variation in the NPC1 receptor alters EBOV ability to infect in humans and bats, so this can be structurally mapped for prediction and downselection of strains (*129*). Signatures of positive selection in bat NPC1 were concentrated at the virus-receptor interface, with the strongest signal at the same residue that controls EBOV infection in *Eidolon helvum* cells. Thus NPC1 is a genetic determinant of filovirus susceptibility in bats, and some NPC1 variations likely reflect host adaptations to reduce filovirus replication and virulence (*130*). Nipah and Hendra use ephrin B2 or ephrin B3 as a receptor, and variation affects its ability to use human vs bat receptor molecules (*101, 131*).

1.5.c. Host-virus evolution and distribution: We will use Bayesian and Maximum Likelihood phylogenetic analyses of RNA-dependent RNA polymerase (RdRp) sequences from PCR screening ~~(or L genes)~~, receptor binding glycoproteins, and/or ~~-~~full genome sequence data (when available) ~~data~~ to reconstruct the evolutionary history of the novel bat SARSr-CoVs, bat MERSr-CoV, ~~filoviruses~~ FV and ~~henipaviruses~~ PMV that we identify. We will run phylogenetic and ancestral state reconstruction analyses **(Fig. 5)** to ~~reconstruct~~identify host taxa and geographic sites of ~~β-CoV, filovirus and henipavirus~~ evolutionary origin~~s~~ within bats, rodents and primates ~~using our expanded dataset and identify the host taxa and geographic regions~~ that together define hotspots of phylo-diversity for these viruses, allowing for more targeted surveillance. For receptor binding glycoprotein

Commented [BRS51]: Any antibodies available? If not we can synthesize them from published co-crystals.

Commented [PD52]: Can someone answer Ralph's question please?

Commented [KJO53]: Make more specific about our plans to sequence NPC1 or Ephrin receptors, etc. Should we frame much of this as "add on projects" pending additional EIDRC-CC funding?

Commented [PD54]: Kevin/Alice – please edit this section to include relevance for filos and henipas as well (and other hosts)

sequence data from characterized viruses, we will apply codon usage analyses and codon adaptation indices, as used recently on Henipaviruses (*132*), to assess the relative contributions of natural selection and mutation pressure in shaping host adaptation and host range. ~~We will rerun MCC analyses **(Fig. 3)** to reconstruct β-CoV evolutionary origins using our expanded dataset. Ecological niche models will be used to predict spatial distribution of PCR-positive species, identify target sites for additional bat sampling, and analyze overlap with people. We will use our viral discovery/accumulation curve approach **(Fig. 4)** to monitor progress towards discovery of >80% of predicted diversity of SARSr-CoVs (lineage 1 & 2) or other virus families within species and sites, halting sampling when this target is reached, and freeing up resources for work other work (*96, 133*).~~

1.5.d. Viral strain prioritization: Of the expected 100-200 novel SARSr-CoV strains, and approximately 600 total CoV strains, we will down-select to prioritize for further characterization based on S genes that are: i) different from SHC014, WIV1, SARS-CoV with diversity ranges of 10-25%; ii) have virus S RBD that could use human/bat receptors; iii) have recombinant chimeric spikes indicative of gene flow between clade I and II strains; iv) have bat ACE2 receptors that might select for spike RBDs that can use human receptors for entry (15/18 conserved residues in human/bat ACE2 molecules that bind SARSs-CoV S RBD domains are likely more efficient receptors than 3/18 conserved sites). Using structural models based on the SARS S glycoprotein, the extent and location of antigenic variation will be annotated onto the structure, further resolving the locations of highly evolving and conserved structural motifs/epitopes that function in protective immunity (*120, 133-135*). For novel ~~henipaviruses~~PMV, we will examine the receptor-binding proteins (e.g. envelope attachment (G) proteins) for sequence and structural homology with particular attention to conversation of amino acids that mediate ephrin receptor binding. We will prioritize strains that are predicted to interact with ephrin-B2 and expression V/W proteins to determine whether these viruses represent zoonotic threats. Using the recombinant Cedar virus (rCedV) platform, we will be able to rescue rCedV that express heterotypic G proteins to explore receptor utilization and tissue dissemination in animal models. We will assess ability to infect human cells using a continuous lung epithelial cell line, Calu3, that our group has previously used to generate data on NiV and HeV growth curves and a variety of host expression changes after infection (*136*). We will also use primary human airway cultures to assess human infection for ~~Henipavirus~~ PMV and MERS- and SARS-related CoV (*137*) (*138*) . There is some evidence for Nipah and Hendra virus replication in mouse lung, low level titers in young animals, 10 fold higher titers in aged mice (*139, 140*). Thus it is likely these models could be improved in the collaborative cross mouse resource at UNC. We have identified CC lines that are highly vulnerable to wildtype SARS and others that develop hemorrhagic disease following mouse adapted Ebola infections (*85-87*).

Commented [KJO55]: I suggested we could get up to 600 CoV "strains" (all seqs, not just SARSr-CoVs) in section 1.4b, but we can reduce that.

1.5.e. Animal models: We will use transgenic mouse models and the UNC Collaborative Cross model to assess spillover potential of viruses. For work on CoVs, we will follow the protocols in the transgenic mouse model used by the Baric lab previously. Briefly, in the BSL3 lab, n=8 10- to 20-week old hACE2 or hDPP4 transgenic mice will be intranasally inoculated with 5 x 10^4 PFU of wildtype WIV-1 or chimeric bat SARSr-CoVs with different spike proteins or MERS-CoV and MERS-related CoV, respectively, then monitored daily for weight loss, morbidity, and clinical signs of disease. Mice will be euthanized at 2, 4, and 6 dpi (endpoint of 14 dpi), organs harvested and virus quantified by NP RT-PCR. After 7 days in formalin, tissues for histopathology will be removed from the BSL3 and stained with H&E, and for immunohistochemistry using polyclonal antibodies against the N protein. We will conduct limited evaluation of existing countermeasures using therapeutic monoclonal antibodies *in vitro* and *in vivo*. Existing SARSr-CoV or MERS-CoV mAbs (*141, 142*) will be diluted in DMEM starting at 1:20, and serial 2-fold dilutions mixed with an equal volume of 50 PFU of chimeric bat SARSr-CoVs with different spike proteins, then incubated on Vero E6 cells at 37°C for 1 h, then changed to a semi-solid medium (DMEM containing 1% methylcellulose and 2% FBS). Antibody neutralization titers will be evaluated by plaque quantification at 4-5dpi. hACE2 transgenic mice will be injected with SARS-CoV mAbs, and infected with chimeric bat SARSr-CoVs. Clinical signs and morbidity will be assessed and tissue pathology examined and compared with mice without treatment of mAbs to determine the therapeutic effect on SARSr-CoV infection, and protection of SARSr-CoV by wildtype SARS-S based vaccines assessed as described (*119, 143*). We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-CoV cross neutralization, synthetically reconstruct a small subset (1-2) and

evaluate the ability of nucleoside analogues to inhibit growth in HAE cultures and/or *in vivo* (*61, 119*). For the Collaborative Cross model, we will….

Finally, for more detailed pathogenesis studies in the natural reservoir, we will use the bat models (natural and transgenic mouse model) at Duke-NUS. Both animal systems can be used to test susceptibility, pathogenesis and immune responses of any newly discovered viruses.

1.6. Potential problems/alternative approaches: Challenges with logistics or obtaining permission ~~to~~ for wildlife sampling in sites we select~~sample bats in sites or provinces we select~~. We have a >20-year track record of successful field work in Malaysia, ~~Thailand and~~ and >10 years in ThailandSingapore, and have ~~worked with~~strong established partnerships with local wildlife authorities and communities to ensure access. We have existing permissions in place which will be renewed at the start of this project. Due to the abundance of wildlife habitats in the region, if access to a site is withdrawn, we will rapidly identify a suitable, complementary site with similar bat species composition and abundance. Finally, we have already collected tens of thousands of wildlife samples from the region and will focus on these while waiting for permission. **We may not identify novel viruses in our sample wildlife species due to seasonality of viral shedding or other factors.** We have calculated sample sizes based ~~on a range of PCR data and are fairly conservative in our approach~~on realistic detection rates from our extensive preliminary data, and are confident that we will detect and identify large numbers of potentially zoonotic pathogens, particularly for CoVs and PMVs. ~~However, as a back-up, we already have identified dozens of novel viruses that are near-neighbors to known high-impact agents, and will continue characterizing these should discovery efforts yield few novel viruses~~We acknowledge that FV strain diversity may be harder to capture, given relatively low seroprevalence in bat populations – suggesting lower levels of viral circulations (*40, 41*). However, our team was the first and among the only groups to sequence FV RNA from Asian wildlife species(*44, 45, 55*), and we are confident that with our targeted sampling and testing strategy will identify additional strains of Ebola and related viruses. The sampling regions we have selected are subtropical, and our previous data, and even published studies in temperate regions (*144*), do not suggest a strong pattern of seasonality in CoV shedding, ~~although there are studies that suggest this~~and where seasonal patterns do occurs, i.e. for ~~in~~ henipaviruses ~~and filovirus (MARV?) shedding (REFS?)~~in Thailand (*145*) or can be predicted from wildlife serology data, we will be sure to ~~. Nonetheless to account for this we will~~ conduct sampling ~~evenly on quarterly basis within each province~~at different timepoints throughout the year to account for this.

Aim 2: Test evidence of viral spillover in high-risk communities using novel serological assays.

Country, site	Lead	# enrolled, focus	Findings
Peninsular Malaysia PREDICT	Hughes	1,390 Orang Asli indigenous population, 9813 samples for viral PCR/serol.	~~25+ novel~~ Ongoing. 4 known CoVs, 1 influenza, Nipah ab+ve, filovirus ab+ve

Commented [PD56]: Ralph – please insert a couple of sentences on how we'll use the cc mouse and what we'd expect

Commented [PD57]: Linfa/Danielle – could you put a couple of sentences in on what we might do with the models. Remember – this could be an additional short project from the EIDCC extra funding.

Commented [KJO58]: Emily and Noam reworking this section significantly, so not touching it much.

Commented [T59]: These serology findings were from UM OA samples not our OA samples. We have not started screening ours yet.

Malaysia Sabah	Kamruddin	1,283 serology	40% JE, 5% ZIKV ab+ve
Malaysia Sabah	William	10,800 zoonotic malaria	High burden of macaque-origin malaria
~~Malaysia Sabah~~	~~Hughes~~	~~150 bat cave workers~~	~~Ongoing~~
Malaysia Sarawak	Tan	500 Bidayuh and Iban people	8% PCR prevalence HPV in women
~~Malaysia PREDICT~~	~~Hughes~~	~~9,933 high zoonotic-risk communities, viral PCR, serology~~	~~Ongoing. Multiple known and novel CoVs, paramyxoviruses, others.~~
Thailand	Wacharaplu-esadee	1,000 bat guano harvesters/villagers	Novel HKU1-CoV assoc. clinical findings
Thailand PREDICT	Wacharaplu-esadee	9,269 high zoonotic-risk communities, viral PCR, serology	Ongoing. Multiple known and novel CoVs, paramyxoviruses, others.
Singapore	Wang	856, Melaka virus	7-11% ab+ve

2.1 Rationale/Innovation: Rapid response requires early detection. Thus, assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities that interface frequently with wildlife that harbor high risk emerging viral strains. To enhance the low statistical probability of identifying these rare events, we will target rural human communities inhabiting regions of high wildlife biodiversity, that also engage in practices that increase the risk of spillover (e.g. hunting and butchering wildlife). In Aim 2 we will build and expand on our preliminary biological sampling and enroll large cross-sectional samples in human populations with high behavioral risk of exposure to wildlife-origin viruses and that live close to sites identified in Aim 1 as having high viral diversity in wildlife. We will initially identify 2 sites in ~~each of~~ Thailand, 3 in Peninsular Malaysia, 1 in Sarawak, and 1 in Sabah for repeated biological sampling. We will design and deploy behavioral risk factor questionnaires, and specific and sensitive serological assays to identify the baseline frequency, and causes of, spillover of known and related novel viral pathogens in these populations. In participants where symptoms are reported in the last 10 days, paired serum specimens (acute and 21 days after fever) will be collected to determine titer of positive antibody. Samples will be marked for additional PCR-based assays to identify presence of known and novel CoVs, ~~henipaviruses~~ PMV and ~~filoviruses~~FV, and for isolation and biological characterization of potential pathogens, using the approach laid out in Aim 1.

2.2 Preliminary data on high-zoonotic risk human community surveillance: 2.2.a Biological sample collection in SE Asia: Our longterm collaboration in the region has included qualitative and quantitative survey data, and collecting large archives of banked human sera and other biological samples from high zoonotic-risk populations. For example, under the USAID-PREDICT project, EHA has collected 9,~~933~~ 813 and 9,269 specimens in Malaysia and Thailand respectively from people living in zoonotic disease interfaces, all of which will be available for retrospective serological testing by EID-SEARCH. Our core group has collected and tested many thousand additional specimens from other important community cohorts **(Table 2)**, and some of these will continue under EID-SEARCH **(See section 2.4)**. Of particular interest is the access to indigenous communities in Peninsular Malaysia (Orang Asli) and Sarawak (Bidayuh, Iban people), and the bat guano harvesters in Thailand. These communities hunt, butcher and eat wildlife daily, and work in supremely high-risk zoonotic interfaces (e.g. bat caves). One of our indigenous community sites is experiencing an ongoing outbreak of unknown etiology that we are investigating currently **(See section 3.2.a)**.

Table 2: Current or recent biological sample collection from healthy populations conducted by members of our consortium in EID-SEARCH hub countries

2.2.b Human Contact Risk Factors: EHA is the lead organization across all 27 USAID-PREDICT project countries for collecting data on, and analyzing the human aspects of, zoonotic spillover risk (*146, 147*). We conducted exploratory studies using standardized one-on-one semi-structured interviews and site observation data with people living in hotspot areas or engaged in high-zoonotic spillover risk activities, using these data to assess local social and cultural norms underlying wildlife contact (*147*). We then developed a risk factor survey to accompany human biological sampling to evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. We conducted cross-sectional studies in high risk populations in 27 countries, and study participants provided biological samples (oral/oropharyngeal swab, nasal/nasopharyngeal

Commented [T60]: This is something we are proposing to do under this proposal not something we have started.

Commented [KJO61]: Check these numbers, don't think we have 9000 people sampled, if this represents individuals! Per below, we say we have 9000 specimens, not people, so need to adjust.

Commented [KJO62]: Emily: Will we be sampling participants more than once?

Commented [PD63]: I think that would be good, depending on the sample size

Commented [KJO64]: Need to decide if we do PCR on community samples, we can add in the questionnaire if you've been sick in the last 10 days and if so, then maybe test only this subset.

Commented [PD65]: Kevin's comment makes sense to me

Commented [T66R65]: Agree

Commented [T67]: 9813 samples from OA
120 samples from Syndromic Surveillance in Sabah

Commented [KJO68]: Don't match table numbers, need to change table header to # specimens, not # enrolled.

Commented [T69]: Community near one of our sites and we are not investigating just provided some informal advice on sampling, PPE etc

Commented [T70]: This needs to go under table – I can't fix formatting.

swab, urine sample/urogenital swab, fecal sample/rectal swab, whole blood, serum, plasma). For example, survey and biological samples were collected from: 1,585 participants from 7 sites in Yunnan, Guangxi, and Guangdong provinces, China; 1,400 participants from 4 sites in Malaysia; and 678 participants from 4 sites in Thailand. Samples were tested with novel serological assays and results analyzed against risk factors from survey data (*12*). In China, CoV-seropositive individuals were mostly farmers/peasants (8/9), living in Yunnan province (7/9), 41-60 yrs old (7/9), with domestic contact with rodents (6/9), that are male (6/9). Although these results are preliminary and don't provide detailed information on routes of exposure, they identify key subpopulations to target that would likely increase the opportunity for identifying evidence of spillover for other CoVs, ~~paramyxoviruses~~ PMV and ~~filoviruses~~FV. Data from PREDICT surveys will be used to help identify target populations in Aim 2, identify strategies to better target at-risk people, and conduct focused **human animal contact surveys and serosurveys to produce statistically significant findings for ~~henipaviruses~~PMV, CoVs and ~~filoviruses~~FV.** In Aim 2, we will use combined biological sampling and risk factor surveillance in targeted populations within the community to 1) identify risk factors correlated with seropositivity to ~~henipaviruses~~PMV, ~~filoviruses~~ FV and CoVs; 2) assess possible health effects of infection in people; and 3) where recent illness is reported, obtain clinical samples for PCR testing and potential viral isolation and characterization. Obtaining this information could be a significant step in understanding the likelihood of recent 'hidden' spillover events and their public health impacts, as well as the risk of future emergence of viral disease.

2.2.c. Risk factors for illness: Our preliminary data from PREDICT in Thailand, Malaysia and China revealed that frequent contact with wild (e.g. bats, rodents, ~~non-human primates~~NHP) and domestic animals (e.g. poultry, swine) among local communities was associated with a high percentage of self-reported severe/acute respiratory illness (SARI/ARI) and Influenza-like illness (ILI) symptoms. In addition, people involved in crop production, hunting and butchering of wildlife reported statistically significantly higher SARI and ILI symptoms (Li *et al.*, in prep.). **These preliminary data suggest a target population and sites for current proposal with the correct targets for serological surveillance and risk factor surveys in Aim 2 to produce statistically significant findings**. Coupled with further refining serological tools, we will be able to identify the likely routes of exposure to known or novel ~~coronaviruses~~COV, ~~henipaviruses~~PMV, and ~~filoviruses~~FV in the study region, as well as build data on the illnesses they cause in people.

2.2.d. Serological platform for community surveillance: One of the present challenges in emerging disease surveillance is that viremia can be short-lived, complicating viral-RNA detection (i.e. traditional PCR-based approaches), especially in zoonotic reservoir hosts, and requiring large samples sizes to understand infection patterns or host range. By contrast, antibodies are typically long-lived and can be used to assess prior virus exposure or infection with much smaller samples sizes (*148*). However, most serological assays only target a single protein, and it's not always clear which antigen is the most immunodominant during an adaptive humoral immune response – i.e. which is the best target for a serological assay. ~~Henipaviruses~~PMV, ~~filovirus~~ FV and CoVs express envelope receptor-binding proteins (e.g. CoV=spike (S) protein, ~~filoviruses~~FV / ~~henipaviruses~~PMV=glycoprotein (GP/G)), which are the target of protective antibody responses. The Broder lab at USU, a leader in the expression and purification of native-like virus surface proteins for immunoassay application (*149*)?, developing monoclonal antibodies (*150, 151*) and as subunit vaccines (*152, 153*), is presently developing a novel, multiplex microsphere immunoassay (MMIA) allowing serum samples to be simultaneously tested for antibodies reactive with ~~henipaviruses~~PMV, ~~filoviruses~~FV and ~~coronaviruses~~CoV. This MMIA uses a Luminex-based multiplex platform and custom designed virus receptor-binding proteins expressed in a mammalian cell-culture system and purified as native-like oligomeric proteins (*149*). These oligomeric antigens retain post-translational modifications and quaternary structures. This structural advantage over peptide antigens, allows the capture of conformational-dependent antibodies raised during a natural infection, which represent the majority of the protective humoral response.

Commented [T71]: This includes 10 people from Syndromic surveillance in Sabah

1390 participants from 3 districts in Peninsular Malaysia – not yet serologically screened but will be this year.

Commented [KJO72]: Peter, sent over LASSO figure for ILI from Thailand and Malaysia for you to consider.

Commented [PD73]: Chris/Eric Is this the right ref?

Commented [KJO74]: See my comment in Aim 1 that some of that serology platform stuff can be moved here.

Serological platforms have been historically limited by the starting antigen, and cross-reactivity between known and unknown related viruses not included in the assay. The known diversity of ~~henipaviruses~~ PMV includes five presently described viruses, NiV, HeV, CedV, GhV and MojV and this assay has been expanded to include all described species **(Fig. XXX)**. In the past, our collaborators have used NiV and HeV G protein antigens in a MMIA assay to identify a serological footprint of NiV-related African henipaviruses and exposure to virus(es) antigenically-related to NiV in livestock sampled in Bangladesh (*76, 77*). To strengthen serological data interpretation, the G protein from each virus is integrated into the MMIA so that virus-specific versus henipavirus cross-reactive antibodies can be detected and address specificity of past virus exposure. Extensive cross-reactivity exists within ebolaviruses and between ebolaviruses and marburgviruses, highlighted by studies examining antibody binding between heterologous viruses (*154-156*). Ongoing work is aimed at validating the MMIA pan-filovirus assay for specificity. The majority of ebolaviruses are endemic to Africa, however the discovery of Měnglà virus in Yunnan, China increased the further demonstrated that a diversity of Asiatic filoviruses with unknown pathogenicity and zoonotic potential exists. Our past work in collaboration with Duke-NUS demonstrated that three under-sampled fruit bat species had serological evidence of past exposure to a filovirus(es) most antigenically-related to ebolaviruses (e.g. Ebola virus, Bundibugyo virus), but unreactive with Reston virus GP, suggesting that novel filoviruses circulate within bat hosts (*41*).

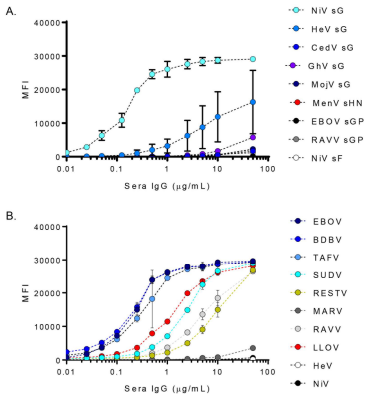


Fig. XXX: Validation of multiplex microsphere immunoassay (MMIA) specificity and identification of immunologically cross-reactive viruses for Nipah (A) and Ebola (B).

2.3 General Approach/Innovation: We will use a dual study design to gain in depth understanding of exposure and risk factors for viral spillover **(Fig. XX)**. In Aim 2 we will conduct community-based surveillance using a brief, focused questionnaire to identify the prevalence and frequency of risk-factors for viral spillover in tandem with collection of high quality biological samples to determine the seroprevalence of spillover viruses in at-risk human populations. We will use generalized linear models to analyze correlation between biological results and risk-factors for viral spillover. In Aim 3, we will conduct clinic-based syndromic surveillance and biological sampling at clinics/hospitals that are the catchment healthcare facilities for the communities sampled in Aim 2. Both community-based and clinic-based syndromic surveillance programs are cross sectional case-control studies designed with the sample sizes necessary to statistically quantify (with a power of 80%) risk factors and health impacts for viral spillover, linked to serological status and PCR prevalence in symptomatic patients.

2.4 Target population & sample sizes: We will target populations in the same geographic regions as those identified in Aim 1 that harbor wildlife with high potential zoonotic viral diversity, that are EID hotspots, and that are well-connected through regional travel and trade hubs to the global travel network **(Fig X)**. We will target specific communities based on our analysis of previously-collected PREDICT behavioral questionnaire data, to assess key at-risk populations and occupations with high exposure to wildlife, as well as using other publicly available data (e.g. wildlife hunting licenses sold in a region, number of guano caves accessed for fertilizer etc.). We will identify populations at two sites in each of the following regions, building on our preliminary data **(Table 2)**: *Thailand (Co-I Wacharapluesadee):* We will continue surveillance in bat guano harvesters and their villages in Ratchaburi province where we have identified MERS-related CoV in bat guano and rectal swabs (*48, 157*) and serological evidence of α-CoVs, HKU1-CoV in people (Wacharapluesadee in prep.). Our second site will be in Chonburi province where we have found Nipah virus (99% identity of whole genome sequence to NiV from a Bangladesh patient) in flying foxes but no outbreaks have yet been reported (~~REF~~Thai MOPH, unpublished), and have found several novel paramyxoviruses and CoVs have been found in bat feces roosting there ((*158, 159*) Wacharapluesadee in prep.).

Commented [PD75]: Hongying -Need a new figure that lays out the community/clinical approach – small one with pretty images.

Commented [PD76]: Supaporn/Kevin/ -Need sample sizes for both populations and power calculations for serology for a typical virus from one of the three families we're targeting

Commented [KJO77R76]: I pasted in info from Supaporn below.

Commented [PD78]: Supaporn – please insert a reference into this comment box

Commented [KJO79]: Added in a couple more of her refs.

Sample size: Serological exposure likely low due to low risk of onward transmission. High risk occoupational groups vs. community sample at each site. Need to stratify sampling in villages based on

Thailand: 100 subjects of healthy high-risk community from 2 study sites (Chonburi and Ratchaburi) will enrolled on Year 2 and tested for antibody, and specimen from ill subject will be collected and teat by PCR and serology assays. Specimens from same subject will be collected on Y3 and Y4 for serology re-testing.

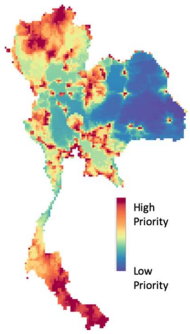
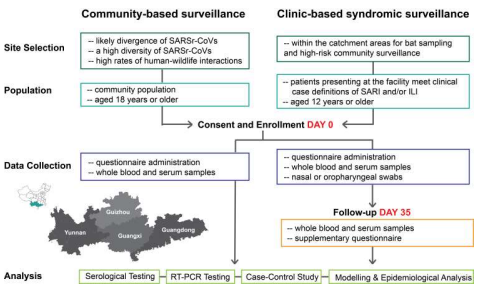


Fig. X: . Geographic analysis to target high-risk sites for human zoonotic disease surveillance in Thailand. Our spatial analysis from Aim 1 will be extended to optimize sites for human community surveillance in Thailand and Malaysia by ranking areas with high numbers of expected viruses in wildlife, greater spillover probability based on EID risk factors, and higher levels of connectivity (measured by road density data) that may facilitate person-person spread.

Peninsular Malaysia (Co-I Hughes, CM Ltd.): We will expand our sampling of the Orang Asli indigenous communities to include additional communities in the 3 Districts we have already sampled, and additional Districts in the States of Kelantan Perak, Pahang, and ~~Kelantan~~Kedah. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife (mainly bats) will be sampled. Human samples will include serum, whole blood, throat swabs, and nasal swabs, ~~urine, and fecal sample or rectal swab.~~ Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and extracted at PERHILITAN's NWFL and tested at NPHL. Participants who hunt, butcher or consume wildlife, or who rear animals will be considered suitable for enrolment. Participants will complete a consent form and questionnaire in addition to having samples collected. During these community trips the field team will conduct community meetings to help inform the Orang Asli about the public health risk of zoonoses. *Sarawak (Co-I Tan, Faculty of Medicine and Health Sciences UNIMAS):* We will expand our sampling of the Bidayuh and Iban indigenous people to include the Dayak and Orang Ulu ("people of the interior") because this group has a more intimate connection to remote wildlife-rich areas than the other two, albeit all are hunters. Human sampling will be led by UniMAS in collaboration with CM Ltd / EHA. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife (mainly bats) will be sampled, following the same protocols as for Peninsular Malaysia. *Sabah:* We will conduct sampling at the Madai bat and bird cave that has 200 permanent residents who harvest bird's nests for soup, and >1,000 during the harvesting season and at Gomantong cave – one of the largest bat and cave swiftlet nesting sites in the world. Protocols follow those for Peninsular Malaysia. *Singapore:* Active human surveillance will be not be conducted in Singapore, but archived human specimens e.g. from our Melaka virus serosurvey (*4*), will be made available to conduct retrospective serosurveys for specific pathogens that are identified as high interest to the EIDRC, NIH, and EID-CC.

Sample sizes: From our previous work we anticipate that 10-30% of the community population will have had exposure to wildlife allowing us to capture highly exposed and non-exposure individuals at each site. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock farming, transportation, selling, or slaughtering wildlife in the surveyed areas will be targeted so that they make up ≥30% of the sampled population in each community. We will stratify sampling to ensure appropriate representation of sex, demographic, and socio-economic factors at each community site. Estimating 5% overall seroprevalence in these high-exposure populations, these sample sizes are sufficient to estimate effect sizes of risk factors of 2X or greater with 80% power. For community-based surveillance, we will enroll xxxx individuals per



Commented [S80]: I think the animal and human specimen collection should be in a same format for Thailand and Malaysia.

Commented [PD81]: Need this figure for Singapore, Pen. Malaysia, Sarawak, Sabah as well – all together is best

Commented [KJO82R81]: Sam is going to send a combined figure tomorrow AM.

Commented [T83]: We don't think we will be able to collect urine or fecal samples from OA communities. Might be possible in Sarawak we have no idead about Madai.

county/province, pooled across two sites for each, allowing us to make county/province-level comparisons of differing effects.

Fig. XX: Human survey and sampling study design overview (Aim 2 and 3)

2.5 Data & sample collection: Following enrollment of eligible participants and completion of the consent procedure, biological specimens (up to 32ml of blood (on average we collect 15ml separated into 1ml of whole blood and 3-5ml of serum, 2 throat swabs and 2 nasal swabs (one in VTM and one in Trizol) 5ml will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 2.5ml whole blood; two 500 µL serum samples) will be collected and a questionnaire will be administered by trained project staff that speak appropriate local language to all participants. Through the serological findings and the survey we will investigate the details of five risk factors related to high risk wildlife exposure, to maximize the power of the analyses, based on continuing analysis of our previous work, and will focus on: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With additional consent considerations for participants enrolled from clinical settings we will review clinical records to collect recent data on medical history, clinical syndromes, and patient etiology.

2.6: Laboratory analysis: 2.6.a Serological testing:

In a previous R01, we expressed his-tagged nucleocapsid protein (NP) of SARSr-CoV Rp3 in *E.coli* and developed a SARSr-CoV specific ELISA for serosurveillance using the purified Rp3 NP. The specificity of this assay was evaluated using polyclonal antibodies against HKU1, OC43, 229E, NL63, MERS-CoV and EBOV and no significant cross-activity detected (*12*). **This suggests that if we can expand our serology tests to cover other bat CoVs, and that doing so may identify many more seropositive individuals.** We will therefore use two serological testing approaches. First, we will expand test all human sera collected from both community- and clinic- based sampling for a panel of bat CoVs that will include SARS-CoV (outbreak strain), a range of lineage 2 bat SARSr-CoVs (including WIV1 and SHC014), lineage 1 HKU3r-CoVs, MERS-CoV, and the α-CoVs SADS-CoV and HKU10. We previously found serological evidence of human exposure to HKU10 (*12*), and HKU10 is known to jump from one host bat species to another (*160*) so is likely to have infected people more widely. It is possible that non-neutralizing cross reactive epitopes exist that afford an accurate measure of cross reactivity between clade1 and clade 2 strains which would allow us to target exposure to strains of 15-25% divergence from SARS-CoV, at a phylogenetic distance where therapeutic or vaccine efficacy may falter. Additionally, we will test samples for antibodies to common human CoVs (HCoV NL63, OC43 – see potential pitfalls/solutions below). Secondly, CoVs have a high propensity to recombine. To serologically target 'novel' recombinant virus exposure, we will conduct 1) ELISA screening with SARSr-CoV S or S1 (n-terminal domain); 2) confirm these results by Western blot; then 3) use NP based ELISA and LIPS assays with a diversity of SARSr-CoV NP. For NP ELISA, micro-titer plates will be coated with 100 ng/well of recombinant batCoV NP and incubated with human sera in duplicates followed by detection with HRP labeled goat anti-human IgG antibodies. For confirmation, all ELISA positive samples will be subjected to Western blot at a dilution of 1:100 by using batCoV-NP as antigen. We will use an S protein-based ELISA to distinguish the lineage of SARSr-CoV. As new SARSr-CoVs are discovered, we will rapidly design specific Luciferase immunoprecipitation system (LIPS) assays targeting the S1 genes of bat-CoV strains for follow-up serological surveillance, as per our previous work (*13*). Additionally, in Thailand, as an initial project on our EIDRC, we will develop serology assays to the novel CoVs found in bats at the bat guano site using LIPS assays and viral genomic data, and will screen archived specimens from 230 participants (2 years' collection in 2017-2018, a subset of the subjects have been sampled at multiple timepoints to allow us to assess seroconversion).

2.6.b RT-PCR testing. Specimens will be screened using RT-PCR for the RdRp gene (See section 1~~.3.b~~.4.c for details). Positive samples will be subjected to full genome sequencing and RT-PCR amplification of the S glycoprotein gene. Samples from the clinic-based syndromic surveillance will also be tested using RT-PCR for Influenza A & B, HCoV NL63, OC43, HKU1, SARS-CoV & 229E, NiV, HeV, Ebola (WHO PCR protocol) based on the symptom as rule-outs, and SADS-CoV and HKU10 as an opportunistic survey for potential spillover these CoVs as proposed above.

Commented [PD84]: Hongying– this figure is from our CoV R01 renewal. Please modify to make relevant to SE Asia and henipas, filos and CoVs.

Commented [T85]: Malaysia protocol – I suggest we don't change so easy to amend ethical approval

Commented [PD86]: Emily/Hongying Please check

Commented [PD87]: Chris, Eric, Linfa, Dani etc. please draft some language here…

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2.6.c Biological characterization of viruses identified: Novel viruses identified in humans will be recovered by cultivation or from full length genome sequences using reverse genetic approaches. Viruses will be characterized for growth in primary human cell lines and in mouse models, including the Collaborative Cross Mice or humanized mice expressing human receptors from related strains. The approaches are discussed in more detail under Aim 1.

2.7 Epidemiological analysis: We will conduct a cross sectional case-control study to identify risk factors for ~~Henipavirus~~PMV, ~~Filoviruses~~FV, and CoVs spillover. "Cases" are defined as participants whose samples tested positive for ~~Henipavirus~~PMV, ~~Filoviruses~~FV, or CoVs by serological tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative for ~~Henipavirus~~PMV, ~~Filoviruses~~FV, or CoVs. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between biological results, serological/PCR status, and risk factors related to high risk wildlife exposure including: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) home or work proximity to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms lifetime and past 12mo. Additionally, we will use this procedure to determine how clinical presentation differs between ~~Henipavirus~~PMV, ~~Filoviruses~~FV, or CoVs in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms.

2.8 Potential problems/alternative approaches: Rarity of spillover events means it may be difficult to identify sufficient seropositives to statistically analyze human risk profiles. First, we will be targeting our community-based surveillance to subpopulations with high-levels of wildlife exposure, at sites selected for diverse and prevalent viruses to increase likelihood of finding positive individuals. Second, our serology testing will include a panel of assays for a large diversity of viruses, increasing likelihood of positives. For example, in previous work our overall bat CoV PCR prevalence in the community was, 11.8%; β-CoV, 3.4%; α-CoV, 9.1%). Thus, using this broad serological panel to screen individuals prone to contact with species of higher zoonotic risk increases the potential for detecting spillover with enough power for statistical analyses, and will shed light on behaviors that predispose to spillover. Third, we will include common human CoVs, ~~paramyxoviruses~~PMV, and influenza viruses in our panel, so that even if low prevalence of wildlife viruses is found, we will be able to conduct a valuable cross-sectional study of the seroprevalence of more common human viruses. Finally, we will be able to assess relative measures of human-wildlife contact from our survey work. We will analyze intensity of contact with high-risk taxa against other risk factors to provide useful proxy information for spillover risk. **Serological testing may not match known viruses due to recombination events, particularly for CoVs.** We will use the three-tiered serological testing system outline in **2.6.a** to try to identify these 'novel' viruses, however, we will also remain flexible on interpretation of data to ensure we account for recombination.

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Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts.

3.1 Rationale/Innovation: Our previous research **(Table 1)** and other data suggests that there are substantial numbers of outbreaks or clinical cases for which the etiologic agent is not diagnosed, e.g. (*161, 162*). To investigate this in SE. Asia, we will work directly with clinics and outbreak control organizations to identify the causes of some 'cryptic' outbreaks or cases in the region. Specifically, we will expand on current syndromic surveillance activities to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and who present at regional clinics or hospitals with symptoms typical of high impact zoonotic viruses. We will collect detailed survey data to assess their contact type and frequency with wildlife and livestock and conduct molecular and serological diagnostic assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. We conduct novel viral discovery assays on patients who present with symptoms indicative of a high-risk zoonotic virus. If novel pathogens are identified by PCR, we will attempt to isolate, obtain full genome sequence and/or biologically characterize the pathogen, using the *in vitro* and *in vivo* strategy laid out in Aim 1.

3.2 Preliminary data clinical surveillance: 3.2.a. Clinical surveys and outbreak investigations: Our ~~longterm~~long-term collaboration in the region has included the following activities investigating clinical syndromes: *Peninsular Malaysia*: **At the time of writing, there is an outbreak of a suspected undiagnosed**

viral ~~undiagnosed~~ illness in the ~~indigenous~~ Batek people, one of Malaysia's Orang Asli (indigenous) ~~("people of the forest")~~ communities~~y~~ living ~~at~~ in the Gua Musang ~~("civet cat cave")~~ district, in Kelantan, Malaysia, close to Taman Negara – a National Park containing the oldest known rainforest. This Orang Asli community ~~is~~ neighbors one where we have conducted prior routine surveillance and biological sampling, demonstrating proof-of-concept that our two-pronged high-risk community/clinic approach will work. Dozens of people are affected and the outbreak is currently being investigated by our collaborators at the Malaysian NPHL. The Orang Asli~~-~~ continue to practice traditional and in many cases subsistence hunting of wild animals – including bats, rodents, nonhuman primates, and raise domestic animals such as chickens and hunting dogs around their villages. These communities are remotely located, in heavily forested areas, with limited access to medical services. ~~live in remote villages in the rainforest of Malaysia, with limited access to modern health care and extensive contact hunting, butchering, and eating wildlife as their primary source of protein.~~ This outbreak, and others that will continue to emerge just like it, underscore the critical need for our EIDRC - to improve the technological tools, training, and human capacity for rapid EID surveillance, diagnosis, and response at the frontlines of disease emergence in SE Asia. ~~**Investigating this outbreak be a key priority if EID-SEARCH is funded.**~~ *Sabah*: Co-I Kamruddin (BMHRC) is leading an asymptomatic leptospirosis study on sanitation workers, an ILI study in a Kota Kinabalu clinic and diarrheal study in children under 5 yrs. Co-Is William, ~~Tan~~ Rajahram and ~~others~~ Yeo have conducted clinical observational studies to determine the etiology of central nervous system infections and acute undifferentiated febrile illness in Sabah. Co-Is ~~Tan~~Yeo, William have collaborated with Co-I Wang to enroll 90 adult and over 100 pediatric participants with central nervous system infections, negative tests for malaria and dengue from 3 district hospitals in Sabah to determine the various etiologies. While a ~~high~~ proportion tested positive for rickettsial agents, the majority had no clear diagnosis. *Sarawak:* Dr Tan (UNIMAS) is conducting a survey of high-risk female Kelabit indigenous people and has found 8% with HPV52 as the dominant strain. He is working on clinical cohorts of hand, foot and mouth disease and will recruit patients from that study in the current proposed work. Finally, he is conducting a long-term study of rotavirus and other viral etiologies of acute gastroenteritis in Kuching, Sarawak in collaboration with Co-Is Kamruddin that will include ~~swine herders and~~pig farmers. These are an important group in Malaysia because pig farmers are ~~shunned by~~wary of the government and ~~local villagers to they~~ are placed far away from town centers, ~~deep in~~on the edge of the forest, and it is this scenario that led to the emergence of Nipah virus in 1998-9 (*18, 163*). *Thailand:* The Chulalongkorn Univ. TRC-EID laboratory works in coordination with the Thailand Ministry of Health to investigate outbreak etiology, including the first case of imported MERS-CoV in Thailand, **for which we produced sequence confirmation within 24 hours from acquiring the specimen ~~(Fig. X)~~**. Three imported MERS cases have been detected in Thailand since 2015, and all cases were confirmed at the TRC-EID. We worked during the Zika outbreak to identify > 500 PCR-positive Zika patients with sequence confirmation. We have also worked on returning and suspected Ebola travelers, syndromic surveillance for SARI, Encephalitis, Hand Foot and Mouth disease in children and Viral diarrhea. Working with the US CDC (Thailand), between 1998 and 2002 we enrolled a large cohort of children from Kamphaeng Phet Province to examine Dengue virus and JE epidemiology (*164, 165*). Among the 784/2,574 convalescent sera Henipavirus-specific antibodies were detected in 13 serum samples (1.7% seroprevalence), suggesting prior exposure to endemic henipaviruses or a related paramyxovirus in rural Thailand. *Singapore:* Duke-NUS has worked with the Ministry of Health to investigate Zika cases (*166*), and with EcoHealth and others, Duke-NUS has developed novel CoV assays for clinical surveillance, and for expedited analysis during outbreaks. Our pipeline of novel virus detection, reservoir host identification, serological assay development, and serological testing is as rapid as several months, with the development of novel serological assays from sequence data in 7 days (*13*). **For example, with the discovery of SADS-CoV, we identified and fully sequenced a new virus, screened archived wildlife specimens to identify the natural reservoir host species (*Rhinolophus* spp. bats), developed LIPS serological assays, and tested human and swine samples to assess seroprevelance in less than 3 months (*13*).**

3.2.b Analysis of self-reported illness: In addition to biological outcomes we have developed a standardized approach in PREDICT for analyzing data on self-reported symptoms related to targeted illnesses; of fever with cough and shortness of breath or difficulty breathing (severe acute respiratory illness - SARI), fever with muscle aches (fevers of unknown origin (FUO) and, cough or sore throat (influenza-like illness - ILI) from study participants. We used a least absolute shrinkage and selection operator (LASSO) regression to identify associations between participants who reported ILI and/or SARI symptoms and demographics, occupation,

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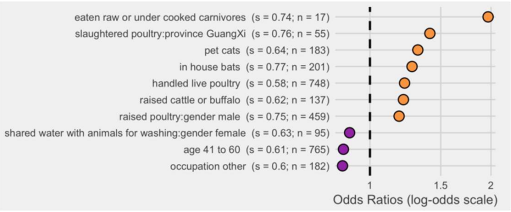
and contact with animals in the last year. Results have clear biological relevance. In Yunnan, China, salient associated variables or combination of variables were, in descending order of odds ratio: 1) having eaten raw or undercooked carnivores; 2) having slaughtered poultry and being a resident of Guangxi province; 3) having had contact with cats or bats in the house; and 4) being a male who has raised poultry **(Fig. 11).** We will expand this approach for all syndromes in Aim 3, and use more granular targeted questions designed to assess patient's exposure to wildlife in terms that are relevant to the specific country.

Fig. 11: Associated variables of self-reported ILI and/or SARI in prior 12 months (s = bootstrap support; n = number +ve out of 1,585 respondents). **Orange circles** = odds ratios > 1 (positively associated with the outcome); **purple** = odds ratios <1 (negatively associated with the outcome).



3.3 General Approach: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent is not identified. To capture these, we will expand on our current syndromic surveillance to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and present at regional clinics with influenza-like illness, severe respiratory illness, encephalitis, and other symptoms typical of high-impact emerging viruses. We will collect survey data tailored to the region to assess contact with wildlife, conduct molecular and serological assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. If novel pathogens are identified by PCR, we will isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work. We will then use behavioral survey data, ecological and phylogeographic analyses to assess the likely reservoir hosts to inform intervention programs.

3.4 Clinical cohorts. 3.4.a Cohort selection: We will initiate active clinic-based syndromic surveillance at 2 town-level level clinics and 2 provincial-level hospitals in Thailand, ~~Peninsular Malaysia,~~\ and two hospitals in Sabah ~~and Sarawak~~. These all serve as the catchment healthcare facility for people in our community-based surveillance of Aim 2 and are inclusive of wildlife sampling sites in Aim 1. Patients ≥ 12 years old presenting at the health facility who meet the syndromic and clinical case definitions for SARI, ILI, encephalitis, fevers of unknown origin, or hemorrhagic fever will be recruited into the study. We will enroll a total of xxxx individuals for clinical syndromic surveillance, which accounts for up to 40% loss from follow-up. Study data will be pooled across country sites, as clinical patients are limited by the number of individuals presenting at hospitals. Sites: *Thailand:* Two new district hospitals at the same site of high-risk community will be included to the study to collect specimens (PREDICT sample protocol) from patients with respiratory and/or encephalitis symptom during 3 years of the study (Year 2 to Year 4), at least 100 patients from each hospital will be enrolled and laboratory tested by PCR and serology assays. The second blood after 21 days of fever will be collected for serology testing. ~~XXXXX~~ *Peninsular Malaysia:* ~~Focusing immediately on the current outbreak in Orang Asli, we will work closely with the Malaysian Ministry of Health to support their investigation. We will also work with 6 Orang Asli clinics which focus solely on this indigenous community.~~No clinical study. *Sarawak:* No clinical study.~~XXXXXX~~ *Sabah:* Clinical study at QEH and HUMS: Patients will be identified in the intake area, the emergency room, in the ward, or in the intensive care unit of each participating hospital by clinic staff according to standard operating procedures at Queen Elizabeth Hospital and Hospital UMS. Staff will identify potential participants that fit undiagnosed syndromes in patients. Patients will be screened for eligibility according to the inclusion / exclusion criteria based on available clinical information, and the research study will be explained to participants by trained staff. Biological samples will be collected from the patients, and the patients or his/her designate will complete a short questionnaire. Over two years this study aims to enroll 200 patients at QEH and 100 at HUMS. Collection of patient's specimens must take place whilst the patient is **still in the viraemic / febrile phase of the disease**. Therefore, this study is relying on the clinical judgement of the treating clinicians to make the decision whether to include the patients into the study at the earliest point possible so that sampling process can take place during the targeted viraemic / febrile phase while not interrupting the treatment of the patients. Patients will complete consent form and answer a short questionnaire. Human

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Commented [T97]: I would make this over 18 for Malaysia it is what we have approval for and dealing with minors is a huge complication – we need to keep this study as simple as possible for hospital staff or they won't recruit people if it is too complicated and time consuming.

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samples will include serum, whole blood, throat swabs, nasal swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored at QEH until there is a sufficient number to move to BMHRC for screening.

~~XXXXXXXXX~~ *Singapore:* ~~XXXXX~~ Given budget constraints we will not do clinical cohorts, however in the ~~e~~avent of a suspected outbreak we have close ~~relationshtips~~relationships with xxxxx xxxxx hospitals and central reference lab.

3.4.b Behavioral and clinical interviews: 2.5 Clinic enrollment and follow-up: We will recruit inpatients and outpatients to participate in the study after initial screening to determine if they meet the clinical case definition of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever of unknown etiology; or 6) severe diarrhea. Once enrolled, biological samples will be collected and a questionnaire administered by trained hospital staff that speak appropriate local language. Samples will be taken concurrently when collecting samples for normative diagnostics. For inpatients, samples will be collected no more than 10 days of reported onset of illness to increase the chance of PCR detection of viruses (*167*). We~~re possible we~~ will follow up 35 days after enrollment to collect an additional two 500 µL serum samples conduct a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. 35 days gives adequate time for development of IgG, which occurs <28 days after onset of symptoms for SARS patients (*168*). These samples may prove valuable to collaborators in that the PBMC could be used in production of therapeutic antibodies.

3.4.c Sampling:

Following enrollment with signed consent form, biological specimens ((up to 23ml of blood (on average we collect 15ml separated into 1ml of whole blood and 3-5ml of serum, 2 x Oropharyngeal swab or throat swab, 2 x Nasopharyngeal swab or nasal swab, urine sample or two urogenital swabs 2 x Rectal swab or Fecal sample (for all swabs one in VTM and one in Trizol) 5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples) including two nasal or oropharyngeal swabs will be collected from all eligible participants, and a questionnaire will be administered. We will investigate five risk factors, to maximize the power of the analyses, all related to high risk wildlife exposure, based on continuing analysis of our previous work, and will include: 11) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With permission from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology.

3.5 Sample testing: PCR, Serology to link symptoms to etiologic agents

The objective is to detect novel CoV, PMV and FV viruses that are causing diseases in patients without known aetiology. Therefore, the tests carried out for this project cannot be relied on as a diagnostic tool, nor should it overlap or duplicate any of the routine diagnostic testing carried at the hospitals or clinics. Since the usually-suspected diseases such as dengue, leptospirosis and malaria will be diagnosed within 6 hours, recruitment of patients will happen only after these diseases have been ruled out by the routine rapid diagnosis. If a patient is not diagnosed through rapid diagnostics and is enrolled in the study but is later diagnosed through routine diagnostics the participant will remain in the study and will have their samples tested regardless of any normative diagnosis result, as we know that coinfection is possible and could be scientifically interesting.

Collection of patient's specimens must take place whilst the patient is still in the viraemic / febrile phase of the disease. Therefore, this study is relying on the clinical judgement of the treating clinicians to make the decision whether to include the patients into the study at the earliest point possible so that sampling process can take place during the targeted viraemic / febrile phase while not interrupting the treatment of the patients. PCR and serology results will often not be available for months after patient is admitted. The results will help us to detect and prevent potential epidemics involving emerging zoonotic agents, as well as to eventually counsel communities on practices that could reduce exposure and related health risks. These sentinel populations will be very important in providing cost-effective and timely information on disease emergence. The study will also

Commented [PD101]: Tom, Kevin – we need information.

Commented [T102R101]: We do not have budget to do clinical study in PM or Sarawak.

Commented [KJO103]: Linfa/Dani?

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Commented [PD105]: Need data for Nipah and filovirus patients

Commented [PD106]: Ralph: PBMC could be valuable for making therapeutic antibodies through collaborations?

Commented [T107]: Malaysia protocol suggest we stick to this for Malaysia as we have approval for this.

Commented [KJO108]: Blood just during 35 day follow up, or both? I guess both ideally, but weigh against IRB.

increase understanding of the conditions and human activities associated with the introduction of zoonotic infections into human populations, which may have implications for disease control worldwide.

The standard syndromic diagnostic PCR assay for the common pathogens will be conducted and the results will be shared to the hospital with 48 hours. Specimens will be further tested for 3 viral families; CoV, PmV and filovirus by consensus PCR. Appropriate specimen from dead case will be further conducted by NGS if the previous PCR tests are negative to identified cause of infection. The serology panel assay will be conducted from paired serum.

Serology: Panel serology assay will be performed from paired serum to determine the four fold raising antibody. The specific primer PCR of the virus antibody positive case will be further tested from acute specimens to confirm for infection.

3.5 Assessing potential for pandemic spread: Building off our spillover risk characterization in Aim 1, ~~W~~we will use statistical models built from collated biological, ecological, and genetic data to ~~predict~~ further assess the likelihood of human infection ~~(or spillover)~~ and also pathogenicity for ~~a~~ the range of diverse viruses we will characterize. The aim is to quickly identify and assess which viruses pose the most significant risk to human health and thus prioritize additional resources to more fully characterize, better understand pathogenicity, and invest in therapeutic development for those pathogens. We will combine two independent but related statistical models to predict pathogen spillover risk and pathogenicity. First, we will expand a simple generalized additive modeling approach (*90*) to assess spillover risk probability to a new virus that is currently not known to infect people, when we have only limited genetic data from initial PCR screening and sequencing. Additional data from receptor binding protein homology and cell line infection experiments ~~can~~ will be incorporated into the model framework as available. Second, given high likelihood to spillover and infect humans, or direct evidence of human infection from our clinical surveillance, we will then predict pathogenicity using nearest neighbor phylogenetic analyses as a first approximation and by incorporating more detailed data from genomic characterization and animal model experiments (Aim 1). ~~For the pathogenicity model, h~~Human epidemiological trait data for ~300 viral species known to infect people (e.g. case fatality rate, global estimates of number of human cases per year or decade, R_0, infectious period, and primary symptoms) ~~will be aggregated including from~~have been collated by recent studies ~~previous~~ and will be used as ~~reviews [Woolhouse ref] to use as~~ predictor variables. Thirdly, we will ~~build off~~apply tools already developed, and being refined by EHA under's ~~previous~~ DTRA and DHS supported research, to predict pandemic spread for ~~viurses~~viruses ~~we identify by integrating surveillance site data,~~using global flight models (*169*), as well as additional datasets ~~(road networks, shipping routes, cell phone data) to measure~~ on human movement and connectivity across Southeast Asia.

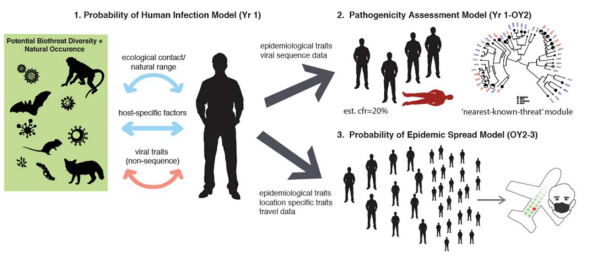


Fig. XXXXX: Conceptual framework highlighting 3 underlying models to estimate: **1)** probability of human infection, or spillover, the first necessary step to understand pathogenicity when status of human infection is unknown; **2)** pathogenicity, i.e. probability of causing clinical signs, case fatality rate estimates, and other metrics relevant to humans; and 3) probability of pathogen spread based on ecological, epidemiological, and airline connectivity and human migration data.

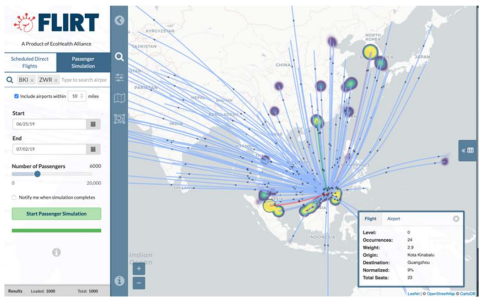
Commented [PD109]: This is v. weak – can someone strengthen it please!

Commented [KJO110]: Text from Supaporn

Commented [PD111]: This is v. weak – can someone strengthen it please!

Commented [PD112]: Hongying – please work with Kevin to come up with something of relevance here. Also, make the text brief, bold and large so it's readable

Commented [KJO113]: See also FLIRT flight simulator figure below. Peter, let's decide if I should rework this figure to combine both, or how to proceed with this section.



3.7 Potential problems/alternative approaches: Patients visiting clinics may have cleared virus, but not yet developed IgG antibodies, reducing seropositive cases. Our 35 day follow-up sampling should avoid this because the maximum lag time between SARS infection and IgG development was ~28 days (*167*). We also expect that patients in rural communities will only visit clinics when symptoms have progressed, likely coinciding with late illness and onset of IgG. We will also have data from our community study, so won't be completely reliant on hospital data to identify PCR- or seropositives. Finally, the risk is outweighed by the potential public health importance of discovering active spillover of a new henipavirus, filovirus or CoV infection.

Commented [PD114]: Add data for Nipah and filo infections

Additional Instructions – Specific to the EIDRC FOA:

Commented [PD115]: All – I've started putting a few notes in this section, but it will need substantial work. Please insert language from other proposals to fit these sections if you have them

4. Administrative Plan

In a clearly labeled section entitled **"Administrative Plan":**

- Describe the administrative and organizational structure of the EIDCRC Administrative and Leadership Team. Include the unique features of the organizational structure that serve to facilitate accomplishment of the long range goals and objectives.

Chula TRC-EID is National reference laboratory for diagnostic and confirmation of EIDs pathogen along with the National public health laboratory at the Department of Medical Science and the WHO Collaborating Centre for Research and Training on Viral Zoonoses. We have built a EID laboratory network in-country among three Ministry including Ministry of Public Health (Department of Medical Science and Department of Disease Control), Department of Livestock and Development, and Department of National Parks, Wildlife and Plant Conservation and Universities and among SEARO countries. Our clinicians provide case consultation for Thailand and SEARO countries. The laboratory and clinic training are regularly conducted.

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For Peninsular Malaysia this project will be administered through the Zoonosis Technical Working Committee established under the PREDICT project – a working technical committee comprising appointed and authorized officers from MOH, DVS, PERHILITAN, EHA who will work together in coordinating and monitoring the implementation of the Study as well as to prepare a report on the progress of the Study pursuant to the existing MOA. **NPHL –** is National reference laboratory for diagnostic and confirmation of EIDs pathogen. Molecular and serological screening (BioPlex) of PM Orang Asli samples, serological screening of syndromic samples from Sabah (already approved) and Sarawak (would need approval). **PERHILITAN molecular zoonosis laboratory, at the National Wildlife Forensic Laboratory — Storage ad extraction of all PM samples.** Molecular and serological screening (BioPlex) of PM wildlife samples. **UPM Faculty of Veterinary Medicine -** Molecular and serological screening (BioPlex) of PM livestock samples

For Sabah this project will be administered through the Sabah Zoonotic Diseases Committee. A working

technical committee comprising appointed and authorized officers from SSHD, DVS, SWD, UMS and EHA who will work together in coordinating and monitoring the implementation of the Study as well as to prepare a report on the progress of the Study pursuant to the existing agreement. **KKPHL -** Molecular screening of syndromic samples from Sabah (already done) and Sarawak (would need approval)**. SWD WHGFL -** Molecular screening of Sabah wildlife samples**. BMHRC –** Once the lab is renovated the BMHRC will be used for the m~~M~~olecular and serological screening (BioPlex) of Sabah livestock samples, serological screening (BioPlex) of Sabah wildlife samples, Molecular and serological screening of syndromic samples from Sabah (already approved) and Sarawak (would need approval). BMHRC will need renovations and BioPlex to do this work – Menzies team might have funds for BioPlex that they would base here as part of DTRA project.

Coordination between PM, Sabah and Sarawak will follow the model used for PREDICT. For PM the relevant authority approves the results for release for example for Livestock samples this is DVS, these results will then be shared with the Ministry of Health and PERHILITAN under the terms of our agreement. After Ministry of Health and PERHILITAN approves the release, these results will be shared with Sabah and Sarawak partners and more widely through the EIDRC network. For Sabah once wildlife results are approved for release by SWD they will be shared with all partners and once human results are approved for release by Sabah State Health Department they will be shared with all partners. It is assumed that a similar arrangement will be made in Sarawak.

Results from human screening will be shared with participants when they become available. Only country coordinator will know which participant number links to which name and the results. A doctor from each District for community surveillance and from QEH and HUMS will be provided with patient name, project ID number or IC number of positive participants so they can look up these individual results in the t report and share results with patient and update their record.

- Describe how communications will be planned, implemented, and provided to collaborators, teams, or sites. In addition, describe plans to achieve synergy and interaction within the EIDRC to ensure efficient cooperation, communication and coordination.
- Specifically address how the EIDRC Team will communicate with other EIDRCs, the EIDRC CC and NIAID and how the EIDRC will implement plans and guidance from the EIDRC CC to ensure coordination and collaboration across the Emerging Infectious Disease Program as a whole. Describe how the study site will provide information, if any, back to the local health authorities and the study subjects as needed. Note any adjustments or changes needed for rapid and flexible responses to outbreaks in emerging infectious diseases.

List out all of our involvement with outbreak investigations, ministries of health surveillance etc.

If possible the clean or summarized answers from the questionnaire should be shared to the hospital Infectious Control team and Bureau of Epidemiology for their information for controlling the outbreak.

Commented [PD117]: From Supaporn – do same for all countries

- Describe the overall management approach, including how resources will be managed, organized and prioritized, including pilot research projects.
- Highlight how the proposed staffing plan incorporates scientific and administrative expertise to fulfill the goal to develop the flexibility and capacity to respond rapidly and effectively to outbreaks in emerging infectious diseases in an international setting.

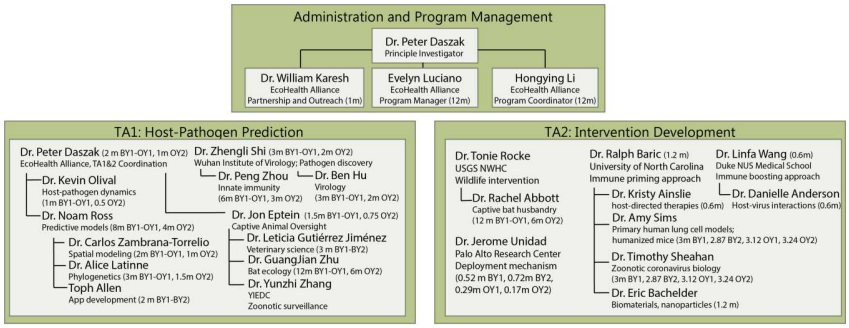
CM/EHA helped establish and manages with SWD the Wildlife Health, Genetic and Forensic Laboratory (certified as a BSL- 2 laboratory according to the US standard for laboratory specifications) that has all the equipment necessary to store samples, run extractions, PCR and analysis on biological samples for wildlife disease surveillance. The lab is used to conduct health checks on rescued and relocated wildlife before being released into new areas or sanctuaries, to screen samples for the PREDICT and Deep Forest Project and for genetic research and forensic investigations. CM/EHA also helped establish the new molecular zoonosis laboratories (certified as a BSL- 2 laboratory according to the US standard for laboratory specifications), at DWNP's National Wildlife Forensic Laboratory. The lab is used to screen samples for the PREDICT & DTRA

projects. In addition CM/EHA also has access to NPHL and KKPHL for screening human samples and the Virology lab at FVMUPM for screening Livestock samples.

The Borneo Medical and Health Research Centre (BMHRC) is a centre of excellence that conducts teaching and scientific researches on communicable diseases, outbreak investigations, and ethnomedicine. BMHRC has offices space including a meeting room and seven laboratories which including preparation lab, parasitology lab, cell culture lab, bacteriology lab, natural product lab, virology lab, and molecular biology lab. BMHRC is supported by an administration staff, medical lab technologist, and research assistants who are available to work on projects. Through this project we will help to establish a certified BSL-2 lab at the BMHRC. This lab can then be used for human testing to elevate pressure at KKPHL and QEH labs. It would also be an ideal location for a Luminex platform as human, wildlife and livestock samples could all be screened here. Co-Is Hughes and Kamruddin will be starting outbreak response training in August 2019 with a team from Sabah CDC, KKPHL and BMHRC. (Dr. Maria Suleiman now in PM But still collaborating with us, Dr. Muhammad Jikal current Director CDC working with us and Dato' Dr Cristina Rundi Director Sabah state health Dept is supportive) this collaborative group is working to develop a Sabah outbreak response team that will help support SSHD/ CDC outbreak team and also conduct sampling for disease surveillance to be based at BMHRC and we will develop this as part of this proposal. In addition the UMS hospital will come in line in 2021 providing additional opportunities for cohort studies and capacity building.

FROM DARPA grant:

Section 1.03 MANAGEMENT PLAN



Project DEFUSE lead institution is EcoHealth Alliance, New York, an international research organization focused on emerging zoonotic diseases. The PI, Dr. Daszak, has 25+ years' experience managing lab, field and modeling research projects on emerging zoonoses. Dr. Daszak will commit 2 months annually (one funded by DEFUSE, one funded by core EHA funds) to oversee and coordinate all project activities, with emphasis on modeling and fieldwork in TA1. Dr. Karesh has 40+ years' experience leading zoonotic and wildlife disease projects, and will commit 1 month annually to manage partnership activities and outreach. Dr. Epstein, with 20 years' experience working emerging bat zoonoses will coordinate animal trials across partners. Drs. Olival and Ross will manage modeling approaches for this project. Support staff includes a field surveillance team, modeling analysts, developers, data managers, and field consultants in Yunnan Province, China. The EHA team has worked extensively with other collaborators: Prof. Wang (15+ yrs); Dr. Shi (15+ yrs); Prof. Baric (5+ yrs) and Dr. Rocke (15+ yrs).

Subcontracts: #1 to Prof. Baric, UNC, to oversee reverse engineering of SARSr-CoVs, BSL-3 humanized mouse experimental infections, design and testing of targeted immune boosting treatments; **#2** to Prof. Wang, Duke-NUS, to oversee broadscale immune boosting, captive bat experiments, and analyze immunological and virological responses to immune modulators; **#3** to Dr. Shi, Wuhan Inst. Virol., to conduct PCR testing, viral discovery and isolation from bat samples collected in China, spike protein binding assays, humanized mouse work, and experimental trials on *Rhinolophus* bats; **#4** to Dr. Rocke, USGS NWHC, to refine delivery mechanisms for immune boosting treatments. Dr. Rocke will use a captive colony of bats at NWHC for initial

Commented [KJO118]: Figure like this would be good, but instead of breaking down by TA, maybe by country or Specific Aim. Per FOA, Need to also show how we'll link in with the Coordinating Center and other EIDRCs.

trials, and oversee cave experiments in the US and China. **#5** to Dr. Unidad, PARC, to develop innovative aerosal platform into a field-deployable device for large-scale inoculation of the bats. Dr. Unidad will collaborate closely with Dr. Rocke in developing a field deployable prototype for both initial trails and cave experiments in China.

Collaborator Coordination: All key personnel will join regularly-scheduled web conferencing and conference calls in addition to frequent ad hoc email and telephone interactions between individuals and groups of investigators. Regular calls will include:

- Weekly meetings between the PI and Program Manager (on project and task status)
- Weekly web/phone meetings between Program Manager and subawardee admin. staff
- Monthly web/phone conferences between EHA PIs and all subawardee PIs.
- Monthly web conferences between key personnel (research presentations/coordination)
- Four in-person partner meetings annually with key personnel at EHA and two in-person partner meetings annually between subawardees.
- Annual in-person meeting among all key personnel

Evaluation metrics will include the generation of high-quality data, successful achievement of milestones and timelines, scientific interaction and collaboration, the generation of high quality publications, and effective budget management. The PI and subawardee PIs will attend a kickoff meeting and the PI will meet regularly with DAPRA at headquarters and at site visits.

Problem identification and resolution: Regular planning, monitoring, and evaluation meetings will be the primary mechanisms for problem identification. Minor problems (e.g. delays in sample availability or test results) will be dealt with internally through appropriate action and resolution monitored by Dr. Daszak and the Project Manager. In the event of significant problems, such as prolonged poor productivity, inadequate scientific collaboration, or major disputes regarding research direction or resource allocation, EHA will assist with resolving the problem through negotiation. Should a resolution not be forthcoming, consultation with our technical advisors and DARPA Program staff may be warranted.

Risk management: Maintaining a timeline and meeting milestones will require strict and continuous oversight of all project phases, frequent and regularly scheduled communication, and the ability to make decisions and implement strategies. A project of this nature requires a different mindset from that typically associated with basic research activities that move at an incremental pace with investigators gradually optimizing experimental systems, refining data, or waiting for additional data before moving ahead with an analysis approach. To maintain our timeline, we will continually evaluate these trade-offs to make decisions about when iteration is appropriate and when necessary to move forward with current information.

Biographies:

Dr. Peter Daszak is President and Chief Scientist of EcoHealth Alliance, Chair of the NASEM Forum on Microbial Threats, member of the Executive Committee and the EHA institutional lead for the $130 million USAID-EPT-PREDICT. His >300 scientific papers include the first global map of EID hotspots, estimates of unknown viral diversity, predictive models of virus-host relationships, and evidence of the bat origin of SARS-CoV and other emerging viruses.

Prof. Ralph Baric is a Professor in the UNC-Chapel Hill Dept. of Epidemiology and Dept. of Microbiology & Immunology. His work focuses on coronaviruses as models to study RNA virus transcription, replication, persistence, cross species transmission and pathogenesis. His group has developed a platform strategy to assess and evaluate countermeasures of the potential “pre-epidemic” risk associated with zoonotic virus cross species transmission.

Prof. Linfa Wang is the Emerging Infectious Diseases Programme Director at Duke-NUS Medical School. His research focuses on emerging bat viruses, including SARS-CoV, SADS-CoV, henipaviruses, and others and genetic work linking bat immunology, flight, and viral tolerance. A 2014 recipient of the Eureka Prize for Research in Infectious Diseases, he currently heads a Singapore National Research Foundation grant “Learning from bats” ($9.7M SGD).

Tom Hughes is trained in ecology, international development and public health. For the past 10 years he has acted as the Malaysian Project Coordinator for EcoHealth Alliance. He has designed, initiated and managed

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collaborative projects on surveillance, viral discovery and ecology of wildlife reservoirs of zoonoses. He has worked closely with partners from the Ministry of Health, the Department of Wildlife and National Parks, and the Department of Veterinary Services, over the last 14 years and with Sabah Wildife Department, Sabah State Health Department and UMS over the last 8 years to develop personnel and laboratory capacity and establish sustainable disease surveillance systems for wildlife and people with high exposure to wildlife.

Current list of contacts at key human (especially) and wildlife orgs in every SE Asian country from map above – please add to this if we've missed any

Indonesia – EHA: Eijkman (Dodi Safari), IPB (Imung Joko Pamungkas); **CM Ltd:** IPB (Imung Joko Pamungkas), Bogor Agricultural University (Diah Iskandriati)

SE China – EHA: Yunnan CDC (XX), Wuhan Inst. Virol. (Zhengli Shi);

Myanmar – Chulalongkorn (Thiravat and Supaporn) have collaborated extensively with Win Min Thit, MD (Yangoon General Hospital, Myanmar); Khin Yi Oo (National Health Laboratory, Myanmar) and also in training PREDICT Myanmar veterinarians in bat sampling under PREDICT.

Japan – **BMHRC**: Oita University (Hidekatsu Iha, Kiyoshi Aoyagi), Nagaskai University (Koichi Morita, Kazuhiko Moji)

Cambodia -
Laos - **CM Ltd:** Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit (Matthew Robinson)
Bangladesh – EHA: IEDCR (Meerjady Sabrina Flora, Ariful Islam), icddr,b (Mohammed Ziaur)

India – EHA: Jon's collaborators;

Vietnam – Wellcome CRU ? Liaison with Zhengli Shi for Serol assays etc.?

Philippines – EHA, CM Ltd: Biodiversity Management Bureau, Department of Environment and Natural Resources (Anson Tagtag), Bureau of Animal Industry (Davinio Catbagan), Research Institute of Tropical Medicine (Catalino Demetria).

Malaysia – **CM Ltd:** Borneo Medical and Health Research Center (Ahmed Kamruddin), Department of Wildlife and National Parks Peninsular Malaysia (Frankie Sitam, Jeffrine Japning, Rahmat Topani, Kadir Hashim, Hatta Musa), Hospital Universiti Malaysia Sabah (Helen Lasimbang), Faculty of Medicine, Universiti of Malaya (Sazaly Abu Bakar), Kota Kinabalu Public Health Laboratory (Jiloris Dony, Joel Jaimin), Ministry of Health (Chong Chee Kheong, Khebir Verasahib, Maria Suleiman), National Public Health Laboratory (Hani Mat Hussin, Noorliza Noordin, Yu Kie Chem), Queen Elizabeth Hospital (Giri Rajahram, Heng Gee Lee), Sabah State Health Department (Christina Rundi, Mohammad Jikal), Sabah Wildlife Department (Rosa Sipangkui, Senthilvel Nathan, Augustine Tuuga, Jumrafiah Sukor), Gleneagles Hospital Kota Kinabalu (Timothy Wiliam); **BMHRC:** CM Ltd (Tom Hughes), HQEII (Shahnaz Sabri), Kota Kinabalu Public Health Laboratory (Jiloris Dony), Sabah State Health Department (Mohammad Jikal)

Thailand – **CM Ltd:** Department of Disease Control, Ministry of Public Health (Rome Buathong, Pawinee Doungngern, Pongtorn Chartpituck), Department of National Park, Wildlife and Plant Conservation (Pattarapol Manee-On), Faculty of Forestry, Kasetsart University (Prateep Duengkae), Mahidol-Oxford Tropical Medicine Research Unit, (Stuart Blacksell, Richard Maude),Chulalongkorn University (Supaporn Wacharapluesadee).

Chulalongkorn lab is a WHO Collaborating Centre for Research and Training on Viral Zoonoses, we work closely with WHO SEARO and SEARO countries including Bangladesh, Bhutan, Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, (Thailand), Timor-Leste.

We had conducted the PREDICT lab training for Veterinary lab in the Southeast Asia region for FAO. We conducted (transferred?) the bat sampling technique for scientists from Malaysia, Myanmar, the Philippines, and China.

5. Data Management Plan

Data and Specimen Management: All research procedures will be conducted in accordance with local laws and IRB regulations (and/or other relevant governing authority regulations for human ethics as appropriate). Only the project coordinator in each country will have access to identifiable human subjects' data. All questionnaire data and biological samples will be labeled with a unique alphanumeric identification code, assigned to each enrolled, sampled individual that does not identify the individual from whom data are collected. No personal identifying information will be recorded on the sample vials or on the questionnaires.

For community sampling, biological samples will be stored in liquid nitrogen dry shippers following collection in the field and during transported to the corresponding Laboratory as detailed above. Samples will be stored in a locked -80C freezer at corresponding Laboratory. Only trained staff and project personnel will have access to the specimens. Subject privacy and confidentiality practices will be addressed during training of all study personnel.

Safety Precautions: The project has standardized protocols, SOPs, and training to ensure the safety of staff involved in research activities, including biological specimen collection. Specimens will be collected by trained clinic staff wearing appropriate PPE, including gloves, fitted and tested N95 masks, gowns, and closed toed shoes. Following agreement from government partners, any specimens transported by air to regional or reference laboratories for testing that cannot be carried out in country will follow safety and International Air Transport Association (IATA) guidelines provided to staff. Samples will be packed frozen into dry shippers designed for shipment approved by IATA with appropriate permits and approvals to allow for shipping of biological specimens and infectious substances, under which shipping of samples must also comply with the Safe Sample Transport guidelines.

Training oversight will be provided by the Project Coordinators in each country. All project personnel handling specimens and involved in their transport are trained annually with documentation of training maintained by PREDICT through the PREDICT internal database, the Emerging Infectious Disease Information and Technology Hub (EIDITH).

Data Quality Control: All data are examined at entry and later upon integration into the study database, for complete de-identification, completeness, accuracy, and logical consistency. Once all test results (e.g. initial detection by PCR, subsequent sequencing of viruses, and serology) are available for a given specimen, the results are interpreted in light of all available scientific literature and previous findings by team scientists.

Data Identification: Data will be identified by a unique identification code assigned to each sampled individual. Participants' names and codes will be recorded in the confidential participant logbook, which is completed in the field by field staff and retained and locked in the office of the country Coordinator. All records that contain names or other personal identifiers, such as informed consent forms and the confidential participant logbook, will be stored separately from study records. All personal information will be stored securely at the country headquarters in locked filing cabinets, with access limited to study staff. Only the Project Coordinator will have access to identifiable human subjects' data. All other collaborators will have access only to coded data that cannot be linked to identifiable information.

Data and Specimen Protection during Transport: All data collected on paper forms will be transported from the study site to the local collaborating laboratory or collaborating institute in secured containers. A password-protected laptop will be used to collect coded data only. Personal identifying information will not be stored in electronic form. All data transmitted electronically will be 128-bit encrypted. Email will not be used to transmit personal identifiable data. All coded electronic data will be backed-up to secure servers maintained by a secure, 128-bit encrypted and password-protected centralized database.

Data Storage:

Human specimens will be banked at the corresponding Laboratory and locked in an ultralow laboratory freezer. Access is restricted to trained personnel. Human questionnaire responses will have restricted access and will only be accessed by trained project personnel. Data will utilize the secure, 128-bit encrypted and password-protected centralized database being developed by EHA to store data for access by the consortium (whose access has been approved by our Malaysian, Thai and Singapore government partners) only for surveillance data analysis. The database is maintained by the EHA information management team. There is no public access to the database.

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Data and/or Specimen Banking: Specimen and data will be stored following data collection in secure research facilities. Personal identifying information will be kept in a secure manner and no personally identifiable information will be stored with participants' questionnaire or biological specimen data. Participants will be given a study code. Participants' names and study codes will be kept in a separate locked file from any questionnaire or biological data. Blood and swab samples will be stored securely in labs and labeled with the de-identified study code number,

Data and specimens will be securely stored for up to ten years with personal identifying information kept in a secure manner. This study could be extended by five-year increments in perpetuity. If participants agreed during the consent process, they may be contacted about having their samples or questionnaire data used for future separate studies about new animal infections discovered in the future, and factors that may affect their chances of getting these animal infections. No data will be released for other purposes without full consent from participants.

Upon completion of the project, personal identifying information will be destroyed unless this protocol is extended or amended for a maximum of ten years of sample and data storage.

Provision to Monitor the Data to Ensure the Safety of Subjects: The data collected and the procedures performed are within the scope of Good Clinical Practice and pose no more than minimal risk to the volunteer. Trained health professionals will perform all specimen collection under the supervision of the country Coordinator. The personnel tasked with conducting these procedures will be well-trained in project protocols and in Universal Precaution Practices to ensure safe sample collection, and to ensure that the sample collection process causes the least discomfort possible to the participant. In addition, the administration of questionnaires will be supervised as appropriate. These individuals will be trusted members of the community and will have often treated the participants or their family members before. Every effort will be made by trained project teams to make sure the respondent is comfortable when answering sensitive questions. Participation in this study will not alter the medical evaluation or therapy provided to the participant by the clinical or medical officer and will be based on the typical standards of care.

As described above, no identifying information will be stored with or paired with questionnaire data or biological specimens. As the data samples will be coded within the database for the life of the study and the on-site data log will be stored in a secure manner, the risk of a loss of confidentiality is minimized for the study volunteers. Results will be shared with the Ministries of Health in the form of the official government report generated by EHA that contains all test results but no identifiable information. When questionnaires are moved to the country headquarters, records will only contain

coded data to ensure the safety and confidentiality of participants and will be maintained in a secure database. The only documents that will link the participant with a unique ID number is the first page of the questionnaire that will be separated in the field and kept with the consent form and Participant Log (which will be stored in a locked file separately from participant data in the offices of the Country Project Coordinator).

At the completion of each surveillance period, generally on a calendar year schedule, a data and safety review will be conducted at each site with the clinical staff and the EcoHealth Alliance country team. At this review, safety information and adverse effects collected during the performance period will be discussed and addressed. Data may include case report forms, notes from study visits, and or any telephone calls to the PI from participants. Adverse effects from sampling protocols are expected to be exceedingly rare, but if any unusual conditions are observed by the team, it will trigger a notification within 5 working days, to assure that additional data collection and investigations are initiated.

Report summaries of the data generated from the project will be provided to the Ministries of Health and to other in-country collaborating investigators, at their request. Adverse or serious adverse events included in report summaries to the Ministry of Health and other in-country collaborators.

In a clearly labeled section entitled **"Data Management Plan":**

- Describe internal and external data acquisition strategies to achieve harmonization of systems and procedures for data management, data quality, data analyses, and dissemination for all data and data-related materials generated by the research study with the EIDRC CC.
- Within the plan, indicate the extent to which dedicated systems or procedures will be utilized to harmonize the acquisition, curation, management, inventory and storage of data and samples. Describe how training for the data and sample collection, in terms of the use of electronic data capture systems, will be provided to all staff including those at enrollment sites.
- Describe the quality control procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens.

This includes sample labeling etc. Get a bar-coding system, everyone has a scanner (cf. PREDICT) – Ralph has this for select agents and MERS. Also need a software system

Language from NIH CoV grant:

Data Sharing Plan: Data will be available to the public and researchers without cost or registration, and be released under a CC0 license, as soon as related publications are in press. Data will be deposited for in long-term public scientific repositories – all sequence data will be made publicly available via GenBank, species location data via the Knowledge Network for Biodiversity, and other data will be deposited in appropriate topic-specific or general repositories. Computer code for modeling and statistical analysis will be made available on a code-hosting site (GitHub), and archived in the Zenodo repository under an open-source, unrestrictive MIT license. Limited human survey and clinical data will be released following anonymization and aggregation per IRB requirements. Publications will be released as open-access via deposition to PubMed commons.

Viral isolates will remain at the Wuhan Institute of Virology initially. Isolates, reagents and any other products, should they be developed, will be made available to other NIH-funded researchers via applicable Wuhan Institute of Virology and EcoHealth Alliance Material Transfer Agreements and/or licensing agreements.

Sharing Model Organisms: We do not anticipate the development of any model organisms from this study. Should any be developed, they will be made available to other NIH-funded researchers via applicable Wuhan Institute of Virology and EcoHealth Alliance Material Transfer Agreements and/or licensing agreements.

Genomic Data Sharing: We anticipate obtaining genetic sequence data for 100s of novel coronavirus genotypes, including RNA-dependent RNA polymerase (RdRp) and Spike genes for all strains/genotypes. In addition, we will generate full viral genomes for a subset of the bat SARSr-CoVs that we identify. All sequence data will be deposited in the NIH genetic sequence database, GenBank. We will ensure that all meta-data associated with these sequences, including collection locality lat/long, species-level host identification, date of collection, and sequencing protocols will also submitted. The genotype data will be made publicly available no later than the date of initial publication or six months after the receipt of final sequencing data, whichever comes first. We anticipate sequence generation will occur over the 5 year proposed project period.

Genome Wide Association Studies (GWAS): Not applicable.

Commented [KJO121]: From CoV NIH grant

Thailand TRC-EID Chulalongkorn lab:Specimen management and BioBank: The software called PACS (Pathogen Asset Control System) supported by BTRP DTRA, is now used for managing the specimen from specimen registration (barcoding, and may move to use QR code system soon), aliquoting, tracking, short patient history record, data summary, searching, test reporting and etc. Our biobank system contains 28 freezers (-80C) and liquid N2 system. Its size is around 120 sq meters. There is a separate electric generator for our lab and biobank. There are CC TV cameras at the biobank and laboratory.

Chula TRC-EID has our own server for data management and analysis. We have the necessary software including Bioinformatic tools (Blast Standalone, SAMTOOL, GATK, AliVIEW, RaxML, FigTREE, MAFFT, Guppy, Megan, PoreChop, MEGA), Database (MySQL, MongoDB) Web, jQuery, Node.js, Express.js React, API, PHP, Ruby, Python, Java, C#, HTML5, Bootstrap, CSS, Responsive, Cloud, Linux Command, PACS (sample management system), Virtualbox virtual machines, R programming languages, Perl scripts, Bash shell scripts, Open source software, Microsoft Office running on both Apple Mac OS X, Ubuntu, Linux, and Windows Operating Systems. Chula TRC-EID has a dedicated 16-core Ram 64GB Linux server with 4TB hard drives, dual quad-core Mac Pro Server with 2TB hard drives and another dedicated iMac Pro (Retina 4K, 21.5-inch, 2017) Processor 3.6GHz quad-core Intel Core i7 (Turbo Boost up to 4.2GHz) Ra, 16GB with 512GB hard drives. TRC-EID also has HPC Linux server CPU: 4 x 26cores Intel Xeon = 104 cores 2.1GHz with 52 TB HHD and 2x3.84TB SSD with GPU 2x Tesla v100 (CUDA core = 5120 cores/GPU, Tenser core = 640 cores/GPU). TRC-EID has a dedicated 10/100/1000 of LAN (Local Area Network), 2.4G / 5G Gigabit Wi-Fi 802.11ac.

Commented [KJO122]: From Supaporn for their lab, some may go in Facilities doc?

From DARPA:

Data Management and Sharing: EcoHealth Alliance will maintain a central database of data collected and generated via all project field, laboratory, and modeling work. The database will use secure cloud hosting services and enable export to archival and platform-independent formats. It will ensure data and metadata compatibility between project components, track data versioning and annotations, and enable compliance with DARPA data requests and disclosure requirements. All archived human sample data will be de-identified. Partners will provide raw and processed data to the central database throughout the course of the project. Project partners will have access to the data they generate in the database at all times, and maintain control over local copies. Release of any data from the database to non-DARPA outside parties or for public release or publication will occur only after consultations with all project partners. EHA has extensive experience in managing data for multi-partner partner projects (PREDICT, USAID IDEEAL, the Global Ranavirus Reporting System).

Commented [KJO123]: Text from DARPA Preempt

6. Clinical Management Plan

Clinical site selection

Our consortium partners have been conducting lab and human surveillance research, inclusive of human surveillance during outbreaks, for over the past two decades and in that time have developed strong relationships with local clinical facilities and processes. The plan for selection of clinical sites will begin in the same geographic regions as those identified in Aim 1. These are regions where we will sample wildlife due to the high potential of zoonotic viral diversity. Additionally, these will be clinical sites that serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2. In PREDICT we developed successful working relationships with the major healthcare facilities the hotspot areas in 6 different countries, including Thailand and Malaysia and we will continue to work with these sites going forward. Continuing to work with these hospitals means we will begin this project with successfully established partners and we can begin data collection quickly. This also reduces the number of additional sites needed to meet our goals within our geographic range. The capacity and capability requirements for clinical sites for this study is limited. All that is required of sites will be the ability to enroll patients that meet the clinical case definitions of interest, capacity to collect and temporarily store biological samples, and follow standards for data management and protection. We will be enrolling inpatient and outpatient participants within 10 days of the presentation of symptoms to increase the chance of PCR detection of viruses so we will not wait for advanced normative diagnostic tests, that are likely unavailable in the more remote sites, to be completed. We will work with clinical sites to determine the best process for project staff, in most cases supporting the time of currently site staff. These will be persons who are locally trained and certified to collect biological specimen from participants including blood draw technique, e.g. doctor or nurse. Last, the clinical site will need the capacity to securely store all personal health information and personally identifiable research data, this will include locked offices with locked filing cabinets to store all paper records and an encrypted computer. Training on project procedures will be provided for all local project staff by local country project management staff and partners from headquarters. The study at clinical sites will begin by developing relationships with local stakeholders at the hospitals and clinics in our geographic areas of interest that serve as catchment facilities for people of within the community surveillance study. We will recruit hospital staff that will be extensively trained for project procedures including enrollment of patients, sample types to be collected, storage of samples, administration of the questionnaire, and data management plans. During the Development and onboarding of new clinical sites we will assess the physical needs of the site and what supplies will be required for collection of data (e.g. locked filing cabinet, dry shipper, secure laptop). [Did not specifically address CONDUCT]. Oversight for clinical sites will be managed by the country coordinator for each country. This will be done with regular site visits to the clinical sites and annual visits by headquarter research staff. Site visits will include monitoring and evaluating the process for patient enrollment, collecting and storage of samples, administration of the questionnaire, and secure and data management procedures for secure personal health information and research data. During these visits any additional training will also be provided.

Commented [EH124]: Is that true

Standardized approach oversight and implementation

Management and oversite for all study sites will be managed by the local country coordinator who is supported by staff at headquarters. Implementation of a standardized approach across all study sites, community and clinical, will begin by extensive training of staff on procedures on requirements of the project, this includes: enrollment, data collection, and data management. Our research team has over 5 years of experience building capacity on human subjects' research and has developed training resources and materials for standardized implementation of community and clinical research. This includes SOPs for screening, enrollment, and retention of participants. In addition to yearly monitory reports the country coordinator will regularly visit each clinical site to ensure quality standards and compliance of procedures are being met. Through our work with clinical sites in PREDICT we have developed culturally appropriate screening measures for clinal sites that do not disrupt the flow or quality of medical treatment received by the patient. This was done by working collectively with clinical research staff to evaluate current procedures and patient flow at the site to determine the most efficient while minimal invasive inclusion of our study into the daily working of the clinal site. Most efficiently this was done by adding minimal basic screening questions to the current clinal intake forms, which allows the clinal research officer to quickly scan charts or logs for potential patients to enroll, allowing for

minimal disruption and prevents potential enrollees from being overlooked if the research staff is not on duty. At clinical sites that do scheduled outpatient days we may have a trained member of the research staff with the intake nurse to monitor the patients coming to the clinic more rapidly to no delay screening or enrollment in such a fast pace environment. Using the screening tools, we will recruit and enroll patients who present with symptoms that meet the clinical case definitions of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever, of unknown etiology within the past 10 days. These patients will be enrolled, samples collected and survey conducted to assess the participants contact with wildlife through: 1) occupational exposures; 2) animal contact; 3) environment biosecurity. With permission from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology. The clinical cohort will be split into cases and controls. “Cases” are defined as participants whose samples tested positive for either coronaviruses, henipavirus, or filoviruses, serological tests. “Controls” will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between serological/PCR status and risk factors. In clinical study, we will use this procedure to determine how clinical presentation differs between virus-exposed and -unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. We will model the outcomes to analyze correlation between biological results and risk factors related to high risk wildlife exposure. Additionally, we will be following up with clinical participants to determine how clinical presentation differs between coronaviruses, henipavirus, or filoviruses in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. [How do these cohorts be leveraged for new emerging pathogens] Achieving the adequate numbers will be attained through duration of the as clinical participant enrollment. While patient’s enrollment is limited by the number of individuals presenting at hospitals, during the PREDICT project we had an average of 105 patients enrolled per year, ranging from 77-244. The country coordinator will be continually monitoring the project database to be sure that we are on track to reach our target.

Clinical cohort setup, recruitment, enrollment

We will recruit inpatients and outpatients from clinical sites to participate in the study after initial screening to determine if they meet the clinical case definitions for 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever, of unknown etiology for symptoms reported onset of illness to increase the chance of PCR detection of viruses. Once enrolled, biological samples will be collected by trained hospital staff. When possible, samples will be taken concurrently with samples being collected for normative diagnostics. We will collect 2* 5mL of blood for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples and two nasal or oropharyngeal swabs will be collected. The cases are defined as participants whose samples tested positive for Henipaviruses, Filoviruses, or CoVs by PCR or serological tests. The controls will be selected from the pool of participants admitted to the studies and whose samples tested negative for coronaviruses, henipavirus, or filoviruses. We will follow up 35 days after enrollment to collect an additional 5mL blood draw for collection of two 500 µL serum samples and a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. Following up 35 days post initial collection gives adequate time for 1) development of IgG and IgM, which occurs <28 days after onset of symptoms for coronaviruses, henipavirus, or filoviruses infected patients to evaluate the immunological progression of disease and 2) further the risk assessment of the participants to monitor contact with wildlife, people, and to assess the likely reservoir hosts to collect information to inform potential intervention programs.

Utilization of collected data

Sampling of wildlife and the enrollment of community surveillance participants and a clinical cohort of participants give us the ability to assess the viruses the are circulating in each of the three populations in a similar geographic region. We will use phylogenetic analysis to compare the relationship between wildlife viruses found and viruses fond in human participants. Additionally, the questionnaire allows us to assess

Commented [EH125]: Will these cohort fulfill the goals of the EIDRC.

Commented [EH126]: PREDICT we also make the stipulation that we will collective relatvive samples if avialble from treatmean collection, eg CSF if the MD is doing a spinal tap we will request an aliqot if possible

Commented [EH127]: Please add any information that might address: types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.

relative measures of human-wildlife contact from our survey work that we will analyze the intensity of contact with species known to be at a higher risk for spillover against other risk to provide useful proxy information for spillover risk.

Commented [EH128]: Please add any information that might address: Describe methods that might be applied to elucidate a zoonotic source

The biological specimens to be collected (2*5mL whole blood for whole blood and serum analysis and nasal or oropharyngeal swabs) from all eligible participants and follow up specimen 35 days (3mL of whole blood for serum) after enrollment with the standardized questionnaire. Capacity for specimen is collection is not more than standard phlebotomy skills and we will collected by locally trained and certified personnel that are part of the research team in country. Staff we will trainline on ethical guidelines for conducing human subjects research and survey data collection methods. Collection of whole blood, serum, and nasal or oropharyngeal swabs allows us to test the samples for PCR to detect viruses in our priority viral families, serologically test for immunological response and historical exposure to zoonotic diseases of relevance, and the questionnaire will assess individuals evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. With this collective data set we aim to quantify and detect novel viruses likely to spillover regularly to people, are often unreported or misdiagnosed as their clinical manifestations are unknown to detect cryptic outbreaks causing previously 'hidden' clinical syndromes in people. Our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence.

Potential expansion
Expansion of the research project to other clinical sites or research areas could happen as new information is gathered, either through our research or an outbreak in the region. If expansion is required we could rapidly implement research activities in additional clinical or community sites through the same process we on-boarded the initial locations. First, by working with local stakeholders to create a working relationship and on-boarding new clinical research staff through advanced trainings of project aims, SOPs on data collection and storage, and ethical guidelines. The network of infectious disease researchers in the community and hospital settings would allow for an accelerated response to any potential infectious disease outbreaks as staff are well trained on ethical guidelines for working with human subjects, data and specimen collection, storage, and transpiration, project staff would be on the front lines ready for the call to action. Ideally, our testing for viruses in priority viral families may lead to the discovery cryptic outbreaks before it spreads in the general public. Biological results coupled with survey data on key risk factors will provided necessary information for granular understand of disease spread.

In a clearly labeled section entitled **"Clinical Management Plan":**

- Describe plans, processes, and procedures for the following activities: clinical site selection including feasibility, capacity, and capabilities, and study development, conduct, and oversight.
- Describe strategies for oversight and implementation of standardized approaches in the recruitment and clinical characterization of adequate numbers of relevant patient populations to ensure the prompt screening, enrollment, retention and completion of studies. Describe novel approaches and solutions to study enrollment. including the clinical meta-data that will be captured and describe how the staff at each enrollment/collection site will be trained and comply with the quality standards for data and sample collection and compliance with the standardized procedures. Provide a general description of the methods for establishing clinical cohorts and how cohorts may be leveraged for new emerging pathogens.
- Provide general approaches to identification of the types of clinical cohorts needed to fulfill the goals of the EIDRC. Discuss types of clinical cohorts and clinical assessments that will be utilized to study natural history and pathophysiology of disease, including characteristics of the cohort, site for recruitment, types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be

utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.
- Describe methods that might be applied to either elucidate a zoonotic source or a vector of transmission.

Our research team incredibly diverse and capable of working with a group of other viral families: Lay out all our additional vector experience incl. my WNV work, our work on Chik etc.
Baric: Flavi, Noro,
Linfa: …
EHA: …

- Describe capacity for and approaches to clinical, immunologic and biologic data and specimen collection, and how these data and specimens will address and accomplish the overall goals and objectives of the research study.

Ralph

1. Norovirus: collaboration with surveillance cohort CDC; first one to publish antigenic characterization of each new pandemic wave virus; and phase 2 and 3 trial of therapeutics
2. Tekada Sanofi Pasteur dengue
3. Nih tetravalent vaccine

- Provide plans for the potential addition of new sites and expanding scientific areas of research when needed for an accelerated response to an outbreak or newly emerging infectious disease.

Linfa – will launch VirCap platform on outbreak samples.

7. Statistical Analysis Plan

Analysis of questionnaire data will involve calculation of metrics of contact for each risk group under study, such as the proportion of respondents indicating they have butchered live animals, seen wildlife in their homes, been bitten or scratched by animals, etc. Comparisons of metrics of contact between men and women, as well as different study communities will be conducted in order to explore the environmental and social factors (gender, age, occupation/religion, socioeconomic status (SES)) that influence contact with animals and to determine who is most at risk. Statistics will be computed to identify differences between groups that are significant at $P < 0.05$. Various metrics of contact related to different activities within specific groups will be compared to determine the activities that put groups at most risk. Finally, multivariate analysis as appropriate for the outcome measure (e.g. ordinary linear regression, logistic regression, generalized estimating equations that correct for cluster effects and non- normal distributions of outcome, etc.) will be employed to explore the relationship between key metrics of contact and the factors that influence frequency and types of human-animal contact.

Test results and viral sequences obtained from human and animal (wildlife and livestock) samples will be compared and phylogenetic analysis performed to document viral sharing between animals and humans. The test results data will be analyzed in the context of the questionnaire data to identify high-risk human-animal interactions and behaviors or practices that are associated with viral spillover and/or sharing. Specifically, we will calculate odds ratios for demographic and behavioral risk factors associated with the outcomes of viral infection or exposure among communities sampled. Self-report of medical history and illnesses collected through the questionnaires will also be evaluated for associations with demographic and behavioral risk factors. Regression analyses will allow for statistical assessment of both categorical and continuous predictor variables and hierarchical aggregation of data to adjust for correlated factors within communities. Data on self-reporting of illnesses will be triangulated with biological data from the concurrent animal and human sampling to identify symptoms and illnesses that could be correlated with potential pathogens.

In a clearly labeled section within the Research Strategy entitled **"Statistical Analysis Plan":**

- Describe the overall plan for statistical analysis of preliminary research data, including observational cohort data.
- Describe the overall staffing plan for biostatistical support and systems available for following functions: 1) preliminary data analyses, 2) estimates of power and sample size, 3) research study design and protocol development.
- Describe the quality control and security procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens. Describe any unique or innovative approaches to statistical analysis that may be utilized.

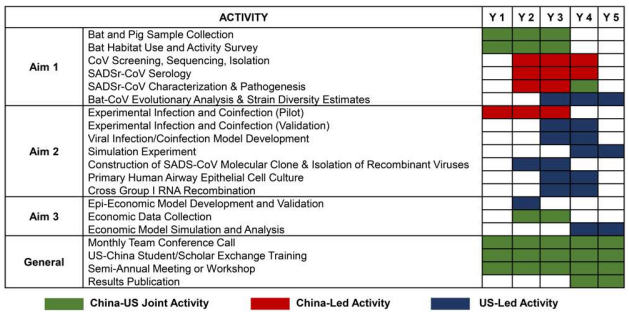
8. Project Milestones and Timelines

In a clearly labeled section entitled **"Project Milestones and Timelines":**

- Describe specific quantifiable milestones by annum and include annual timelines for the overall research study and for tracking progress from individual sites, collaborators, and Teams. Milestones must specify the outcome(s) for each activity. Milestones should be quantifiable and scientifically justified, and include the completion of major research study activities, including, for example, protocol development, case report forms, scheduled clinical visits, obtaining clearances study completion, and analysis of final data. Milestone criteria should not simply be a restatement of the specific aims. Using a Gantt chart or equivalent tool, describe the associated timelines and identified outcomes for the research study.

Show that for this proposal, we won't be doing the Specific Aims sequentially. We'll begin the human work (SA 2, 3) at the beginning of the project, along with SA1. We'll do the diagnostic assay development initially in Ralph's, Linfa's, Chris's lab, but then begin tech transfer in Y1….

Project Management & Timeline



PI Daszak will oversee all aspects of the project. Dr. Daszak and has two decades of experience managing international, multidisciplinary research in disease ecology, including successful multi-year projects funded by NSF, NIH and USAID. He will conduct monthly conference calls with all co-PIs, including the China team **(see Activity schedule below)**. He will be assisted by EHA Sr. Personnel Li, who is a US-based Chinese national with fluent Mandarin. Co-PI Olival will oversee Aim 1 working closely with China PI Tong, who will test samples. Co-PI Ma (China team) will coordinate and lead all Chinese experimental infections, with guidance from co-PI Saif, who will coordinate and lead all US experiments with co-PI Qiuhong Wang. Sr. Personnel Ross will conduct all modeling activities in Aim 2, working closely with co-PI Saif to parameterize the models. Co-PI Baric and Sr. Personnel Sims will lead infectious clone, recombinant viral and culture experiments. Consultant Linfa Wang will advise on serology, PCR and other assays. China team co-PIs Shi and Zhou will oversee viral characterization in Aim 1. Sr. Personnel Feferholtz will oversee the economic modeling in close collaboration with Ross.

Commented [KJO129]: Text and figure From NSF-NIH SADS grant. Peter will adapt

Another Example of Milestones and specific tasks from a prev DHS (unfunded) proposal:

Expand existing databases to include predictor variables for pathogenicity risk model

Task 1: Collate available human clinical information from the literature for ~300 viral pathogens known to infect humans (Contract Award Date to 6 month)

Task 2: Update EHA host-viral association database to include new data for mammalian and avian species from literature 2015-present (Contract Award Date to 3 month)
Task 3: Extract and curate full and partial genomic data from Genbank for 5-6 viral families with a high proportion of human-infectious pathogens for phylogenetic analyses – e.g. 'nearest-known-threat module'. (Month 2 to 6 month)

Develop underlying pathogenicity and spillover risk assessment models
Task 4: Fit generalized linear models in Stan for multiple response variables to measure pathogen impact (Month 7 to 10 month)
Task 5: Fit generalized linear models to estimate risk of human infection (spillover) based on host, ecological, and location specific traits (Month 4 to 8 month)
Task 6: Validate model effectiveness by simulating early-outbreak, limited-information scenarios from known pandemics to confirm model behavior (Month 8 to 10 month)

Commented [KJO130]: Just example, but probably just want to use a Gantt chart format per most of our grants, depending on space.

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II. Research Strategy:

1. Significance:

Southeast Asia is a critical hotspot for emerging diseases due to its high biodiversity of wildlife and their viruses, and the presence of key ecological and socioeconomic drivers of emergence **(Fig. 1)** (*1*). These include dense human populations living in networks of rural and urban communities, with strong cultural and occupational connection to wildlife and livestock farming and trading, and intimate connection to global travel networks (*2*). The region is also undergoing rapid environmental and demographic change, both of which increase the risk of disease emergence and spread.

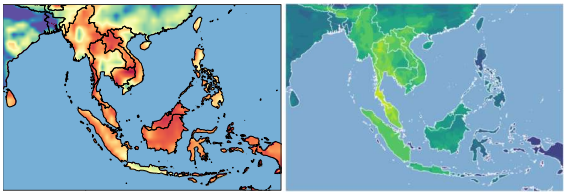


Fig. 1: Southeast Asia, and in particular, parts of Thailand and Malaysia are hotspots of risk for disease emergence **(left)**, and predicted high diversity of 'missing' or as-yet undiscovered viruses, yellow = highest diversity **(left)**. From (*1, 2*).

Commented [MOU1]: Should include Olival et al. 2017 for missing zoonoses, not Jones. Or Jones, Allen, and Olival papers.

Not surprisingly a number of novel viruses, including near-neighbors of known agents, have emerged in the region leading to sometimes unusual clinical presentations **(Table 1)**.These factors are also behind a significant background burden of repeated Dengue virus outbreaks in the region (*16*), and over 30 known *Flavivirus* species circulating throughout S. and SE Asia (*17*). The events in **Table 1** are dominated by three viral families: coronaviruses, henipaviruses and filoviruses, which serve as initial examples of our team's research focus and capabilities. These viral groups have led to globally important emerging zoonoses (*18-29*). Their outbreaks have caused tens of thousands of deaths, and billions of dollars in economic damages (*30-32*). Furthermore, relatives and near-neighbors of known pathogens in these families have been reported throughout the region, including by members of our consortium: henipaviruses in bats in Thailand, Cambodia, Philippines, Laos, Indonesia, China, Malaysia, Bangladesh and India (*33-39*); a novel henipavirus, Mojiang virus, in wild rats in Yunnan (*10*); serological evidence of filoviruses in bats in Bangladesh (*40*) and Singapore (*41*); Nipah- and Ebola-like viruses in *Hipposideros*, *Cynopterus* and *Rhinolophus* species in Malaysia (Hughes *et al.*, in prep.); evidence of novel filoviruses in bats in China (*42-44*), including Měnglà virus that appears capable of infecting human cells (*45*); novel paramyxoviruses in bats and rodents consumed as bushmeat in Vietnam (*46*); a lineage C β-CoV in bats in China that uses the same cell receptor as MERS-CoV and can infect human cells *in vitro* (*47*); MERSr-CoVs in dry bat guano being harvested as fertilizer in Thailand (*48*), and directly in bats (Wacharapluesadee *et al.*, in prep.);

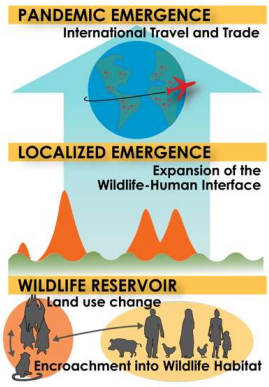
Viral agent	Site, date	Impact	Novelty of event	Ref.
Melaka & Kampar virus	Malaysia 2006	SARI in family group, individual	1st disease due to bat-origin reoviruses, also Singapore, Vietnam etc.	(*3-6*)
Reston filovirus	Philippines 2008	Seropositive people killed pigs,	No prior findings of filoviruses in pigs	(*7*)
Thrombocytopenia Syndrome virus	E. Asia 2009	100s of deaths in people	Novel tick-borne zoonosis with large caseload	(*8*)
Reston filovirus	Shanghai 2011	PCR positive pigs	Further evidence of pig RESTV infection	(*9*)
Mojiang virus	Yunnan 2012	Death of 3 mineworkers	1st evidence of rodent origin henipavirus in people	(*10*)
Nipah-like virus	Philippines 2015	Killed horses	No prior horse infection for NiV, known for HeV	(*11*)
SARSr-CoV & HKU10-CoV	S. China 2015	Seropositive people	1st evidence human infection HKU10 & SARSr-CoV	(*12*)
SADS-CoV	China 2017	>25,000 pig deaths seronegative people	Novel emergence of bat-origin CoV	(*13*)
Nipah virus	Kerala 2018, 2019	Killed 17/19 people 2018, 1 infected 2019	1st outbreak of NiV outside Bangladesh, W. Bengal focus	(*14, 15*)

Table 1: Recent emergence events in SE Asia indicating potential for novel pathways of emergence, or unusual presentations for known or close relatives of known viruses.

Commented [MOU2]: Shouldn't we add the emergence of Nipah in Malaysia Singapore in 1999 at the top of the table!?

172 novel β-CoVs (52 novel SARSr-CoVs) and a new β-CoV clade ("lineage E") in bat species in S. China that are also found throughout the region (*20, 47, 49, 50*); and 9 and 27 novel wildlife-origin CoVs and

paramyxoviruses in Thailand (respectively) and 9 novel CoVs in Malaysia identified as part our work under USAID-PREDICT funding (*49, 51*). These discoveries underscore the clear and present danger of future zoonotic events in the region. Furthermore, the wide diversity of potentially pathogenic viral strains has significant potential to help in the development of broadly active vaccines, immunotherapeutics and drugs (*47*). Most high-impact zoonotic viruses originate in wildlife reservoirs, sometimes spilling over first into livestock 'amplifier' hosts, or directly into localized human populations with high levels of animal contact **(Fig. 2)**. Efforts to prevent emerging zoonoses have targeted these high-risk populations in regions prone to disease emergence (*29, 52*). However, these regions tend to be in the developing world, where nations often lack the resources to investigate novel zoonotic events, or wildlife reservoirs of zoonoses, and prioritize more immediate public health concerns such as Dengue virus or NTDs (*16*). Zoonotic disease surveillance and control is also hampered by inadequate information on basic disease ecology (e.g. range of wildlife reservoirs, risk factors for spillover) and pathogen biology (viral diversity, pathogenesis), and by logistical challenges sharing novel diagnostics, animal models, samples and reagents at sites where outbreaks begin (*53*). Countermeasure and vaccine development is challenged by the small number of isolates available, and the presence of closely-related strains, some with evidence of transmission to people but with unknown pathogenicity. For example, using NIH and other funding, we found serological evidence of exposure to African henipaviruses in 3-4% of a sample of people in Cameroon, with a significant risk factor being butchering of bats; and serological responses to bat SARSr-CoVs in 6/209 (2.87%) and bat HKU10-CoV in x/xx (x%) people living close to a bat cave in Yunnan, all novel viruses with unknown clinical impact (*12, 54*). We discovered two novel bat-borne reoviruses, Melaka and Pulau viruses, and showed that 14/109 (13%) of people living in close proximity to bat roosts on Tioman Island, Malaysia were exposed to these viruses (*3*) as well as 12/856 (1.4%) people screened in Singapore (*4*). Previous studies in the Philippines found famers and butchers who had contact with sick pigs tested positive for Reston ebolavirus antibodies but had not experienced illness (*7*), and it was our team that discovered Philippine bats as natural reservoir hosts for Reston virus (*55*). In pPreliminary screening in Malaysia with funding from DTRA ~~for serological surveillance of Henipaviruses and Filoviruses spillover found serological evidence of past exposure to Nipah and Ebola antigenically-related viruses in non-human primates (NHPs) and people (Hughes, in prep.).~~ we found serological evidence of exposure to Henipaviruses (Hendra, Nipah and Cedar) in 14 bats and 6 human samples, and Ebola (Zaire and Sudan) antigenically-related viruses in 11 bats, 2 NHP and 3 human samples (Hughes, in prep.).



Even when spillover leads to outbreaks of illness and mortality, they are often unreported, undiagnosed or misdiagnosed. Under a prior NIH R01, we initiated targeted syndromic surveillance of encephalitis patients in Bangladesh clinics and showed that NiV causes repeated annual outbreaks with an overall mortality rate of ~70% (*56*). Nipah virus has been reported in people in West Bengal, India, which borders Bangladesh (*26*), in bats in Central North India (*57*), and now in people in Kerala, South India in 2018 and 2019, raising the specter of future spillover at other sites in the region (*14, 58*).

Fig. 2: Our Early Warning Approach: Most zoonotic viruses emerge from wildlife hosts, sometimes via livestock (Stage 1, lower panel), to cause small scale outbreaks (green) or larger chains (red) of human-to-human transmission (Stage 2, middle). In some cases, these spread more widely via air travel (Stage 3, upper). Our EIDRC proposal targets each stage: SA1 examines the diversity and spillover potential of viral threats in targeted SE Asian wildlife; SA2 seeks evidence of their spillover into focused high-risk human populations (stage 2); SA3 identifies their capacity to cause illness and to spread more widely (stage 3). Figure from (*52*).

Under a current NIAID R01, we have shown that we can use analytical approaches to target surveillance of wildlife and people and identify likely sites of spillover. We used phylogenetic analysis of bat hosts and their CoVs to identify a key cave complex in SW Yunnan Province that harbors all the genetic elements of SARS-CoV (*59*), as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics (*20, 47, 49, 50, 60-62*). We conducted behavioral risk surveys and biological sampling of human populations living close to this cave and found evidence of spillover (xx serology) in people highly exposed to wildlife. **This work provides proof-of-**

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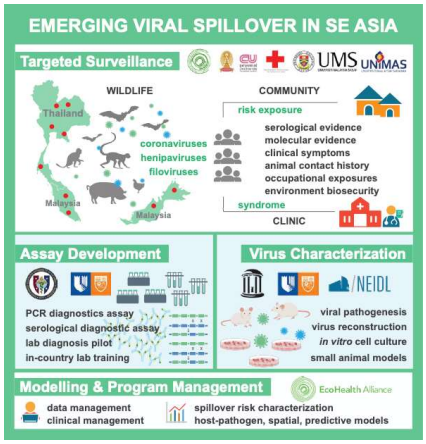
concept for an early warning approach that we will expand in this EIDRC to target key viral groups in one of the world's most high-risk EID hotspots.

The overall premise for this EIDRC proposal is that there is substantial evidence that: 1) diverse CoVs, henipaviruses and filoviruses related to known zoonotic pathogens are circulating in wildlife reservoirs in Southeast Asia; **2)** these viruses likely spillover regularly to people, are often unreported or misdiagnosed, and their clinical manifestations and potential to cause pandemics are unknown and underestimated; and **3)** our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence. To scale-up this approach, we propose to launch **EID-SEARCH** (the **E**merging **I**nfectious **D**iseases - **S**outh **E**ast **A**sia **R**esearch **C**ollaboration **H**ub), to better understand the risk of zoonotic viral emergence in Southeast Asia. We will initially focus on the diversity of known and novel CoVs, henipaviruses, and filoviruses in wildlife reservoirs. We will use this work to build our center's interactive network throughout the region, bridge barriers, provide early focal point for basic and translational studies, develop sample handling and storage routines, communication networks, cohort management, infrastructure development, and cross-training in surveillance and novel diagnostic platforms. We will develop and transfer technology to test these viruses' capacity to infect human cells and mouse models, develop new specific and sensitive serological and molecular diagnostic tools, conduct surveillance of human communities with high risk of exposure to wildlife, and clinical cohorts to identify evidence of spillover of viruses causing previously 'cryptic' clinical syndromes in people. We will test the capacity of novel viruses we isolate, or genetically characterize, to infect human cells and/or mouse models, and thus asses potential for spillover and spread which will feed into our risk models. Our group also has extensive experience in other viruses, including vector-borne flaviviruses, and alphaviruses, and will use this experience to provide robust long-term surveillance, diagnostics, human and basic science opportunities. While the current proposal focuses its basic and applied research agendas on this group of viral EID, we note that the EID-SEARCH is poised to rapidly respond to any new emerging outbreak. Thus, filoviruses, henipaviruses and coronaviruses are examples of sentinel surveillance systems in place that can capture, identify and initiate a rapid response targeting any novel microbial pathogen that emerges in this region.

2. Innovation: Previous work by our consortium has developed strategies to target surveillance in wildlife and people to better anticipate and identify early spillover events and pre-empt outbreaks of emerging viruses. In the EID-SEARCH we scale up this approach in a central research "hub" made up of leading laboratories, clinics and surveillance sites in three critically high-risk EID hotspot countries in SE Asia. EID-SEARCH's reach is expanded through its regional network of collaborators that covers nearly all of the greater SE Asia region. The innovation of the EID-SEARCH is in: **1)** Its multidisciplinary approach that combines modeling to target geographic and taxonomic sampling targets with on-the-ground zoonotic disease surveillance in human and wildlife populations; **2)** the biological characterization approach that identifies how likely viruses are to be able to spillover into people, and enables evaluation of existing countermeasure technologies; an approach that is grounded in our successful work on SARSr-CoVs; and **3)** the strategic sampling of both high-risk communities with extensive animal contact at the front-lines of viral spillover *and* syndromic clinical cohorts that present with signs of novel viral emergence, to identify risk of viral spillover in an early warning system approach **(Fig. 2)**. **In Aim 1, we will target viruses in archived and newly collected wildlife samples, and characterize their risk of spillover to people**. We will use spatial and phylogeographic analyses to identify the geography and species of wildlife to test samples from, and to further characterize viruses we have recently discovered. We will use *in silico* methods (novel ecological-phylogenetic risk ranking and antigenic mapping of receptor binding domains), cell culture, and animal models to assess their potential for spillover into



Commented [BRS5]: Statement should be in the aims. …Our center covers the worlds most high risk EID hostspot?

Commented [KJO6]: Hongying to add in "genetic sequencing" under Virus Characterization, and also Aim 1 + 3 for Virus Characterization and Aim 1 + 2 for Assay Development

Commented [KJO7]: And pathogeneicity? Could argue for those we use animal models on.

Fig. 2: EID-SEARCH approach, core members, and roles.

high-risk human populations. **In Aim 2, we will conduct ~~focused,~~ targeted cross-sectional surveys of ~~and sampling of~~ human communities with high levels of animal contact ~~exposure to wildlife and other animals~~to identify occupational and other risk factors for zoonotic virus exposure.** We will use serological tests targeting viruses identified in Aim 1 to identify the baseline spillover of known or novel CoVs, henipaviruses and filoviruses in these populations. Serological findings will be analyzed together with clinical metadata (including a simple animal-contact survey, location data, viral sequence/isolates etc.) to identify zoonotic risk factors and routes of exposure from specific wildlife or vector species. **In Aim 3, we will use syndromic surveillance of clinical cohorts to identify evidence of viral etiology for otherwise 'cryptic' outbreaks.** We will enroll and collect biological samples from patients in local clinics and hospitals who present with syndromes previously linked to known viral agents and live within communities at high risk for disease emergence. We will conduct molecular and follow-up serological diagnostic assays to identify likely viral etiology of syndromes that may represent otherwise cryptic events. Where viruses are identified, we will attempt to isolate and characterize them, and use survey data, ecological and phylogeographic analysis to identify likely reservoir hosts and inform intervention programs. Our US partner BSL-4 laboratory, NEIDL, will attempt isolation and characterize any viruses requiring high levels of containment (e.g. any novel filoviruses discovered).

Commented [PD8]: Hongying – Figure needs edits to text: top panel, 'wildlife' should read "Aim 1: WILDLIFE", "AIM 2: COMMUNITY", "AIM 3: CLINIC". Right hand side: "Clinical symptoms" should be "Clinical history", "Occupational exposures" should be "Occupational exposure", "environment biosecurity" should be "environmental risk factors"

Middle panel: "Assay Development" and "Virus Characterization" should have "(Aim 1)" after them. Left hand side: should read "PCR diagnostics", "serology", "piloting diagnostics" and the last one is ok. Right hand side bullets should be "receptor binding characterization" "in vitro characterization" and "mouse models"

Bottom panel: all good

Commented [PD9]: PD will get stronger letter from Jerry Keusch: opportunity for visiting scholars to be certified for BSL-4 research, strong Ebola person at NEIDL to conduct characterization if we find filos.

3. Approach

Research team: The EID-SEARCH builds on long-term collaboration among world leaders in emerging virus research with proven experience collaborating internationally on field, lab and human surveillance research **(Fig. 2)**. Over the past two decades, our consortium partners have conducted high profile research on EIDs within the region and globally, including identifying the wildlife reservoirs for Nipah and Hendra virus, MERS- and SARS-CoVs (*20, 25, 57, 59, 63-65*), discovering SADS-CoV (*13*), and developing an array of serological, molecular, *in vitro* and *in vivo* approaches to characterizing high-risk CoVs, henipaviruses, and filoviruses (*60-62, 66-79*). Our team has substantial experience conducting human surveillance in the community and during outbreaks **(See sections 2.x.x, 3.x.x)**, including: USAID-PREDICT, and DTRA-funded viral surveillance of indigenous communities in Peninsular Malaysia and syndromic surveillance of hospital patients in Sabah (Co-I Hughes); collection of >2,000 serum samples from healthy blood donors and sanitation workers, febrile and influenza like illness (ILI), and diarrheal stool samples from children under 5 years of age (Co-I Kamruddin); collaboration on the MONKEYBAR project with the London School of Hygiene and Tropical Medicine to analyze risk and prevalence of the zoonotic malaria *Plasmodium knowlesi* (Co-Is William, Tock Hing); case control and cross-sectional studies in Sabah villages (~800, 10,000 participants resp.) (Co-Is GR, TYW); and community-based surveillance of hundreds of bat-exposed villagers in Thailand (Co-Is Wacharapluesedee and Hemachudha). As a proof-of-concept of how our team can rapidly scale up resources for diagnostics and surveillance in the face of a new EID, in 2016, we discovered a novel bat-origin HKU-2 CoV (*80, 81*) killing >20,000 pigs in S. China, designed PCR and LIPS serology tests, then surveyed pig farmers for evidence of exposure, and conducted experimental infections, pathogenesis and molecular studies, **all in the span of 3 months** (*13, 82*).

The administration of this center **(See section X)** will be led by PI Daszak, who has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5-20 yrs on NIAID- and USAID-funded research. He will oversee all activities in this EIDRC. Drs. Olival and Zambrana are leaders in analytical approaches to targeting surveillance and head the modeling and analytics work for USAID-PREDICT. Drs. Wang, Baric, Broder, Anderson, and Laing have developed a unique array of *in vitro* cell culture, serological and molecular reagents to identify and characterize emerging viruses in people and animals (henipaviruses and other paramyxoviruses, CoVs, filoviruses and many others). Dr. Wacharapluesedee, Hughes and collaborators have coordinated surveillance of people, wildlife, and livestock within Thailand and Malaysia for the past 10 years, in collaboration with Ministries of Health, Agriculture and Environment. Finally, this consortium has extensive external funding from NIH, DoD, USAID, DHS and other USG agencies that supports a range of laboratory, analytical and field studies directly related to the current proposal, and will bring substantial leverage to our EIDRC.

Geographical focus: The three core (hub) countries for this proposed EIDRC are Thailand, Malaysia and Singapore, three contiguous EID hotspot countries that stretch through one of the most significant foci of EID origins globally. Our proposed work includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sarawak and Sabah, and thus contains overlapping wildlife biogeography with India, Bangladesh, Myanmar, SW China, Laos, Cambodia, Vietnam, Philippines, and Indonesia. It encompasses diverse cultural and socioeconomic backgrounds which have correspondingly diverse and intensive interactions with wildlife reservoirs and the global travel network. We have already begun sampling of wildlife hunters, livestock workers, wildlife farmers and market workers, rural villagers living close to high risk wildlife species, people otherwise highly exposed to animals in these countries, and clinical cohorts, as part of current/prior NIH, DTRA, and USAID funding. To develop a larger catchment area for emerging zoonoses, we will deploy our core member's extensive network of collaborators in clinics, research institutes and public health laboratories in almost every other Southeast Asian country. We will conduct regular meetings with key research, public health and community leaders, including >50 leading laboratories/institutions in ~~XX~~ 10 countries that are currently collaborating with core members of our consortium via other funded work (**Fig. 3**). We will use these, and our annual meetings, to share information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks across the region.

Fig.3 : Map of Southeast Asia indicating three hub countries for this proposed EIDRC (Red: Thailand, Singapore, Malaysia) and countries in which Key Personnel have collaborating partners (Green), indicating that EID-SEARCH provides access to key collaborators throughout the region.

Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife

1.1 Rationale/Innovation: The vast majority of emerging zoonotic pathogens are linked directly or indirectly to wildlife host, particularly from important mammalian groups like bats, rodents and non-human primates (*83*). Our previous work on wildlife origin viruses demonstrates that zoonotic viral emergence tends to occur in specific regions with high diversity of wildlife that harbor viruses of zoonotic potential, and human populations that have close contact to these wildlife **(Fig. 1)** (*2*). In Aim 1, we will strategically conduct EID surveillance by applying risk analytics to prioritize the regions, wildlife species, and human populations that have a particularly high risk of viral spillover. We will target high-risk zoonotic host reservoirs (bats, rodents and primates) in these regions and apply novel predictive models to estimate viral diversity and host range to identify wildlife species that have been ignored or under-sampled by previous zoonotic disease surveillance efforts. We will strategically collect specimens from these species and screen them, together with some of the tens of thousands of recently collected samples we have archived in freezers in our labs, for known and novel agents. We will begin screening using conserved PCR assays to identify known and novel virus in high risk RNA viral families, *Coronaviridae (CoV)*, *Paramyxoviridae (PMV)*, and *Filoviridae (FV)*. We will expand to other groups of pathogens, including arboviruses, pending available resources and priorities established with the EIDRC coordinating center (EIDRC-CC) and other regional EIDRCs. Using risk ranking algorithms and a series of ecological and virological risk factors, we will triage the wildlife viruses we discover for further genomic characterization and viral isolation. On this subset of viruses, we will then use *in vitro* and *in vivo* approaches (viral rescue, cell lines, gene edited humanized transgenic mice and the collaborative cross genetic reference mouse population) previously developed and widely used by our team to predict capacity of novel viruses to infect people and spillover (*61, 84-87*). These high-risk viruses and their close relatives will be targets for human community serosurveys and clinical sampling in Aim 2 and 3, respectively.

1.2 Preliminary data: 1.2.a. Geographic targeting: EcoHealth Alliance has led the field in conducting analyses to indicate where the highest risk of zoonotic spillover is across the globe, identifying SE Asia as a

Commented [KJO10]: Including those in green on map, plus Australia.

Commented [PD11]: Hongying – "EIDRC Program Countries" should be "EID-SEARCH core countries" and "External Partner Countries" should be "Collaborating Partners"

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major high risk location for pathogen emergence (*1, 2*). For Southeast Asia, these EID hotspots are sites where high wildlife biodiversity, human population density and rapid ecologic change collide **(Fig 1)**. Using host and ICTV viral data for all known mammalian viruses, and generalized additive models to correct for sampling bias, we are able to predict the relative number of yet-to-be-described viruses that a species harbors (*2*). Using these data to map unknown viral diversity demonstrates substantial undiscovered viral diversity in mammals across Southeast Asia, and particularly in regions of Thailand, Singapore and Malaysia **(Fig. 1)**. In a separate paper on risk of viruses emerging from bat hosts, we found that factors driving spillover (host diversity, climate) differ from those driving spread in human populations (population density, bushmeat hunting, livestock production) (*88*). We therefore analyze capacity for viral spread as a separate risk factor, using demographic and air travel data and flight model software we have produced with Dept Homeland Security funds (*89*). In the current proposed work, we will use all the above approaches to target wildlife sampling (archive and new field collections), and to inform sites for human sampling in Aim 2, to areas of high risk for spillover and spread.

1.2.b. Reservoir host species targeting: Our analysis of all known mammalian host-viral associations demonstrates that bats, rodents and primates represent the three most important reservoirs for zoonoses, responsible for the highest proportion of known and predicted viral diversity of zoonotic potential(*90*). Thus, the bulk of our proposed wildlife sampling and testing will consist of these three taxa with a focus on bats, because this group is the known or hypothesized reservoir for the majority of high-impact zoonotic CoVs, ~~henipaviruses and filovirusesPMVs and FVs~~ (*48, 91-93*). Bats also have the highest proportion of predicted zoonotic viruses in any mammalian group (*2*), and are exceedingly diverse in Southeast Asia (~500 species). To further prioritize taxonomic sampling targets within these diverse geographic hotspots, we have used a novel, Bayesian phylogeographic analysis to identify host taxa that are the most important centers of evolutionary diversity for a given viral group, e.g. bat genera for β-CoVs ~~using an extensive CoV sequence dataset from our NIH-funded research~~ **(Fig. 5)** (*49*). This approach will be extended to other mammal taxa and viral families of interest, allowing us to identify the ancestral wildlife hosts of specific zoonotic viral groups, where their diversity is likely highest.

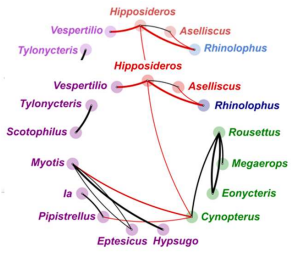

Fig 5: Preliminary analysis to identify the most important bat genera in Southeast Asia ~~as hosts~~ for beta-CoV evolutionary ~~diversitys~~ using ~~our previously-collected CoV~~ sequence data collected under our NIH-funded research. Line thickness is proportional to the probability of virus sharing between two genera, with~~,~~ in ~~In~~ter-family switches ~~are highlighted~~ in red. We will apply this Bayesian discrete phylogeographic model to identify taxonomic and geographic centers of viral diversification and sharing for relevant ~~paramyxovirus~~PMV, CoV ~~, coronavirus,~~ and other virus clades.

Finally, we have designed a strategy to estimate sampling gaps, using a viral 'mark-recapture' approach we previously published (*94, 95*) **(Fig. 6)**. Using prior data for bat, rodent and primate viral discovery vs. sampling rates, we can identify the likely total viral diversity for specific host groups in a region, and use this to estimate sample sizes to discovering the majority of unknown viruses within a specific family or genus. **In the current proposal,** we will use these analyses to estimate geographic and species-specific sampling targets **to more effectively identify new strains to support experimental infection studies and risk assessment.**

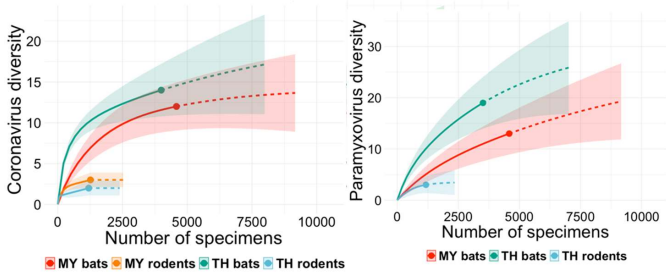

Fig. 6: Estimated ~~coronavirus~~ CoV **(left)** and ~~paramyxovirus~~ PMV **(right)** ~~putative~~ viral 'species' diversity in bats and rodents ~~for~~ from Thailand and Malaysia, using data from PCR screening and RdRp ~~gene~~ sequences ~~preliminary data~~ from >103,000 ~~PCR tests~~specimens in bats and 4,500 ~~tests~~in ~~in~~ rodents. Bats in Thailand and Malaysia have 4X the number of viruses than rodent species, controlling for sampling effort. ~~CoV and paramyxovirus diversity estimates are comparable, but discovery has not yet saturated in any taxonomic group or location.~~ We estimate that additional collection

Commented [KJO15]: Wrong ref! Should be Olival et al. 2017.

Commented [KJO16]: This is currently in Aim 3.

Commented [KJO17]: Olival et al appears twice in refs, fix.

Commented [BRS18]: I would say we focus on these three species (plus vectors?). To demonstrate the capability of the team, this proposal will focus on bat surveillance (for a variety of reasons), noting that this approach will also be applied to other zoonotic vectors during the 5 year program throughout the region.

Commented [PD19]: Ralph – have we done this well enough now? If not, please add text/edit

Commented [PD20]: Kevin - Can you get rid of the shading on the background to this figure and change the colors so the circles and genus names are more visible (esp. the green). Colors should be darker and more contrasty

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Commented [KJO22]: We can show just a single curve per country (aggregating all wildlife species) for simplicity, and could fit CoVs and PMVs on one graph.

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of 5k-9k bat specimens ~~sampling of ~5,000 bats~~ and testing of our archived rodents specimens alone will identify >80% of remaining CoV and ~~paramyxovirus~~ PMV viral species in these key reservoirs, yielding >800 unique viral strains. ~~We will use this approach to define sampling targets for other wildlife taxa, and for viral *strain* diversity.~~

1.2.c. Sample testing to identify known and novel viruses: We have conducted extensive wildlife surveillance for novel viruses across Southeast Asia in prior NIAID funded work and as part of the USAID PREDICT project. Under the 10-year PREDICT program globally, we identified 912 novel and 210 known viruses, more than the total number of viruses previously recognized in mammals by the International Committee on Taxonomy of Viruses. This work included collecting nearly 300,000 individual mammal samples from 14 EHA-led PREDICT countries and PCR-screening more than 60,000 individual specimens(*51*). In southern China alone, this sampling lead to the identification of 178 β-CoVs, of which 172 were novel (52 novel SARSr-CoVs). Included were members of a new β-CoV clade, "lineage E" (41), diverse HKU3-related CoVs (179 sequences) within a 'sister' clade to the SARS-CoV lineage, and the wildlife origin of a new virus, SADS-CoV, responsible for killing >20,000 pigs in Guangdong Province (*13*). ~~Many of the identified bat species are found across the region.~~ We have collected 28,957 samples from bats, rodents and primates in Thailand and 51,373 in Malaysia, conducting 146,527 PCR tests on a large proportion of these specimens. We have **archived duplicate samples which are now available for use in this project**, including fecal, oral, urogenital and serum samples from bats, rodents, and non-human primates, and primate biopsies. In Thailand this screening has identified 100 novel viruses and 37 known viruses in wildlife reservoirs. In Malaysia this screening has identified 77 novel viruses and 23 known viruses in wildlife reservoirs (66 novel and 19 known in Sabah and 11 novel and 9 known from Peninsular Malaysia). ~~We used~~Using family level primers for ~~henipaviruses~~PMV, ~~filoviruses~~ FV, and CoVs, we ~~and~~ have already discovered 9 novel and 7 known CoVs and 26 novel and 2 known paramyxoviruses in Thailand; and . ~~This is in addition to~~ 13 novel and 5 known CoVs and 15 novel and 1 known paramyxovirus in Malaysia from wildlife. In the current proposed work, we will attempt to sequence, isolate and characterize those viruses that our analyses below suggest are most likely to be able to infect humans.

Our collaborative group has worked together on these projects, as well as other DTRA funded projects in Singapore/Thailand (Co-Is Broder, Wacharapleusadee, Wang) to design, cross-train, and use novel serological and molecular platforms to investigate virus infection dynamics in bats and examine potential spillover events with or without human disease (see Aim 2). For serology, Co-Is Wang and Anderson (Duke-NUS) will develop a phage-display method to screen antibodies for all known and related bat-borne viruses, focusing on henipaviruses, filoviruses and coronaviruses. Once various "trends" are discovered, additional platforms (including Luminex and LIPS) will be used for verification and expanded surveillance. For molecular investigation, we will combine targeted PCR approach with a discovery-driven enrichment NGS strategy to not only survey currently known viruses, but also to discover potential novel viruses. Both the phage-display serological and enrichment platforms can be rapidly updated to include new viruses identified. In Malaysia (Co-Is Hughes, Broder, Laing) we have transferred the Luminex serology platform to partner laboratories for screening of wildlife, livestock and human samples to investigate the spillover of henipaviruses and filoviruses at high risk interfaces. These interfaces include farms near the forest and indigenous communities with lots of subsistence hunting in Peninsular Malaysia, and preliminary screening has found serological evidence of past exposure to viruses antigenically-related to Nipah and Ebola in humans, bats and non-human primates (NHPs). In Thailand, Co-Is Broder, Laing and Wacharapluesadee have transferred a Luminex-based serology platform for detecting exposure to novel Asiatic filoviruses and known henipaviruses in wildlife and human samples, with testing underway and currently being validated in our Chulalongkorn University laboratory **(See also section 2.2.d)**.

1.2.d. In vitro & in vivo *characterization of viral potential for human infection:* Using Coronaviridae as an example, we have conducted *in vitro* and *in vivo* experiments to characterize the pathogenic potential of novel SARSr-CoVs from bats. We isolated three SARSr-CoVs from bat feces: WIV1, WIV16 and Rs4874, with S protein sequences that diverged from SARS-CoV by 3-7% (*20, 59, 96*). We conducted full-length genome sequencing of 12 other novel SARSr-CoVs, some highly similar to outbreak SARS-CoV in the most variable genes: N-terminal domain and receptor binding domain (RBD) of the S gene, ORF8 and ORF3 (*59*). Using our reverse genetics system, we constructed chimeric viruses with SARSr-CoV WIV1 backbone and the S gene of

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Commented [MOU25R24]: These are correct, based on EIDITH P1 + P2 for EHA countries.

Commented [KJO26]: Leave this out as it may look like we've already done a bulk of the work?

Commented [MOU27]: Think we need to abbreviate these viral families throughout, should Find/Replace. Also, henipa is not a viral family, so changing back to PMVs.

Commented [KJO28]: Move to Aim 2? I think it's good to have some flexibility to test wildlife sera using these assays, but not readily clear how this fits in with our PCR screening? We could do a broader survey of a smaller number of wildlife samples from more species, and those with high seroprev, we can follow up w molecular assays – though not sure this is necessary. All the serology in this section can prob go to Aim 2.

two different variants. All 3 SARSr-CoV isolates and the two chimeric viruses replicated efficiently in Vero E6 cells and in HeLa cells expressing hACE2, but not in those without ACE2 (*20, 59, 96*). We used the SARS-CoV reverse genetics system (*70*) to generate a chimeric virus with a mouse-adapted SARS-CoV backbone expressing SHC014 S protein with 10% sequence divergence from SARS-CoV S. This chimera replicated in primary human airway epithelium, using the human ACE2 receptor to enter into cells (*60*). Thus, **SARSr-CoVs with diverse variants of SL-CoV S protein without deletions in their RBD can use human ACE2 as receptor for cell entry.** The Baric lab has a well-established hACE2 and DPP4 transgenic mouse model that we will use this to characterize the capacity of specific SARSr-CoVs to infect (*84, 97*). We used this chimera approach to demonstrate that pathogenicity of bat SARSr-CoVs in humanized mice differs with divergent S proteins, **confirming the value of this model in assessing novel SARSr-CoV pathogenicity**. Infection of rWIV1-SHC014S caused mild SARS-like clinical signs in the transgenic hACE2 mouse model **that weren't reduced by immune-therapeutic monoclonals that attenuate SARS-CoV pathogenicity, nor after challenge following vaccination against SARS-CoV** (*60*). We repeated this virus characterization approach with chimeras using HKU3r-CoV S proteins that are ~25% divergent from SARS-CoV S, **and found that they are unable to use the ACE2 receptor**. Additionally, we were unable to culture HKU3r-CoVs in Vero E6 cells, or human cell lines.

A similar approach to the above pipeline for CoVs will be applied to novel henipaviruses and filoviruses we discover during our research. The broad mammalian tropism of Hendra virus and Nipah virus (*66*) is likely mediated by their usage of highly conserved ephrin ligands for cell entry (*15, 98*). A third isolated henipavirus, Cedar virus does not cause pathogenesis in animal models. Recently, **Co-Is Broder and Laing** have developed a reverse genetics system and rescued a recombinant CedV to test ephrin receptor usage/tropism and pathogenicity during henipavirus infections (*75*). CedV is unable to use the Ephrin-B3 receptor (*75, 99*) which is found in spinal cord and may underlie NiV encephalitis (*100*) and that pathogenesis also involves virulence factors V and W proteins. The putative henipaviruses, Ghana virus and Mojiang virus, predict V and W protein expression, with GhV able to bind to ephrin-B2, but not -B2 (*101*), and the receptor for MoJV remaining unknown (*102*) but is likely ephrin-B2 (*103*). Primary human lung endothelial cells are highly susceptible to Ebolavirus infection (*104*). Huh7 cells are good candidates for ebola in the liver, oftentimes used to isolate virus from clinical samples (*105*) Huh7 cells also offer advantages for reverse genetic recovery of novel filoviruses (*106*). Thus far, paramyxovirus attachment glycoproteins have been divided into three major groups: hemagglutinin (H), hemagglutinin-neuraminidase (HN), and attachment glycoproteins (G).

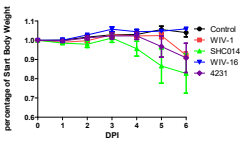

Fig. 7: Body weight change in hACE2 transgenic mice after SARSr-CoV infection, demonstrating that bat-origin SARSr-CoVs cause similar clinical presentation to outbreak strain (4231). From (*60, 61*).

Models of Outbred Populations. For novel viruses across all families, we will use the UNC collaborative cross mouse that captures 90% of the diversity within all mouse groups to assess the most suitable model for further experiments (*107*). We have used this model for CoV, ~~filo~~ FV (Ebola), Flaviviruses, and alphaviruses infections. In subpopulations it reproduces SARS weight loss, hemorrhage in EBOV, enceph in WNV, acute or chronic arthiritis in Chikungunya infection (*85-87, 108-111*). *Bat Models*. Duke-NUS has developed two models for bat *in vivo* studies: a colony of *Eonycteris spelaea* (cave dwelling small fruit bat) and a bat mouse model, which contains the bat immune system in an immunodeficient mouse (*112*).

Commented [PD29]: Ralph/Danielle/Eric/Chris – need something substantial here on filovirus binding that shows we have a valid approach for novel filos

Commented [PD30]: Ralph – I think I mis-spoke in this fig. description – please correct.

Commented [PD31]: Ralph – please add data from the CC mouse re. filoviruses, to beef up the image of us as a filovirus group

Commented [PD32]: Linfa/Danielle – please draft a brief para explaining how we'll use these two models

1.3. General Approach: We will conduct analyses to geographically- and taxonomically-target testing of samples from wild mammals that are most likely to harbor known high-profile zoonotic pathogens, or close relatives with potential to infect people. This includes selecting from tens of thousands of samples collected on recent projects and stored in freezers in our laboratories, and new sampling of wildlife in high risk locales. We will use viral family level PCR ~~screening~~ assays to identify and partially characterize viruses, then follow up sequencing of relevant receptor binding proteins to assess homology and predict binding affinity, then attempt to isolate and biologically characterize viruses that phylogenetic and other data suggest have high spillover and pandemic potential. We will follow this with *in vitro* binding assays and cell culture experiments, and *in vivo* animal model infections to assess their potential to infect people and cause disease **(Fig. 8)**. EHA will lead the study design, targeted sampling, and data analysis; Co-Is Hughes and Wacharapluesadee will lead field sampling and in-country testing; Duke-NUS, UNC, USUS will lead the serological and PCR diagnostic development and the *in vitro* and *in vivo* analyses, as well as cross-training and tech transfer to the hub labs in Thailand and Malaysia. This will include via support of one Malaysian Ph.D student, Mei-Ho Lee (CM Ltd.) who has worked with the EID-SEARCH collaborators for 9 years. The focus of her study will be infection potential of novel CoVs and paramyxoviruses already found in bats in Malaysia under preliminary data for this proposal.

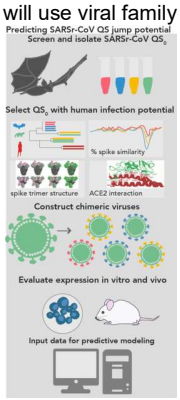

Fig. 8: Schematic showing EID-SEARCH approach to selection of sampling, viral testing, prioritization and characterization to identify novel, high zoonotic-risk viruses in wildlife.

Commented [PD33]: Hongying – this is a placeholder from DARPA proposal. We need a new one with Stages: 1) Geographic and taxonomic modeling to maximize discovery of potential zoonotic viruses; 2) PCR screening and partial RNA sequencing; 3) Full genome or RBD and Glycoprotien sequencing to model receptor binding based on sequence similarity; 4) Cell line infections (for prioritized viruses); 5) animal model infections (for prioritized); 6) High priority viruses ranked based on data (red, yellow, green) schematic.

1.4 Wildlife samples: 1.4.a. Geographic and taxonomic targeting for newly collected wildlife samples: We will prioritize sites within each country that rank highest for both the risk of zoonotic disease emergence (*113*) and the predicted number of 'missing' zoonotic viruses (*90*). Our preliminary analysis **(Fig. 1)** suggests priority areas include: the Kra Isthmus ~~of Kra~~ (S. Thailand and N. Peninsular Malaysia), NW Thailand (near the Myanmar-Laos border), and the lowland rainforests of Sarawak and Sabah. In each of these 'hottest of the hotspots' regions, we will identify 2-4 distinct sampling sites based on the geographic distribution of key hosts we prioritize for ~~intensive~~ additional wildlife sampling. We will priority rank bat, rodent, and primate species using ~~analysis of host trait data for the highest predicted number of viruses based~~host trait-based models (*90*). We will also identify species predicted to be centers of evolutionary diversity for Corona-, Paramyxo-, and Filoviridae using ancestral host reconstruction of available sequence data and Bayesian discrete phylogeographic models (*114-116*). ~~We will ensure wildlife sampling is complimentary to specimens archived in our extensive freezer banks. We will collect only the additional specimens projected from each prioritized taxa to achieve statistically robust sample sizes given predicted levels of viral richness and rates of discovery **(see 1.4.b)**.~~ We will leverage our strong collaborations with wildlife experts in Malaysia and Thailand to ground-truth geographic and taxonomic model outputs with local expert knowledge to ensure the sampling plan is realistic based on current species distributions, population size estimates, seasonality, and accessibility to sites.

Recognizing that most zoonotic viruses are multi-host, i.e. naturally harbored by multiple wildlife species, and that the phylogenetic relatedness of those hosts is predictable and varies across viral families (*90*) – we will use a combined network analysis and a phylogeographic model ~~also apply a generalizable phylogenetic and spatial modeling approach~~ to rapidly predict and prioritize new (unsampled) hosts for important, novel viruses we discover during our research. ~~We will use a combined network analysis and a phylogeographic model to estimate a viruses' host range and to~~We have shown this relatively simple model ~~prioritize additional wildlife species for sampling and testing for viruses we discover. Our recently developed model of viral sharing uses just two phylogeographic traits~~ (using just wildlife species range overlap and phylogenetic similarity between host species) is both generalizable to estimate host range for all mammalian and avian viruses *and* robust – accurately predicting hosts 98% of the time when cross-validated using data from known viruses ~~to successfully predict host species in the top 2% of all 4,200 possible mammal species~~ (*117*).

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1.4.b. Sample size justifications for testing new and archived wildlife specimens: We calculate initial ~~We will~~sample sizes targets using ~~e~~ our preliminary data on average PCR prevalence from screening tens of thousands of bat, rodent and primate specimens for CoV and ~~Paramyxoviruses~~ PMVs under the USAID-PREDICT project in Malaysia and Thailand. We will refine these sample size targets, in real-time, over the course of our project using viral discovery curve analyses **(Fig. 6)** to either scale-up or scale-back new sampling and testing of archived specimens for each species depending on ~~their~~ viral ~~diversity~~capture rates. We will only collect additional specimens projected from each prioritized taxa to achieve statistically robust sample sizes and to complement our archived specimens. For example, ~~*Rousettus* spp. bats, known filovirus~~ reservoirs, were not adequately sampled under PREDICT research in Thailand. Preliminary analysis indicates we will require ~14,000 new samples to identify 80% of remaining viral species diversity in our study region, ~5,000 samples from Thailand and ~9,000 samples from Malaysia **(Fig. 9).** Viral strain ~~discovery~~versity from a sampling effort this size is expected to number in the hundreds of strains. For example, given ~6% prevalence of CoVs in bat species previously sampled under the PREDICT project, a target of 14,000 individuals would yield ~800 PCR positive individuals, representing, ~~conservatively,~~ an estimated ~~~200~~600 novel ~~CoV strains~~sequences/strains. Similarly, for paramyxoviruses, which have an average PCR detection prevalence of ~1.5%, sampling of 14,000 individuals would yield ~200 positives and an estimated ~~~50~~150 novel ~~paramyxovirus~~ strains. From each mammal, we will collect serum, whole blood, throat swabs, urine and fecal samples or rectal swab using our previously-validated ~~For example, given 5-12% prevalence of CoVs in the most common bat species we previously-sampled in Thailand and Malaysia, a target of XXX individuals per species would yield XX (± x) PCR positive individual bats, and an estimated ~xx novel strains. Similarly, for paramyxoviruses with an average PCR detection rate of X%, we would need XX individuals per species to detect 80% of predicted viral strain diversity with 80% power. Preliminary analysis indicates we will require XX,000 samples to identify 80% of remaining viral diversity **(Fig. 9).**~~ ~~All wildlife sampling will be~~ nondestructive methods (see Vertebrate Animals section) [REFS]. In the event that an animal is found dead or needs to be euthanized due to injury we will collect ~~biopsy~~ necropsy samples for screening.

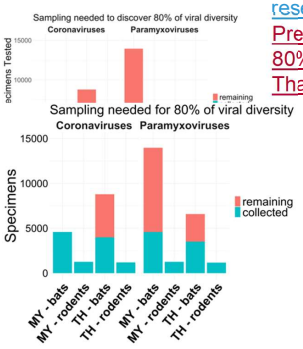

Fig. 9. Stacked bar plot showing number of currently-archived specimens **(blue)** and new specimens required **(orange)** to discover 80% of predicted number of CoVs and PMV species ~~s~~ from high-risk bat and rodent taxa in Malaysia and Thailand

We estimate the following sampling effort, broken down for each region: *Peninsular Malaysia:* Wildlife sampling will be led by CM Ltd / EHA in collaboration with PERHILITAN. Archive bat, rodent and NHP samples will be screened serologically using the BioPlex and other serology techniques identified through this project. New wildlife sampling will focus on bats but it is expected that rodents and NHPs will be sample as well. Wildlife sampling will be conducted around Orang Asli communities sampled as part of aim 2. Three new communities will be identified in the districts we have already sampled in. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife mainly bats will be sampled. Wildlife and domestic animal samples will include serum, whole blood, throat swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and tested at PERHILITAN's NWFL. *Sarawak, Malaysia:* Wildlife sampling will be led by CM Ltd / EHA in collaboration with UniMAS. Wildlife sampling will be conducted around Dyak communities sampled as part of aim 2. One community will be identified. Concurrent sampling trip will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife mainly bats will be sampled. Wildlife and domestic animal samples will include serum, whole blood, throat swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and tested at UniMAS or BMHRC. *Sabah, Malaysia:* Bat sampling will be led by CM Ltd / EHA in collaboration with SWD and WHU. Bat sampling will initially focus on 3 caves. Batu Supu Sabah low disturbance cave and surrounding area, Madai medium disturbance cave and surrounding area, Gomantong cave high disturbance cave and surrounding area. Each cave will be sampled

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Commented [KJO35]: Just assumed that 75% of detections would = unique sequences/strains. We can lower this to be more conservative.

Commented [MOU36]: Kevin will work with Noam and Evan to fill in these numbers.

Commented [KJO37]: PREDICT sampling protocols? Jon's FAO bat book manual, and other published for rodents and primates (from Bangladesh?)

Commented [PD38]: Hongying/KEvin Need less text on the figure, but larger font size, or crisper text to make it all visible at this size.

Commented [T39]: Will dig into budget tomorrow to confirm numbers.

Commented [T40]: With Dr Tan and possibly Dr Faisl – still waiting for Dr Faisl paper work and details of samples he has in storage will discuss with Dr Tan tomorrow.

Commented [T41]: Will dig into budget tomorrow to confirm numbers.

Commented [T42]: Discussing with Tam tomorrow.

twice once in wet season and once in dry season targeting 500 bats at each cave on each trip for a total of 3000 bats. Wildlife samples will include serum, whole blood, throat swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored at WHGFL, molecular testing will be conducted at WHGFL and serology screening at BMHRC. In addition at Madi cave the community that lives in the mouth of the cave will also be sampled targeting 150 people, while at Gomantong cave 25 workers involved in the bird next farming will be enrolled into the study.

Thailand: XXXXXXXXXX New bat specimens will be collected from bat species that was not collected during PREDICT2 study for example *Rousettus* bats (targeted species for Filovirus study), *Taphozous* bat (targeted species for MERS-CoV), *Rhinolophus* (targeted species for SARS-CoV) for serology and PCR assays (n=100 for each species).

~~*Thailand:* XXXXXXXXXX~~

1.4.c. Sample testing, viral isolation: Viral RNA will be extracted from bat fecal pellets/anal swabs ~~with High Pure Viral RNA Kit (Roche)~~. RNA will be aliquoted, and stored at -80C. PCR screening will be performed with pan-coronavirus, filovirus and paramyxovirus primers, in addition to virus specific primers where additional sensitivity is warranted in key viral reservoirs, i.e. Nipah virus specific primers [REF]. PCR products will be gel purified and sequenced. We will attempt isolation on samples that contain viruses of interest (determined in **1.4, below**), using Vero cells and primary cell culture from the wildlife taxonomic order (bat, rodent and primate cell lines). This includes the over 30 bat cell lines maintained at Duke-NUS from four different bat species.

1.4.d. Moving beyond RNA viruses: To detect pathogens from other families, such as non-RNA viruses we will combine conserved PCR assays with NGS. We will perform either unbiased NGS, or use a pan-viral enrichment library to sequence full-length genomes. We will conduct this work via EID-CC research projects or as part of outbreak detection/investigation in the region from DNA viruses or other agents.

1.4.e. Sequencing Spike Glycoproteins: Based on earlier studies, our working hypothesis is that zoonotic coronaviruses, filoviruses and henipaviruses encode receptor binding glycoproteins that elicit efficient infection of primary human cells (e.g., lung, liver, etc.) (*12, 59, 60*). Characterization of these suggests SARSr-CoVs that are up to 10%, but not 25% different in the spike glycoprotein use human and bat ACE2 receptors for docking and entry. Uneven sampling gaps (e.g., no strains were found with 10-25% spike variation) prevent a thorough understanding of the transition point where the most divergent strains lose human ACE2 receptor usage. Our viral discovery curves **(Fig. 4)** suggest further sampling will reveal a rich diversity of as-yet-undiscovered SARSr-CoVs. In this aim we will use phylogenetic and viral discovery analysis to specifically target bat species and regions that are undersampled to allow sufficient power to identify and characterize missing SARSr-CoV strain diversity. Our previous work also suggests that SARSr-CoVs with S proteins that are ~10% divergent from SARS-CoV resist neutralization by therapeutic mAbs and escape SARS-CoV vaccines (*20, 60, 118*), suggesting that existing vaccines and immunotherapeutics could easily fail against future emerging SARS-like CoV. Moreover, viruses with 10-25% divergence in S proteins may bind to human cell receptors, but completely evade therapeutic and vaccine effects, and could therefore be a higher risk for public health. We will sequence the S proteins of novel SARSr-CoVs to prioritize viruses for experimental work in Aim 3 to test this hypothesis. Likewise, we will test the hypothesis that phylogenetic related zoonotic receptor binding protein genes of MERS-related, Ebola-related and Nipah/Hendra-related viruses will also program efficient entry via human ortholog receptors. For all novel virus strains, we will sequence the complete entry spike genes by amplifying overlapping fragments using degenerate primers as shown previously (*20, 59*). Full-length genomes of selected CoV strains (representative across subclades) will be sequenced using NEBNext Ultra II DNA Library Prep Kit for Illumina and sequenced on a MiSeq sequencer. PCR or MinIon sequencing will be performed to fill gaps in the genome. The full length spike gene sequences, including the amount of variation in the receptor binding residues that bind the appropriate human receptor will be used to select strains for Aim 3 experiments.

1.5. Assessing risk for spillover. 1.5.a. Capacity to infect human cells: We will use a series of cell cultures to assess the capacity of target viruses to enter human cells. For CoVs, filoviruses and henipaviruses, we will use primary human ciliated airway epithelial cells (HAE) and lung endothelial cell cultures from the lungs of transplant recipients represent highly differentiated human airway epithelium containing ciliated and non-

Commented [T43]: Will dig into budget tomorrow to confirm numbers.

Commented [PD44]: Kevin – who can write this bits?

Commented [KJO45]: I think all this could be deleted. Doesn't fit with the overall plan, or is redundant. We may want to call out a few specific sites Tom mentions (but won't that depend on the modeling!?), and you may want to keep the partner info Tom provided and call that out - maybe more in Aim 2 since its about people too?

Commented [MOU46]: Supaporn: From my DTRA study, specific Nipah primers and WHO 2C CoV primers is more sensitive than consensus PCR if we want to find the specific virus.

Commented [PD47]: Linfa – can you give a v. brief description of these please, e.g.: "This includes 13 primary lung, 5 primary gut epithelial, and 1 primary liver cell line. In addition we have 12 immortalized cell lines, 7 of which are intestinal, and therefore suitable for CoVs. Cell lines are derived from insectivorous and frugivorous bats (genera……)"

ciliated epithelial and goblet cells, grown on an air-liquid interface for several weeks prior to use (*60, 61, 119*). We will prepare HAE and lung endothelial cultures from three different patient codes in triplicate in collaboration with the tissue procurement facility at the Cystic Fibrosis Center at UNC. Cultures will be inoculated with chimeric bat SARSr-CoVs or other test filoviruses or henipaviruses to assess efficient replication. At 72 hpi, cultures will be fixed for immunofluorescent staining using antisera to the SARS-CoV conserved nucleocapsid protein (N) or to structural genes of the other viruses (*62, 69*). SARSr-CoVs that differ significantly in S protein sequence (11-24%) from epidemic SARS-CoV yet replicate *in vitro*, will also be evaluated for sensitivity to neutralization in Vero cells by PRNT50 assays using broadly SARS-CoV cross reactive human mAbs S227.14, S230.15, S215.17, and S109.8 (*61, 120*) or monoclonal antibodies targeting the Ebola or Marburg E glycoprotein (Available through BEI), or Nipah or Hendra viruses (*121*). As controls or if antisera is not available, the S genes of novel SARSr-CoV or spike entry genes of other novel viruses under investigation will be inserted into VEE 3526 replicon vectors (VRP3526-S), packaged and used to vaccinate mice (*122*). Polyclonal sera against SARS-related viruses will be harvested and tested for ability to cross neutralize SARS-CoV, GD03, WIV-1, SHC014, WIV-16, other novel SARSr-CoV and HKU3-SRBD by PRNT50 assay (*61, 123, 124*). Using PRNT50 titers with sera (n=4 each) among these viruses, antigenic cartography (*125*) will allow comparison of antigenic vs. phylogenetic distance, identifying the transition at which SARSr-CoV strains escape SARS-CoV based vaccines, informing immunotherapeutic and vaccine design strategies (*126-128*). Similar approaches will be applied to novel MERS-related viruses, other CoV, filoviruses or henipaviruses. While most wildlife-origin viruses with unknown transmission potential or pathogenicity in people are BSL-2 or -3, cell culture for potentially BSL-4 pathogens will be undertaken at collaborating institute NEIDL. To facilitate this, duplicate samples in culture medium from animals PCR +ve for filoviruses will be shipped to NEIDL **(See letter of support)** for culture, characterization and sharing with other approved agencies including NIH Rocky Mountain Laboratories ~~RML~~where our EID-SEARCH team has ongoing collaborations. When appropriate of feasible, training opportunities at NEIDL for in-country staff will be given.

1.5.b. Host ACE2, DPPR, Niemann-Pick C1 (NPC1) and EphrinB2/EphrinB3 receptors: We will sequence host ACE2 receptors of different bat species or different bat populations from a single species (e.g. *Rhinolophus sinicus*) to identify relative importance of different hosts of high risk SARSr-CoVs and the *intra*specific scale of host-CoV coevolution. Of particular interest is homology across 18 bat and human orthologue ACE2 contact interface residues that engage the SARS RBD as a potential indicator of SARSr-CoV cross species transmission potential and growth in human cells (*68*). ~~A lot of v~~Variation in the NPC1 receptor alters EBOV ability to infect in humans and bats, so this can be structurally mapped for prediction and downselection of strains (*129*). Signatures of positive selection in bat NPC1 were concentrated at the virus-receptor interface, with the strongest signal at the same residue that controls EBOV infection in *Eidolon helvum* cells. Thus NPC1 is a genetic determinant of filovirus susceptibility in bats, and some NPC1 variations likely reflect host adaptations to reduce filovirus replication and virulence (*130*). Nipah and Hendra use ephrin B2 or ephrin B3 as a receptor, and variation affects its ability to use human vs bat receptor molecules (*101, 131*).

1.5.c. Host-virus evolution and distribution: We will use Bayesian and Maximum Likelihood phylogenetic analyses of RNA-dependent RNA polymerase (RdRp) sequences from PCR screening ~~(or L genes)~~, receptor binding glycoproteins, and/or ~~-~~full genome sequence data (when available) ~~data~~ to reconstruct the evolutionary history of the novel bat SARSr-CoVs, bat MERSr-CoV, filoviruses and henipaviruses that we identify. We will run phylogenetic and ancestral state reconstruction analyses **(Fig. 5)** to ~~reconstruct~~identify host taxa and geographic sites of ~~β-CoV, filovirus and henipavirus~~ evolutionary origins within bats, rodents and primates ~~using our expanded dataset and identify the host taxa and geographic regions~~ that together define hotspots of phylo-diversity for these viruses, allowing for more targeted surveillance. For receptor binding glycoprotein sequence data from characterized viruses, we will apply codon usage analyses and codon adaptation indices, as used recently on Henipaviruses (*132*), to assess the relative contributions of natural selection and mutation pressure in shaping host adaptation and host range. ~~We will rerun MCC analyses **(Fig. 3)** to reconstruct β-CoV evolutionary origins using our expanded dataset. Ecological niche models will be used to predict spatial distribution of PCR-positive species, identify target sites for additional bat sampling, and analyze overlap with people. We will use our viral discovery/accumulation curve approach **(Fig. 4)** to monitor progress towards~~

Commented [BRS48]: Any antibodies available? If not we can synthesize them from published co-crystals.

Commented [PD49]: Can someone answer Ralph's question please?

Commented [KJO50]: Make more specific about our plans to sequence NPC1 or Ephrin receptors, etc. Should we frame much of this as "add on projects" pending additional EIDRC-CC funding?

Commented [PD51]: Kevin/Alice – please edit this section to include relevance for filos and henipas as well (and other hosts)

~~discovery of >80% of predicted diversity of SARSr-CoVs (lineage 1 & 2) or other virus families within species and sites, halting sampling when this target is reached, and freeing up resources for work other work (*96, 133*).~~

1.5.d. Viral strain prioritization: Of the expected 100-200 novel SARSr-CoV strains, and approximately 600 total CoV strains, we will down-select to prioritize for further characterization based on S genes that are: i) different from SHC014, WIV1, SARS-CoV with diversity ranges of 10-25%; ii) have virus S RBD that could use human/bat receptors; iii) have recombinant chimeric spikes indicative of gene flow between clade I and II strains; iv) have bat ACE2 receptors that might select for spike RBDs that can use human receptors for entry (15/18 conserved residues in human/bat ACE2 molecules that bind SARSs-CoV S RBD domains are likely more efficient receptors than 3/18 conserved sites). Using structural models based on the SARS S glycoprotein, the extent and location of antigenic variation will be annotated onto the structure, further resolving the locations of highly evolving and conserved structural motifs/epitopes that function in protective immunity (*120, 133-135*). For novel henipaviruses, we will examine the receptor-binding proteins (e.g. envelope attachment (G) proteins) for sequence and structural homology with particular attention to conversation of amino acids that mediate ephrin receptor binding. We will prioritize strains that are predicted to interact with ephrin-B2 and expression V/W proteins to determine whether these viruses represent zoonotic threats. Using the recombinant Cedar virus (rCedV) platform, we will be able to rescue rCedV that express heterotypic G proteins to explore receptor utilization and tissue dissemination in animal models. We will assess ability to infect human cells using a continuous lung epithelial cell line, Calu3, that our group has previously used to generate data on NiV and HeV growth curves and a variety of host expression changes after infection (*136*). We will also use primary human airway cultures to assess human infection for Henipavirus and MERS- and SARS-related CoV (*137*) (*138*) . There is some evidence for Nipah and Hendra virus replication in mouse lung, low level titers in young animals, 10 fold higher titers in aged mice (*139, 140*). Thus it is likely these models could be improved in the collaborative cross mouse resource at UNC. We have identified CC lines that are highly vulnerable to wildtype SARS and others that develop hemorrhagic disease following mouse adapted Ebola infections (*85-87*).

1.5.e. Animal models: We will use transgenic mouse models and the UNC Collaborative Cross model to assess spillover potential of viruses. For work on CoVs, we will follow the protocols in the transgenic mouse model used by the Baric lab previously. Briefly, in the BSL3 lab, n=8 10- to 20-week old hACE2 or hDPP4 transgenic mice will be intranasally inoculated with 5 x 10^4 PFU of wildtype WIV-1 or chimeric bat SARSr-CoVs with different spike proteins or MERS-CoV and MERS-related CoV, respectively, then monitored daily for weight loss, morbidity, and clinical signs of disease. Mice will be euthanized at 2, 4, and 6 dpi (endpoint of 14 dpi), organs harvested and virus quantified by NP RT-PCR. After 7 days in formalin, tissues for histopathology will be removed from the BSL3 and stained with H&E, and for immunohistochemistry using polyclonal antibodies against the N protein. We will conduct limited evaluation of existing countermeasures using therapeutic monoclonal antibodies *in vitro* and *in vivo*. Existing SARSr-CoV or MERS-CoV mAbs (*141, 142*) will be diluted in DMEM starting at 1:20, and serial 2-fold dilutions mixed with an equal volume of 50 PFU of chimeric bat SARSr-CoVs with different spike proteins, then incubated on Vero E6 cells at 37°C for 1 h, then changed to a semi-solid medium (DMEM containing 1% methylcellulose and 2% FBS). Antibody neutralization titers will be evaluated by plaque quantification at 4-5dpi. hACE2 transgenic mice will be injected with SARS-CoV mAbs, and infected with chimeric bat SARSr-CoVs. Clinical signs and morbidity will be assessed and tissue pathology examined and compared with mice without treatment of mAbs to determine the therapeutic effect on SARSr-CoV infection, and protection of SARSr-CoV by wildtype SARS-S based vaccines assessed as described (*119, 143*). We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-CoV cross neutralization, synthetically reconstruct a small subset (1-2) and evaluate the ability of nucleoside analogues to inhibit growth in HAE cultures and/or *in vivo* (*61, 119*). For the Collaborative Cross model, we will….

Finally, for more detailed pathogenesis studies in the natural reservoir, we will use the bat models (natural and transgenic mouse model) at Duke-NUS. Both animal systems can be used to test susceptibility, pathogenesis and immune responses of any newly discovered viruses.

Commented [KJO52]: I suggested we could get up to 600 CoV "strains" (all seqs, not just SARSr-CoVs) in section 1.4b, but we can reduce that.

Commented [PD53]: Ralph – please insert a couple of sentences on how we'll use the cc mouse and what we'd expect

Commented [PD54]: Linfa/Danielle – could you put a couple of sentences in on what we might do with the models. Remember – this could be an additional short project from the EIDCC extra funding.

1.6. Potential problems/alternative approaches: **Challenges with logistics or obtaining permission ~~to~~ for wildlife sampling in sites we select~~sample bats in sites or provinces we select~~**. We have a >20-year track record of successful field work in Malaysia, ~~Thailand and~~ and >10 years in Thailand~~Singapore~~, and have ~~worked with~~strong established partnerships with local wildlife authorities and communities to ensure access. We have existing permissions in place which will be renewed at the start of this project. Due to the abundance of wildlife habitats in the region, if access to a site is withdrawn, we will rapidly identify a suitable, complementary site with similar bat species composition and abundance. Finally, we have already collected tens of thousands of wildlife samples from the region and will focus on these while waiting for permission. **We may not identify novel viruses in our sample wildlife species due to seasonality of viral shedding or other factors.** We have calculated sample sizes based ~~on a range of PCR data and are fairly conservative in our approach~~on realistic detection rates from our extensive preliminary data, and are confident that we will detect and identify large numbers of potentially zoonotic pathogens, particularly for CoVs and PMVs. ~~However, as a back-up, we already have identified dozens of novel viruses that are near-neighbors to known high-impact agents, and will continue characterizing these should discovery efforts yield few novel viruses~~We acknowledge that FV strain diversity may be harder to capture, given relatively low seroprevalence in bat populations – suggesting lower levels of viral circulations (*40, 41*). However, our team was the first and among the only groups to sequence FV RNA from Asian wildlife species(*44, 45, 55*), and we are confident that with our targeted sampling and testing strategy will identify additional strains of Ebola and related viruses. The sampling regions we have selected are subtropical, and our previous data, and even published studies in temperate regions (*144*), do not suggest a strong pattern of seasonality in CoV shedding, ~~although there are studies that suggest this~~and where seasonal patterns do occur~~s~~, i.e. for ~~in~~ henipavirus~~es~~ ~~and filovirus (MARV?) shedding (REFS?)~~in Thailand (*145*) or can be predicted from wildlife serology data, we will be sure to ~~. Nonetheless to account for this we will~~ conduct sampling ~~evenly on quarterly basis within each province~~at different timepoints throughout the year to account for this.

Aim 2: Test evidence of viral spillover in high-risk communities using novel serological assays.

Commented [KJO55]: Emily and Noam reworking this section significantly, so not touching it much.

Country, site	Lead	# enrolled, focus	Findings
Peninsular Malaysia	Hughes	1,390 Orang Asli indigenous population, PCR/serol.	25+ novel CoVs, influenza, Nipah ab+ve, filovirus ab+ve
Malaysia Sabah	Kamruddin	1,283 serology	40% JE, 5% ZIKV ab+ve
Malaysia Sabah	William	10,800 zoonotic malaria	High burden of macaque-origin malaria
Malaysia Sabah	Hughes	150 bat cave workers	Ongoing
Malaysia Sarawak	Tan	500 Bidayuh and Iban people	8% PCR prevalence HPV in women

Malaysia PREDICT	Hughes	9,933 high zoonotic-risk communities, viral PCR, serology	Ongoing. Multiple known and novel CoVs, paramyxoviruses, others.
Thailand	Wacharaplu-esadee	1,000 bat guano harvesters/villagers	Novel HKU1-CoV assoc. clinical findings
Thailand PREDICT	Wacharaplu-esadee	9,269 high zoonotic-risk communities, viral PCR, serology	Ongoing. Multiple known and novel CoVs, paramyxoviruses, others.
Singapore	Wang	856, Melaka virus	7-11% ab+ve

2.1 Rationale/Innovation: Rapid response requires early detection. Thus, assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities that interface frequently with wildlife that harbor high risk emerging viral strains. To enhance the low statistical probability of identifying these rare events, we will target rural human communities inhabiting regions of high wildlife biodiversity, that also engage in practices that increase the risk of spillover (e.g. hunting and butchering wildlife). In Aim 2 we will build and expand on our preliminary biological sampling and enroll large cross-sectional samples in human populations with high behavioral risk of exposure to wildlife-origin viruses and that live close to sites identified in Aim 1 as having high viral diversity in wildlife. We will initially identify 2 sites in each of Thailand, Peninsular Malaysia, Sarawak, and Sabah for repeated biological sampling. We will design and deploy behavioral risk factor questionnaires, and specific and sensitive serological assays to identify the baseline frequency, and causes of, spillover of known and related novel viral pathogens in these populations. In participants where symptoms are reported in the last 10 days, paired serum specimens (acute and 21 days after fever) will be collected to determine titer of positive antibody. Samples will be marked for additional PCR-based assays to identify presence of known and novel CoVs, henipaviruses and filoviruses, and for isolation and biological characterization of potential pathogens, using the approach laid out in Aim 1.

2.2 Preliminary data on high-zoonotic risk human community surveillance: 2.2.a Biological sample collection in SE Asia: Our longterm collaboration in the region has included qualitative and quantitative survey data, and collecting large archives of banked human sera and other biological samples from high zoonotic-risk populations. For example, under the USAID-PREDICT project, EHA has collected 9,933 and 9,269 specimens in Malaysia and Thailand respectively from people living in zoonotic disease interfaces, all of which will be available for retrospective serological testing by EID-SEARCH. Our core group has collected and tested many thousand additional specimens from other important community cohorts **(Table 2)**, and some of these will continue under EID-SEARCH **(See section 2.4)**. Of particular interest is the access to indigenous communities in Peninsular Malaysia (Orang Asli) and Sarawak (Bidayuh, Iban people), and the bat guano harvesters in Thailand. These communities hunt, butcher and eat wildlife daily, and work in supremely high-risk zoonotic interfaces (e.g. bat caves). One of our indigenous community sites is experiencing an ongoing outbreak of unknown etiology that we are investigating currently **(See section 3.2.a)**.

Table 2: Current or recent biological sample collection from healthy populations conducted by members of our consortium in EID-SEARCH hub countries

2.2.b Human Contact Risk Factors: EHA is the lead organization across all 27 USAID-PREDICT project countries for collecting data on, and analyzing the human aspects of, zoonotic spillover risk (*146, 147*). We conducted exploratory studies using standardized one-on-one semi-structured interviews and site observation data with people living in hotspot areas or engaged in high-zoonotic spillover risk activities, using these data to assess local social and cultural norms underlying wildlife contact (*147*). We then developed a risk factor survey to accompany human biological sampling to evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. We conducted cross-sectional studies in high risk populations in 27 countries, and study participants provided biological samples (oral/oropharyngeal swab, nasal/nasopharyngeal swab, urine sample/urogenital swab, fecal sample/rectal swab, whole blood, serum, plasma). For example, survey and biological samples were collected from: 1,585 participants from 7 sites in Yunnan, Guangxi, and Guangdong provinces, China; 1,400 participants from 4 sites in Malaysia; and 678 participants from 4 sites in Thailand. Samples were tested with novel serological assays and results analyzed against risk factors from survey data (*12*). In China, CoV-seropositive individuals were mostly farmers/peasants (8/9), living in Yunnan

Commented [KJO56]: Check these numbers, don't think we have 9000 people sampled, if this represents individuals! Per below, we say we have 9000 specimens, not people, so need to adjust.

Commented [KJO57]: Emily: Will we be sampling participants more than once?

Commented [PD58]: I think that would be good, depending on the sample size

Commented [KJO59]: Need to decide if we do PCR on community samples, we can add in the questionnaire if you've been sick in the last 10 days and if so, then maybe test only this subset.

Commented [PD60]: Kevin's comment makes sense to me

Commented [KJO61]: Don't match table numbers, need to change table header to # specimens, not # enrolled.

province (7/9), 41-60 yrs old (7/9), with domestic contact with rodents (6/9), that are male (6/9). Although these results are preliminary and don't provide detailed information on routes of exposure, they identify key subpopulations to target that would likely increase the opportunity for identifying evidence of spillover for other CoVs, paramyxoviruses and filoviruses. Data from PREDICT surveys will be used to help identify target populations in Aim 2, identify strategies to better target at-risk people, and conduct focused **human animal contact surveys and serosurveys to produce statistically significant findings for henipaviruses, CoVs and filoviruses.** In Aim 2, we will use combined biological sampling and risk factor surveillance in targeted populations within the community to 1) identify risk factors correlated with seropositivity to henipaviruses, filoviruses and CoVs; 2) assess possible health effects of infection in people; and 3) where recent illness is reported, obtain clinical samples for PCR testing and potential viral isolation and characterization. Obtaining this information could be a significant step in understanding the likelihood of recent 'hidden' spillover events and their public health impacts, as well as the risk of future emergence of viral disease.

2.2.c. Risk factors for illness: Our preliminary data from PREDICT in Thailand, Malaysia and China revealed that frequent contact with wild (e.g. bats, rodents, non-human primates) and domestic animals (e.g. poultry, swine) among local communities was associated with a high percentage of self-reported severe/acute respiratory illness (SARI/ARI) and Influenza-like illness (ILI) symptoms. In addition, people involved in crop production, hunting and butchering of wildlife reported statistically significantly higher SARI and ILI symptoms (Li *et al.*, in prep.). **These preliminary data suggest a target population and sites for current proposal with the correct targets for serological surveillance and risk factor surveys in Aim 2 to produce statistically significant findings**. Coupled with further refining serological tools, we will be able to identify the likely routes of exposure to known or novel coronaviruses, henipaviruses, and filoviruses in the study region, as well as build data on the illnesses they cause in people.

2.2.d. Serological platform for community surveillance: One of the present challenges in emerging disease surveillance is that viremia can be short-lived, complicating viral-RNA detection (i.e. traditional PCR-based approaches), especially in zoonotic reservoir hosts, and requiring large samples sizes to understand infection patterns or host range. By contrast, antibodies are typically long-lived and can be used to assess prior virus exposure or infection with much smaller samples sizes (*148*). However, most serological assays only target a single protein, and it's not always clear which antigen is the most immunodominant during an adaptive humoral immune response – i.e. which is the best target for a serological assay. Henipaviruses, filovirus and CoVs express envelope receptor-binding proteins (e.g. CoV=spike (S) protein, filoviruses/henipaviruses=glycoprotein (GP/G)), which are the target of protective antibody responses. The Broder lab at USU, a leader in the expression and purification of native-like virus surface proteins for immunoassay application (*149*)?, developing monoclonal antibodies (*150, 151*) and as subunit vaccines (*152, 153*), is presently developing a novel, multiplex microsphere immunoassay (MMIA) allowing serum samples to be simultaneously tested for antibodies reactive with henipaviruses, filoviruses and coronaviruses. This MMIA uses a Luminex-based multiplex platform and custom designed virus receptor-binding proteins expressed in a mammalian cell-culture system and purified as native-like oligomeric proteins (*149*). These oligomeric antigens retain post-translational modifications and quaternary structures. This structural advantage over peptide antigens, allows the capture of conformational-dependent antibodies raised during a natural infection, which represent the majority of the protective humoral response.

Commented [KJO62]: Peter, sent over LASSO figure for ILI from Thailand and Malaysia for you to consider.

Commented [PD63]: Chris/Eric Is this the right ref?

Commented [KJO64]: See my comment in Aim 1 that some of that serology platform stuff can be moved here.

Serological platforms have been historically limited by the starting antigen, and cross-reactivity between known and unknown related viruses not included in the assay. The known diversity of henipaviruses includes five presently described viruses, NiV, HeV, CedV, GhV and MojV and this assay has been expanded to include all described species **(Fig. XXX)**. In the past, our collaborators have used NiV and HeV G protein antigens in a MMIA assay to identify a serological footprint of NiV-related African henipaviruses and exposure to virus(es) antigenically-related to NiV in livestock sampled in Bangladesh (*76, 77*). To strengthen serological data interpretation, the G protein from each virus is integrated into the MMIA so that virus-specific versus henipavirus cross-reactive antibodies can be detected and address specificity of past virus exposure. Extensive cross-reactivity exists within ebolaviruses and between ebolaviruses and marburgviruses, highlighted by studies examining antibody binding between heterologous viruses (*154-156*). Ongoing work is aimed at validating the MMIA pan-filovirus assay for specificity. The majority of ebolaviruses are endemic to Africa, however the discovery of Měnglà virus in Yunnan, China increased the further demonstrated that a diversity of Asiatic filoviruses with unknown pathogenicity and zoonotic potential exists. Our past work in collaboration with Duke-NUS demonstrated that three under-sampled fruit bat species had serological evidence of past exposure to a filovirus(es) most antigenically-related to ebolaviruses (e.g. Ebola virus, Bundibugyo virus), but unreactive with Reston virus GP, suggesting that novel filoviruses circulate within bat hosts (*41*).

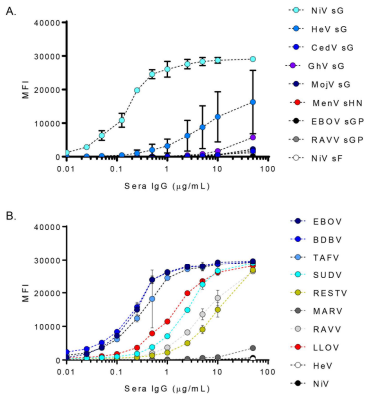


Fig. XXX: Validation of multiplex microsphere immunoassay (MMIA) specificity and identification of immunologically cross-reactive viruses for Nipah (A) and Ebola (B).

2.3 General Approach/Innovation: We will use a dual study design to gain in depth understanding of exposure and risk factors for viral spillover **(Fig. XX)**. In Aim 2 we will conduct community-based surveillance using a brief, focused questionnaire to identify the prevalence and frequency of risk-factors for viral spillover in tandem with collection of high quality biological samples to determine the seroprevalence of spillover viruses in at-risk human populations. We will use generalized linear models to analyze correlation between biological results and risk-factors for viral spillover. In Aim 3, we will conduct clinic-based syndromic surveillance and biological sampling at clinics/hospitals that are the catchment healthcare facilities for the communities sampled in Aim 2. Both community-based and clinic-based syndromic surveillance programs are cross sectional case-control studies designed with the sample sizes necessary to statistically quantify (with a power of 80%) risk factors and health impacts for viral spillover, linked to serological status and PCR prevalence in symptomatic patients.

2.4 Target population & sample sizes: We will target populations in the same geographic regions as those identified in Aim 1 that harbor wildlife with high potential zoonotic viral diversity, that are EID hotspots, and that are well-connected through regional travel and trade hubs to the global travel network **(Fig X)**. We will target specific communities based on our analysis of previously-collected PREDICT behavioral questionnaire data, to assess key at-risk populations and occupations with high exposure to wildlife, as well as using other publicly available data (e.g. wildlife hunting licenses sold in a region, number of guano caves accessed for fertilizer etc.). We will identify populations at two sites in each of the following regions, building on our preliminary data **(Table 2)**: *Thailand (Co-I Wacharapluesadee):* We will continue surveillance in bat guano harvesters and their villages in Ratchaburi province where we have identified MERS-related CoV in bat guano and rectal swabs (*48, 157*) and serological evidence of α-CoVs, HKU1-CoV in people (Wacharapluesadee in prep.). Our second site will be in Chonburi province where we have found Nipah virus (99% identity of whole genome sequence to NiV from a Bangladesh patient) in flying foxes but no outbreaks have yet been reported (~~REF~~Thai MOPH, unpublished), and have found several novel paramyxoviruses and CoVs have been found in bat feces roosting there ((*158, 159*) Wacharapluesadee in prep.).

Commented [PD65]: Hongying -Need a new figure that lays out the community/clinical approach – small one with pretty images.

Commented [PD66]: Supaporn/Kevin/ -Need sample sizes for both populations and power calculations for serology for a typical virus from one of the three families we're targeting

Commented [KJO67R66]: I pasted in info from Supaporn below.

Commented [PD68]: Supaporn – please insert a reference into this comment box

Commented [KJO69]: Added in a couple more of her refs.

Sample size: Serological exposure likely low due to low risk of onward transmission. High risk occoupational groups vs. community sample at each site. Need to stratify sampling in villages based on

Thailand: 100 subjects of healthy high-risk community from 2 study sites (Chonburi and Ratchaburi) will enrolled on Year 2 and tested for antibody, and specimen from ill subject will be collected and teat by PCR and serology assays. Specimens from same subject will be collected on Y3 and Y4 for serology re-testing.

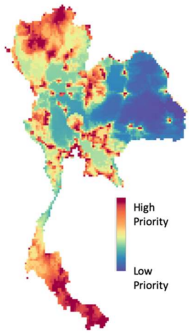
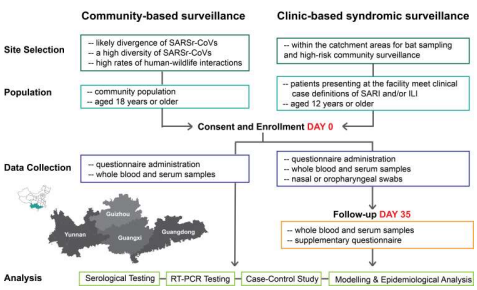


Fig. X: . Geographic analysis to target high-risk sites for human zoonotic disease surveillance in Thailand. Our spatial analysis from Aim 1 will be extended to optimize sites for human community surveillance in Thailand and Malaysia by ranking areas with high numbers of expected viruses in wildlife, greater spillover probability based on EID risk factors, and higher levels of connectivity (measured by road density data) that may facilitate person-person spread.

Peninsular Malaysia (Co-I Hughes, CM Ltd.): We will expand our sampling of the Orang Asli indigenous communities to include additional communities in the 3 Districts we have already sampled, and additional Districts in the States of Kelantan Perak, Pahang, and Kelantan. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife (mainly bats) will be sampled. Human samples will include serum, whole blood, throat swabs, nasal swabs, urine, and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and tested at NPHL. Participants who hunt, butcher or consume wildlife, or who rear animals will be considered suitable for enrolment. Participants will complete a consent form and questionnaire in addition to having samples collected. During these community trips the field team will conduct community meetings to help inform the Orang Asli about the public health risk of zoonoses. *Sarawak (Co-I Tan, UNIMAS):* We will expand our sampling of the Bidayuh and Iban indigenous people to include the Dayak and Orang Ulu ("people of the interior") because this group has a more intimate connection to remote wildlife-rich areas than the other two, albeit all are hunters. Human sampling will be led by UniMAS in collaboration with CM Ltd / EHA. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife (mainly bats) will be sampled, following the same protocols as for Peninsular Malaysia. *Sabah:* We will conduct sampling at the Madai bat and bird cave that has 200 permanent residents who harvest bird's nests for soup, and >1,000 during the harvesting season and at Gomantong cave – one of the largest bat and cave swiftlet nesting sites in the world. Protocols follow those for Peninsular Malaysia. *Singapore:* Active human surveillance will be not be conducted in Singapore, but archived human specimens e.g. from our Melaka virus serosurvey (*4*), will be made available to conduct retrospective serosurveys for specific pathogens that are identified as high interest to the EIDRC, NIH, and EID-CC.

Sample sizes: From our previous work we anticipate that 10-30% of the community population will have had exposure to wildlife allowing us to capture highly exposed and non-exposure individuals at each site. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock farming, transportation, selling, or slaughtering wildlife in the surveyed areas will be targeted so that they make up ≥30% of the sampled population in each community. We will stratify sampling to ensure appropriate representation of sex, demographic, and socio-economic factors at each community site. Estimating 5% overall seroprevalence in these high-exposure populations, these sample sizes are sufficient to estimate effect sizes of risk factors of 2X or greater with 80% power. For community-based surveillance, we will enroll xxxx individuals per county/province, pooled across two sites for each, allowing us to make county/province-level comparisons of differing effects.



Commented [S70]: I think the animal and human specimen collection should be in a same format for Thailand and Malaysia.

Commented [PD71]: Need this figure for Singapore, Pen. Malaysia, Sarawak, Sabah as well – all together is best

Commented [KJO72R71]: Sam is going to send a combined figure tomorrow AM.

Fig. XX: Human survey and sampling study design overview (Aim 2 and 3)

2.5 Data & sample collection: Following enrollment of eligible participants and completion of the consent procedure, biological specimens (5ml will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 2.5ml whole blood; two 500 µL serum samples) will be collected and a questionnaire will be administered by trained project staff that speak appropriate local language to all participants. Through the serological findings and the survey we will investigate the details of five risk factors related to high risk wildlife exposure, to maximize the power of the analyses, based on continuing analysis of our previous work, and will focus on: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With additional consent considerations for participants enrolled from clinical settings we will review clinical records to collect recent data on medical history, clinical syndromes, and patient etiology.

2.6: Laboratory analysis: 2.6.a Serological testing:

In a previous R01, we expressed his-tagged nucleocapsid protein (NP) of SARSr-CoV Rp3 in *E.coli* and developed a SARSr-CoV specific ELISA for serosurveillance using the purified Rp3 NP. The specificity of this assay was evaluated using polyclonal antibodies against HKU1, OC43, 229E, NL63, MERS-CoV and EBOV and no significant cross-activity detected (*12*). **This suggests that if we can expand our serology tests to cover other bat CoVs, and that doing so may identify many more seropositive individuals.** We will therefore use two serological testing approaches. First, we will expand test all human sera collected from both community- and clinic- based sampling for a panel of bat CoVs that will include SARS-CoV (outbreak strain), a range of lineage 2 bat SARSr-CoVs (including WIV1 and SHC014), lineage 1 HKU3r-CoVs, MERS-CoV, and the α-CoVs SADS-CoV and HKU10. We previously found serological evidence of human exposure to HKU10 (*12*), and HKU10 is known to jump from one host bat species to another (*160*) so is likely to have infected people more widely. It is possible that non-neutralizing cross reactive epitopes exist that afford an accurate measure of cross reactivity between clade1 and clade 2 strains which would allow us to target exposure to strains of 15-25% divergence from SARS-CoV, at a phylogenetic distance where therapeutic or vaccine efficacy may falter. Additionally, we will test samples for antibodies to common human CoVs (HCoV NL63, OC43 – see potential pitfalls/solutions below). Secondly, CoVs have a high propensity to recombine. To serologically target 'novel' recombinant virus exposure, we will conduct 1) ELISA screening with SARSr-CoV S or S1 (n-terminal domain); 2) confirm these results by Western blot; then 3) use NP based ELISA and LIPS assays with a diversity of SARSr-CoV NP. For NP ELISA, micro-titer plates will be coated with 100 ng/well of recombinant batCoV NP and incubated with human sera in duplicates followed by detection with HRP labeled goat anti-human IgG antibodies. For confirmation, all ELISA positive samples will be subjected to Western blot at a dilution of 1:100 by using batCoV-NP as antigen. We will use an S protein-based ELISA to distinguish the lineage of SARSr-CoV. As new SARSr-CoVs are discovered, we will rapidly design specific Luciferase immunoprecipitation system (LIPS) assays targeting the S1 genes of bat-CoV strains for follow-up serological surveillance, as per our previous work (*13*). Additionally, in Thailand, as an initial project on our EIDRC, we will develop serology assays to the novel CoVs found in bats at the bat guano site using LIPS assays and viral genomic data, and will screen archived specimens from 230 participants (2 years' collection in 2017-2018, a subset of the subjects have been sampled at multiple timepoints to allow us to assess seroconversion).

2.6.b RT-PCR testing. Specimens will be screened using RT-PCR for the RdRp gene (See section 1~~.3.b~~.4.c for details). Positive samples will be subjected to full genome sequencing and RT-PCR amplification of the S glycoprotein gene. Samples from the clinic-based syndromic surveillance will also be tested using RT-PCR for Influenza A & B, HCoV NL63, OC43, HKU1, SARS-CoV & 229E, NiV, HeV, Ebola (WHO PCR protocol) based on the symptom as rule-outs, and SADS-CoV and HKU10 as an opportunistic survey for potential spillover these CoVs as proposed above.

2.6.c Biological characterization of viruses identified: Novel viruses identified in humans will be recovered by cultivation or from full length genome sequences using reverse genetic approaches. Viruses will be characterized for growth in primary human cell lines and in mouse models, including the Collaborative Cross

Commented [PD73]: Hongying– this figure is from our CoV R01 renewal. Please modify to make relevant to SE Asia and henipas, filos and CoVs.

Commented [PD74]: Emily/Hongying Please check

Commented [PD75]: Chris, Eric, Linfa, Dani etc. please draft some language here…

Commented [PD76]: HELP – please add text for Henipas, filos

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Mice or humanized mice expressing human receptors from related strains. The approaches are discussed in more detail under Aim 1.

2.7 Epidemiological analysis: We will conduct a cross sectional case-control study to identify risk factors for Henipavirus, Filoviruses, and CoVs spillover. "Cases" are defined as participants whose samples tested positive for Henipavirus, Filoviruses, or CoVs by serological tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative for Henipavirus, Filoviruses, or CoVs. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between biological results, serological/PCR status, and risk factors related to high risk wildlife exposure including: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) home or work proximity to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms lifetime and past 12mo. Additionally, we will use this procedure to determine how clinical presentation differs between Henipavirus, Filoviruses, or CoVs in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms.

2.8 Potential problems/alternative approaches: Rarity of spillover events means it may be difficult to identify sufficient seropositives to statistically analyze human risk profiles. First, we will be targeting our community-based surveillance to subpopulations with high-levels of wildlife exposure, at sites selected for diverse and prevalent viruses to increase likelihood of finding positive individuals. Second, our serology testing will include a panel of assays for a large diversity of viruses, increasing likelihood of positives. For example, in previous work our overall bat CoV PCR prevalence in the community was, 11.8%; β-CoV, 3.4%; α-CoV, 9.1%). Thus, using this broad serological panel to screen individuals prone to contact with species of higher zoonotic risk increases the potential for detecting spillover with enough power for statistical analyses, and will shed light on behaviors that predispose to spillover. Third, we will include common human CoVs, paramyxoviruses, and influenza viruses in our panel, so that even if low prevalence of wildlife viruses is found, we will be able to conduct a valuable cross-sectional study of the seroprevalence of more common human viruses. Finally, we will be able to assess relative measures of human-wildlife contact from our survey work. We will analyze intensity of contact with high-risk taxa against other risk factors to provide useful proxy information for spillover risk. **Serological testing may not match known viruses due to recombination events, particularly for CoVs.** We will use the three-tiered serological testing system outline in **2.6.a** to try to identify these 'novel' viruses, however, we will also remain flexible on interpretation of data to ensure we account for recombination.

Commented [KJO78]: Make this more general. There's a greater challenge of interpretation of serological data, esp. if we're using many platforms (each will have its own problems that our lab colleagues are probably more familiar with). Noam may be able to generally comment on a solution here from a statistical perspective.

Commented [PD79]: Kevin – please modify according to your comment

Commented [KJO80R79]: Noam is working on this section, so can review after/tomorrow.

Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts.

3.1 Rationale/Innovation: Our previous research **(Table 1)** and other data suggests that there are substantial numbers of outbreaks or clinical cases for which the etiologic agent is not diagnosed, e.g. (*161, 162*). To investigate this in SE. Asia, we will work directly with clinics and outbreak control organizations to identify the causes of some 'cryptic' outbreaks or cases in the region. Specifically, we will expand on current syndromic surveillance activities to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and who present at regional clinics or hospitals with symptoms typical of high impact zoonotic viruses. We will collect detailed survey data to assess their contact type and frequency with wildlife and livestock and conduct molecular and serological diagnostic assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. We conduct novel viral discovery assays on patients who present with symptoms indicative of a high-risk zoonotic virus. If novel pathogens are identified by PCR, we will attempt to isolate, obtain full genome sequence and/or biologically characterize the pathogen, using the *in vitro* and *in vivo* strategy laid out in Aim 1.

3.2 Preliminary data clinical surveillance: 3.2.a. Clinical surveys and outbreak investigations: Our longterm collaboration in the region has included the following activities investigating clinical syndromes: *Peninsular Malaysia*: **At the time of writing, there is an outbreak of suspected viral undiagnosed illness in the indigenous Batek Orang Asli ("people of the forest") community living at Gua Musang ("civet cat cave") district, in Kelantan, Malaysia, close to Taman Negara – a National Park containing the oldest known rainforest**. This community is one where we have conducted prior routine surveillance and biological

sampling, demonstrating proof-of-concept that our two-pronged high-risk community/clinic approach will work. Dozens of people are affected and the outbreak is currently being investigated by our collaborators at the Malaysian NPHL. The Orang Asli live in remote villages in the rainforest of Malaysia, with limited access to modern health care and extensive contact hunting, butchering, and eating wildlife as their primary source of protein. This outbreak, and others that will continue to emerge just like it, underscore the critical need for our EIDRC - to improve the technological tools, training, and human capacity for rapid EID surveillance, diagnosis, and response at the frontlines of disease emergence in SE Asia. ~~Investigating this outbreak be a key priority if EID-SEARCH is funded.~~ *Sabah*: Co-I Kamruddin (BMHRC) is leading an asymptomatic leptospirosis study on sanitation workers, an ILI study in a Kota Kinabalu clinic and diarrheal study in children under 5 yrs. Co-Is William, Tan and others have conducted clinical observational studies to determine the etiology of central nervous system infections and acute undifferentiated febrile illness in Sabah. Co-Is Tan, William have collaborated with Co-I Wang to enroll 90 adult and over 100 pediatric participants with central nervous system infections, negative tests for malaria and dengue from 3 district hospitals in Sabah to determine the various etiologies. While a high proportion tested positive for rickettsial agents, the majority had no clear diagnosis. *Sarawak:* Dr Tan (UNIMAS) is conducting a survey of high-risk female Kelabit indigenous people and has found 8% with HPV52 as the dominant strain. He is working on clinical cohorts of hand, foot and mouth disease and will recruit patients from that study in the current proposed work. Finally, he is conducting a longterm study of rotavirus and other viral etiologies of acute gastroenteritis in Kuching, Sarawak that will include swine herders and farmers. These are an important group in Malaysia because pig farms are shunned by the government and local villagers to they are placed far away from town centers, deep in the forest, and it is this scenario that led to the emergence of Nipah virus in 1998-9 (*18, 163*). *Thailand:* The Chulalongkorn Univ. TRC-EID laboratory works in coordination with the Thailand Ministry of Health to investigate outbreak etiology, including the first case of imported MERS-CoV in Thailand, **for which we produced sequence confirmation within 24 hours from acquiring the specimen** ~~**(Fig. X)**~~. Three imported MERS cases have been detected in Thailand since 2015, and all cases were confirmed at the TRC-EID. We worked during the Zika outbreak to identify > 500 PCR-positive Zika patients with sequence confirmation. We have also worked on returning and suspected Ebola travelers, syndromic surveillance for SARI, Encephalitis, Hand Foot and Mouth disease in children and Viral diarrhea. Working with the US CDC (Thailand), between 1998 and 2002 we enrolled a large cohort of children from Kamphaeng Phet Province to examine Dengue virus and JE epidemiology (*164, 165*). Among the 784/2,574 convalescent sera Henipavirus-specific antibodies were detected in 13 serum samples (1.7% seroprevalence), suggesting prior exposure to endemic henipaviruses or a related paramyxovirus in rural Thailand. *Singapore:* Duke-NUS has worked with the Ministry of Health to investigate Zika cases (*166*), and with EcoHealth and others, Duke-NUS has developed novel CoV assays for clinical surveillance, and for expedited analysis during outbreaks. Our pipeline of novel virus detection, reservoir host identification, serological assay development, and serological testing is as rapid as several months, with the development of novel serological assays from sequence data in 7 days (*13*). **For example, with the discovery of SADS-CoV, we identified and fully sequenced a new virus, screened archived wildlife specimens to identify the natural reservoir host species (*Rhinolophus* spp. bats), developed LIPS serological assays, and tested human and swine samples to assess seroprevelance in less than 3 months (*13*).**

3.2.b Analysis of self-reported illness: In addition to biological outcomes we have developed a standardized approach in PREDICT for analyzing data on self-reported symptoms related to targeted illnesses; of fever with cough and shortness of breath or difficulty breathing (severe acute respiratory illness - SARI), fever with muscle aches (fevers of unknown origin (FUO) and, cough or sore throat (influenza-like illness - ILI) from study participants. We used a least absolute shrinkage and selection operator (LASSO) regression to identify associations between participants who reported ILI and/or SARI symptoms and demographics, occupation, and contact with animals in the last year. Results have clear biological relevance. In Yunnan, China, salient associated variables or combination of variables were, in descending order of odds ratio: 1) having eaten raw or undercooked carnivores; 2) having slaughtered poultry and being a resident of Guangxi province; 3) having had contact with cats or bats in the house; and 4) being a male who has raised poultry **(Fig. 11).** We will expand this approach for all syndromes in Aim 3, and use more granular targeted questions designed to assess patient's exposure to wildlife in terms that are relevant to the specific country.

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Commented [KJO81]: Deleted sentence below, because reality is, not likely an issue in ~4-6 months when we get funded and seems to be measles. Should play up as an example, but not an actual item for follow up..

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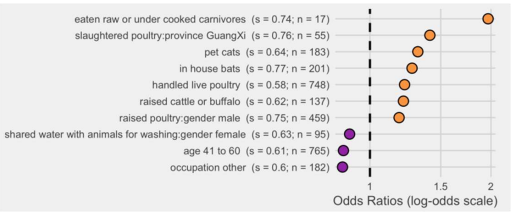
Fig. 11: Associated variables of self-reported ILI and/or SARI in prior 12 months (s = bootstrap support; n = number +ve out of 1,585 respondents). **Orange circles** = odds ratios > 1 (positively associated with the outcome); **purple** = odds ratios <1 (negatively associated with the outcome).



3.3 General Approach: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent is not identified. To capture these, we will expand on our current syndromic surveillance to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and present at regional clinics with influenza-like illness, severe respiratory illness, encephalitis, and other symptoms typical of high-impact emerging viruses. We will collect survey data tailored to the region to assess contact with wildlife, conduct molecular and serological assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. If novel pathogens are identified by PCR, we will isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work. We will then use behavioral survey data, ecological and phylogeographic analyses to assess the likely reservoir hosts to inform intervention programs.

3.4 Clinical cohorts. 3.4.a Cohort selection: We will initiate active clinic-based syndromic surveillance at 2 town-level level clinics and 2 provincial-level hospitals in Thailand, Peninsular Malaysia, Sabah and Sarawak. These all serve as the catchment healthcare facility for people in our community-based surveillance of Aim 2 and are inclusive of wildlife sampling sites in Aim 1. Patients ≥ 12 years old presenting at the health facility who meet the syndromic and clinical case definitions for SARI, ILI, encephalitis, fevers of unknown origin, or hemorrhagic fever will be recruited into the study. We will enroll a total of xxxx individuals for clinical syndromic surveillance, which accounts for up to 40% loss from follow-up. Study data will be pooled across country sites, as clinical patients are limited by the number of individuals presenting at hospitals. Sites: *Thailand:* Two new district hospitals at the same site of high-risk community will be included to the study to collect specimens (PREDICT sample protocol) from patients with respiratory and/or encephalitis symptom during 3 years of the study (Year 2 to Year 4), at least 100 patients from each hospital will be enrolled and laboratory tested by PCR and serology assays. The second blood after 21 days of fever will be collected for serology testing. ~~XXXXX~~ *Peninsular Malaysia:* Focusing immediately on the current outbreak in Orang Asli, we will work closely with the Malaysian Ministry of Health to support their investigation. We will also work with 6 Orang Asli clinics which focus solely on this indigenous community. *Sarawak:* XXXXXX *Sabah:* XXXXXXXXX. *Singapore:* ~~XXXXX~~ Given budget constraints we will not do clinical cohorts, however in the ~~ea~~vent of a suspected outbreak we have close ~~relationshtips~~relationships with xxxxx xxxxx hospitals and central reference lab.

3.4.b Behavioral and clinical interviews: 2.5 Clinic enrollment and follow-up: We will recruit inpatients and outpatients to participate in the study after initial screening to determine if they meet the clinical case definition of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever of unknown etiology; or 6) severe diarrhea. Once enrolled, biological samples will be collected and a questionnaire administered by trained hospital staff that speak appropriate local language. Samples will be taken concurrently when collecting samples for normative diagnostics. For inpatients, samples will be collected no more than 10 days of reported onset of illness to increase the chance of PCR detection of viruses (*167*). We will follow up 35 days after enrollment to collect an additional two 500 µL serum samples conduct a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. 35 days gives adequate time for development of IgG, which occurs <28 days after onset of symptoms for SARS patients (*168*). These samples may prove valuable to collaborators in that the PBMC could be used in production of therapeutic antibodies.

3.4.c Sampling:

Commented [KJO83]: Pasted in from Supaporn.

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Commented [PD85]: Tom, Kevin – we need information.

Commented [KJO86]: Linfa/Dani?

Commented [PD87]: Hongying Emily

Commented [PD88]: Need data for Nipah and filovirus patients

Commented [PD89]: Ralph: PBMC could be valuable for making therapeutic antibodies through collaborations?

Following enrollment with signed consent form, biological specimens (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples) including two nasal or oropharyngeal swabs will be collected from all eligible participants, and a questionnaire will be administered. We will investigate five risk factors, to maximize the power of the analyses, all related to high risk wildlife exposure, based on continuing analysis of our previous work, and will include: 11) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With permission from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology.

3.5 Sample testing: PCR, Serol to link symptoms to etiologic agents

The standard syndromic diagnostic PCR assay for the common pathogens will be conducted and the results will be shared to the hospital with 48 hours. Specimens will be further tested for 3 viral families; CoV, PmV and filovirus by consensus PCR. Appropriate specimen from dead case will be further conducted by NGS if the previous PCR tests are negative to identified cause of infection. The serology panel assay will be conducted from paired serum.

Serology: Panel serology assay will be performed from paired serum to determine the four fold raising antibody. The specific primer PCR of the virus antibody positive case will be further tested from acute specimens to confirm for infection.

3.5 Assessing potential for pandemic spread: Building off our spillover risk characterization in Aim 1, wWe will use statistical models built from collated biological, ecological, and genetic data to predict further assess the likelihood of human infection (or spillover) and also pathogenicity for a the range of diverse viruses we will characterize. The aim is to quickly identify and assess which viruses pose the most significant risk to human health and thus prioritize additional resources to more fully characterize, better understand pathogenicity, and invest in therapeutic development for those pathogens. We will combine two independent but related statistical models to predict pathogen spillover risk and pathogenicity. First, we will expand a simple generalized additive modeling approach (*90*) to assess spillover risk probability to a new virus that is currently not known to infect people, when we have only limited genetic data from initial PCR screening and sequencing. Additional data from receptor binding protein homology and cell line infection experiments can will be incorporated into the model framework as available. Second, given high likelihood to spillover and infect humans, or direct evidence of human infection from our clinical surveillance, we will then predict pathogenicity using nearest neighbor phylogenetic analyses as a first approximation and by incorporating more detailed data from genomic characterization and animal model experiments (Aim 1). For the pathogenicity model, hEuman epidemiological trait data for ~300 viral species known to infect people (e.g. case fatality rate, global estimates of number of human cases per year or decade, R_0, infectious period, and primary symptoms) will be aggregated including fromhave been collated by recent studies previous and will be used as reviews [Woolhouse ref] to use as predictor variables. Thirdly, we will build offapply tools already developed, and being refined by EHA under's previous DTRA and DHS supported research, to predict pandemic spread for viursesviruses we identify by integrating surveillance site data,using global flight models (*169*), as well as additional datasets (road networks, shipping routes, cell phone data) to measure on human movement and connectivity across Southeast Asia.

Commented [KJO90]: Blood just during 35 day follow up, or both? I guess both ideally, but weigh against IRB.

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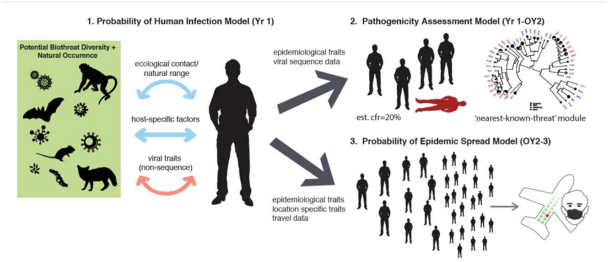
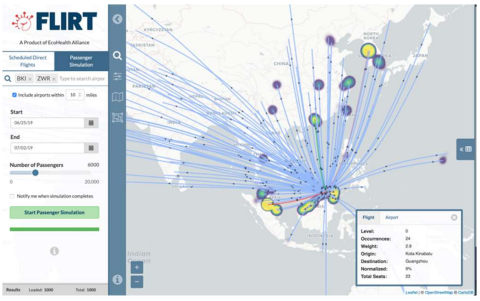


Fig. XXXXX: Conceptual framework highlighting 3 underlying models to estimate: **1)** probability of human infection, or spillover, the first necessary step to understand pathogenicity when status of human infection is unknown; **2)** pathogenicity, i.e. probability of causing clinical signs, case fatality rate estimates, and other metrics relevant to humans; and 3) probability of pathogen spread based on ecological, epidemiological, and airline connectivity and human migration data.

Commented [PD94]: Hongying – please work with Kevin to come up with something of relevance here. Also, make the text brief, bold and large so it's readable

Commented [KJO95]: See also FLIRT flight simulator figure below. Peter, let's decide if I should rework this figure to combine both, or how to proceed with this section.



3.7 Potential problems/alternative approaches: Patients visiting clinics may have cleared virus, but not yet developed IgG antibodies, reducing seropositive cases. Our 35 day follow-up sampling should avoid this because the maximum lag time between SARS infection and IgG development was ~28 days (*167*). We also expect that patients in rural communities will only visit clinics when symptoms have progressed, likely coinciding with late illness and onset of IgG. We will also have data from our community study, so won't be completely reliant on hospital data to identify PCR- or seropositives. Finally, the risk is outweighed by the potential public health importance of discovering active spillover of a new henipavirus, filovirus or CoV infection.

Commented [PD96]: Add data for Nipah and filo infections

Additional Instructions – Specific to the EIDRC FOA:

Commented [PD97]: All – I've started putting a few notes in this section, but it will need substantial work. Please insert language from other proposals to fit these sections if you have them

4. Administrative Plan

In a clearly labeled section entitled **"Administrative Plan":**

- Describe the administrative and organizational structure of the EIDCRC Administrative and Leadership Team. Include the unique features of the organizational structure that serve to facilitate accomplishment of the long range goals and objectives.

Chula TRC-EID is National reference laboratory for diagnostic and confirmation of EIDs pathogen along with the National public health laboratory at the Department of Medical Science and the WHO Collaborating Centre for Research and Training on Viral Zoonoses. We have built a EID laboratory network in-country among three Ministry including Ministry of Public Health (Department of Medical Science and Department of Disease Control), Department of Livestock and Development, and Department of National Parks, Wildlife and Plant Conservation and Universities and among SEARO countries. Our clinicians provide case consultation for Thailand and SEARO countries. The laboratory and clinic training are regularly conducted.

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For Malaysia –

NPHL – Molecular and serological screening (BioPlex) of PM Orang Asli samples, serological screening of syndromic samples from Sabah (already approved) and Sarawak (would need approval)

PERHILITAN molecular zoonosis laboratory, at the National Wildlife Forensic Laboratory - Molecular and serological screening (BioPlex) of PM wildlife samples

UPM Faculty of Veterinary Medicine - Molecular and serological screening (BioPlex) of PM livestock samples

KKPHL - Molecular of syndromic samples from Sabah (already done) and Sarawak (would need approval)

SWD WHGFL - Molecular screening of Sabah wildlife samples

BMHRC - Molecular and serological screening (BioPlex) of Sabah livestock samples, serological screening (BioPlex) of Sabah wildlife samples, Molecular and serological screening of syndromic samples from Sabah (already approved) and Sarawak (would need approval). BMHRC will need renovations and BioPlex to do this work – Menzies team might have funds for BioPlex that they would base here as part of DTRA project.

- Describe how communications will be planned, implemented, and provided to collaborators, teams, or sites. In addition, describe plans to achieve synergy and interaction within the EIDRC to ensure efficient cooperation, communication and coordination.
- Specifically address how the EIDRC Team will communicate with other EIDRCs, the EIDRC CC and NIAID and how the EIDRC will implement plans and guidance from the EIDRC CC to ensure coordination and collaboration across the Emerging Infectious Disease Program as a whole. Describe how the study site will provide information, if any, back to the local health authorities and the study subjects as needed. Note any adjustments or changes needed for rapid and flexible responses to outbreaks in emerging infectious diseases.

List out all of our involvement with outbreak investigations, ministries of health surveillance etc.

If possible the clean or summarized answers from the questionnaire should be shared to the hospital Infectious Control team and Bureau of Epidemiology for their information for controlling the outbreak.

Commented [PD99]: From Supaporn – do same for all countries

- Describe the overall management approach, including how resources will be managed, organized and prioritized, including pilot research projects.
- Highlight how the proposed staffing plan incorporates scientific and administrative expertise to fulfill the goal to develop the flexibility and capacity to respond rapidly and effectively to outbreaks in emerging infectious diseases in an international setting.

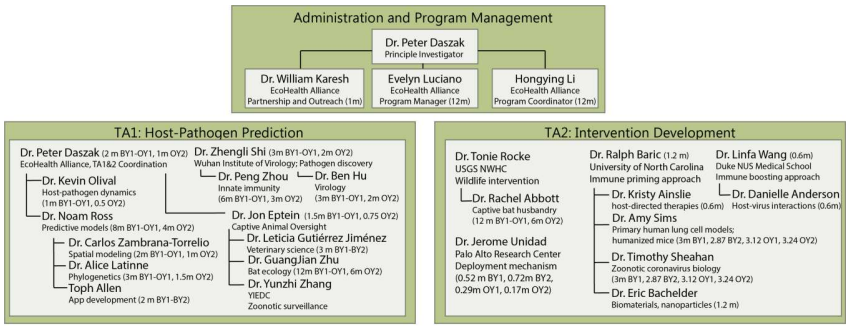
CM/EHA helped establish and manages with SWD the Wildlife Health, Genetic and Forensic Laboratory (certified as a BSL- 2 laboratory according to the US standard for laboratory specifications) that has all the equipment necessary to store samples, run extractions, PCR and analysis on biological samples for wildlife disease surveillance. The lab is used to conduct health checks on rescued and relocated wildlife before being released into new areas or sanctuaries, to screen samples for the PREDICT and Deep Forest Project and for genetic research and forensic investigations. CM/EHA also helped establish the new molecular zoonosis laboratories (certified as a BSL- 2 laboratory according to the US standard for laboratory specifications), at DWNP's National Wildlife Forensic Laboratory. The lab is used to screen samples for the PREDICT & DTRA projects. In addition CM/EHA also has access to NPHL and KKPHL for screening human samples and the Virology lab at FVMUPM for screening Livestock samples.

The Borneo Medical and Health Research Centre (BMHRC) is a centre of excellence that conducts teaching and scientific researches on communicable diseases, outbreak investigations, and ethnomedicine. BMHRC has offices space including a meeting room and seven laboratories which including preparation lab, parasitology lab, cell culture lab, bacteriology lab, natural product lab, virology lab, and molecular biology lab.

BMHRC is supported by an administration staff, medical lab technologist, and research assistants who are available to work on projects. Through this project we will help to establish a certified BSL-2 lab at the BMHRC. This lab can then be used for human testing to elevate pressure at KKPHL and QEH labs. It would also be an ideal location for a Luminex platform as human, wildlife and livestock samples could all be screened here. Co-Is Hughes and Kamruddin will be starting outbreak response training in August 2019 with a team from Sabah CDC, KKPHL and BMHRC. (Dr. Maria Suleiman now in PM But still collaborating with us, Dr. Muhammad Jikal current Director CDC working with us and Dato' Dr Cristina Rundi Director Sabah state health Dept is supportive) this collaborative group is working to develop a Sabah outbreak response team that will help support SSHD/ CDC outbreak team and also conduct sampling for disease surveillance to be based at BMHRC and we will develop this as part of this proposal. In addition the UMS hospital will come in line in 2021 providing additional opportunities for cohort studies and capacity building.

FROM DARPA grant:

Section 1.03 MANAGEMENT PLAN



Project DEFUSE lead institution is EcoHealth Alliance, New York, an international research organization focused on emerging zoonotic diseases. The PI, Dr. Daszak, has 25+ years' experience managing lab, field and modeling research projects on emerging zoonoses. Dr. Daszak will commit 2 months annually (one funded by DEFUSE, one funded by core EHA funds) to oversee and coordinate all project activities, with emphasis on modeling and fieldwork in TA1. Dr. Karesh has 40+ years' experience leading zoonotic and wildlife disease projects, and will commit 1 month annually to manage partnership activities and outreach. Dr. Epstein, with 20 years' experience working emerging bat zoonoses will coordinate animal trials across partners. Drs. Olival and Ross will manage modeling approaches for this project. Support staff includes a field surveillance team, modeling analysts, developers, data managers, and field consultants in Yunnan Province, China. The EHA team has worked extensively with other collaborators: Prof. Wang (15+ yrs); Dr. Shi (15+ yrs); Prof. Baric (5+ yrs) and Dr. Rocke (15+ yrs).

Subcontracts: #1 to Prof. Baric, UNC, to oversee reverse engineering of SARSr-CoVs, BSL-3 humanized mouse experimental infections, design and testing of targeted immune boosting treatments; **#2** to Prof. Wang, Duke-NUS, to oversee broadscale immune boosting, captive bat experiments, and analyze immunological and virological responses to immune modulators; **#3** to Dr. Shi, Wuhan Inst. Virol., to conduct PCR testing, viral discovery and isolation from bat samples collected in China, spike protein binding assays, humanized mouse work, and experimental trials on *Rhinolophus* bats; **#4** to Dr. Rocke, USGS NWHC, to refine delivery mechanisms for immune boosting treatments. Dr. Rocke will use a captive colony of bats at NWHC for initial trials, and oversee cave experiments in the US and China. **#5** to Dr. Unidad, PARC, to develop innovative aerosal platform into a field-deployable device for large-scale inoculation of the bats. Dr. Unidad will collaborate closely with Dr. Rocke in developing a field deployable prototype for both initial trails and cave experiments in China.

Collaborator Coordination: All key personnel will join regularly-scheduled web conferencing and conference calls in addition to frequent ad hoc email and telephone interactions between individuals and groups of investigators. Regular calls will include:

Commented [KJO100]: Figure like this would be good, but instead of breaking down by TA, maybe by country or Specific Aim. Per FOA, Need to also show how we'll link in with the Coordinating Center and other EIDRCs.

- Weekly meetings between the PI and Program Manager (on project and task status)
- Weekly web/phone meetings between Program Manager and subawardee admin. staff
- Monthly web/phone conferences between EHA PIs and all subawardee PIs.
- Monthly web conferences between key personnel (research presentations/coordination)
- Four in-person partner meetings annually with key personnel at EHA and two in-person partner meetings annually between subawardees.
- Annual in-person meeting among all key personnel

Evaluation metrics will include the generation of high-quality data, successful achievement of milestones and timelines, scientific interaction and collaboration, the generation of high quality publications, and effective budget management. The PI and subawardee PIs will attend a kickoff meeting and the PI will meet regularly with DAPRA at headquarters and at site visits.

Problem identification and resolution: Regular planning, monitoring, and evaluation meetings will be the primary mechanisms for problem identification. Minor problems (e.g. delays in sample availability or test results) will be dealt with internally through appropriate action and resolution monitored by Dr. Daszak and the Project Manager. In the event of significant problems, such as prolonged poor productivity, inadequate scientific collaboration, or major disputes regarding research direction or resource allocation, EHA will assist with resolving the problem through negotiation. Should a resolution not be forthcoming, consultation with our technical advisors and DARPA Program staff may be warranted.

Risk management: Maintaining a timeline and meeting milestones will require strict and continuous oversight of all project phases, frequent and regularly scheduled communication, and the ability to make decisions and implement strategies. A project of this nature requires a different mindset from that typically associated with basic research activities that move at an incremental pace with investigators gradually optimizing experimental systems, refining data, or waiting for additional data before moving ahead with an analysis approach. To maintain our timeline, we will continually evaluate these trade-offs to make decisions about when iteration is appropriate and when necessary to move forward with current information.

Biographies:

Dr. Peter Daszak is President and Chief Scientist of EcoHealth Alliance, Chair of the NASEM Forum on Microbial Threats, member of the Executive Committee and the EHA institutional lead for the $130 million USAID-EPT-PREDICT. His >300 scientific papers include the first global map of EID hotspots, estimates of unknown viral diversity, predictive models of virus-host relationships, and evidence of the bat origin of SARS-CoV and other emerging viruses.

Prof. Ralph Baric is a Professor in the UNC-Chapel Hill Dept. of Epidemiology and Dept. of Microbiology & Immunology. His work focuses on coronaviruses as models to study RNA virus transcription, replication, persistence, cross species transmission and pathogenesis. His group has developed a platform strategy to assess and evaluate countermeasures of the potential “pre-epidemic” risk associated with zoonotic virus cross species transmission.

Prof. Linfa Wang is the Emerging Infectious Diseases Programme Director at Duke-NUS Medical School. His research focuses on emerging bat viruses, including SARS-CoV, SADS-CoV, henipaviruses, and others and genetic work linking bat immunology, flight, and viral tolerance. A 2014 recipient of the Eureka Prize for Research in Infectious Diseases, he currently heads a Singapore National Research Foundation grant “Learning from bats” ($9.7M SGD).

Commented [PD101]: Some of references in this section are duplicates

Current list of contacts at key human (especially) and wildlife orgs in every SE Asian country from map above – please add to this if we’ve missed any

Indonesia – EHA: Eijkman (Dodi Safari), IPB (Imung Joko Pamungkas); **CM Ltd:** IPB (Imung Joko Pamungkas), Bogor Agricultural University (Diah Iskandriati)

SE China – EHA: Yunnan CDC (XX), Wuhan Inst. Virol. (Zhengli Shi);

Myanmar – Chulalongkorn (Thiravat and Supaporn) have collaborated extensively with Win Min Thit, MD (Yangoon General Hospital, Myanmar); Khin Yi Oo (National Health Laboratory, Myanmar) and also in training PREDICT Myanmar veterinarians in bat sampling under PREDICT.

Japan – **BMHRC**: Oita University (Hidekatsu Iha, Kiyoshi Aoyagi), Nagaskai University (Koichi Morita, Kazuhiko Moji)

Cambodia -
Laos - **CM Ltd:** Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit (Matthew Robinson)
Bangladesh – EHA: IEDCR (Meerjady Sabrina Flora, Ariful Islam), icddr,b (Mohammed Ziaur)

India – EHA: Jon's collaborators;

Vietnam – Wellcome CRU ? Liaison with Zhengli Shi for Serol assays etc.?

Philippines – EHA, CM Ltd: Biodiversity Management Bureau, Department of Environment and Natural Resources (Anson Tagtag), Bureau of Animal Industry (Davinio Catbagan), Research Institute of Tropical Medicine (Catalino Demetria).

Malaysia – **CM Ltd:** Borneo Medical and Health Research Center (Ahmed Kamruddin), Department of Wildlife and National Parks Peninsular Malaysia (Frankie Sitam, Jeffrine Japning, Rahmat Topani, Kadir Hashim, Hatta Musa), Hospital Universiti Malaysia Sabah (Helen Lasimbang), Faculty of Medicine, Universiti of Malaya (Sazaly Abu Bakar), Kota Kinabalu Public Health Laboratory (Jiloris Dony, Joel Jaimin), Ministry of Health (Chong Chee Kheong, Khebir Verasahib, Maria Suleiman), National Public Health Laboratory (Hani Mat Hussin, Noorliza Noordin, Yu Kie Chem), Queen Elizabeth Hospital (Giri Rajahram, Heng Gee Lee), Sabah State Health Department (Christina Rundi, Mohammad Jikal), Sabah Wildlife Department (Rosa Sipangkui, Senthilvel Nathan, Augustine Tuuga, Jumrafiah Sukor), Gleneagles Hospital Kota Kinabalu (Timothy Wiliam); **BMHRC:** CM Ltd (Tom Hughes), HQEII (Shahnaz Sabri), Kota Kinabalu Public Health Laboratory (Jiloris Dony), Sabah State Health Department (Mohammad Jikal)

Thailand – **CM Ltd:** Department of Disease Control, Ministry of Public Health (Rome Buathong, Pawinee Doungngern, Pongtorn Chartpituck), Department of National Park, Wildlife and Plant Conservation (Pattarapol Manee-On), Faculty of Forestry, Kasetsart University (Prateep Duengkae), Mahidol-Oxford Tropical Medicine Research Unit, (Stuart Blacksell, Richard Maude),Chulalongkorn University (Supaporn Wacharapluesadee).

Chulalongkorn lab is a WHO Collaborating Centre for Research and Training on Viral Zoonoses, we work closely with WHO SEARO and SEARO countries including Bangladesh, Bhutan, Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, (Thailand), Timor-Leste.
We had conducted the PREDICT lab training for Veterinary lab in the Southeast Asia region for FAO.
We conducted (transferred?) the bat sampling technique for scientists from Malaysia, Myanmar, the Philippines, and China.

5. Data Management Plan

In a clearly labeled section entitled **"Data Management Plan":**

- Describe internal and external data acquisition strategies to achieve harmonization of systems and procedures for data management, data quality, data analyses, and dissemination for all data and data-related materials generated by the research study with the EIDRC CC.
- Within the plan, indicate the extent to which dedicated systems or procedures will be utilized to harmonize the acquisition, curation, management, inventory and storage of data and samples. Describe

how training for the data and sample collection, in terms of the use of electronic data capture systems, will be provided to all staff including those at enrollment sites.

- Describe the quality control procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens.

This includes sample labeling etc. Get a bar-coding system, everyone has a scanner (cf. PREDICT) – Ralph has this for select agents and MERS. Also need a software system

Language from NIH CoV grant:

Data Sharing Plan: Data will be available to the public and researchers without cost or registration, and be released under a CC0 license, as soon as related publications are in press. Data will be deposited for in long-term public scientific repositories – all sequence data will be made publicly available via GenBank, species location data via the Knowledge Network for Biodiversity, and other data will be deposited in appropriate topic-specific or general repositories. Computer code for modeling and statistical analysis will be made available on a code-hosting site (GitHub), and archived in the Zenodo repository under an open-source, unrestrictive MIT license. Limited human survey and clinical data will be released following anonymization and aggregation per IRB requirements. Publications will be released as open-access via deposition to PubMed commons.

Viral isolates will remain at the Wuhan Institute of Virology initially. Isolates, reagents and any other products, should they be developed, will be made available to other NIH-funded researchers via applicable Wuhan Institute of Virology and EcoHealth Alliance Material Transfer Agreements and/or licensing agreements.

Sharing Model Organisms: We do not anticipate the development of any model organisms from this study. Should any be developed, they will be made available to other NIH-funded researchers via applicable Wuhan Institute of Virology and EcoHealth Alliance Material Transfer Agreements and/or licensing agreements.

Genomic Data Sharing: We anticipate obtaining genetic sequence data for 100s of novel coronavirus genotypes, including RNA-dependent RNA polymerase (RdRp) and Spike genes for all strains/genotypes. In addition, we will generate full viral genomes for a subset of the bat SARSr-CoVs that we identify. All sequence data will be deposited in the NIH genetic sequence database, GenBank. We will ensure that all meta-data associated with these sequences, including collection locality lat/long, species-level host identification, date of collection, and sequencing protocols will also submitted. The genotype data will be made publicly available no later than the date of initial publication or six months after the receipt of final sequencing data, whichever comes first. We anticipate sequence generation will occur over the 5 year proposed project period.

Genome Wide Association Studies (GWAS): Not applicable.

Commented [KJO102]: From CoV NIH grant

Thailand TRC-EID Chulalongkorn lab:Specimen management and BioBank: The software called PACS (Pathogen Asset Control System) supported by BTRP DTRA, is now used for managing the specimen from specimen registration (barcoding, and may move to use QR code system soon), aliquoting, tracking, short patient history record, data summary, searching, test reporting and etc. Our biobank system contains 28 freezers (-80C) and liquid N2 system. Its size is around 120 sq meters. There is a separate electric generator for our lab and biobank. There are CC TV cameras at the biobank and laboratory.

Chula TRC-EID has our own server for data management and analysis. We have the necessary software including Bioinformatic tools (Blast Standalone, SAMTOOL, GATK, AliVIEW, RaxML, FigTREE, MAFFT, Guppy, Megan, PoreChop, MEGA), Database (MySQL, MongoDB) Web, jQuery, Node.js, Express.js React,

API, PHP, Ruby, Python, Java, C#, HTML5, Bootstrap, CSS, Responsive, Cloud, Linux Command, PACS (sample management system), Virtualbox virtual machines, R programming languages, Perl scripts, Bash shell scripts, Open source software, Microsoft Office running on both Apple Mac OS X, Ubuntu, Linux, and Windows Operating Systems. Chula TRC-EID has a dedicated 16-core Ram 64GB Linux server with 4TB hard drives, dual quad-core Mac Pro Server with 2TB hard drives and another dedicated iMac Pro (Retina 4K, 21.5-inch, 2017) Processor 3.6GHz quad-core Intel Core i7 (Turbo Boost up to 4.2GHz) Ra, 16GB with 512GB hard drives. TRC-EID also has HPC Linux server CPU: 4 x 26cores Intel Xeon = 104 cores 2.1GHz with 52 TB HHD and 2x3.84TB SSD with GPU 2x Tesla v100 (CUDA core = 5120 cores/GPU, Tenser core = 640 cores/GPU). TRC-EID has a dedicated 10/100/1000 of LAN (Local Area Network), 2.4G / 5G Gigabit Wi-Fi 802.11ac.

Commented [KJO103]: From Supaporn for their lab, some may go in Facilities doc?

From DARPA:

Data Management and Sharing: EcoHealth Alliance will maintain a central database of data collected and generated via all project field, laboratory, and modeling work. The database will use secure cloud hosting services and enable export to archival and platform-independent formats. It will ensure data and metadata compatibility between project components, track data versioning and annotations, and enable compliance with DARPA data requests and disclosure requirements. All archived human sample data will be de-identified. Partners will provide raw and processed data to the central database throughout the course of the project. Project partners will have access to the data they generate in the database at all times, and maintain control over local copies. Release of any data from the database to non-DARPA outside parties or for public release or publication will occur only after consultations with all project partners. EHA has extensive experience in managing data for multi-partner partner projects (PREDICT, USAID IDEEAL, the Global Ranavirus Reporting System).

Commented [KJO104]: Text from DARPA Preempt

6. Clinical Management Plan

Clinical site selection

Our consortium partners have been conducting lab and human surveillance research, inclusive of human surveillance during outbreaks, for over the past two decades and in that time have developed strong relationships with local clinical facilities and processes. The plan for selection of clinical sites will begin in the same geographic regions as those identified in Aim 1. These are regions where we will sample wildlife due to the high potential of zoonotic viral diversity. Additionally, these will be clinical sites that serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2. In PREDICT we developed successful working relationships with the major healthcare facilities the hotspot areas in 6 different countries, including Thailand and Malaysia and we will continue to work with these sites going forward. Continuing to work with these hospitals means we will begin this project with successfully established partners and we can begin data collection quickly. This also reduces the number of additional sites needed to meet our goals within our geographic range. The capacity and capability requirements for clinical sites for this study is limited. All that is required of sites will be the ability to enroll patients that meet the clinical case definitions of interest, capacity to collect and temporarily store biological samples, and follow standards for data management and protection. We will be enrolling inpatient and outpatient participants within 10 days of the presentation of symptoms to increase the chance of PCR detection of viruses so we will not wait for advanced normative diagnostic tests, that are likely unavailable in the more remote sites, to be completed. We will work with clinical sites to determine the best process for project staff, in most cases supporting the time of currently site staff. These will be persons who are locally trained and certified to collect biological specimen from participants including blood draw technique, e.g. doctor or nurse. Last, the clinical site will need the capacity to securely store all personal health information and personally identifiable research data, this will include locked offices with locked filing cabinets to store all paper records and an encrypted computer. Training on project procedures will be provided for all local project staff by local country project management staff and partners from headquarters. The study at clinical sites will begin by developing relationships with local stakeholders at the hospitals and clinics in our geographic areas of interest that serve as catchment facilities for people of within the community surveillance study. We will recruit hospital staff that will be extensively trained for project procedures including enrollment of patients, sample types to be collected, storage of samples, administration of the questionnaire, and data

Commented [EH105]: Is that true

management plans. During the Development and onboarding of new clinical sites we will assess the physical needs of the site and what supplies will be required for collection of data (e.g. locked filing cabinet, dry shipper, secure laptop). [Did not specifically address CONDUCT]. Oversight for clinical sites will be managed by the country coordinator for each country. This will be done with regular site visits to the clinical sites and annual visits by headquarter research staff. Site visits will include monitoring and evaluating the process for patient enrollment, collecting and storage of samples, administration of the questionnaire, and secure and data management procedures for secure personal health information and research data. During these visits any additional training will also be provided.

Standardized approach oversight and implementation

Management and oversite for all study sites will be managed by the local country coordinator who is supported by staff at headquarters. Implementation of a standardized approach across all study sites, community and clinical, will begin by extensive training of staff on procedures on requirements of the project, this includes: enrollment, data collection, and data management. Our research team has over 5 years of experience building capacity on human subjects' research and has developed training resources and materials for standardized implementation of community and clinical research. This includes SOPs for screening, enrollment, and retention of participants. In addition to yearly monitory reports the country coordinator will regularly visit each clinical site to ensure quality standards and compliance of procedures are being met. Through our work with clinical sites in PREDICT we have developed culturally appropriate screening measures for clinal sites that do not disrupt the flow or quality of medical treatment received by the patient. This was done by working collectively with clinical research staff to evaluate current procedures and patient flow at the site to determine the most efficient while minimal invasive inclusion of our study into the daily working of the clinal site. Most efficiently this was done by adding minimal basic screening questions to the current clinal intake forms, which allows the clinal research officer to quickly scan charts or logs for potential patients to enroll, allowing for minimal disruption and prevents potential enrollees from being overlooked if the research staff is not on duty. At clinical sites that do scheduled outpatient days we may have a trained member of the research staff with the intake nurse to monitor the patients coming to the clinic more rapidly to no delay screening or enrollment in such a fast pace environment. Using the screening tools, we will recruit and enroll patients who present with symptoms that meet the clinical case definitions of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever, of unknown etiology within the past 10 days. These patients will be enrolled, samples collected and survey conducted to assess the participants contact with wildlife through: 1) occupational exposures; 2) animal contact; 3) environment biosecurity. With permission from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology. The clinical cohort will be split into cases and controls. "Cases" are defined as participants whose samples tested positive for either coronaviruses, henipavirus, or filoviruses, serological tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between serological/PCR status and risk factors. In clinical study, we will use this procedure to determine how clinical presentation differs between virus-exposed and -unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. We will model the outcomes to analyze correlation between biological results and risk factors related to high risk wildlife exposure. Additionally, we will be following up with clinical participants to determine how clinical presentation differs between coronaviruses, henipavirus, or filoviruses in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. [How do these cohorts be leveraged for new emerging pathogens] Achieving the adequate numbers will be attained through duration of the as clinical participant enrollment. While patient's enrollment is limited by the number of individuals presenting at hospitals, during the PREDICT project we had an average of 105 patients enrolled per year, ranging from 77-244. The country coordinator will be continually monitoring the project database to be sure that we are on track to reach our target.

Clinical cohort setup, recruitment, enrollment

We will recruit inpatients and outpatients from clinical sites to participate in the study after initial screening to determine if they meet the clinical case definitions for 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever, of unknown etiology for symptoms reported onset of illness to increase the chance of PCR detection of viruses. Once enrolled, biological samples will be collected by trained hospital staff. When possible, samples will be taken concurrently with samples being collected for normative diagnostics. We will collect 2* 5mL of blood for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples and two nasal or oropharyngeal swabs will be collected. The cases are defined as participants whose samples tested positive for Henipaviruses, Filoviruses, or CoVs by PCR or serological tests. The controls will be selected from the pool of participants admitted to the studies and whose samples tested negative for coronaviruses, henipavirus, or filoviruses. We will follow up 35 days after enrollment to collect an additional 5mL blood draw for collection of two 500 µL serum samples and a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. Following up 35 days post initial collection gives adequate time for 1) development of IgG and IgM, which occurs <28 days after onset of symptoms for coronaviruses, henipavirus, or filoviruses infected patients to evaluate the immunological progression of disease and 2) further the risk assessment of the participants to monitor contact with wildlife, people, and to assess the likely reservoir hosts to collect information to inform potential intervention programs.

Utilization of collected data

Sampling of wildlife and the enrollment of community surveillance participants and a clinical cohort of participants give us the ability to assess the viruses the are circulating in each of the three populations in a similar geographic region. We will use phylogenetic analysis to compare the relationship between wildlife viruses found and viruses fond in human participants. Additionally, the questionnaire allows us to assess relative measures of human-wildlife contact from our survey work that we will analyze the intensity of contact with species known to be at a higher risk for spillover against other risk to provide useful proxy information for spillover risk.

The biological specimens to be collected (2*5mL whole blood for whole blood and serum analysis and nasal or oropharyngeal swabs) from all eligible participants and follow up specimen 35 days (3mL of whole blood for serum) after enrollment with the standardized questionnaire. Capacity for specimen is collection is not more than standard phlebotomy skills and we will collected by locally trained and certified personnel that are part of the research team in country. Staff we will trainline on ethical guidelines for conducing human subjects research and survey data collection methods. Collection of whole blood, serum, and nasal or oropharyngeal swabs allows us to test the samples for PCR to detect viruses in our priority viral families, serologically test for immunological response and historical exposure to zoonotic diseases of relevance, and the questionnaire will assess individuals evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. With this collective data set we aim to quantify and detect novel viruses likely to spillover regularly to people, are often unreported or misdiagnosed as their clinical manifestations are unknown to detect cryptic outbreaks causing previously ‘hidden’ clinical syndromes in people. Our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an ‘early warning system’ to conduct public health interventions and disrupt disease emergence.

Potential expansion

Expansion of the research project to other clinical sites or research areas could happen as new information is gathered, either through our research or an outbreak in the region. If expansion is required we could rapidly implement research activities in additional clinical or community sites through the same process we on-boarded the initial locations. First, by working with local stakeholders to create a working relationship and on-boarding new clinical research staff through advanced trainings of project aims, SOPs on data collection and storage, and ethical guidelines. The network of infectious disease researchers in the community and hospital

Commented [EH106]: Will these cohort fulfill the goals of the EIDRC.

Commented [EH107]: PREDICT we also make the stipulation that we will collective relatvive samples if avialble from treatmean collection, eg CSF if the MD is doing a spinal tap we will request an aliqot if possible

Commented [EH108]: Please add any information that might address: types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.

Commented [EH109]: Please add any information that might address: Describe methods that might be applied to elucidate a zoonotic source

settings would allow for an accelerated response to any potential infectious disease outbreaks as staff are well trained on ethical guidelines for working with human subjects, data and specimen collection, storage, and transpiration, project staff would be on the front lines ready for the call to action. Ideally, our testing for viruses in priority viral families may lead to the discovery cryptic outbreaks before it spreads in the general public. Biological results coupled with survey data on key risk factors will provided necessary information for granular understand of disease spread.

In a clearly labeled section entitled **"Clinical Management Plan":**

- Describe plans, processes, and procedures for the following activities: clinical site selection including feasibility, capacity, and capabilities, and study development, conduct, and oversight.
- Describe strategies for oversight and implementation of standardized approaches in the recruitment and clinical characterization of adequate numbers of relevant patient populations to ensure the prompt screening, enrollment, retention and completion of studies. Describe novel approaches and solutions to study enrollment. including the clinical meta-data that will be captured and describe how the staff at each enrollment/collection site will be trained and comply with the quality standards for data and sample collection and compliance with the standardized procedures. Provide a general description of the methods for establishing clinical cohorts and how cohorts may be leveraged for new emerging pathogens.
- Provide general approaches to identification of the types of clinical cohorts needed to fulfill the goals of the EIDRC. Discuss types of clinical cohorts and clinical assessments that will be utilized to study natural history and pathophysiology of disease, including characteristics of the cohort, site for recruitment, types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.
- Describe methods that might be applied to either elucidate a zoonotic source or a vector of transmission.

Our research team incredibly diverse and capable of working with a group of other viral families: Lay out all our additional vector experience incl. my WNV work, our work on Chik etc.
Baric: Flavi, Noro,
Linfa: …
EHA: …

- Describe capacity for and approaches to clinical, immunologic and biologic data and specimen collection, and how these data and specimens will address and accomplish the overall goals and objectives of the research study.

Ralph

1. Norovirus: collaboration with surveillance cohort CDC; first one to publish antigenic characterization of each new pandemic wave virus; and phase 2 and 3 trial of therapeutics
2. Tekada Sanofi Pasteur dengue
3. Nih tetravalent vaccine

- Provide plans for the potential addition of new sites and expanding scientific areas of research when needed for an accelerated response to an outbreak or newly emerging infectious disease.

Linfa – will launch VirCap platform on outbreak samples.

7. Statistical Analysis Plan

In a clearly labeled section within the Research Strategy entitled **"Statistical Analysis Plan":**

- Describe the overall plan for statistical analysis of preliminary research data, including observational

cohort data.

- Describe the overall staffing plan for biostatistical support and systems available for following functions: 1) preliminary data analyses, 2) estimates of power and sample size, 3) research study design and protocol development.
- Describe the quality control and security procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens. Describe any unique or innovative approaches to statistical analysis that may be utilized.

8. Project Milestones and Timelines

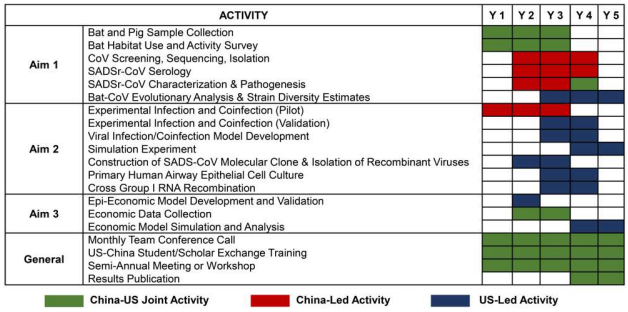
In a clearly labeled section entitled **"Project Milestones and Timelines":**

- Describe specific quantifiable milestones by annum and include annual timelines for the overall research study and for tracking progress from individual sites, collaborators, and Teams. Milestones must specify the outcome(s) for each activity. Milestones should be quantifiable and scientifically justified, and include the completion of major research study activities, including, for example, protocol development, case report forms, scheduled clinical visits, obtaining clearances study completion, and analysis of final data. Milestone criteria should not simply be a restatement of the specific aims. Using a Gantt chart or equivalent tool, describe the associated timelines and identified outcomes for the research study.

Show that for this proposal, we won't be doing the Specific Aims sequentially. We'll begin the human work (SA 2, 3) at the beginning of the project, along with SA1. We'll do the diagnostic assay development initially in Ralph's, Linfa's, Chris's lab, but then begin tech transfer in Y1….

Project Management & Timeline

PI Daszak will oversee all aspects of the project. Dr. Daszak and has two decades of experience managing international, multidisciplinary research in disease ecology, including successful multi-year projects funded by NSF, NIH and USAID. He will conduct monthly conference calls with all co-PIs, including the China team **(see Activity schedule below)**. He will be assisted by EHA Sr. Personnel Li, who is a US-based Chinese national with fluent Mandarin. Co-PI Olival will oversee Aim 1 working closely with China PI Tong, who will test samples. Co-PI Ma (China team) will coordinate and lead all Chinese experimental infections, with guidance from co-PI Saif, who will coordinate and lead all US experiments with co-PI Qiuhong Wang. Sr. Personnel Ross will conduct all modeling activities in Aim 2, working closely with co-PI Saif to parameterize the models. Co-PI Baric and Sr. Personnel Sims will lead infectious clone, recombinant viral and culture experiments. Consultant Linfa Wang will advise on serology, PCR and other assays. China team co-PIs Shi and Zhou will oversee viral characterization in Aim 1. Sr. Personnel Feferholtz will oversee the economic modeling in close collaboration with Ross.



Another Example of Milestones and specific tasks from a prev DHS (unfunded) proposal:

Expand existing databases to include predictor variables for pathogenicity risk model

Task 1: Collate available human clinical information from the literature for ~300 viral pathogens known to infect humans (Contract Award Date to 6 month)

Commented [KJO110]: Text and figure From NSF-NIH SADS grant. Peter will adapt

Task 2: Update EHA host-viral association database to include new data for mammalian and avian species from literature 2015-present (Contract Award Date to 3 month)
Task 3: Extract and curate full and partial genomic data from Genbank for 5-6 viral families with a high proportion of human-infectious pathogens for phylogenetic analyses – e.g. 'nearest-known-threat module'. (Month 2 to 6 month)

Develop underlying pathogenicity and spillover risk assessment models
Task 4: Fit generalized linear models in Stan for multiple response variables to measure pathogen impact (Month 7 to 10 month)
Task 5: Fit generalized linear models to estimate risk of human infection (spillover) based on host, ecological, and location specific traits (Month 4 to 8 month)
Task 6: Validate model effectiveness by simulating early-outbreak, limited-information scenarios from known pandemics to confirm model behavior (Month 8 to 10 month)

Commented [KJO111]: Just example, but probably just want to use a Gantt chart format per most of our grants, depending on space.

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II. Research Strategy:

1. Significance:

Southeast Asia is a critical hotspot for emerging diseases due to its high biodiversity of wildlife and their viruses, and the presence of key ecological and socioeconomic drivers of emergence **(Fig. 1)** (*1*). These include dense human populations living in networks of rural and urban communities, with strong cultural and occupational connection to wildlife and livestock farming and trading, and intimate connection to global travel networks (*2*). The region is also undergoing rapid environmental and demographic change, both of which increase the risk of disease emergence and spread.

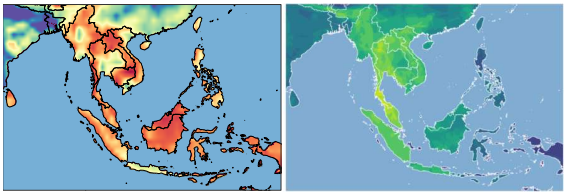

Fig. 1: Southeast Asia, and in particular, parts of Thailand and Malaysia are hotspots of risk for disease emergence **(left)**, and predicted high diversity of 'missing' or as-yet undiscovered viruses, yellow = highest diversity **(left)**. From (*1, 2*).

Not surprisingly a number of novel viruses, including near-neighbors of known agents, have emerged in the region leading to sometimes unusual clinical presentations **(Table 1)**.These factors are also behind a significant background burden of repeated Dengue virus outbreaks in the region (*16*), and over 30 known *Flavivirus* species circulating throughout S. and SE Asia (*17*). The events in **Table 1** are dominated by three viral families: coronaviruses, henipaviruses and filoviruses, which serve as initial examples of team research focus and capabilities. These viral groups have led to globally important emerging zoonoses (*18-29*). Their outbreaks have caused tens of thousands of deaths, and billions of dollars in economic damages (*30-32*). Furthermore, relatives and near-neighbors of known pathogens in these families have been reported throughout the region, including by members of our consortium: henipaviruses in bats in Thailand, Cambodia, Philippines, Laos, Indonesia, China, Malaysia, Bangladesh and India (*33-39*); a novel henipavirus, Mojiang virus, in wild rats in Yunnan (*10*); serological evidence of filoviruses in bats in Bangladesh (*40*) and Singapore (*41*); Nipah- and Ebola-like viruses in *Hipposideros*, *Cynopterus* and *Rhinolophus* species in Malaysia (Hughes *et al.*, in prep.); evidence of novel filoviruses in bats in China (*42-44*), including Měnglà virus that appears capable of infecting human cells (*45*); novel paramyxoviruses in bats and rodents consumed as bushmeat in Vietnam (*46*); a lineage C β-CoV in bats in China that uses the same cell receptor as MERS-CoV and can infect human cells *in vitro* (*47*); MERSr-CoVs in dry bat guano being harvested as fertilizer in Thailand (*48*), and directly in bats (Wacharapluesadee *et al.*, in prep.);

Viral agent	Site, date	Impact	Novelty of event	Ref.
Melaka & Kampar virus	Malaysia 2006	SARI in family group, individual	1st disease due to bat-origin reoviruses, also Singapore, Vietnam etc.	(*3-6*)
Reston filovirus	Philippines 2008	Seropositive people killed pigs,	No prior findings of filoviruses in pigs	(*7*)
Thrombocytopenia Syndrome virus	E. Asia 2009	100s of deaths in people	Novel tick-borne zoonosis with large caseload	(*8*)
Reston filovirus	Shanghai 2011	PCR positive pigs	Further evidence of pig RESTV infection	(*9*)
Mojiang virus	Yunnan 2012	Death of 3 mineworkers	1st evidence of rodent origin henipavirus in people	(*10*)
Nipah-like virus	Philippines 2015	Killed horses	No prior horse infection for NiV, known for HeV	(*11*)
SARSr-CoV & HKU10-CoV	S. China 2015	Seropositive people	1st evidence human infection HKU10 & SARSr-CoV	(*12*)
SADS-CoV	China 2017	>25,000 pig deaths seronegative people	Novel emergence of bat-origin CoV	(*13*)
Nipah virus	Kerala 2018, 2019	Killed 17/19 people 2018, 1 infected 2019	1st outbreak of NiV outside Bangladesh, W. Bengal focus	(*14, 15*)

Table 1: Recent emergence events in SE Asia indicating potential for novel pathways of emergence, or unusual presentations for known or close relatives of known viruses.

172 novel β-CoVs (52 novel SARSr-CoVs) and a new β-CoV clade ("lineage E") in bat species in S. China that are also found throughout the region (*20, 47, 49, 50*); and 9 and 27 novel wildlife-origin CoVs and

paramyxoviruses in Thailand (respectively) and 9 novel CoVs in Malaysia identified as part our work under USAID-PREDICT funding (*49, 51*). These discoveries underscore the clear and present danger of future zoonotic events in the region. Furthermore, the wide diversity of potentially pathogenic viral strains has significant potential to help in the development of broadly active vaccines, immunotherapeutics and drugs (*47*).

Most high-impact zoonotic viruses originate in wildlife reservoirs, sometimes spilling over first into livestock 'amplifier' hosts, or directly into localized human populations with high levels of animal contact **(Fig. 2)**. Efforts to prevent emerging zoonoses have targeted these high-risk populations in regions prone to disease emergence (*29, 52*). However, these regions tend to be in the developing world, where nations often lack the resources to investigate novel zoonotic events, or wildlife reservoirs of zoonoses, and prioritize more immediate public health concerns such as Dengue virus or NTDs (*16*). Zoonotic disease surveillance and control is also hampered by inadequate information on basic disease ecology (e.g. range of wildlife reservoirs, risk factors for spillover) and pathogen biology (viral diversity, pathogenesis), and by logistical challenges sharing novel diagnostics, animal models, samples and reagents at sites where outbreaks begin (*53*). Countermeasure and vaccine development is challenged by the small number of isolates available, and the presence of closely-related strains, some with evidence of transmission to people but with unknown pathogenicity. For example, using NIH and other funding, we found serological evidence of exposure to African henipaviruses in 3-4% of a sample of people in Cameroon, with a significant risk factor being butchering of bats; and serological responses to bat SARSr-CoVs in 6/209 (2.87%) and bat HKU10-CoV in x/xx (x%) people living close to a bat cave in Yunnan, all novel viruses with unknown clinical impact (*12, 54*). We discovered two novel bat-borne reoviruses, Melaka and Pulau viruses, and showed that 14/109 (13%) of people living in close proximity to bat roosts on Tioman Island, Malaysia were exposed to these viruses (*3*) as well as 12/856 (1.4%) people screened in Singapore (*4*). Previous studies in the Philippines found famers and butchers who had contact with sick pigs tested positive for Reston ebolavirus antibodies but had not experienced illness (*7*), and it was our team that discovered Philippine bats as natural reservoir hosts for Reston virus (*55*). Preliminary screening in Malaysia with funding from DTRA for serological surveillance of Henipaviruses and Filoviruses spillover found serological evidence of past exposure to Nipah and Ebola antigenically-related viruses in non-human primates (NHPs) and people (Hughes, in prep.). Even when spillover leads to outbreaks of illness and mortality, they are often unreported, undiagnosed or misdiagnosed. Under a prior NIH R01, we initiated targeted syndromic surveillance of encephalitis patients in Bangladesh clinics and showed that NiV causes repeated annual outbreaks with an overall mortality rate of ~70% (*56*). Nipah virus has been reported in people in West Bengal, India, which borders Bangladesh (*26*), in bats in Central North India (*57*), and now in people in Kerala, South India in 2018 and 2019, raising the specter of future spillover at other sites in the region (*14, 58*).

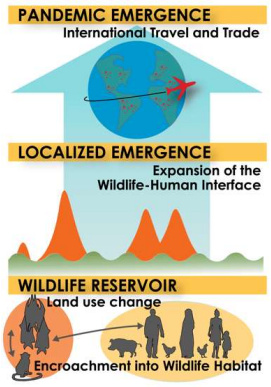

Fig. 2: Our Early Warning Approach: Most zoonotic viruses emerge from wildlife hosts, sometimes via livestock (Stage 1, lower panel), to cause small scale outbreaks (green) or larger chains (red) of human-to-human transmission (Stage 2, middle). In some cases, these spread more widely via air travel (Stage 3, upper). Our EIDRC proposal targets each stage: SA1 examines the diversity and spillover potential of viral threats in targeted SE Asian wildlife; SA2 seeks evidence of their spillover into focused high-risk human populations (stage 2); SA3 identifies their capacity to cause illness and to spread more widely (stage 3). Figure from (*52*).

Under a current NIAID R01, we have shown that we can use analytical approaches to target surveillance of wildlife and people and identify likely sites of spillover. We used phylogenetic analysis of bat hosts and their CoVs to identify a key cave complex in SW Yunnan Province that harbors all the genetic elements of SARS-CoV (*59*), as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics (*20, 47, 49, 50, 60-62*). We conducted behavioral risk surveys and biological sampling of human populations living close to this cave and found evidence of spillover (xx serology) in people highly exposed to wildlife. **This work provides proof-of-concept for an early warning approach that we will expand in this EIDRC to target key viral groups in**

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one of the world's most high-risk EID hotspots.

The overall premise for this EIDRC proposal is that there is substantial evidence that: 1) diverse CoVs, henipaviruses and filoviruses related to known zoonotic pathogens are circulating in wildlife reservoirs in Southeast Asia; **2)** these viruses likely spillover regularly to people, are often unreported or misdiagnosed, and their clinical manifestations and potential to cause pandemics are unknown and underestimated; and **3)** our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence. To scale-up this approach, we propose to launch **EID-SEARCH** (the **E**merging **I**nfectious **D**iseases - **S**outh **E**ast **A**sia **R**esearch **C**ollaboration **H**ub), to better understand the risk of zoonotic viral emergence in Southeast Asia. We will initially focus on the diversity of known and novel CoVs, henipaviruses, and filoviruses in wildlife reservoirs. We will use this work to build our center's interactive network throughout the region, bridge barriers, provide early focal point for basic and translational studies, develop sample handling and storage routines, communication networks, cohort management, infrastructure development, and cross-training in surveillance and novel diagnostic platforms. We will develop and transfer technology to test these viruses' capacity to infect human cells and mouse models, develop new specific and sensitive serological and molecular diagnostic tools, conduct surveillance of human communities with high risk of exposure to wildlife, and clinical cohorts to identify evidence of spillover of viruses causing previously 'cryptic' clinical syndromes in people. We will test the capacity of novel viruses we isolate, or genetically characterize, to infect human cells and/or mouse models, and thus asses potential for spillover and spread which will feed into our risk models. Our group also has extensive experience in other viruses, including vector-borne flaviviruses, and alphaviruses, and will use this experience to provide robust long-term surveillance, diagnostics, human and basic science opportunities. While the current proposal focuses its basic and applied research agendas on this group of viral EID, we note that the EID-SEARCH is poised to rapidly respond to any new emerging outbreak. Thus, filoviruses, henipaviruses and coronaviruses are examples of sentinel surveillance systems in place that can capture, identify and initiate a rapid response targeting any novel microbial pathogen that emerges in this region.

2. Innovation: Previous work by our consortium has developed strategies to target surveillance in wildlife and people to better anticipate and identify early spillover events and pre-empt outbreaks of emerging viruses. In the EID-SEARCH we scale up this approach in a central research "hub" made up of leading laboratories, clinics and surveillance sites in three critically high-risk EID hotspot countries in SE Asia. EID-SEARCH's reach is expanded through its regional network of collaborators that covers nearly all of the greater SE Asia region. The innovation of the EID-SEARCH is in: **1)** Its multidisciplinary approach that combines modeling to target geographic and taxonomic sampling targets with on-the-ground zoonotic disease surveillance in human and wildlife populations; **2)** the biological characterization approach that identifies how likely viruses are to be able to spillover into people, and enables evaluation of existing countermeasure technologies; an approach that is grounded in our successful work on SARSr-CoVs; and **3)** the strategic sampling of both high-risk communities with extensive animal contact at the front-lines of viral spillover *and* syndromic clinical cohorts that present with signs of novel viral emergence, to identify risk of viral spillover in an early warning system approach **(Fig. 2)**. **In Aim 1, we will target viruses in archived and newly collected wildlife samples, and characterize their risk of spillover to people**. We will use spatial and phylogeographic analyses to identify the geography and species of wildlife to test samples from, and to further characterize viruses we have recently discovered. We will use *in silico* methods (inovel ecological-phylogenetic risk ranking and antigenic mapping of receptor binding domains), cell culture, and animal models to assess their potential for spillover into

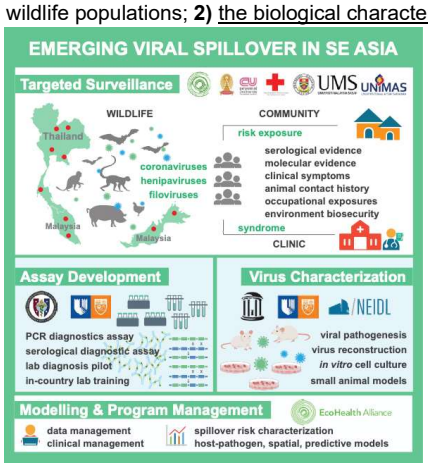

Fig. 2: EID-SEARCH approach, core members, and roles.

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Commented [PD4]: Hongying – Figure needs edits to text: top panel, 'wildlife' should read "Aim 1: WILDLIFE", "AIM 2: COMMUNITY", "AIM 3: CLINIC". Right hand side: "Clinical symptoms" should be "Clinical history", "Occupational exposures" should be "Occupational exposure", "environment biosecurity" should be "environmental risk factors"

Middle panel: "Assay Development" and "Virus Characterization" should have "(Aim 1)" after them. Left hand side: should read "PCR diagnostics", "serology", "piloting diagnostics" and the last one is ok. Right hand side bullets should be "receptor binding characterization" "in vitro characterization" and "mouse models"

Bottom panel: all good

high-risk human populations. **In Aim 2, we will conduct focused, targeted surveys and sampling of human communities with high exposure to wildlife and other animals.** We will use serological tests targeting viruses identified in Aim 1 to identify the baseline spillover of known or novel CoVs, henipaviruses and filoviruses in these populations. Serological findings will be analyzed together with clinical metadata (including a simple animal-contact survey, location data, viral sequence/isolates etc.) to identify zoonotic risk factors and routes of exposure from specific wildlife or vector species. **In Aim 3, we will use syndromic surveillance of clinical cohorts to identify evidence of viral etiology for otherwise 'cryptic' outbreaks.** We will enroll and collect biological samples from patients in local clinics and hospitals who present with syndromes previously linked to known viral agents and live within communities at high risk for disease emergence. We will conduct molecular and serological diagnostic assays to identify likely viral etiology of syndromes that may represent otherwise cryptic events. Where viruses are identified, we will attempt to isolate and characterize them, and use survey data, ecological and phylogeographic analysis to identify likely reservoir hosts and inform intervention programs. Our US partner BSL-4 laboratory, NEIDL, will attempt isolation and characterize any viruses requiring high levels of containment (e.g. any novel filoviruses discovered).

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3. Approach

Research team: The EID-SEARCH builds on long-term collaboration among world leaders in emerging virus research with proven experience collaborating internationally on field, lab and human surveillance research **(Fig. 2)**. Over the past two decades, our consortium partners have conducted high profile research on EIDs within the region and globally, including identifying the wildlife reservoirs for Nipah and Hendra virus, MERS- and SARS-CoVs (*20, 25, 59, 63-66*), discovering SADS-CoV (*13*), and developing an array of serological, molecular, *in vitro* and *in vivo* approaches to characterizing high-risk CoVs, henipaviruses, and filoviruses (*60-62, 67-80*). Our team has substantial experience conducting human surveillance in the community and during outbreaks **(See sections 2.x.x, 3.x.x)**, including: USAID-PREDICT, and DTRA-funded viral surveillance of indigenous communities in Peninsular Malaysia and syndromic surveillance of hospital patients in Sabah (Co-I Hughes); collection of >2,000 serum samples from healthy blood donors and sanitation workers, febrile and influenza like illness (ILI), and diarrheal stool samples from children under 5 years of age (Co-I Kamruddin); collaboration on the MONKEYBAR project with the London School of Hygiene and Tropical Medicine to analyze risk and prevalence of the zoonotic malaria *Plasmodium knowlesi* (Co-Is William, Tock Hing); case control and cross-sectional studies in Sabah villages (~800, 10,000 participants resp.) (Co-Is GR, TYW). As a proof-of-concept of how our team can rapidly scale up resources for diagnostics and surveillance in the face of a new EID, in 2016, we discovered a novel bat-origin HKU-2 CoV (*81, 82*) killing >20,000 pigs in S. China, designed PCR and LIPS serology tests, then surveyed pig farmers for evidence of exposure, and conducted experimental infections, pathogenesis and molecular studies, **all in the span of 3 months** (*13, 83*).

The administration of this center **(See section X)** will be led by PI Daszak, who has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5- 20 yrs on NIAID- and USAID-funded research. He will oversee all activities in this EIDRC. Drs. Olival and Zambrana are leaders in analytical approaches to targeting surveillance and head the modeling and analytics work for USAID-PREDICT. Drs. Wang, Baric, Broder, Anderson, and Laing have developed a unique array of *in vitro* cell culture, serological and molecular reagents to identify and characterize emerging viruses in people and animals (henipaviruses and other paramyxoviruses, CoVs, filoviruses and many others). Dr. Wacharapluesedee, Hughes and collaborators have coordinated surveillance of people, wildlife, and livestock within Thailand and Malaysia for the past 10 years, in collaboration with Ministries of Health, Agriculture and Environment. Finally, this consortium has extensive external funding from NIH, DoD, USAID, DHS and other USG agencies that supports a range of laboratory, analytical and field studies directly related to the current proposal, and will bring substantial leverage to our EIDRC.

Geographical focus: The three core (hub) countries for this proposed EIDRC are Thailand, Malaysia and Singapore, three contiguous EID hotspot countries that stretch through one of the most significant foci of EID origins globally. Our proposed work includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sarawak and Sabah, and thus contains overlapping wildlife biogeography with India, Bangladesh, Myanmar, SW China, Laos, Cambodia, Vietnam, Philippines, and Indonesia. It encompasses diverse cultural and

socioeconomic backgrounds which have correspondingly diverse and intensive interactions with wildlife reservoirs and the global travel network. We have already begun sampling of wildlife hunters, livestock workers, wildlife farmers and market workers, rural villagers living close to high risk wildlife species, people otherwise highly exposed to animals in these countries, and clinical cohorts, as part of current/prior NIH, DTRA, and USAID funding. To develop a larger catchment area for emerging zoonoses, we will deploy our core member's extensive network of collaborators in clinics, research institutes and public health laboratories in almost every other Southeast Asian country. We will conduct regular meetings with key research, public health and community leaders, including >50 leading laboratories/institutions in XX countries that are currently collaborating with core members of our consortium via other funded work (**Fig. 3**). We will use these, and our annual meetings, to share information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks across the region.

Fig.3 : Map of Southeast Asia indicating three hub countries for this proposed EIDRC (Red: Thailand, Singapore, Malaysia) and countries in which Key Personnel have collaborating partners (Green), indicating that EID-SEARCH provides access to key collaborators throughout the region.

Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife

1.1 Rationale/Innovation: The vast majority of emerging zoonotic pathogens are linked directly or indirectly to wildlife host, particularly from important mammalian groups like bats, rodents and non-human primates (*84*). Our previous work on wildlife origin viruses demonstrates that zoonotic viral emergence tends to occur in specific regions with high diversity of wildlife that harbor viruses of zoonotic potential, and human populations that have close contact to these wildlife **(Fig. 1)** (*2*). In Aim 1, we will strategically conduct EID surveillance by applying risk analytics to prioritize the regions, wildlife species, and human populations that have a particularly high risk of viral spillover. We will target high-risk zoonotic host reservoirs (bats, rodents and primates) in these regions and apply novel predictive models to estimate viral diversity and host range to identify wildlife species that have been ignored or under-sampled by previous zoonotic disease surveillance efforts. We will strategically collect specimens from these species and screen them, together with some of the tens of thousands of recently collected samples we have archived in freezers in our labs, for known and novel agents. We will begin screening using conserved PCR assays to identify known and novel virus in high risk RNA viral families, *Coronaviridae*, *Paramyxoviridae*, and *Filoviridae*. We will expand to other groups of pathogens, pending available resources and priorities established with the EIDRC coordinating center (EIDRC-CC) and other regional EIDRCs. Using risk ranking algorithms and a series of ecological and virological risk factors, we will triage the wildlife viruses we discover for further genomic characterization and viral isolation. On this subset of viruses, we will then use *in vitro* and *in vivo* approaches (viral rescue, cell lines, gene edited humanized transgenic mice and the collaborative cross genetic reference mouse population) previously developed and widely used by our team to predict capacity of novel viruses to infect people and spillover (*61, 85-88*). These high-risk viruses and their close relatives will be targets for human community and clinical sampling in Aim 2 and 3, respectively.

1.2 Preliminary data: 1.2.a. Geographic targeting: EcoHealth Alliance has led the field in conducting analyses to indicate where the highest risk of zoonotic spillover is across the globe, identifying SE Asia as a major high risk location for pathogen emergence (*1, 2*). For Southeast Asia, these EID hotspots are sites where high wildlife biodiversity, human population density and rapid ecologic change collide **(Fig 1)**. Using host and ICTV viral data for all known mammalian viruses, and generalized additive models to correct for sampling bias, we are able to predict the relative number of yet-to-be-described viruses that a species harbors (*2*). Using these data to map unknown viral diversity demonstrates substantial undiscovered viral diversity in mammals across Southeast Asia, and particularly in regions of Thailand, Singapore and Malaysia **(Fig. 1)**. In a separate paper on risk of viruses emerging from bat hosts, we found that factors driving spillover (host diversity, climate)

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differ from those driving spread in human populations (population density, bushmeat hunting, livestock production) (*89*). We therefore analyze capacity for viral spread as a separate risk factor, using demographic and air travel data and flight model software we have produced with Dept Homeland Security funds (*90*). In the current proposed work, we will use all the above approaches to target wildlife sampling (archive and new field collections), and to inform sites for human sampling in Aim 2, to areas of high risk for spillover and spread.

1.2.b. Reservoir host species targeting: Our analysis of all known mammalian host-viral associations demonstrates that bats, rodents and primates represent the three most important reservoirs for zoonoses, responsible for the highest proportion of known and predicted viral diversity of zoonotic potential(*91*). Thus, the bulk of our proposed wildlife sampling and testing will consist of these three taxa with a focus on bats, because this group is the known or hypothesized reservoir for the majority of high-impact zoonotic CoVs, henipaviruses and filoviruses (*48, 92-94*). Bats also have the highest proportion of predicted zoonotic viruses in any mammalian group (*2*), and are exceedingly diverse in Southeast Asia (~500 species). To further prioritize taxonomic sampling targets within these diverse geographic hotspots, we have used a novel, Bayesian phylogeographic analysis to identify host taxa that are the most important centers of evolutionary diversity for a given viral group, e.g. bat genera for β-CoVs using an extensive CoV sequence dataset from our NIH-funded research **(Fig. 5)** (*49*). This approach will be extended to other mammal taxa and viral families of interest, allowing us to identify the ancestral wildlife hosts of specific zoonotic viral groups, where their diversity is likely highest.

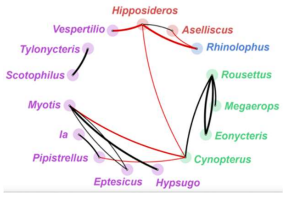

Fig 5: Preliminary analysis to identify the most important bat genera in Southeast Asia as hosts for beta-CoVs using our previously-collected CoV sequence data. Line thickness is proportional to the probability of virus sharing between two genera. Inter-family switches are highlighted in red. We will apply this Bayesian discrete phylogeographic model to identify taxonomic and geographic centers of viral diversification and sharing for relevant paramyxovirus, coronavirus, and other virus clades.

Finally, we have designed a strategy to estimate sampling gaps, using a viral 'mark-recapture' approach we previously published (*95, 96*) **(Fig. 6)**. Using prior data for bat, rodent and primate viral discovery vs. sampling rates, we can identify the likely total viral diversity for specific host groups in a region, and use this to estimate sample sizes to discovering the majority of unknown viruses within a specific family or genus. **In the current proposal,** we will use these analyses to estimate geographic and species-specific sampling targets **to more effectively identify new strains to support experimental infection studies and risk assessment.**

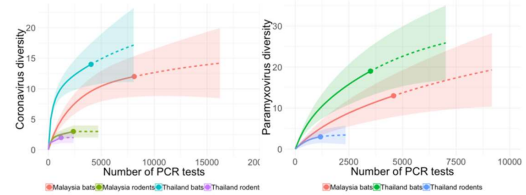

Fig. 6: Estimated coronavirus **(left)** and paramyxovirus **(right)** putative viral 'species' diversity in bats and rodents for Thailand and Malaysia, using RdRp gene sequence preliminary data from >13,000 PCR tests in bats and 4,500 in rodents. Bats in Thailand and Malaysia have 4X the number of viruses than rodent species, controlling for sampling effort. CoV and paramyxovirus diversity estimates are comparable, but discovery has not yet saturated in any taxonomic group or location. We estimate that additional sampling of ~5,000 bats and testing of our archived rodents specimens alone will identify >80% of remaining CoV and paramyxovirus viral species in these key reservoirs. We will use this approach to define sampling targets for other wildlife taxa, and for viral *strain* diversity.

1.2.c. Sample testing to identify known and novel viruses: We have conducted extensive wildlife surveillance for novel viruses across Southeast Asia in prior NIAID funded work and as part of the USAID PREDICT project. Under the 10-year PREDICT program globally, we identified 912 novel and 210 known viruses, more than the total number of viruses previously recognized in mammals by the International Committee on Taxonomy of Viruses. This work included collecting nearly 300,000 individual mammal samples from 14 EHA-led PREDICT countries and PCR-screening more than 60,000 individual specimens(*51*). In southern China alone, this sampling lead to the identification of 178 β-CoVs, of which 172 were novel (52 novel SARSr-CoVs). Included were members of a new β-CoV clade, "lineage E" (41), diverse HKU3-related CoVs (179 sequences) within a 'sister' clade to the SARS-CoV lineage, and the wildlife origin of a new virus, SADS-CoV, responsible for killing >20,000 pigs in Guangdong Province (*13*). Many of the identified bat species are

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found across the region. We have collected 28,957 samples from bats, rodents and primates in Thailand and 51,373 in Malaysia, conducting 146,527 PCR tests on a large proportion of these specimens. We have **archived duplicate samples which are now available for use in this project**, including fecal, oral, urogenital and serum samples from bats, rodents, and non-human primates, and primate biopsies. In Thailand this screening has identified 100 novel viruses and 37 known viruses in wildlife reservoirs. In Malaysia this screening has identified 77 novel viruses and 23 known viruses in wildlife reservoirs (66 novel and 19 known in Sabah and 11 novel and 9 known from Peninsular Malaysia). We used family level primers for henipaviruses, filoviruses and CoVs and have already discovered 9 novel and 7 known CoVs and 26 novel and 2 known paramyxoviruses in Thailand. This is in addition to 13 novel and 5 known CoVs and 15 novel and 1 known paramyxovirus in Malaysia from wildlife. In the current proposed work, we will attempt to sequence, isolate and characterize those viruses that our analyses below suggest are most likely to be able to infect humans.

Our collaborative group has worked together on these projects, as well as other DTRA funded projects in Singapore/Thailand (Co-Is Broder, Wacharapleusadee, Wang) to design, cross-train, and use novel serological and molecular platforms to investigate virus infection dynamics in bats and examine potential spillover events with or without human disease. For serology, Co-Is Wang and Anderson (Duke-NUS) will develop a phage-display method to screen antibodies for all known and related bat-borne viruses, focusing on henipaviruses, filoviruses and coronaviruses. Once various "trends" are discovered, additional platforms (including Luminex and LIPS) will be used for verification and expanded surveillance. For molecular investigation, we will combine targeted PCR approach with a discovery-driven enrichment NGS strategy to not only survey currently known viruses, but also to discover potential novel viruses. Both the phage-display serological and enrichment platforms can be rapidly updated to include new viruses identified. In Malaysia (Co-Is Hughes, Broder, Laing) we have transferred the Luminex serology platform to partner laboratories for screening of wildlife, livestock and human samples to investigate the spillover of henipaviruses and filoviruses at high risk interfaces. These interfaces include farms near the forest and indigenous communities with lots of subsistence hunting in Peninsular Malaysia, and preliminary screening has found serological evidence of past exposure to viruses antigenically-related to Nipah and Ebola in humans, bats and non-human primates (NHPs). In Thailand, Co-Is Broder, Laing and Wacharapluesadee have transferred a Luminex-based serology platform for detecting exposure to novel Asiatic filoviruses and known henipaviruses in wildlife and human samples, with testing underway and currently being validated in our Chulalongkorn University laboratory **(See also section 2.2.d)**.

1.2.d. <u>In vitro</u> & <u>in vivo</u> *characterization of viral potential for human infection:* Using Coronaviridae as an example, we have conducted *in vitro* and *in vivo* experiments to characterize the pathogenic potential of novel SARSr-CoVs from bats. We isolated three SARSr-CoVs from bat feces: WIV1, WIV16 and Rs4874, with S protein sequences that diverged from SARS-CoV by 3-7% (*20, 59, 97*). We conducted full-length genome sequencing of 12 other novel SARSr-CoVs, some highly similar to outbreak SARS-CoV in the most variable genes: N-terminal domain and receptor binding domain (RBD) of the S gene, ORF8 and ORF3 (*59*). Using our reverse genetics system, we constructed chimeric viruses with SARSr-CoV WIV1 backbone and the S gene of two different variants. All 3 SARSr-CoV isolates and the two chimeric viruses replicated efficiently in Vero E6 cells and in HeLa cells expressing hACE2, but not in those without ACE2 (*20, 59, 97*). We used the SARS-CoV reverse genetics system (*71*) to generate a chimeric virus with a mouse-adapted SARS-CoV backbone expressing SHC014 S protein with 10% sequence divergence from SARS-CoV S. This chimera replicated in primary human airway epithelium, using the human ACE2 receptor to enter into cells (*60*). Thus, **SARSr-CoVs with diverse variants of SL-CoV S protein without deletions in their RBD can use human ACE2 as receptor for cell entry.** The Baric lab has a well-established hACE2 and DPP4 transgenic mouse model that we will use this to characterize the capacity of specific SARSr-CoVs to infect (*85, 98*). We used this chimera approach to demonstrate that pathogenicity of bat SARSr-CoVs in humanized mice differs with divergent S proteins, **confirming the value of this model in assessing novel SARSr-CoV pathogenicity**. Infection of rWIV1-SHC014S caused mild SARS-like clinical signs in the transgenic hACE2 mouse model **that weren't reduced by immune-therapeutic monoclonals that attenuate SARS-CoV pathogenicity, nor after challenge following vaccination against SARS-CoV** (*60*). We repeated this virus characterization approach with chimeras using HKU3r-CoV S proteins that are ~25% divergent from SARS-CoV S, **and found that they**

are unable to use the ACE2 receptor. Additionally, we were unable to culture HKU3r-CoVs in Vero E6 cells, or human cell lines.

A similar approach to the above pipeline for CoVs will be applied to novel henipaviruses and filoviruses we discover during our research. The broad mammalian tropism of Hendra virus and Nipah virus (*67*) is likely mediated by their usage of highly conserved ephrin ligands for cell entry (*15, 99*). A third isolated henipavirus, Cedar virus does not cause pathogenesis in animal models. Recently, **Co-Is Broder and Laing** have developed a reverse genetics system and rescued a recombinant CedV to test ephrin receptor usage/tropism and pathogenicity during henipavirus infections (*76*). CedV is unable to use the Ephrin-B3 receptor (*76, 100*) which is found in spinal cord and may underlie NiV encephalitis (*101*) and that pathogenesis also involves virulence factors V and W proteins. The putative henipaviruses, Ghana virus and Mojiang virus, predict V and W protein expression, with GhV able to bind to ephrin-B2, but not -B2 (*102*), and the receptor for MoJV remaining unknown (*103*) but is likely ephrin-B2 (*104*). Primary human lung endothelial cells are highly susceptible to Ebolavirus infection (*105*). Huh7 cells are good candidates for ebola in the liver, oftentimes used to isolate virus from clinical samples (*106*) Huh7 cells also offer advantages for reverse genetic recovery of novel filoviruses (*107*). Thus far, paramyxovirus attachment glycoproteins have been divided into three major groups: hemagglutinin (H), hemagglutinin-neuraminidase (HN), and attachment glycoproteins (G).

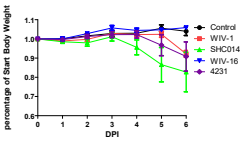

Fig. 7: Body weight change in hACE2 transgenic mice after SARSr-CoV infection, demonstrating that bat-origin SARSr-CoVs cause similar clinical presentation to outbreak strain (4231). From (*60, 61*).

Models of Outbred Populations. For novel viruses across all families, we will use the UNC collaborative cross mouse that captures 90% of the diversity within all mouse groups to assess the most suitable model for further experiments (*108*). We have used this model for CoV, filo (Ebola), Flaviviruses, and alphaviruses infections. In subpopulations it reproduces SARS weight loss, hemorrhage in EBOV, enceph in WNV, acute or chronic arthiritis in Chikungunya infection (*86-88, 109-112*). *Bat Models*. Duke-NUS has developed two models for bat *in vivo* studies: a colony of *Eonycteris spelaea* (cave dwelling small fruit bat) and a bat mouse model, which contains the bat immune system in an immunodeficient mouse (*113*).

1.3. General Approach: We will conduct analyses to geographically- and taxonomically-target testing of samples from wild mammals that are most likely to harbor known high-profile zoonotic pathogens, or close relatives with potential to infect people. This includes selecting from tens of thousands of samples collected on recent projects and stored in freezers in our laboratories, and new sampling of wildlife in high risk locales. We will use viral family level PCR screening to identify and partially characterize viruses, then follow up sequencing of relevant receptor binding proteins to assess homology and predict binding affinity, then attempt to isolate and biologically characterize viruses that phylogenetic and other data suggest have high spillover and pandemic potential. We will follow this with *in vitro* binding assays and cell culture experiments, and *in vivo* animal model infections to assess their potential to infect people and cause disease **(Fig. 8)**. EHA will lead the study design, targeted sampling, and data analysis; Co-Is Hughes and Wacharapluesadee will lead field sampling and in-country testing; Duke-NUS, UNC, USUS will lead the serological and PCR diagnostic development and the *in vitro* and *in vivo* analyses, as well as cross-training and tech transfer to the hub labs in Thailand and Malaysia. This will include via support of one Malaysian Ph.D student, Mei-Ho Lee (CM Ltd.) who has worked with the EID-SEARCH collaborators for 9 years. The focus of her study will be infection potential of novel CoVs and paramyxoviruses already found in bats in Malaysia under preliminary data for this proposal.

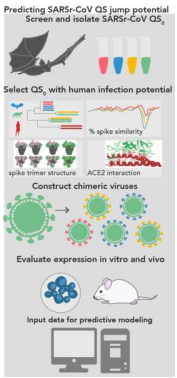

Fig. 8: Schematic showing EID-SEARCH approach to selection of sampling, viral testing, prioritization and characterization to identify novel, high zoonotic-risk viruses in wildlife.

1.4 Wildlife samples: 1.4.a. Geographic and taxonomic targeting for newly collected wildlife samples: We will prioritize sites within each country that rank highest for both the risk of zoonotic disease emergence

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Commented [PD20]: Ralph – please add data from the CC mouse re. filoviruses, to beef up the image of us as a filovirus group

Commented [PD21]: Linfa/Danielle – please draft a brief para explaining how we'll use these two models

Commented [PD22]: Hongying – this is a placeholder from DARPA proposal. We need a new one with Stages: 1) Geographic and taxonomic modeling to maximize discovery of potential zoonotic viruses; 2) PCR screening and partial RNA sequencing; 3) Full genome or RBD and Glycoprotien sequencing to model receptor binding based on sequence similarity; 4) Cell line infections (for prioritized viruses); 5) animal model infections (for prioritized); 6) High priority viruses ranked based on data (red, yellow, green) schematic.

(*114*) and the predicted number of 'missing' zoonotic viruses (*91*). Our preliminary analysis **(Fig. 1)** suggests priority areas include: the Isthmus of Kra (S. Thailand and N. Peninsular Malaysia), NW Thailand (near the Myanmar-Laos border), and the lowland rainforests of Sarawak and Sabah. In each of these 'hottest of the hotspots' regions, we will identify 2-4 distinct sampling sites based on the geographic distribution of key hosts we prioritize for intensive wildlife sampling. We will priority rank bat, rodent, and primate species using analysis of host trait data for the highest predicted number of viruses based (*91*). We will also identify species predicted to be centers of evolutionary diversity for Corona-, Paramyxo-, and Filoviridae using ancestral host reconstruction of available sequence data and Bayesian discrete phylogeographic models (*115-117*). We will ensure wildlife sampling is complimentary to specimens archived in our extensive freezer banks. We will collect only the additional specimens projected from each prioritized taxa to achieve statistically robust sample sizes given predicted levels of viral richness and rates of discovery **(see 1.4.b)**. We will leverage our strong collaborations with wildlife experts in Malaysia and Thailand to ground-truth geographic and taxonomic model outputs with local expert knowledge to ensure the sampling plan is realistic based on current species distributions, population size estimates, seasonality, and accessibility to sites.

Recognizing that most zoonotic viruses are multi-host, i.e. naturally harbored by multiple wildlife species, and that the phylogenetic relatedness of those hosts is predictable and varies across viral families (*91*) – we will also apply a generalizable phylogenetic and spatial modeling approach to rapidly predict new (unsampled) hosts for novel viruses we discover during our research. We will use a combined network analysis and a phylogeographic model to estimate a viruses' host range and to prioritize additional wildlife species for sampling and testing for viruses we discover. Our recently developed model of viral sharing uses just two phylogeographic traits (wildlife species range overlap and phylogenetic similarity between host species) to successfully predict host species in the top 2% of all 4,200 possible mammal species (*118*).

1.4.b. Sample size justifications for testing new and archived wildlife specimens: We will use our preliminary data on average PCR prevalence from screening tens of thousands of bat, rodent and primate specimens for CoV and Paramyxoviruses under the USAID-PREDICT project in Malaysia and Thailand. We will refine these sample size targets, in real-time, over the course of our project using viral discovery curve analyses **(Fig. 6)** to either scale-up or scale-back sampling and testing of archived specimens for each species depending on their viral diversity. For example, given 5-12% prevalence of CoVs in the most common bat species we previously-sampled in Thailand and Malaysia, a target of XXX individuals per species would yield XX (± x) PCR positive individual bats, and an estimated ~xx novel strains. Similarly, for paramyxoviruses with an average PCR detection rate of X%, we would need XX individuals per species to detect 80% of predicted viral strain diversity with 80% power. Preliminary analysis indicates we will require XX,000 samples to identify 80% of remaining viral diversity **(Fig. 9)**. All wildlife sampling will be nondestructive. In the event that an animal is found dead or needs to be euthanized due to injury we will collect biopsy samples for screening.

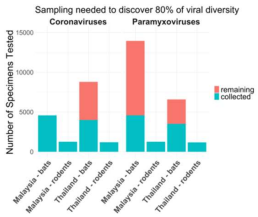

Fig. 9. Stacked bar plot showing number of currently-archived specimens **(blue)** and new specimens required **(orange)** to discover 80% of predicted number of CoVs and PMVs from high-risk bat and rodent taxa in Malaysia and Thailand

We estimate the following sampling effort, broken down for each region: *Peninsular Malaysia:* Wildlife sampling will be led by CM Ltd / EHA in collaboration with PERHILITAN. Archive bat, rodent and NHP samples will be screened serologically using the BioPlex and other serology techniques identified through this project. New wildlife sampling will focus on bats but it is expected that rodents and NHPs will be sample as well. Wildlife sampling will be conducted around Orang Asli communities sampled as part of aim 2. Three new communities will be identified in the districts we have already sampled in. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife mainly bats will be sampled. Wildlife and domestic animal samples will include serum, whole blood, throat swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and tested at PERHILITAN's NWFL. *Sarawak, Malaysia:* Wildlife sampling will be led by CM Ltd / EHA in collaboration with UniMAS. Wildlife sampling will be conducted around Dyak communities sampled as part of aim 2. One community will be identified. Concurrent sampling trip will be

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conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife mainly bats will be sampled. Wildlife and domestic animal samples will include serum, whole blood, throat swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and tested at UniMAS or BMHRC. *Sabah, Malaysia:* Bat sampling will be led by CM Ltd / EHA in collaboration with SWD and WHU. Bat sampling will initially focus on 3 caves. Batu Supu Sabah low disturbance cave and surrounding area, Madai medium disturbance cave and surrounding area, Gomantong cave high disturbance cave and surrounding area. Each cave will be sampled twice once in wet season and once in dry season targeting 500 bats at each cave on each trip for a total of 3000 bats. Wildlife samples will include serum, whole blood, throat swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored at WHGFL, molecular testing will be conducted at WHGFL and serology screening at BMHRC. In addition at Madi cave the community that lives in the mouth of the cave will also be sampled targeting 150 people, while at Gomantong cave 25 workers involved in the bird next farming will be enrolled into the study. *Thailand:* XXXXXXXXXX

1.4.c. Sample testing, viral isolation: Viral RNA will be extracted from bat fecal pellets/anal swabs with High Pure Viral RNA Kit (Roche). RNA will be aliquoted, and stored at -80C. PCR will be performed with pan-coronavirus, filovirus and paramyxovirus primers. PCR products will be gel purified and sequenced. We will attempt isolation on samples that contain viruses of interest (determined in **1.4, below**), using Vero cells and primary cell culture from the wildlife taxonomic order (bat, rodent and primate cell lines). This includes the over 30 bat cell lines maintained at Duke-NUS from four different bat species.

1.4.d. Moving beyond RNA viruses: To detect pathogens from other families, such as non-RNA viruses we will combine conserved PCR assays with NGS. We will perform either unbiased NGS, or use a pan-viral enrichment library to sequence full-length genomes. We will conduct this work via EID-CC research projects or as part of outbreak detection/investigation in the region from DNA viruses or other agents.

1.4.e. Sequencing Spike Glycoproteins: Based on earlier studies, our working hypothesis is that zoonotic coronaviruses, filoviruses and henipaviruses encode receptor binding glycoproteins that elicit efficient infection of primary human cells (e.g., lung, liver, etc.) (*12, 59, 60*). Characterization of these suggests SARSr-CoVs that are up to 10%, but not 25% different in the spike glycoprotein use human and bat ACE2 receptors for docking and entry. Uneven sampling gaps (e.g., no strains were found with 10-25% spike variation) prevent a thorough understanding of the transition point where the most divergent strains lose human ACE2 receptor usage. Our viral discovery curves **(Fig. 4)** suggest further sampling will reveal a rich diversity of as-yet-undiscovered SARSr-CoVs. In this aim we will use phylogenetic and viral discovery analysis to specifically target bat species and regions that are undersampled to allow sufficient power to identify and characterize missing SARSr-CoV strain diversity. Our previous work also suggests that SARSr-CoVs with S proteins that are ~10% divergent from SARS-CoV resist neutralization by therapeutic mAbs and escape SARS-CoV vaccines (*20, 60, 119*), suggesting that existing vaccines and immunotherapeutics could easily fail against future emerging SARS-like CoV. Moreover, viruses with 10-25% divergence in S proteins may bind to human cell receptors, but completely evade therapeutic and vaccine effects, and could therefore be a higher risk for public health. We will sequence the S proteins of novel SARSr-CoVs to prioritize viruses for experimental work in Aim 3 to test this hypothesis. Likewise, we will test the hypothesis that phylogenetic related zoonotic receptor binding protein genes of MERS-related, Ebola-related and Nipah/Hendra-related viruses will also program efficient entry via human ortholog receptors. For all novel virus strains, we will sequence the complete entry spike genes by amplifying overlapping fragments using degenerate primers as shown previously (*20, 59*). Full-length genomes of selected CoV strains (representative across subclades) will be sequenced using NEBNext Ultra II DNA Library Prep Kit for Illumina and sequenced on a MiSeq sequencer. PCR or MinIon sequencing will be performed to fill gaps in the genome. The full length spike gene sequences, including the amount of variation in the receptor binding residues that bind the appropriate human receptor will be used to select strains for Aim 3 experiments.

1.5. Assessing risk for spillover. 1.5.a. Capacity to infect human cells: We will use a series of cell cultures to assess the capacity of target viruses to enter human cells. For CoVs, filoviruses and henipaviruses, we will

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Commented [PD32]: Linfa – can you give a v. brief description of these please, e.g.: "This includes 13 primary lung, 5 primary gut epithelial, and 1 primary liver cell line. In addition we have 12 immortalized cell lines, 7 of which are intestinal, and therefore suitable for CoVs. Cell lines are derived from insectivorous and frugivorous bats (genera……)"

use primary human ciliated airway epithelial cells (HAE) and lung endothelial cell cultures from the lungs of transplant recipients represent highly differentiated human airway epithelium containing ciliated and non-ciliated epithelial and goblet cells, grown on an air-liquid interface for several weeks prior to use (*60, 61, 120*). We will prepare HAE and lung endothelial cultures from three different patient codes in triplicate in collaboration with the tissue procurement facility at the Cystic Fibrosis Center at UNC. Cultures will be inoculated with chimeric bat SARSr-CoVs or other test filoviruses or henipaviruses to assess efficient replication. At 72 hpi, cultures will be fixed for immunofluorescent staining using antisera to the SARS-CoV conserved nucleocapsid protein (N) or to structural genes of the other viruses (*62, 70*). SARSr-CoVs that differ significantly in S protein sequence (11-24%) from epidemic SARS-CoV yet replicate *in vitro*, will also be evaluated for sensitivity to neutralization in Vero cells by PRNT50 assays using broadly SARS-CoV cross reactive human mAbs S227.14, S230.15, S215.17, and S109.8 (*61, 121*) or monoclonal antibodies targeting the Ebola or Marburg E glycoprotein (Available through BEI), or Nipah or Hendra viruses (*122*). As controls or if antisera is not available, the S genes of novel SARSr-CoV or spike entry genes of other novel viruses under investigation will be inserted into VEE 3526 replicon vectors (VRP3526-S), packaged and used to vaccinate mice (*123*). Polyclonal sera against SARS-related viruses will be harvested and tested for ability to cross neutralize SARS-CoV, GD03, WIV-1, SHC014, WIV-16, other novel SARSr-CoV and HKU3-SRBD by PRNT50 assay (*61, 124, 125*). Using PRNT50 titers with sera (n=4 each) among these viruses, antigenic cartography (*126*) will allow comparison of antigenic vs. phylogenetic distance, identifying the transition at which SARSr-CoV strains escape SARS-CoV based vaccines, informing immunotherapeutic and vaccine design strategies (*127-129*). Similar approaches will be applied to novel MERS-related viruses, other CoV, filoviruses or henipaviruses. While most wildlife-origin viruses with unknown transmission potential or pathogenicity in people are BSL-2 or -3, cell culture for potentially BSL-4 pathogens will be undertaken at collaborating institute NEIDL. To facilitate this, duplicate samples in culture medium from animals PCR +ve for filoviruses will be shipped to NEIDL **(See letter of support)** for culture, characterization and sharing with other approved agencies including NIH RML. When appropriate of feasible, training opportunities at NEIDL for in-country staff will be given.

1.5.b. Host ACE2, DPPR, Niemann-Pick C1 (NPC1) and EphrinB2/EphrinB3 receptors: We will sequence host ACE2 receptors of different bat species or different bat populations from a single species (e.g. *Rhinolophus sinicus*) to identify relative importance of different hosts of high risk SARSr-CoVs and the *intra*specific scale of host-CoV coevolution. Of particular interest is homology across 18 bat and human orthologue ACE2 contact interface residues that engage the SARS RBD as a potential indicator of SARSr-CoV cross species transmission potential and growth in human cells (*69*). A lot of variation in the NPC1 receptor alters EBOV ability to infect in humans and bats, so this can be structurally mapped for prediction and downselection of strains (*130*). Signatures of positive selection in bat NPC1 were concentrated at the virus-receptor interface, with the strongest signal at the same residue that controls EBOV infection in *Eidolon helvum* cells. Thus NPC1 is a genetic determinant of filovirus susceptibility in bats, and some NPC1 variations likelyreflect host adaptations to reduce filovirus replication and virulence (*131*). Nipah and Hendra use ephrin B2 or ephrin B3 as a receptor, and variation affects its ability to use human vs bat receptor molecules (*102, 132*).

1.5.c. Host-virus evolution and distribution: We will use Bayesian and Maximum Likelihood phylogenetic analyses of RdRp (or L genes), receptor binding glycoproteins, and full genome sequence (when available) data to reconstruct the evolutionary history of the novel bat SARSr-CoVs, bat MERSr-CoV, filoviruses and henipaviruses that we identify. We will rerun MCC analyses **(Fig. 3)** to reconstruct β-CoV evolutionary origins using our expanded dataset. Ecological niche models will be used to predict spatial distribution of PCR-positive species, identify target sites for additional bat sampling, and analyze overlap with people. We will use our viral discovery/accumulation curve approach **(Fig. 4)** to monitor progress towards discovery of >80% of predicted diversity of SARSr-CoVs (lineage 1 & 2) or other virus families within species and sites, halting sampling when this target is reached, and freeing up resources for work other work (*96, 133*).

1.5.d. Viral strain prioritization: Of the expected 100-200 novel SARSr-CoV strains, we will down-select to prioritize for further characterization based on S genes that are: i) different from SHC014, WIV1, SARS-CoV with diversity ranges of 10-25%; ii) have virus S RBD that could use human/bat receptors; iii) have

Commented [BRS33]: Any antibodies available? If not we can synthesize them from published co-crystals.

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recombinant chimeric spikes indicative of gene flow between clade I and II strains; iv) have bat ACE2 receptors that might select for spike RBDs that can use human receptors for entry (15/18 conserved residues in human/bat ACE2 molecules that bind SARSs-CoV S RBD domains are likely more efficient receptors than 3/18 conserved sites). Using structural models based on the SARS S glycoprotein, the extent and location of antigenic variation will be annotated onto the structure, further resolving the locations of highly evolving and conserved structural motifs/epitopes that function in protective immunity (*121, 134-136*). For novel henipaviruses, we will examine the receptor-binding proteins (e.g. envelope attachment (G) proteins) for sequence and structural homology with particular attention to conversation of amino acids that mediate ephrin receptor binding. We will prioritize strains that are predicted to interact with ephrin-B2 and expression V/W proteins to determine whether these viruses represent zoonotic threats. Using the recombinant Cedar virus (rCedV) platform, we will be able to rescue rCedV that express heterotypic G proteins to explore receptor utilization and tissue dissemination in animal models. We will assess ability to infect human cells using a continuous lung epithelial cell line, Calu3, that our group has previously used to generate data on NiV and HeV growth curves and a variety of host expression changes after infection (*137*). We will also use primary human airway cultures to assess human infection for Henipavirus and MERS- and SARS-related CoV (*138*) (*139*) . There is some evidence for Nipah and Hendra virus replication in mouse lung, low level titers in young animals, 10 fold higher titers in aged mice (*140, 141*). Thus it is likely these models could be improved in the collaborative cross mouse resource at UNC. We have identified CC lines that are highly vulnerable to wildtype SARS and others that develop hemorrhagic disease following mouse adapted Ebola infections (*86-88*).

1.5.e. Animal models: We will use transgenic mouse models and the UNC Collaborative Cross model to assess spillover potential of viruses. For work on CoVs, we will follow the protocols in the transgenic mouse model used by the Baric lab previously. Briefly, in the BSL3 lab, n=8 10- to 20-week old hACE2 or hDPP4 transgenic mice will be intranasally inoculated with 5 x 10^4 PFU of wildtype WIV-1 or chimeric bat SARSr-CoVs with different spike proteins or MERS-CoV and MERS-related CoV, respectively, then monitored daily for weight loss, morbidity, and clinical signs of disease. Mice will be euthanized at 2, 4, and 6 dpi (endpoint of 14 dpi), organs harvested and virus quantified by NP RT-PCR. After 7 days in formalin, tissues for histopathology will be removed from the BSL3 and stained with H&E, and for immunohistochemistry using polyclonal antibodies against the N protein. We will conduct limited evaluation of existing countermeasures using therapeutic monoclonal antibodies *in vitro* and *in vivo*. Existing SARSr-CoV or MERS-CoV mAbs (*142, 143*) will be diluted in DMEM starting at 1:20, and serial 2-fold dilutions mixed with an equal volume of 50 PFU of chimeric bat SARSr-CoVs with different spike proteins, then incubated on Vero E6 cells at 37°C for 1 h, then changed to a semi-solid medium (DMEM containing 1% methylcellulose and 2% FBS). Antibody neutralization titers will be evaluated by plaque quantification at 4-5dpi. hACE2 transgenic mice will be injected with SARS-CoV mAbs, and infected with chimeric bat SARSr-CoVs. Clinical signs and morbidity will be assessed and tissue pathology examined and compared with mice without treatment of mAbs to determine the therapeutic effect on SARSr-CoV infection, and protection of SARSr-CoV by wildtype SARS-S based vaccines assessed as described (*120, 144*). We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-CoV cross neutralization, synthetically reconstruct a small subset (1-2) and evaluate the ability of nucleoside analogues to inhibit growth in HAE cultures and/or *in vivo* (*61, 120*). For the Collaborative Cross model, we will….

Finally, for more detailed pathogenesis studies in the natural reservoir, we will use the bat models (natural and transgenic mouse model) at Duke-NUS. Both animal systems can be used to test susceptibility, pathogenesis and immune responses of any newly discovered viruses.

1.6. Potential problems/alternative approaches: **to sample bats in sites or provinces we select**. We have a >20-year track record of successful field work in Malaysia, Thailand and >10 years in Singapore, and have worked with local authorities and communities to ensure access. We have existing permissions in place which will be renewed at the start of this project. Due to the abundance of wildlife habitats in the region, if access to a site is withdrawn, we will rapidly identify a suitable, complementary site with similar bat species composition and abundance. Finally, we have already collected tens of thousands of wildlife samples from the region and will focus on these while waiting for permission. **We may not identify novel viruses in our sample wildlife species due to seasonality of viral shedding or other factors.** We have calculated sample

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sizes based on a range of PCR data and are fairly conservative in our approach. However, as a back-up, we already have identified dozens of novel viruses that are near-neighbors to known high-impact agents, and will continue characterizing these should discovery efforts yield few novel viruses. The sampling regions we have selected are subtropical, and our previous data, and even published studies in temperate regions (*145*), do not suggest a strong pattern of seasonality in CoV shedding, although there are studies that suggest this occurs in henipavirus and filovirus (MARV?) shedding (REFS?). Nonetheless to account for this we will conduct sampling evenly on quarterly basis within each province.

Aim 2: Test evidence of viral spillover in high-risk communities using novel serological assays.

2.1 Rationale/Innovation: Rapid response requires early detection. Thus, assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities that interface frequently with wildlife that harbor high risk emerging viral strains. To enhance the low statistical probability of identifying these rare events, we will target rural human communities inhabiting regions of high wildlife biodiversity, that also engage in practices that increase the risk of spillover (e.g. hunting and butchering wildlife). In Aim 2 we will build and expand on our preliminary biological sampling and enroll large cross-sectional samples in human populations with high behavioral risk of exposure to wildlife-origin viruses and that live close to sites identified in Aim 1 as having high viral diversity in wildlife. We will initially identify 2 sites in each of Thailand, Peninsular Malaysia, Sarawak, and Sabah for repeated biological sampling. We will design and deploy behavioral risk factor questionnaires, and specific and sensitive serological assays to identify the baseline frequency, and causes of, spillover of known and related novel viral pathogens in these populations. In participants where symptoms are reported in the last 10 days, paired serum specimens (acute and 21 days after fever) will be collected to determine titer of positive antibody. Samples will be marked for additional PCR-based assays to identify presence of known and novel CoVs, henipaviruses and filoviruses, and for isolation and biological characterization of potential pathogens, using the approach laid out in Aim 1.

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Commented [PD41]: I think that would be good, depending on the sample size

Commented [KJO42]: Need to decide if we do PCR on community samples, we can add in the questionnaire if you've been sick in the last 10 days and if so, then maybe test only this subset.

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Country, site	Lead	# enrolled, focus	Findings
Peninsular Malaysia	Hughes	1,390 Orang Asli indigenous population, PCR/serol.	25+ novel CoVs, influenza, Nipah ab+ve, filovirus ab+ve
Malaysia Sabah	Kamruddin	1,283 serology	40% JE, 5% ZIKV ab+ve
Malaysia Sabah	William	10,800 zoonotic malaria	High burden of macaque-origin malaria
Malaysia Sabah	Hughes	150 bat cave workers	Ongoing
Malaysia Sarawak	Tan	500 Bidayuh and Iban people	8% PCR prevalence HPV in women
Malaysia PREDICT	Hughes	9,933 high zoonotic-risk communities, viral PCR, serology	Ongoing. Multiple known and novel CoVs, paramyxoviruses, others.
Thailand	Wacharaplu-esadee	1,000 bat guano harvesters/villagers	Novel HKU1-CoV assoc. clinical findings
Thailand PREDICT	Wacharaplu-esadee	9,269 high zoonotic-risk communities, viral PCR, serology	Ongoing. Multiple known and novel CoVs, paramyxoviruses, others.
Singapore	Wang	856, Melaka virus	7-11% ab+ve

2.2 Preliminary data on high-zoonotic risk human community surveillance: 2.2.a Biological sample collection in SE Asia: Our longterm collaboration in the region has included qualitative and quantitative survey data, and collecting large archives of banked human sera and other biological samples from high zoonotic-risk populations. For example, under the USAID-PREDICT project, EHA has collected 9,933 and 9,269 specimens in Malaysia and Thailand respectively from people living in zoonotic disease interfaces, all of which will be available for retrospective serological testing by EID-SEARCH. Our core group has collected and tested many thousand additional specimens from other important community cohorts **(Table 2)**, and some of these will continue under EID-SEARCH **(See section 2.4)**. Of particular interest is the access to indigenous communities in Peninsular Malaysia (Orang Asli) and Sarawak (Bidayuh, Iban people), and the bat guano harvesters in Thailand. These communities hunt, butcher and eat wildlife daily, and work in supremely high-risk zoonotic interfaces (e.g. bat caves). One of our indigenous community sites is experiencing an ongoing outbreak of unknown etiology that we are investigating currently **(See section 3.2.a)**.

Table 2: Current or recent biological sample collection from healthy populations conducted by members of our consortium in EID-SEARCH hub countries

2.2.b Human Contact Risk Factors: EHA is the lead organization across all 27 USAID-PREDICT project countries for collecting data on, and analyzing the human aspects of, zoonotic spillover risk (*146, 147*). We conducted exploratory studies using standardized one-on-one semi-structured interviews and site observation data with people living in hotspot areas or engaged in high-zoonotic spillover risk activities, using these data to assess local social and cultural norms underlying wildlife contact (*147*). We then developed a risk factor survey to accompany human biological sampling to evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. We conducted cross-sectional studies in high risk populations in 27 countries, and study participants provided biological samples (oral/oropharyngeal swab, nasal/nasopharyngeal swab, urine sample/urogenital swab, fecal sample/rectal swab, whole blood, serum, plasma). For example, survey and biological samples were collected from: 1,585 participants from 7 sites in Yunnan, Guangxi, and Guangdong provinces, China; 1,400 participants from 4 sites in Malaysia; and 678 participants from 4 sites in Thailand. Samples were tested with novel serological assays and results analyzed against risk factors from survey data (*12*). In China, CoV-seropositive individuals were mostly farmers/peasants (8/9), living in Yunnan province (7/9), 41-60 yrs old (7/9), with domestic contact with rodents (6/9), that are male (6/9). Although these results are preliminary and don't provide detailed information on routes of exposure, they identify key subpopulations to target that would likely increase the opportunity for identifying evidence of spillover for other CoVs, paramyxoviruses and filoviruses. Data from PREDICT surveys will be used to help identify target populations in Aim 2, identify strategies to better target at-risk people, and conduct focused **human animal contact surveys and serosurveys to produce statistically significant findings for henipaviruses, CoVs and filoviruses.** In Aim 2, we will use combined biological sampling and risk factor surveillance in targeted populations within the community to 1) identify risk factors correlated with seropositivity to henipaviruses, filoviruses and CoVs; 2) assess possible health effects of infection in people; and 3) where recent illness is reported, obtain clinical samples for PCR testing and potential viral isolation and characterization. Obtaining this information could be a significant step in understanding the likelihood of recent 'hidden' spillover events and their public health impacts, as well as the risk of future emergence of viral disease.

2.2.c. Risk factors for illness: Our preliminary data from PREDICT in Thailand, Malaysia and China revealed that frequent contact with wild (e.g. bats, rodents, non-human primates) and domestic animals (e.g. poultry, swine) among local communities was associated with a high percentage of self-reported severe/acute respiratory illness (SARI/ARI) and Influenza-like illness (ILI) symptoms. In addition, people involved in crop production, hunting and butchering of wildlife reported statistically significantly higher SARI and ILI symptoms (Li *et al.*, in prep.). **These preliminary data suggest a target population and sites for current proposal with the correct targets for serological surveillance and risk factor surveys in Aim 2 to produce statistically significant findings**. Coupled with further refining serological tools, we will be able to identify the likely routes of exposure to known or novel coronaviruses, henipaviruses, and filoviruses in the study region, as well as build data on the illnesses they cause in people.

2.2.d. Serological platform for community surveillance: One of the present challenges in emerging disease surveillance is that viremia can be short-lived, complicating viral-RNA detection (i.e. traditional PCR-based approaches), especially in zoonotic reservoir hosts, and requiring large samples sizes to understand infection patterns or host range. By contrast, antibodies are typically long-lived and can be used to assess prior virus exposure or infection with much smaller samples sizes (*148*). However, most serological assays only target a single protein, and it's not always clear which antigen is the most immunodominant during an adaptive humoral immune response – i.e. which is the best target for a serological assay. Henipaviruses, filovirus and CoVs express envelope receptor-binding proteins (e.g. CoV=spike (S) protein, filoviruses/henipaviruses=glycoprotein (GP/G)), which are the target of protective antibody responses. The Broder lab at USU, a leader in the expression and purification of native-like virus surface proteins for immunoassay application (*149*)?, developing monoclonal antibodies (*150, 151*) and as subunit vaccines (*152, 153*), is presently developing a novel, multiplex microsphere immunoassay (MMIA) allowing serum samples to be simultaneously tested for antibodies reactive with henipaviruses, filoviruses and coronaviruses. This MMIA uses a Luminex-based

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multiplex platform and custom designed virus receptor-binding proteins expressed in a mammalian cell-culture system and purified as native-like oligomeric proteins (*149*). These oligomeric antigens retain post-translational modifications and quaternary structures. This structural advantage over peptide antigens, allows the capture of conformational-dependent antibodies raised during a natural infection, which represent the majority of the protective humoral response.

Serological platforms have been historically limited by the starting antigen, and cross-reactivity between known and unknown related viruses not included in the assay. The known diversity of henipaviruses includes five presently described viruses, NiV, HeV, CedV, GhV and MojV and this assay has been expanded to include all described species **(Fig. XXX)**. In the past, our collaborators have used NiV and HeV G protein antigens in a MMIA assay to identify a serological footprint of NiV-related African henipaviruses and exposure to virus(es) antigenically-related to NiV in livestock sampled in Bangladesh (*77, 78*). To strengthen serological data interpretation, the G protein from each virus is integrated into the MMIA so that virus-specific versus henipavirus cross-reactive antibodies can be detected and address specificity of past virus exposure. Extensive cross-reactivity exists within ebolaviruses and between ebolaviruses and marburgviruses, highlighted by studies examining antibody binding between heterologous viruses (*154-156*). Ongoing work is aimed at validating the MMIA pan-filovirus assay for specificity. The majority of ebolaviruses are endemic to Africa, however the discovery of Měnglà virus in Yunnan, China increased the further demonstrated that a diversity of Asiatic filoviruses with unknown pathogenicity and zoonotic potential exists. Our past work in collaboration with Duke-NUS demonstrated that three under-sampled fruit bat species had serological evidence of past exposure to a filovirus(es) most antigenically-related to ebolaviruses (e.g. Ebola virus, Bundibugyo virus), but unreactive with Reston virus GP, suggesting that novel filoviruses circulate within bat hosts (*41*).

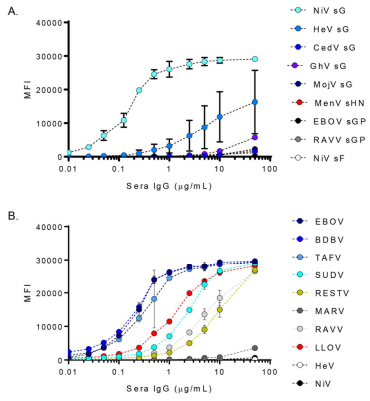

Fig. XXX: Validation of multiplex microsphere immunoassay (MMIA) specificity and identification of immunologically cross-reactive viruses for Nipah (A) and Ebola (B).

2.3 General Approach/Innovation: We will use a dual study design to gain in depth understanding of exposure and risk factors for viral spillover (**Fig. XX**). In Aim 2 we will conduct community-based surveillance using a brief, focused questionnaire to identify the prevalence and frequency of risk-factors for viral spillover in tandem with collection of high quality biological samples to determine the seroprevalence of spillover viruses in at-risk human populations. We will use generalized linear models to analyze correlation between biological results and risk-factors for viral spillover. In Aim 3, we will conduct clinic-based syndromic surveillance and biological sampling at clinics/hospitals that are the catchment healthcare facilities for the communities sampled in Aim 2. Both community-based and clinic-based syndromic surveillance programs are cross sectional case-control studies designed with the sample sizes necessary to statistically quantify (with a power of 80%) risk factors and health impacts for viral spillover, linked to serological status and PCR prevalence in symptomatic patients.

Commented [PD45]: Hongying -Need a new figure that lays out the community/clinical approach – small one with pretty images.

2.4 Target population & sample sizes: We will target populations in the same geographic regions as those identified in Aim 1 that harbor wildlife with high potential zoonotic viral diversity, that are EID hotspots, and that are well-connected through regional travel and trade hubs to the global travel network **(Fig X)**. We will target specific communities based on our analysis of previously-collected PREDICT behavioral questionnaire data, to assess key at-risk populations and occupations with high exposure to wildlife, as well as using other publicly available data (e.g. wildlife hunting licenses sold in a region, number of guano caves accessed for fertilizer etc.). We will identify populations at two sites in each of the following regions, building on our preliminary data **(Table 2)**: *Thailand (Co-I Wacharapluesadee):* We will continue surveillance in bat guano harvesters and their villages in Ratchaburi province where we have identified MERS-related CoV in bat guano and rectal swabs

(*48, 157*) and serological evidence of α-CoVs, HKU1-CoV in people (Wacharapluesadee in prep.). Our second site will be in Chonburi province where we have found Nipah virus (99% identity of whole genome sequence to NiV from a Bangladesh patient) in flying foxes but no outbreaks have yet been reported (REF), and have found several novel paramyxoviruses and CoVs have been found in bat feces roosting there (Wacharapluesadee in prep.).

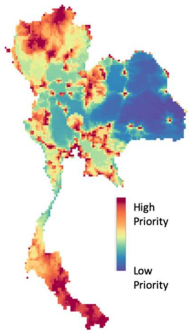

Fig. X: . Geographic analysis to target high-risk sites for human zoonotic disease surveillance in Thailand. Our spatial analysis from Aim 1 will be extended to optimize sites for human community surveillance in Thailand and Malaysia by ranking areas with high numbers of expected viruses in wildlife, greater spillover probability based on EID risk factors, and higher levels of connectivity (measured by road density data) that may facilitate person-person spread.

Peninsular Malaysia (Co-I Hughes, CM Ltd.): We will expand our sampling of the Orang Asli indigenous communities to include additional communities in the 3 Districts we have already sampled, and additional Districts in the States of Kelantan Perak, Pahang, and Kelantan. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife (mainly bats) will be sampled. Human samples will include serum, whole blood, throat swabs, nasal swabs, urine, and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and tested at NPHL. Participants who hunt, butcher or consume wildlife, or who rear animals will be considered suitable for enrolment. Participants will complete a consent form and questionnaire in addition to having samples collected. During these community trips the field team will conduct community meetings to help inform the Orang Asli about the public health risk of zoonoses. *Sarawak (Co-I Tan, UNIMAS):* We will expand our sampling of the Bidayuh and Iban indigenous people to include the Dayak and Orang Ulu ("people of the interior") because this group has a more intimate connection to remote wildlife-rich areas than the other two, albeit all are hunters. Human sampling will be led by UniMAS in collaboration with CM Ltd / EHA. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife (mainly bats) will be sampled, following the same protocols as for Peninsular Malaysia. *Sabah:* We will conduct sampling at the Madai bat and bird cave that has 200 permanent residents who harvest bird's nests for soup, and >1,000 during the harvesting season and at Gomantong cave – one of the largest bat and cave swiftlet nesting sites in the world. Protocols follow those for Peninsular Malaysia. *Singapore:* Active human surveillance will be not be conducted in Singapore, but archived human specimens e.g. from our Melaka virus serosurvey (*4*), will be made available to conduct retrospective serosurveys for specific pathogens that are identified as high interest to the EIDRC, NIH, and EID-CC.

Sample sizes: From our previous work we anticipate that 10-30% of the community population will have had exposure to wildlife allowing us to capture highly exposed and non-exposure individuals at each site. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock farming, transportation, selling, or slaughtering wildlife in the surveyed areas will be targeted so that they make up ≥30% of the sampled population in each community. We will stratify sampling to ensure appropriate representation of sex, demographic, and socio-economic factors at each community site. Estimating 5% overall seroprevalence in these high-exposure populations, these sample sizes are sufficient to estimate effect sizes of risk factors of 2X or greater with 80% power. For community-based surveillance, we will enroll xxxx individuals per county/province, pooled across two sites for each, allowing us to make county/province-level comparisons of differing effects.

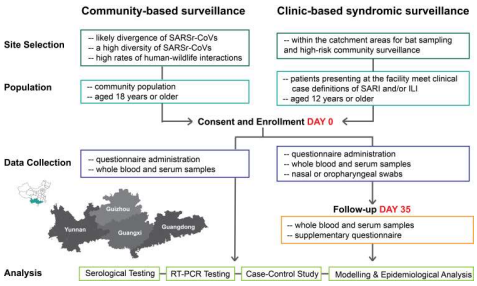

Fig. XX: Human survey and sampling study design overview (Aim 2 and 3)

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2.5 Data & sample collection: Following enrollment of eligible participants and completion of the consent procedure, biological specimens (5ml will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 2.5ml whole blood; two 500 µL serum samples) will be collected and a questionnaire will be administered by trained project staff that speak appropriate local language to all participants. Through the serological findings and the survey we will investigate the details of five risk factors related to high risk wildlife exposure, to maximize the power of the analyses, based on continuing analysis of our previous work, and will focus on: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With additional consent considerations for participants enrolled from clinical settings we will review clinical records to collect recent data on medical history, clinical syndromes, and patient etiology.

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2.6: Laboratory analysis: 2.6.a Serological testing:

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In a previous R01, we expressed his-tagged nucleocapsid protein (NP) of SARSr-CoV Rp3 in *E.coli* and developed a SARSr-CoV specific ELISA for serosurveillance using the purified Rp3 NP. The specificity of this assay was evaluated using polyclonal antibodies against HKU1, OC43, 229E, NL63, MERS-CoV and EBOV and no significant cross-activity detected (*12*). **This suggests that if we can expand our serology tests to cover other bat CoVs, and that doing so may identify many more seropositive individuals.** We will therefore use two serological testing approaches. First, we will expand test all human sera collected from both community- and clinic- based sampling for a panel of bat CoVs that will include SARS-CoV (outbreak strain), a range of lineage 2 bat SARSr-CoVs (including WIV1 and SHC014), lineage 1 HKU3r-CoVs, MERS-CoV, and the α-CoVs SADS-CoV and HKU10. We previously found serological evidence of human exposure to HKU10 (*12*), and HKU10 is known to jump from one host bat species to another (*158*) so is likely to have infected people more widely. It is possible that non-neutralizing cross reactive epitopes exist that afford an accurate measure of cross reactivity between clade1 and clade 2 strains which would allow us to target exposure to strains of 15-25% divergence from SARS-CoV, at a phylogenetic distance where therapeutic or vaccine efficacy may falter. Additionally, we will test samples for antibodies to common human CoVs (HCoV NL63, OC43 – see potential pitfalls/solutions below). Secondly, CoVs have a high propensity to recombine. To serologically target 'novel' recombinant virus exposure, we will conduct 1) ELISA screening with SARSr-CoV S or S1 (n-terminal domain); 2) confirm these results by Western blot; then 3) use NP based ELISA and LIPS assays with a diversity of SARSr-CoV NP. For NP ELISA, micro-titer plates will be coated with 100 ng/well of recombinant batCoV NP and incubated with human sera in duplicates followed by detection with HRP labeled goat anti-human IgG antibodies. For confirmation, all ELISA positive samples will be subjected to Western blot at a dilution of 1:100 by using batCoV-NP as antigen. We will use an S protein-based ELISA to distinguish the lineage of SARSr-CoV. As new SARSr-CoVs are discovered, we will rapidly design specific Luciferase immunoprecipitation system (LIPS) assays targeting the S1 genes of bat-CoV strains for follow-up serological surveillance, as per our previous work (*13*). Additionally, in Thailand, as an initial project on our EIDRC, we will develop serology assays to the novel CoVs found in bats at the bat guano site using LIPS assays and viral genomic data, and will screen archived specimens from 230 participants (2 years' collection in 2017-2018, a subset of the subjects have been sampled at multiple timepoints to allow us to assess seroconversion).

2.6.b RT-PCR testing. Specimens will be screened using RT-PCR for the RdRp gene (See section 1.3.b for details). Positive samples will be subjected to full genome sequencing and RT-PCR amplification of the S glycoprotein gene. Samples from the clinic-based syndromic surveillance will also be tested using RT-PCR for Influenza A & B, HCoV NL63, OC43, HKU1, SARS-CoV & 229E as rule-outs, and SADS-CoV and HKU10 as an opportunistic survey for potential spillover these CoVs as proposed above.

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2.6.c Biological characterization of viruses identified: Novel viruses identified in humans will be recovered by cultivation or from full length genome sequences using reverse genetic approaches. Viruses will be characterized for growth in primary human cell lines and in mouse models, including the Collaborative Cross Mice or humanized mice expressing human receptors from related strains. The approaches are discussed in more detail under Aim 1.

2.7 Epidemiological analysis: We will conduct a cross sectional case-control study to identify risk factors for Henipavirus, Filoviruses, and CoVs spillover. "Cases" are defined as participants whose samples tested positive for Henipavirus, Filoviruses, or CoVs by serological tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative for Henipavirus, Filoviruses, or CoVs. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between biological results, serological/PCR status, and risk factors related to high risk wildlife exposure including: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) home or work proximity to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms lifetime and past 12mo. Additionally, we will use this procedure to determine how clinical presentation differs between Henipavirus, Filoviruses, or CoVs in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms.

2.8 Potential problems/alternative approaches: Rarity of spillover events means it may be difficult to identify sufficient seropositives to statistically analyze human risk profiles. First, we will be targeting our community-based surveillance to subpopulations with high-levels of wildlife exposure, at sites selected for diverse and prevalent viruses to increase likelihood of finding positive individuals. Second, our serology testing will include a panel of assays for a large diversity of viruses, increasing likelihood of positives. For example, in previous work our overall bat CoV PCR prevalence in the community was, 11.8%; β-CoV, 3.4%; α-CoV, 9.1%). Thus, using this broad serological panel to screen individuals prone to contact with species of higher zoonotic risk increases the potential for detecting spillover with enough power for statistical analyses, and will shed light on behaviors that predispose to spillover. Third, we will include common human CoVs, paramyxoviruses, and influenza viruses in our panel, so that even if low prevalence of wildlife viruses is found, we will be able to conduct a valuable cross-sectional study of the seroprevalence of more common human viruses. Finally, we will be able to assess relative measures of human-wildlife contact from our survey work. We will analyze intensity of contact with high-risk taxa against other risk factors to provide useful proxy information for spillover risk. **Serological testing may not match known viruses due to recombination events, particularly for CoVs.** We will use the three-tiered serological testing system outline in **2.6.a** to try to identify these 'novel' viruses, however, we will also remain flexible on interpretation of data to ensure we account for recombination.

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Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts.

3.1 Rationale/Innovation: Our previous research **(Table 1)** and other data suggests that there are substantial numbers of outbreaks or clinical cases for which the etiologic agent is not diagnosed, e.g. (*159, 160*). To investigate this in SE. Asia, we will work directly with clinics and outbreak control organizations to identify the causes of some 'cryptic' outbreaks or cases in the region. Specifically, we will expand on current syndromic surveillance activities to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and who present at regional clinics or hospitals with symptoms typical of high impact zoonotic viruses. We will collect detailed survey data to assess their contact type and frequency with wildlife and livestock and conduct molecular and serological diagnostic assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. We conduct novel viral discovery assays on patients who present with symptoms indicative of a high-risk zoonotic virus. If novel pathogens are identified by PCR, we will attempt to isolate, obtain full genome sequence and/or biologically characterize the pathogen, using the *in vitro* and *in vivo* strategy laid out in Aim 1.

3.2 Preliminary data clinical surveillance: 3.2.a. Clinical surveys and outbreak investigations: Our longterm collaboration in the region has included the following activities investigating clinical syndromes: *Peninsular Malaysia*: **At the time of writing, there is an outbreak of suspected viral undiagnosed illness in the indigenous Batek Orang Asli ("people of the forest") community living at Gua Musang ("civet cat cave") district, in Kelantan, Malaysia, close to Taman Negara – a National Park containing the oldest known rainforest**. This community is one where we have conducted prior routine surveillance and biological sampling, demonstrating proof-of-concept that our two-pronged high-risk community/clinic approach will work. Dozens of people are affected and the outbreak is currently being investigated by our collaborators at the

Malaysian NPHL. The Orang Asli live in remote villages in the rainforest of Malaysia, with limited access to modern health care and extensive contact hunting, butchering, and eating wildlife as their primary source of protein. This outbreak, and others that will continue to emerge just like it, underscore the critical need for our EIDRC - to improve the technological tools, training, and human capacity for rapid EID surveillance, diagnosis, and response at the frontlines of disease emergence in SE Asia. **Investigating this outbreak be a key priority if EID-SEARCH is funded.** *Sabah*: Co-I Kamruddin (BMHRC) is leading an asymptomatic leptospirosis study on sanitation workers, an ILI study in a Kota Kinabalu clinic and diarrheal study in children under 5 yrs. Co-Is William, Tan and others have conducted clinical observational studies to determine the etiology of central nervous system infections and acute undifferentiated febrile illness in Sabah. Co-Is Tan, William have collaborated with Co-I Wang to enroll 90 adult and over 100 pediatric participants with central nervous system infections, negative tests for malaria and dengue from 3 district hospitals in Sabah to determine the various etiologies. While a high proportion tested positive for rickettsial agents, the majority had no clear diagnosis. *Sarawak:* Dr Tan (UNIMAS) is conducting a survey of high-risk female Kelabit indigenous people and has found 8% with HPV52 as the dominant strain. He is working on clinical cohorts of hand, foot and mouth disease and will recruit patients from that study in the current proposed work. Finally, he is conducting a longterm study of rotavirus and other viral etiologies of acute gastroenteritis in Kuching, Sarawak that will include swine herders and farmers. These are an important group in Malaysia because pig farms are shunned by the government and local villagers to they are placed far away from town centers, deep in the forest, and it is this scenario that led to the emergence of Nipah virus in 1998-9 (*18, 161*). *Thailand:* The Chulalongkorn Univ. TRC-EID laboratory works in coordination with the Thailand Ministry of Health to investigate outbreak etiology, including the first case of imported MERS-CoV in Thailand, **for which we produced sequence confirmation within 24 hours from acquiring the specimen (Fig. X)**. Three imported MERS cases have been detected in Thailand since 2015, and all cases were confirmed at the TRC-EID. We worked during the Zika outbreak to identify > 500 PCR-positive Zika patients with sequence confirmation. We have also worked on returning and suspected Ebola travelers, syndromic surveillance for SARI, Encephalitis, Hand Foot and Mouth disease in children and Viral diarrhea. Working with the US CDC (Thailand), between 1998 and 2002 we enrolled a large cohort of children from Kamphaeng Phet Province to examine Dengue virus and JE epidemiology (*162, 163*). Among the 784/2,574 convalescent sera Henipavirus-specific antibodies were detected in 13 serum samples (1.7% seroprevalence), suggesting prior exposure to endemic henipaviruses or a related paramyxovirus in rural Thailand. *Singapore:* Duke-NUS has worked with the Ministry of Health to investigate Zika cases (*164*), and with EcoHealth and others, Duke-NUS has developed novel CoV assays for clinical surveillance, and for expedited analysis during outbreaks. Our pipeline of novel virus detection, reservoir host identification, serological assay development, and serological testing is as rapid as several months, with the development of novel serological assays from sequence data in 7 days (*13*). **For example, with the discovery of SADS-CoV, we identified and fully sequenced a new virus, screened archived wildlife specimens to identify the natural reservoir host species (*Rhinolophus* spp. bats), developed LIPS serological assays, and tested human and swine samples to assess seroprevelance in less than 3 months (*13*).**

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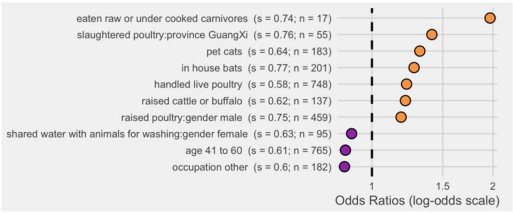
3.2.b Analysis of self-reported illness: In addition to biological outcomes we have developed a standardized approach in PREDICT for analyzing data on self-reported symptoms related to targeted illnesses; of fever with cough and shortness of breath or difficulty breathing (severe acute respiratory illness - SARI), fever with muscle aches (fevers of unknown origin (FUO) and, cough or sore throat (influenza-like illness - ILI) from study participants. We used a least absolute shrinkage and selection operator (LASSO) regression to identify associations between participants who reported ILI and/or SARI symptoms and demographics, occupation, and contact with animals in the last year. Results have clear biological relevance. In Yunnan, China, salient associated variables or combination of variables were, in descending order of odds ratio: 1) having eaten raw or undercooked carnivores; 2) having slaughtered poultry and being a resident of Guangxi province; 3) having had contact with cats or bats in the house; and 4) being a male who has raised poultry **(Fig. 11).** We will expand this approach for all syndromes in Aim 3, and use more granular targeted questions designed to assess patient's exposure to wildlife in terms that are relevant to the specific country.

Fig. 11: Associated variables of self-reported ILI and/or SARI in prior 12 months (s = bootstrap support; n = number +ve out of 1,585 respondents). **Orange circles** = odds ratios > 1 (positively associated with the outcome); **purple** = odds ratios <1 (negatively associated with the outcome).



3.3 General Approach: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent is not identified. To capture these, we will expand on our current syndromic surveillance to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and present at regional clinics with influenza-like illness, severe respiratory illness, encephalitis, and other symptoms typical of high-impact emerging viruses. We will collect survey data tailored to the region to assess contact with wildlife, conduct molecular and serological assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. If novel pathogens are identified by PCR, we will isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work. We will then use behavioral survey data, ecological and phylogeographic analyses to assess the likely reservoir hosts to inform intervention programs.

3.4 Clinical cohorts. 3.4.a Cohort selection: We will initiate active clinic-based syndromic surveillance at 2 town-level level clinics and 2 provincial-level hospitals in Thailand, Peninsular Malaysia, Sabah and Sarawak. These all serve as the catchment healthcare facility for people in our community-based surveillance of Aim 2 and are inclusive of wildlife sampling sites in Aim 1. Patients ≥ 12 years old presenting at the health facility who meet the syndromic and clinical case definitions for SARI, ILI, encephalitis, fevers of unknown origin, or hemorrhagic fever will be recruited into the study. We will enroll a total of xxxx individuals for clinical syndromic surveillance, which accounts for up to 40% loss from follow-up. Study data will be pooled across country sites, as clinical patients are limited by the number of individuals presenting at hospitals. Sites: *Thailand:* XXXXX *Peninsular Malaysia:* Focusing immediately on the current outbreak in Orang Asli, we will work closely with the Malaysian Ministry of Health to support their investigation. We will also work with 6 Orang Asli clinics which focus solely on this indigenous community. *Sarawak:* XXXXXX *Sabah:* XXXXXXXXX. *Singapore:* XXXXX

3.4.b Behavioral and clinical interviews: 2.5 Clinic enrollment and follow-up: We will recruit inpatients and outpatients to participate in the study after initial screening to determine if they meet the clinical case definition of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever of unknown etiology; or 6) severe diarrhea. Once enrolled, biological samples will be collected and a questionnaire administered by trained hospital staff that speak appropriate local language. Samples will be taken concurrently when collecting samples for normative diagnostics. For inpatients, samples will be collected no more than 10 days of reported onset of illness to increase the chance of PCR detection of viruses (*165*). We will follow up 35 days after enrollment to collect an additional two 500 µL serum samples conduct a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. 35 days gives adequate time for development of IgG, which occurs <28 days after onset of symptoms for SARS patients (*166*). These samples may prove valuable to collaborators in that the PBMC could be used in production of therapeutic antibodies.

3.4.c Sampling:

Following enrollment with signed consent form, biological specimens (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples) including two nasal or oropharyngeal swabs will be collected from all eligible participants, and a questionnaire will be administered. We will investigate five risk factors, to maximize the power of the analyses, all related to high risk wildlife exposure, based on continuing analysis of our previous work, and will include: 11) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With permission from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology.

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3.5 Sample testing: PCR, Serol to link symptoms to etiologic agents

The standard syndromic diagnostic PCR assay for the common pathogens will be conducted and the results will be shared to the hospital with 48 hours. Specimens will be further tested for 3 viral families; CoV, PmV and filovirus by consensus PCR. Appropriate specimen from dead case will be further conducted by NGS if the previous PCR tests are negative to identified cause of infection. The serology panel assay will be conducted from paired serum.

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3.5 Assessing potential for pandemic spread: We will use statistical models built from collated biological, ecological, and genetic data to predict the likelihood of human infection (or spillover) and pathogenicity for a the range of diverse viruses we will characterize. The aim is to quickly identify and assess which viruses pose the most significant risk to human health and thus prioritize additional resources to more fully characterize, better understand pathogenicity, and invest in therapeutic development for those pathogens. We will combine two independent but related statistical models to predict pathogen spillover risk and pathogenicity. First, we will expand a simple generalized additive modeling approach (*91*) to assess spillover risk probability to a new virus that is currently not known to infect people, when we have only limited genetic data from initial PCR screening and sequencing. Additional data from receptor binding protein homology and cell line infection experiments can be incorporated into the model framework as available. Second, given high likelihood to spillover and infect humans, or direct evidence of human infection from our clinical surveillance, we will then predict pathogenicity using nearest neighbor phylogenetic analyses as a first approximation and by incorporating more detailed data from genomic characterization and animal model experiments. For the pathogenicity model, human epidemiological data for ~300 viral species known to infect people (case fatality rate, global estimates of number of human cases per year or decade, R_0, infectious period, and primary symptoms) will be aggregated including from previous reviews [Woolhouse ref] to use as predictor variables. Thirdly, we will build off EHA's previous DTRA and DHS supported research to predict pandemic spread for viurses we identify by integrating surveillance site data, global flight models (*167*), as well as additional datasets (road networks, shipping routes, cell phone data) to measure human movement and connectivity across Southeast Asia.

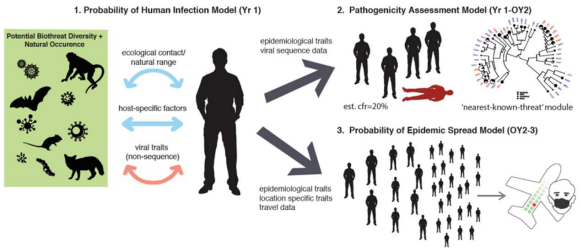

Fig. XXXXX: Conceptual framework highlighting 3 underlying models to estimate: **1)** probability of human infection, or spillover, the first necessary step to understand pathogenicity when status of human infection is unknown; **2)** pathogenicity, i.e. probability of causing clinical signs, case fatality rate estimates, and other metrics relevant to humans; and 3) probability of pathogen spread based on ecological, epidemiological, and airline connectivity and human migration data.

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3.7 Potential problems/alternative approaches: Patients visiting clinics may have cleared virus, but not yet developed IgG antibodies, reducing seropositive cases. Our 35 day follow-up sampling should avoid this because the maximum lag time between SARS infection and IgG development was ~28 days (*165*). We also expect that patients in rural communities will only visit clinics when symptoms have progressed, likely coinciding with late illness and onset of IgG. We will also have data from our community study, so won't be completely reliant on hospital data to identify PCR- or seropositives. Finally, the risk is outweighed by the potential public health importance of discovering active spillover of a new henipavirus, filovirus or CoV infection.

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Additional Instructions – Specific to the EIDRC FOA:

4. Administrative Plan

In a clearly labeled section entitled **"Administrative Plan":**

- Describe the administrative and organizational structure of the EIDCRC Administrative and Leadership Team. Include the unique features of the organizational structure that serve to facilitate accomplishment of the long range goals and objectives.

Chula TRC-EID is National reference laboratory for diagnostic and confirmation of EIDs pathogen along with the National public health laboratory at the Department of Medical Science and the WHO Collaborating Centre for Research and Training on Viral Zoonoses. We have built a EID laboratory network in-country among three Ministry including Ministry of Public Health (Department of Medical Science and Department of Disease Control), Department of Livestock and Development, and Department of National Parks, Wildlife and Plant Conservation and Universities and among SEARO countries. Our clinicians provide case consultation for Thailand and SEARO countries. The laboratory and clinic training are regularly conducted.

For Malaysia –
NPHL – Molecular and serological screening (BioPlex) of PM Orang Asli samples, serological screening of syndromic samples from Sabah (already approved) and Sarawak (would need approval)

PERHILITAN molecular zoonosis laboratory, at the National Wildlife Forensic Laboratory - Molecular and serological screening (BioPlex) of PM wildlife samples

UPM Faculty of Veterinary Medicine - Molecular and serological screening (BioPlex) of PM livestock samples

KKPHL - Molecular of syndromic samples from Sabah (already done) and Sarawak (would need approval)

SWD WHGFL - Molecular screening of Sabah wildlife samples

BMHRC - Molecular and serological screening (BioPlex) of Sabah livestock samples, serological screening (BioPlex) of Sabah wildlife samples, Molecular and serological screening of syndromic samples from Sabah (already approved) and Sarawak (would need approval). BMHRC will need renovations and BioPlex to do this work – Menzies team might have funds for BioPlex that they would base here as part of DTRA project.

- Describe how communications will be planned, implemented, and provided to collaborators, teams, or sites. In addition, describe plans to achieve synergy and interaction within the EIDRC to ensure efficient cooperation, communication and coordination.
- Specifically address how the EIDRC Team will communicate with other EIDRCs, the EIDRC CC and NIAID and how the EIDRC will implement plans and guidance from the EIDRC CC to ensure coordination and collaboration across the Emerging Infectious Disease Program as a whole. Describe how the study site will provide information, if any, back to the local health authorities and the study subjects as needed. Note any adjustments or changes needed for rapid and flexible responses to outbreaks in emerging infectious diseases.

List out all of our involvement with outbreak investigations, ministries of health surveillance etc.
If possible the clean or summarized answers from the questionnaire should be shared to the hospital Infectious Control team and Bureau of Epidemiology for their information for controlling the outbreak.

- Describe the overall management approach, including how resources will be managed, organized and prioritized, including pilot research projects.

Commented [PD64]: All – I've started putting a few notes in this section, but it will need substantial work. Please insert language from other proposals to fit these sections if you have them

Commented [PD65]: From Supaporn

Commented [PD66]: From Supaporn – do same for all countries

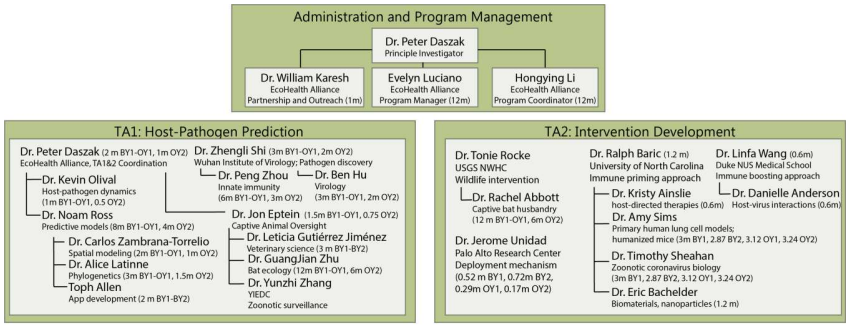
- Highlight how the proposed staffing plan incorporates scientific and administrative expertise to fulfill the goal to develop the flexibility and capacity to respond rapidly and effectively to outbreaks in emerging infectious diseases in an international setting.

CM/EHA helped establish and manages with SWD the Wildlife Health, Genetic and Forensic Laboratory (certified as a BSL- 2 laboratory according to the US standard for laboratory specifications) that has all the equipment necessary to store samples, run extractions, PCR and analysis on biological samples for wildlife disease surveillance. The lab is used to conduct health checks on rescued and relocated wildlife before being released into new areas or sanctuaries, to screen samples for the PREDICT and Deep Forest Project and for genetic research and forensic investigations. CM/EHA also helped establish the new molecular zoonosis laboratories (certified as a BSL- 2 laboratory according to the US standard for laboratory specifications), at DWNP's National Wildlife Forensic Laboratory. The lab is used to screen samples for the PREDICT & DTRA projects. In addition CM/EHA also has access to NPHL and KKPHL for screening human samples and the Virology lab at FVMUPM for screening Livestock samples.

The Borneo Medical and Health Research Centre (BMHRC) is a centre of excellence that conducts teaching and scientific researches on communicable diseases, outbreak investigations, and ethnomedicine. BMHRC has offices space including a meeting room and seven laboratories which including preparation lab, parasitology lab, cell culture lab, bacteriology lab, natural product lab, virology lab, and molecular biology lab. BMHRC is supported by an administration staff, medical lab technologist, and research assistants who are available to work on projects. Through this project we will help to establish a certified BSL-2 lab at the BMHRC. This lab can then be used for human testing to elevate pressure at KKPHL and QEH labs. It would also be an ideal location for a Luminex platform as human, wildlife and livestock samples could all be screened here. Co-Is Hughes and Kamruddin will be starting outbreak response training in August 2019 with a team from Sabah CDC, KKPHL and BMHRC. (Dr. Maria Suleiman now in PM But still collaborating with us, Dr. Muhammad Jikal current Director CDC working with us and Dato' Dr Cristina Rundi Director Sabah state health Dept is supportive) this collaborative group is working to develop a Sabah outbreak response team that will help support SSHD/ CDC outbreak team and also conduct sampling for disease surveillance to be based at BMHRC and we will develop this as part of this proposal. In addition the UMS hospital will come in line in 2021 providing additional opportunities for cohort studies and capacity building.

FROM DARPA grant:

Section 1.03 MANAGEMENT PLAN



Project DEFUSE lead institution is EcoHealth Alliance, New York, an international research organization focused on emerging zoonotic diseases. The PI, Dr. Daszak, has 25+ years' experience managing lab, field and modeling research projects on emerging zoonoses. Dr. Daszak will commit 2 months annually (one funded by DEFUSE, one funded by core EHA funds) to oversee and coordinate all project activities, with emphasis on modeling and fieldwork in TA1. Dr. Karesh has 40+ years' experience leading zoonotic and wildlife disease projects, and will commit 1 month annually to manage partnership activities and

Commented [KJO67]: Figure like this would be good, but instead of breaking down by TA, maybe by country or Specific Aim. Per FOA, Need to also show how we'll link in with the Coordinating Center and other EIDRCs.

outreach. Dr. Epstein, with 20 years' experience working emerging bat zoonoses will coordinate animal trials across partners. Drs. Olival and Ross will manage modeling approaches for this project. Support staff includes a field surveillance team, modeling analysts, developers, data managers, and field consultants in Yunnan Province, China. The EHA team has worked extensively with other collaborators: Prof. Wang (15+ yrs); Dr. Shi (15+ yrs); Prof. Baric (5+ yrs) and Dr. Rocke (15+ yrs).

Subcontracts: #1 to Prof. Baric, UNC, to oversee reverse engineering of SARSr-CoVs, BSL-3 humanized mouse experimental infections, design and testing of targeted immune boosting treatments; **#2** to Prof. Wang, Duke-NUS, to oversee broadscale immune boosting, captive bat experiments, and analyze immunological and virological responses to immune modulators; **#3** to Dr. Shi, Wuhan Inst. Virol., to conduct PCR testing, viral discovery and isolation from bat samples collected in China, spike protein binding assays, humanized mouse work, and experimental trials on *Rhinolophus* bats; **#4** to Dr. Rocke, USGS NWHC, to refine delivery mechanisms for immune boosting treatments. Dr. Rocke will use a captive colony of bats at NWHC for initial trials, and oversee cave experiments in the US and China. **#5** to Dr. Unidad, PARC, to develop innovative aerosal platform into a field-deployable device for large-scale inoculation of the bats. Dr. Unidad will collaborate closely with Dr. Rocke in developing a field deployable prototype for both initial trails and cave experiments in China.

Collaborator Coordination: All key personnel will join regularly-scheduled web conferencing and conference calls in addition to frequent ad hoc email and telephone interactions between individuals and groups of investigators. Regular calls will include:

- Weekly meetings between the PI and Program Manager (on project and task status)
- Weekly web/phone meetings between Program Manager and subawardee admin. staff
- Monthly web/phone conferences between EHA PIs and all subawardee PIs.
- Monthly web conferences between key personnel (research presentations/coordination)
- Four in-person partner meetings annually with key personnel at EHA and two in-person partner meetings annually between subawardees.
- Annual in-person meeting among all key personnel

Evaluation metrics will include the generation of high-quality data, successful achievement of milestones and timelines, scientific interaction and collaboration, the generation of high quality publications, and effective budget management. The PI and subawardee PIs will attend a kickoff meeting and the PI will meet regularly with DAPRA at headquarters and at site visits.

Problem identification and resolution: Regular planning, monitoring, and evaluation meetings will be the primary mechanisms for problem identification. Minor problems (e.g. delays in sample availability or test results) will be dealt with internally through appropriate action and resolution monitored by Dr. Daszak and the Project Manager. In the event of significant problems, such as prolonged poor productivity, inadequate scientific collaboration, or major disputes regarding research direction or resource allocation, EHA will assist with resolving the problem through negotiation. Should a resolution not be forthcoming, consultation with our technical advisors and DARPA Program staff may be warranted.

Risk management: Maintaining a timeline and meeting milestones will require strict and continuous oversight of all project phases, frequent and regularly scheduled communication, and the ability to make decisions and implement strategies. A project of this nature requires a different mindset from that typically associated with basic research activities that move at an incremental pace with investigators gradually optimizing experimental systems, refining data, or waiting for additional data before moving ahead with an analysis approach. To maintain our timeline, we will continually evaluate these trade-offs to make decisions about when iteration is appropriate and when necessary to move forward with current information.

Biographies:

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Dr. Peter Daszak is President and Chief Scientist of EcoHealth Alliance, Chair of the NASEM Forum on Microbial Threats, member of the Executive Committee and the EHA institutional lead for the $130 million USAID-EPT-PREDICT. His >300 scientific papers include the first global map of EID hotspots, estimates of unknown viral diversity, predictive models of virus-host relationships, and evidence of the bat origin of SARS-CoV and other emerging viruses.

Prof. Ralph Baric is a Professor in the UNC-Chapel Hill Dept. of Epidemiology and Dept. of Microbiology & Immunology. His work focuses on coronaviruses as models to study RNA virus transcription, replication, persistence, cross species transmission and pathogenesis. His group has developed a platform strategy to assess and evaluate countermeasures of the potential "pre-epidemic" risk associated with zoonotic virus cross species transmission.
Prof. Linfa Wang is the Emerging Infectious Diseases Programme Director at Duke-NUS Medical School. His research focuses on emerging bat viruses, including SARS-CoV, SADS-CoV, henipaviruses, and others and genetic work linking bat immunology, flight, and viral tolerance. A 2014 recipient of the Eureka Prize for Research in Infectious Diseases, he currently heads a Singapore National Research Foundation grant "Learning from bats" ($9.7M SGD).

Current list of contacts at key human (especially) and wildlife orgs in every SE Asian country from map above – please add to this if we've missed any

Indonesia – EHA: Eijkman (Dodi Safari), IPB (Imung Joko Pamungkas); **CM Ltd:** IPB (Imung Joko Pamungkas), Bogor Agricultural University (Diah Iskandriati)

SE China – EHA: Yunnan CDC (XX), Wuhan Inst. Virol. (Zhengli Shi);

Myanmar – Chulalongkorn (Thiravat and Supaporn) have collaborated extensively with Win Min Thit, MD (Yangoon General Hospital, Myanmar); Khin Yi Oo (National Health Laboratory, Myanmar) and also in training PREDICT Myanmar veterinarians in bat sampling under PREDICT.

Japan – **BMHRC**: Oita University (Hidekatsu Iha, Kiyoshi Aoyagi), Nagaskai University (Koichi Morita, Kazuhiko Moji)

Cambodia -
Laos - **CM Ltd:** Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit (Matthew Robinson)
Bangladesh – EHA: IEDCR (Meerjady Sabrina Flora, Ariful Islam), icddr,b (Mohammed Ziaur)

India – EHA: Jon's collaborators;

Vietnam – Wellcome CRU ? Liaison with Zhengli Shi for Serol assays etc.?

Philippines – EHA, CM Ltd: Biodiversity Management Bureau, Department of Environment and Natural Resources (Anson Tagtag), Bureau of Animal Industry (Davinio Catbagan), Research Institute of Tropical Medicine (Catalino Demetria).

Malaysia – **CM Ltd:** Borneo Medical and Health Research Center (Ahmed Kamruddin), Department of Wildlife and National Parks Peninsular Malaysia (Frankie Sitam, Jeffrine Japning, Rahmat Topani, Kadir Hashim, Hatta Musa), Hospital Universiti Malaysia Sabah (Helen Lasimbang), Faculty of Medicine, Universiti of Malaya (Sazaly Abu Bakar), Kota Kinabalu Public Health Laboratory (Jiloris Dony, Joel Jaimin), Ministry of Health (Chong Chee Kheong, Khebir Verasahib, Maria Suleiman), National Public Health Laboratory (Hani Mat Hussin, Noorliza Noordin, Yu Kie Chem), Queen Elizabeth Hospital (Giri Rajahram, Heng Gee Lee), Sabah State Health Department (Christina Rundi, Mohammad Jikal), Sabah Wildlife Department (Rosa Sipangkui, Senthilvel Nathan, Augustine Tuuga, Jumrafiah Sukor), Gleneagles Hospital Kota Kinabalu (Timothy Wiliam); **BMHRC:** CM Ltd (Tom Hughes), HQEII (Shahnaz Sabri), Kota Kinabalu Public Health Laboratory (Jiloris Dony), Sabah State Health Department (Mohammad Jikal)

Thailand – **CM Ltd:** Department of Disease Control, Ministry of Public Health (Rome Buathong, Pawinee Doungngern, Pongtorn Chartpituck), Department of National Park, Wildlife and Plant Conservation (Pattarapol

Manee-On), Faculty of Forestry, Kasetsart University (Prateep Duengkae), Mahidol-Oxford Tropical Medicine Research Unit, (Stuart Blacksell, Richard Maude),Chulalongkorn University (Supaporn Wacharapluesadee).

Chulalongkorn lab is a WHO Collaborating Centre for Research and Training on Viral Zoonoses, we work closely with WHO SEARO and SEARO countries including Bangladesh, Bhutan, Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, (Thailand), Timor-Leste.
We had conducted the PREDICT lab training for Veterinary lab in the Southeast Asia region for FAO.
We conducted (transferred?) the bat sampling technique for scientists from Malaysia, Myanmar, the Philippines, and China.

5. Data Management Plan

In a clearly labeled section entitled **"Data Management Plan":**

- Describe internal and external data acquisition strategies to achieve harmonization of systems and procedures for data management, data quality, data analyses, and dissemination for all data and data-related materials generated by the research study with the EIDRC CC.
- Within the plan, indicate the extent to which dedicated systems or procedures will be utilized to harmonize the acquisition, curation, management, inventory and storage of data and samples. Describe how training for the data and sample collection, in terms of the use of electronic data capture systems, will be provided to all staff including those at enrollment sites.
- Describe the quality control procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens.

This includes sample labeling etc. Get a bar-coding system, everyone has a scanner (cf. PREDICT) – Ralph has this for select agents and MERS. Also need a software system

Language from NIH CoV grant:

Data Sharing Plan: Data will be available to the public and researchers without cost or registration, and be released under a CC0 license, as soon as related publications are in press. Data will be deposited for in long-term public scientific repositories – all sequence data will be made publicly available via GenBank, species location data via the Knowledge Network for Biodiversity, and other data will be deposited in appropriate topic-specific or general repositories. Computer code for modeling and statistical analysis will be made available on a code-hosting site (GitHub), and archived in the Zenodo repository under an open-source, unrestrictive MIT license. Limited human survey and clinical data will be released following anonymization and aggregation per IRB requirements. Publications will be released as open-access via deposition to PubMed commons.

Viral isolates will remain at the Wuhan Institute of Virology initially. Isolates, reagents and any other products, should they be developed, will be made available to other NIH-funded researchers via applicable Wuhan Institute of Virology and EcoHealth Alliance Material Transfer Agreements and/or licensing agreements.

Sharing Model Organisms: We do not anticipate the development of any model organisms from this study. Should any be developed, they will be made available to other NIH-funded researchers via applicable Wuhan Institute of Virology and EcoHealth Alliance Material Transfer Agreements and/or licensing agreements.

Genomic Data Sharing: We anticipate obtaining genetic sequence data for 100s of novel coronavirus genotypes, including RNA-dependent RNA polymerase (RdRp) and Spike genes for all strains/genotypes. In

addition, we will generate full viral genomes for a subset of the bat SARSr-CoVs that we identify. All sequence data will be deposited in the NIH genetic sequence database, GenBank. We will ensure that all meta-data associated with these sequences, including collection locality lat/long, species-level host identification, date of collection, and sequencing protocols will also submitted. The genotype data will be made publicly available no later than the date of initial publication or six months after the receipt of final sequencing data, whichever comes first. We anticipate sequence generation will occur over the 5 year proposed project period.

Genome Wide Association Studies (GWAS): Not applicable.

Commented [KJO69]: From CoV NIH grant

Thailand TRC-EID Chulalongkorn lab:Specimen management and BioBank: The software called PACS (Pathogen Asset Control System) supported by BTRP DTRA, is now used for managing the specimen from specimen registration (barcoding, and may move to use QR code system soon), aliquoting, tracking, short patient history record, data summary, searching, test reporting and etc. Our biobank system contains 28 freezers (-80C) and liquid N2 system. Its size is around 120 sq meters. There is a separate electric generator for our lab and biobank. There are CC TV cameras at the biobank and laboratory.

Chula TRC-EID has our own server for data management and analysis. We have the necessary software including Bioinformatic tools (Blast Standalone, SAMTOOL, GATK, AliVIEW, RaxML, FigTREE, MAFFT, Guppy, Megan, PoreChop, MEGA), Database (MySQL, MongoDB) Web, jQuery, Node.js, Express.js React, API, PHP, Ruby, Python, Java, C#, HTML5, Bootstrap, CSS, Responsive, Cloud, Linux Command, PACS (sample management system), Virtualbox virtual machines, R programming languages, Perl scripts, Bash shell scripts, Open source software, Microsoft Office running on both Apple Mac OS X, Ubuntu, Linux, and Windows Operating Systems. Chula TRC-EID has a dedicated 16-core Ram 64GB Linux server with 4TB hard drives, dual quad-core Mac Pro Server with 2TB hard drives and another dedicated iMac Pro (Retina 4K, 21.5-inch, 2017) Processor 3.6GHz quad-core Intel Core i7 (Turbo Boost up to 4.2GHz) Ra, 16GB with 512GB hard drives. TRC-EID also has HPC Linux server CPU: 4 x 26cores Intel Xeon = 104 cores 2.1GHz with 52 TB HHD and 2x3.84TB SSD with GPU 2x Tesla v100 (CUDA core = 5120 cores/GPU, Tenser core = 640 cores/GPU). TRC-EID has a dedicated 10/100/1000 of LAN (Local Area Network), 2.4G / 5G Gigabit Wi-Fi 802.11ac.

Commented [KJO70]: From Supaporn for their lab, some may go in Facilities doc?

From DARPA:

Data Management and Sharing: EcoHealth Alliance will maintain a central database of data collected and generated via all project field, laboratory, and modeling work. The database will use secure cloud hosting services and enable export to archival and platform-independent formats. It will ensure data and metadata compatibility between project components, track data versioning and annotations, and enable compliance with DARPA data requests and disclosure requirements. All archived human sample data will be de-identified. Partners will provide raw and processed data to the central database throughout the course of the project. Project partners will have access to the data they generate in the database at all times, and maintain control over local copies. Release of any data from the database to non-DARPA outside parties or for public release or publication will occur only after consultations with all project partners. EHA has extensive experience in managing data for multi-partner partner projects (PREDICT, USAID IDEEAL, the Global Ranavirus Reporting System).

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6. Clinical Management Plan

Clinical site selection

Our consortium partners have been conducting lab and human surveillance research, inclusive of human surveillance during outbreaks, for over the past two decades and in that time have developed strong relationships with local clinical facilities and processes. The plan for selection of clinical sites will begin in the same geographic regions as those identified in Aim 1. These are regions where we will sample wildlife due to the high potential of zoonotic viral diversity. Additionally, these will be clinical sites that serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2. In PREDICT we developed

successful working relationships with the major healthcare facilities the hotspot areas in 6 different countries, including Thailand and Malaysia and we will continue to work with these sites going forward. Continuing to work with these hospitals means we will begin this project with successfully established partners and we can begin data collection quickly. This also reduces the number of additional sites needed to meet our goals within our geographic range. The capacity and capability requirements for clinical sites for this study is limited. All that is required of sites will be the ability to enroll patients that meet the clinical case definitions of interest, capacity to collect and temporarily store biological samples, and follow standards for data management and protection. We will be enrolling inpatient and outpatient participants within 10 days of the presentation of symptoms to increase the chance of PCR detection of viruses so we will not wait for advanced normative diagnostic tests, that are likely unavailable in the more remote sites, to be completed. We will work with clinical sites to determine the best process for project staff, in most cases supporting the time of currently site staff. These will be persons who are locally trained and certified to collect biological specimen from participants including blood draw technique, e.g. doctor or nurse. Last, the clinical site will need the capacity to securely store all personal health information and personally identifiable research data, this will include locked offices with locked filing cabinets to store all paper records and an encrypted computer. Training on project procedures will be provided for all local project staff by local country project management staff and partners from headquarters. The study at clinical sites will begin by developing relationships with local stakeholders at the hospitals and clinics in our geographic areas of interest that serve as catchment facilities for people of within the community surveillance study. We will recruit hospital staff that will be extensively trained for project procedures including enrollment of patients, sample types to be collected, storage of samples, administration of the questionnaire, and data management plans. During the Development and onboarding of new clinical sites we will assess the physical needs of the site and what supplies will be required for collection of data (e.g. locked filing cabinet, dry shipper, secure laptop). [Did not specifically address CONDUCT]. Oversight for clinical sites will be managed by the country coordinator for each country. This will be done with regular site visits to the clinical sites and annual visits by headquarter research staff. Site visits will include monitoring and evaluating the process for patient enrollment, collecting and storage of samples, administration of the questionnaire, and secure and data management procedures for secure personal health information and research data. During these visits any additional training will also be provided.

Commented [EH72]: Is that true

Standardized approach oversight and implementation

Management and oversite for all study sites will be managed by the local country coordinator who is supported by staff at headquarters. Implementation of a standardized approach across all study sites, community and clinical, will begin by extensive training of staff on procedures on requirements of the project, this includes: enrollment, data collection, and data management. Our research team has over 5 years of experience building capacity on human subjects' research and has developed training resources and materials for standardized implementation of community and clinical research. This includes SOPs for screening, enrollment, and retention of participants. In addition to yearly monitory reports the country coordinator will regularly visit each clinical site to ensure quality standards and compliance of procedures are being met. Through our work with clinical sites in PREDICT we have developed culturally appropriate screening measures for clinal sites that do not disrupt the flow or quality of medical treatment received by the patient. This was done by working collectively with clinical research staff to evaluate current procedures and patient flow at the site to determine the most efficient while minimal invasive inclusion of our study into the daily working of the clinal site. Most efficiently this was done by adding minimal basic screening questions to the current clinal intake forms, which allows the clinal research officer to quickly scan charts or logs for potential patients to enroll, allowing for minimal disruption and prevents potential enrollees from being overlooked if the research staff is not on duty. At clinical sites that do scheduled outpatient days we may have a trained member of the research staff with the intake nurse to monitor the patients coming to the clinic more rapidly to no delay screening or enrollment in such a fast pace environment. Using the screening tools, we will recruit and enroll patients who present with symptoms that meet the clinical case definitions of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever, of unknown etiology within the past 10 days. These patients will be enrolled, samples collected and survey conducted to assess the

participants contact with wildlife through: 1) occupational exposures; 2) animal contact; 3) environment biosecurity. With permission from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology. The clinical cohort will be split into cases and controls. “Cases” are defined as participants whose samples tested positive for either coronaviruses, henipavirus, or filoviruses, serological tests. “Controls” will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between serological/PCR status and risk factors. In clinical study, we will use this procedure to determine how clinical presentation differs between virus-exposed and -unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. We will model the outcomes to analyze correlation between biological results and risk factors related to high risk wildlife exposure. Additionally, we will be following up with clinical participants to determine how clinical presentation differs between coronaviruses, henipavirus, or filoviruses in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. [How do these cohorts be leveraged for new emerging pathogens] Achieving the adequate numbers will be attained through duration of the as clinical participant enrollment. While patient’s enrollment is limited by the number of individuals presenting at hospitals, during the PREDICT project we had an average of 105 patients enrolled per year, ranging from 77-244. The country coordinator will be continually monitoring the project database to be sure that we are on track to reach our target.

Clinical cohort setup, recruitment, enrollment

We will recruit inpatients and outpatients from clinical sites to participate in the study after initial screening to determine if they meet the clinical case definitions for 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever, of unknown etiology for symptoms reported onset of illness to increase the chance of PCR detection of viruses. Once enrolled, biological samples will be collected by trained hospital staff. When possible, samples will be taken concurrently with samples being collected for normative diagnostics. We will collect 2* 5mL of blood for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples and two nasal or oropharyngeal swabs will be collected. The cases are defined as participants whose samples tested positive for Henipaviruses, Filoviruses, or CoVs by PCR or serological tests. The controls will be selected from the pool of participants admitted to the studies and whose samples tested negative for coronaviruses, henipavirus, or filoviruses. We will follow up 35 days after enrollment to collect an additional 5mL blood draw for collection of two 500 µL serum samples and a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. Following up 35 days post initial collection gives adequate time for 1) development of IgG and IgM, which occurs <28 days after onset of symptoms for coronaviruses, henipavirus, or filoviruses infected patients to evaluate the immunological progression of disease and 2) further the risk assessment of the participants to monitor contact with wildlife, people, and to assess the likely reservoir hosts to collect information to inform potential intervention programs.

Utilization of collected data

Sampling of wildlife and the enrollment of community surveillance participants and a clinical cohort of participants give us the ability to assess the viruses the are circulating in each of the three populations in a similar geographic region. We will use phylogenetic analysis to compare the relationship between wildlife viruses found and viruses fond in human participants. Additionally, the questionnaire allows us to assess relative measures of human-wildlife contact from our survey work that we will analyze the intensity of contact with species known to be at a higher risk for spillover against other risk to provide useful proxy information for spillover risk.

The biological specimens to be collected (2*5mL whole blood for whole blood and serum analysis and nasal or oropharyngeal swabs) from all eligible participants and follow up specimen 35 days (3mL of whole blood for serum) after enrollment with the standardized questionnaire. Capacity for specimen is collection is not more

Commented [EH73]: Will these cohort fulfill the goals of the EIDRC.

Commented [EH74]: PREDICT we also make the stipulation that we will collective relatvive samples if avialble from treatmean collection, eg CSF if the MD is doing a spinal tap we will request an aliqot if possible

Commented [EH75]: Please add any information that might address: types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.

Commented [EH76]: Please add any information that might address: Describe methods that might be applied to elucidate a zoonotic source

than standard phlebotomy skills and we will collected by locally trained and certified personnel that are part of the research team in country. Staff we will trainline on ethical guidelines for conducing human subjects research and survey data collection methods. Collection of whole blood, serum, and nasal or oropharyngeal swabs allows us to test the samples for PCR to detect viruses in our priority viral families, serologically test for immunological response and historical exposure to zoonotic diseases of relevance, and the questionnaire will assess individuals evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. With this collective data set we aim to quantify and detect novel viruses likely to spillover regularly to people, are often unreported or misdiagnosed as their clinical manifestations are unknown to detect cryptic outbreaks causing previously 'hidden' clinical syndromes in people. Our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence.

Potential expansion
Expansion of the research project to other clinical sites or research areas could happen as new information is gathered, either through our research or an outbreak in the region. If expansion is required we could rapidly implement research activities in additional clinical or community sites through the same process we on-boarded the initial locations. First, by working with local stakeholders to create a working relationship and on-boarding new clinical research staff through advanced trainings of project aims, SOPs on data collection and storage, and ethical guidelines. The network of infectious disease researchers in the community and hospital settings would allow for an accelerated response to any potential infectious disease outbreaks as staff are well trained on ethical guidelines for working with human subjects, data and specimen collection, storage, and transpiration, project staff would be on the front lines ready for the call to action. Ideally, our testing for viruses in priority viral families may lead to the discovery cryptic outbreaks before it spreads in the general public. Biological results coupled with survey data on key risk factors will provided necessary information for granular understand of disease spread.

In a clearly labeled section entitled **"Clinical Management Plan":**

- Describe plans, processes, and procedures for the following activities: clinical site selection including feasibility, capacity, and capabilities, and study development, conduct, and oversight.
- Describe strategies for oversight and implementation of standardized approaches in the recruitment and clinical characterization of adequate numbers of relevant patient populations to ensure the prompt screening, enrollment, retention and completion of studies. Describe novel approaches and solutions to study enrollment. including the clinical meta-data that will be captured and describe how the staff at each enrollment/collection site will be trained and comply with the quality standards for data and sample collection and compliance with the standardized procedures. Provide a general description of the methods for establishing clinical cohorts and how cohorts may be leveraged for new emerging pathogens.
- Provide general approaches to identification of the types of clinical cohorts needed to fulfill the goals of the EIDRC. Discuss types of clinical cohorts and clinical assessments that will be utilized to study natural history and pathophysiology of disease, including characteristics of the cohort, site for recruitment, types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.
- Describe methods that might be applied to either elucidate a zoonotic source or a vector of transmission.

Our research team incredibly diverse and capable of working with a group of other viral families: Lay out all our additional vector experience incl. my WNV work, our work on Chik etc.
Baric: Flavi, Noro,
Linfa: …

EHA: …

- Describe capacity for and approaches to clinical, immunologic and biologic data and specimen collection, and how these data and specimens will address and accomplish the overall goals and objectives of the research study.

Ralph

1. Norovirus: collaboration with surveillance cohort CDC; first one to publish antigenic characterization of each new pandemic wave virus; and phase 2 and 3 trial of therapeutics
2. Tekada Sanofi Pasteur dengue
3. Nih tetravalent vaccine

- Provide plans for the potential addition of new sites and expanding scientific areas of research when needed for an accelerated response to an outbreak or newly emerging infectious disease.

Linfa – will launch VirCap platform on outbreak samples.

7. Statistical Analysis Plan

In a clearly labeled section within the Research Strategy entitled **"Statistical Analysis Plan":**

- Describe the overall plan for statistical analysis of preliminary research data, including observational cohort data.
- Describe the overall staffing plan for biostatistical support and systems available for following functions: 1) preliminary data analyses, 2) estimates of power and sample size, 3) research study design and protocol development.
- Describe the quality control and security procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens. Describe any unique or innovative approaches to statistical analysis that may be utilized.

8. Project Milestones and Timelines

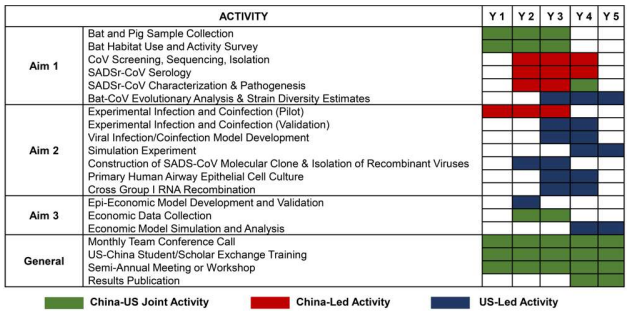
In a clearly labeled section entitled **"Project Milestones and Timelines":**

- Describe specific quantifiable milestones by annum and include annual timelines for the overall research study and for tracking progress from individual sites, collaborators, and Teams. Milestones must specify the outcome(s) for each activity. Milestones should be quantifiable and scientifically justified, and include the completion of major research study activities, including, for example, protocol development, case report forms, scheduled clinical visits, obtaining clearances study completion, and analysis of final data. Milestone criteria should not simply be a restatement of the specific aims. Using a Gantt chart or equivalent tool, describe the associated timelines and identified outcomes for the research study.

Show that for this proposal, we won't be doing the Specific Aims sequentially. We'll begin the human work (SA 2, 3) at the beginning of the project, along with SA1. We'll do the diagnostic assay development initially in Ralph's, Linfa's, Chris's lab, but then begin tech transfer in Y1….

Project Management & Timeline

PI Daszak will oversee all aspects of the project. Dr. Daszak and has two decades of experience managing international, multidisciplinary research in disease ecology, including successful multi-year projects funded by NSF, NIH and USAID. He will conduct monthly



conference calls with all co-PIs, including the China team **(see Activity schedule below)**. He will be assisted by EHA Sr. Personnel Li, who is a US-based Chinese national with fluent Mandarin. Co-PI Olival will oversee Aim 1 working closely with China PI Tong, who will test samples. Co-PI Ma (China team) will coordinate and lead all Chinese experimental infections, with guidance from co-PI Saif, who will coordinate and lead all US experiments with co-PI Qiuhong Wang. Sr. Personnel Ross will conduct all modeling activities in Aim 2, working closely with co-PI Saif to parameterize the models. Co-PI Baric and Sr. Personnel Sims will lead infectious clone, recombinant viral and culture experiments. Consultant Linfa Wang will advise on serology, PCR and other assays. China team co-PIs Shi and Zhou will oversee viral characterization in Aim 1. Sr. Personnel Feferholtz will oversee the economic modeling in close collaboration with Ross.

Commented [KJO77]: Text and figure From NSF-NIH SADS grant. Peter will adapt

Another Example of Milestones and specific tasks from a prev DHS (unfunded) proposal:

Expand existing databases to include predictor variables for pathogenicity risk model
Task 1: Collate available human clinical information from the literature for ~300 viral pathogens known to infect humans (Contract Award Date to 6 month)
Task 2: Update EHA host-viral association database to include new data for mammalian and avian species from literature 2015-present (Contract Award Date to 3 month)
Task 3: Extract and curate full and partial genomic data from Genbank for 5-6 viral families with a high proportion of human-infectious pathogens for phylogenetic analyses – e.g. 'nearest-known-threat module'. (Month 2 to 6 month)

Develop underlying pathogenicity and spillover risk assessment models
Task 4: Fit generalized linear models in Stan for multiple response variables to measure pathogen impact (Month 7 to 10 month)
Task 5: Fit generalized linear models to estimate risk of human infection (spillover) based on host, ecological, and location specific traits (Month 4 to 8 month)
Task 6: Validate model effectiveness by simulating early-outbreak, limited-information scenarios from known pandemics to confirm model behavior (Month 8 to 10 month)

Commented [KJO78]: Just example, but probably just want to use a Gantt chart format per most of our grants, depending on space.

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From: Laing, Eric on behalf of Laing, Eric <eric.laing@usuhs.edu>
To: Tom Hughes
Cc: Peter Daszak; Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Supaporn Wacharapluesadee; Aleksei Avery Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross; Kevin Olival
Subject: Re: EID-SEARCH v4
Date: Wednesday, June 26, 2019 12:59:39 PM
Attachments: EIDRC Southeast Asia v4-CCB-EDL.docx

Hi Peter,

Built on top of Chris' suggestions. Included a prelimin data figure from Hughes et al, in prep. Double check that is ok with Tom?

- Eric

Eric D. Laing, Ph.D.
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On Wed, Jun 26, 2019 at 11:26 AM Tom Hughes <tom.hughes@ecohealthalliance.org> wrote:

> Hi Peter,
>
> I have added my edits and comments to Kevin's.
>
> Please let me know if you ave any questions or need more details.
>
> Thanks.
>
> Tom
>
> ---
>
> **From:** Kevin Olival <olival@ecohealthalliance.org>
> **Sent:** 26 June 2019 2:51 PM
> **To:** Peter Daszak
> **Cc:** Wang Linfa; Danielle E. ANDERSON PhD; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris

Broder; Eric Laing; Thomas Hughes; Supaporn Wacharapluesadee; Aleksei Avery Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross
Subject: Re: EID-SEARCH v4

Peter, v4 with my edits, some revised figs, and additional comments.

Cheers,
Kevin

Kevin J. Olival, PhD
Vice President for Research

EcoHealth Alliance
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> On Jun 25, 2019, at 9:26 AM, Peter Daszak <daszak@ecohealthalliance.org> wrote:
>
> Dear All,
>
> Here's version 4. Apologies for the delay. It's in better shape now, but there are still lots of areas that need your attention. Please put in time today, and tomorrow to edit and firm up the text. I've put your names in some of the comment boxes where I need specific help but I also need you to read this critically now and edit and tighten it up. We have 30 pages of space, and our research plan is now 21 pages, so we need to lose maybe 3 or 4 pages. There are also some weak points, specifically on Filoviruses, on the details of where we'll do our clinical work, and on what we'll do with the animal models for henipas and filos.
>
> Please get comments back with me before Wednesday 26th 4pm New York time, so that I have Wendesday evening and all day Thursday to incorporate them. In the meantime, I'll be working with folks here on better figures, and all the extra sections that come after the research plan. I'll need you all to help with those on Thursday while I'm finishing off the draft.
>
> Thanks to all of you in advance and look forward to the next round.

Cheers,

Peter

Peter Daszak
President

EcoHealth Alliance
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New York, NY 10001

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Twitter: @PeterDaszak

EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

From: Peter Daszak
Sent: Thursday, June 20, 2019 9:41 PM
To: Wang Linfa; Danielle Anderson (danielle.anderson@duke-nus.edu.sg); Ralph Baric (rbaric@email.unc.edu); Baric, Toni C; Sims, Amy C; Christopher Broder (christopher.broder@usuhs.edu); Eric Laing; 'Tom Hughes'
Cc: Aleksei Chmura (chmura@ecohealthalliance.org); Alison Andre; Luke Hamel (hamel@ecohealthalliance.org); Kevin Olival, PhD (olival@ecohealthalliance.org)
Subject: EIDRC-SEA v.3
Importance: High

Dear all,

Thanks to everyone for comments and text for the earlier draft at the beginning of the week. Kevin's spent the last couple of days pulling all the comments together into one draft and inserting his changes, and I've now attached a v3 of the draft. It's still rough, and now too long, but it's getting there. I wanted you to look at this rough version because I'm stuck in Liberia in meetings Friday, then flying back home Saturday. I'll continue editing this draft on the plane and will be working solely on this grant from home on Sunday, Monday, Tues, Wed, Thurs.

Please work on this rapidly if possible. If you can all push your comments through, using track changes, and get them back to me **by Sunday evening New York Time**, I

will turn round a polished draft by Tuesday, giving us time for significant last go-over on Wed, Thurs. It's tight, but unfortunately, it is what it is, so please commit the time if you can!! As well as general edits (using track changes, with references in comment boxes), please do the following…

Linfa/Danielle – We especially need your input in section 1.1, 1.3, 1.4 and 2.5

Ralph/Amy - Please work your magic on this draft, inserting text, editing and reducing text, and making sure the technical details of the characterization are correct and fit the proposed work on CoVs, PMVs and Henipaviruses

Chris/Eric – Eric – congratulations on your (b) (6) and thanks for working on it at this time! Chris – please help and edit/finesse the language in the relevant sections for you

Tom – Your edits crossed over with Kevin's changes, so please insert all the bits that were new in the last version you sent to me, into this version. I think these are mainly the sections and comments from the other Malaysian colleagues, but please check and re-insert all key text. We especially need to be specific about which partner in Malaysia is doing what cohorts, and where, and about who's doing the testing

Thanks again all, and I'm looking forward to non-stop work on this as soon as I get on my flight in 24 hours…

Cheers,

Peter

Peter Daszak
President

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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

<EIDRC Southeast Asia v4.docx>

II. Research Strategy:

1. Significance:

Southeast Asia is a critical hotspot for emerging diseases due to its high biodiversity of wildlife and their viruses, and the presence of key ecological and socioeconomic drivers of emergence **(Fig. 1)** (*1*). These include dense human populations living in networks of rural and urban communities, with strong cultural and occupational connection to wildlife and livestock farming and trading, and intimate connection to global travel networks (*2*). The region is also undergoing rapid environmental and demographic change, both of which increase the risk of disease emergence and spread.

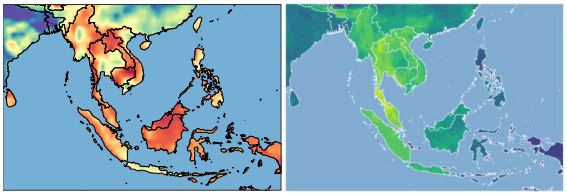


Fig. 1: Southeast Asia, and in particular, parts of Thailand and Malaysia are hotspots of risk for disease emergence **(left)**, and predicted high diversity of 'missing' or as-yet undiscovered viruses, yellow = highest diversity **(left)**. From (*1, 2*).

Not surprisingly a number of novel viruses, including near-neighbors of known agents, have emerged in the region leading to sometimes unusual clinical presentations **(Table 1)**.These factors are also behind a significant background burden of repeated Dengue virus outbreaks in the region (*16*), and over 30 known *Flavivirus* species circulating throughout S. and SE Asia (*17*). The events in **Table 1** are dominated by three viral families: coronaviruses, henipaviruses and filoviruses, which serve as initial examples of our team's research focus and capabilities. These viral groups have led to globally important emerging zoonoses (*18-29*). Their outbreaks have caused tens of thousands of deaths, and billions of dollars in economic damages (*30-32*). Furthermore, relatives and near-neighbors of known pathogens in these families have been reported throughout the region, including by members of our consortium: henipaviruses in bats in Thailand, Cambodia, Philippines, Laos, Indonesia, China, Malaysia, Bangladesh and India (*33-39*); a novel henipavirus, Mojiang virus, in wild rats in Yunnan (*10*); serological evidence of filoviruses in bats in Bangladesh (*40*) and Singapore (*41*); Nipah- and Ebola-like viruses in *Hipposideros*, *Cynopterus* and *Rhinolophus* species in Malaysia (Hughes *et al.*, in prep.); evidence of novel filoviruses in bats in China (*42-44*), including Měnglà virus that appears capable of infecting human cells (*45*); novel paramyxoviruses in bats and rodents consumed as bushmeat in Vietnam (*46*); a lineage C β-CoV in bats in China that uses the same cell receptor as MERS-CoV and can infect human cells *in vitro* (*47*); MERSr-CoVs in dry bat guano being harvested as fertilizer in Thailand (*48*), and directly in bats (Wacharapluesadee *et al.*, in prep.);

Viral agent	Site, date	Impact	Novelty of event	Ref.
Melaka & Kampar virus	Malaysia 2006	SARI in family group, individual	1st disease due to bat-origin reoviruses, also Singapore, Vietnam etc.	(*3-6*)
Reston filovirus	Philippines 2008	Seropositive people killed pigs,	No prior findings of filoviruses in pigs	(*7*)
Thrombocytopenia Syndrome virus	E. Asia 2009	100s of deaths in people	Novel tick-borne zoonosis with large caseload	(*8*)
Reston filovirus	Shanghai 2011	PCR positive pigs	Further evidence of pig RESTV infection	(*9*)
Mojiang virus	Yunnan 2012	Death of 3 mineworkers	1st evidence of rodent origin henipavirus in people	(*10*)
Nipah-like virus	Philippines 2015	Killed horses	No prior horse infection for NiV, known for HeV	(*11*)
SARSr-CoV & HKU10-CoV	S. China 2015	Seropositive people	1st evidence human infection HKU10 & SARSr-CoV	(*12*)
SADS-CoV	China 2017	>25,000 pig deaths seronegative people	Novel emergence of bat-origin CoV	(*13*)
Nipah virus	Kerala 2018, 2019	Killed 17/19 people 2018, 1 infected 2019	1st outbreak of NiV outside Bangladesh, W. Bengal focus	(*14, 15*)

Table 1: Recent emergence events in SE Asia indicating potential for novel pathways of emergence, or unusual presentations for known or close relatives of known viruses.

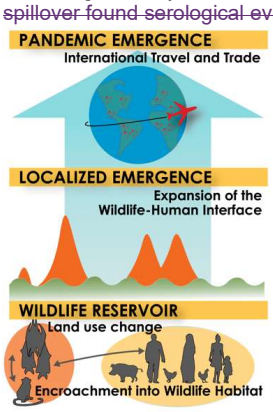
172 novel β-CoVs (52 novel SARSr-CoVs) and a new β-CoV clade ("lineage E") in bat species in S. China that are also found throughout the region (*20, 47, 49, 50*); and 9 and 27 novel wildlife-origin CoVs and

Commented [MOU1]: Should include Olival et al. 2017 for missing zoonoses, not Jones. Or Jones, Allen, and Olival papers.

Commented [MOU2]: Shouldn't we add the emergence of Nipah in Malaysia Singapore in 1999 at the top of the table!?

Commented [DA3]: Yes.
PMID: 10827955
PMID: 10520634

paramyxoviruses in Thailand (respectively) and 9 novel CoVs in Malaysia identified as part our work under USAID-PREDICT funding (*49, 51*). These discoveries underscore the clear and present danger of future zoonotic events in the region. Furthermore, the wide diversity of potentially pathogenic viral strains has significant potential to help in the development of broadly active vaccines, immunotherapeutics and drugs (*47*). Most high-impact zoonotic viruses originate in wildlife reservoirs, sometimes spilling over first into livestock 'amplifier' hosts, or directly into localized human populations with high levels of animal contact **(Fig. 2)**. Efforts to prevent emerging zoonoses have targeted these high-risk populations in regions prone to disease emergence (*29, 52*). However, these regions tend to be in the developing world, where nations often lack the resources to investigate novel zoonotic events, or wildlife reservoirs of zoonoses, and prioritize more immediate public health concerns such as Dengue virus or NTDs (*16*). Zoonotic disease surveillance and control is also hampered by inadequate information on basic disease ecology (e.g. range of wildlife reservoirs, risk factors for spillover) and pathogen biology (viral diversity, pathogenesis), and by logistical challenges sharing novel diagnostics, animal models, samples and reagents at sites where outbreaks begin (*53*). Countermeasure and vaccine development is challenged by the small number of isolates available, and the presence of closely-related strains, some with evidence of transmission to people but with unknown pathogenicity. For example, using NIH and other funding, we found serological evidence of exposure to African henipaviruses in 3-4% of a sample of people in Cameroon, with a significant risk factor being butchering of bats; and serological responses to bat SARSr-CoVs in 6/209 (2.87%) and bat HKU10-CoV in x/xx (x%) people living close to a bat cave in Yunnan, all novel viruses with unknown clinical impact (*12, 54*). We discovered two novel bat-borne reoviruses, Melaka and Pulau viruses, and showed that 14/109 (13%) of people living in close proximity to bat roosts on Tioman Island, Malaysia were exposed to these viruses (*3*) as well as 12/856 (1.4%) people screened in Singapore (*4*). Previous studies in the Philippines found famers and butchers who had contact with sick pigs tested positive for Reston ebolavirus antibodies but had not experienced illness (*7*), and it was our team that discovered Philippine bats as natural reservoir hosts for Reston virus (*55*). In pPreliminary screening in Malaysia with funding from DTRA ~~for serological surveillance of Henipaviruses and Filoviruses spillover found serological evidence of past exposure to Nipah and Ebola antigenically-related viruses in non-human primates (NHPs) and people (Hughes, in prep.).~~ we found serological evidence of exposure to ~~H~~henipaviruses (Hendra (HeV), Nipah- (NiV) and Cedar (CedV)) in 14 bats and 6 human samples, and Ebola (Zaire and Sudan) antigenically-related viruses in 11 bats, 2 NHP and 3 human samples (Hughes, in prep.).



Even when spillover leads to outbreaks of illness and mortality, they are often unreported, undiagnosed or misdiagnosed. Under a prior NIH R01, we initiated targeted syndromic surveillance of encephalitis patients in Bangladesh clinics and showed that NiV causes repeated annual outbreaks with an overall mortality rate of ~70% (*56*). Nipah virus has been reported in people in West Bengal, India, which borders Bangladesh (*26*), in bats in Central North India (*57*), and now in people in Kerala, South India in 2018 and 2019, raising the specter of future spillover at other sites in the region (*14, 58*).

Fig. 2: Our Early Warning Approach: Most zoonotic viruses emerge from wildlife hosts, sometimes via livestock (Stage 1, lower panel), to cause small scale outbreaks (green) or larger chains (red) of human-to-human transmission (Stage 2, middle). In some cases, these spread more widely via air travel (Stage 3, upper). Our EIDRC proposal targets each stage: SA1 examines the diversity and spillover potential of viral threats in targeted SE Asian wildlife; SA2 seeks evidence of their spillover into focused high-risk human populations (stage 2); SA3 identifies their capacity to cause illness and to spread more widely (stage 3). Figure from (*52*).

Under a current NIAID R01, we have shown that we can use analytical approaches to target surveillance of wildlife and people and identify likely sites of spillover. We used phylogenetic analysis of bat hosts and their CoVs to identify a key cave complex in SW Yunnan Province that harbors all the genetic elements of SARS-CoV (*59*), as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics (*20, 47, 49, 50, 60-62*). We conducted behavioral risk surveys and biological sampling of human populations living close to this cave and found evidence of spillover (xx serology) in people highly exposed to wildlife. **This work provides proof-of-**

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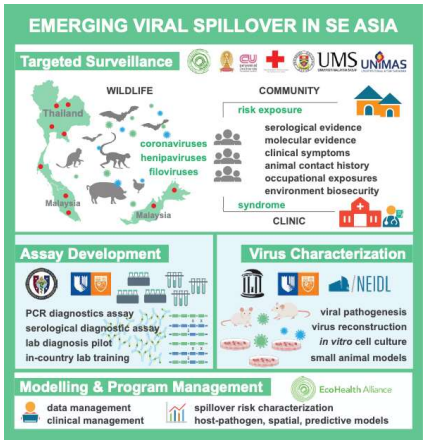
concept for an early warning approach that we will expand in this EIDRC to target key viral groups in one of the world's most high-risk EID hotspots.

The overall premise for this EIDRC proposal is that there is substantial evidence that: 1) diverse CoVs, henipaviruses and filoviruses related to known zoonotic pathogens are circulating in wildlife reservoirs in Southeast Asia; **2)** these viruses likely spillover regularly to people, are often unreported or misdiagnosed, and their clinical manifestations and potential to cause pandemics are unknown and underestimated; and **3)** our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence. To scale-up this approach, we propose to launch **EID-SEARCH** (the **E**merging **I**nfectious **D**iseases - **S**outh **E**ast **A**sia **R**esearch **C**ollaboration **H**ub), to better understand the risk of zoonotic viral emergence in Southeast Asia. We will initially focus on the diversity of known and novel CoVs, henipaviruses, and filoviruses in wildlife reservoirs. We will use this work to build our center's interactive network throughout the region, bridge barriers, provide early focal point for basic and translational studies, develop sample handling and storage routines, communication networks, cohort management, infrastructure development, and cross-training in surveillance and novel diagnostic platforms. We will develop and transfer technology to test these viruses' capacity to infect human cells and mouse models, develop new specific and sensitive serological and molecular diagnostic tools, conduct surveillance of human communities with high risk of exposure to wildlife, and clinical cohorts to identify evidence of spillover of viruses causing previously 'cryptic' clinical syndromes in people. We will test the capacity of novel viruses we isolate, or genetically characterize, to infect human cells and/or mouse models, and thus asses potential for spillover and spread which will feed into our risk models. Our group also has extensive experience in other viruses, including vector-borne flaviviruses, and alphaviruses, and will use this experience to provide robust long-term surveillance, diagnostics, human and basic science opportunities. While the current proposal focuses its basic and applied research agendas on this group of viral EID, we note that the EID-SEARCH is poised to rapidly respond to any new emerging outbreak. Thus, filoviruses, henipaviruses and coronaviruses are examples of sentinel surveillance systems in place that can capture, identify and initiate a rapid response targeting any novel microbial pathogen that emerges in this region.

2. Innovation: Previous work by our consortium has developed strategies to target surveillance in wildlife and people to better anticipate and identify early spillover events and pre-empt outbreaks of emerging viruses. In the EID-SEARCH we scale up this approach in a central research "hub" made up of leading laboratories, clinics and surveillance sites in three critically high-risk EID hotspot countries in SE Asia. EID-SEARCH's reach is expanded through its regional network of collaborators that covers nearly all of the greater SE Asia region. The innovation of the EID-SEARCH is in: **1)** Its multidisciplinary approach that combines modeling to target geographic and taxonomic sampling targets with on-the-ground zoonotic disease surveillance in human and wildlife populations; **2)** the biological characterization approach that identifies how likely viruses are to be able to spillover into people, and enables evaluation of existing countermeasure technologies; an approach that is grounded in our successful work on SARSr-CoVs; and **3)** the strategic sampling of both high-risk communities with extensive animal contact at the front-lines of viral spillover *and* syndromic clinical cohorts that present with signs of novel viral emergence, to identify risk of viral spillover in an early warning system approach **(Fig. 2)**. **In Aim 1, we will target viruses in archived and newly collected wildlife samples, and characterize their risk of spillover to people**. We will use spatial and phylogeographic analyses to identify the geography and species of wildlife to test samples from, and to further characterize viruses we have recently discovered. We will use *in silico* methods (novel ecological-phylogenetic risk ranking and antigenic mapping of receptor binding domains), cell culture, and animal models to assess their potential for spillover into



Commented [BRS6]: Statement should be in the aims. …Our center covers the worlds most high risk EID hostspot?

Commented [KJO7]: Hongying to add in "genetic sequencing" under Virus Characterization, and also Aim 1 + 3 for Virus Characterization and Aim 1 + 2 for Assay Development

Commented [KJO8]: And pathogeneicity? Could argue for those we use animal models on.

Fig. 2: EID-SEARCH approach, core members, and roles.

high-risk human populations. **In Aim 2, we will conduct ~~focused,~~ targeted cross-sectional surveys of ~~and sampling of~~ human communities with high levels of animal contact ~~exposure to wildlife and other animals~~to identify occupational and other risk factors for zoonotic virus exposure.** We will use serological tests targeting viruses identified in Aim 1 to identify the baseline spillover of known or novel CoVs, henipaviruses and filoviruses in these populations. Serological findings will be analyzed together with clinical metadata (including a simple animal-contact survey, location data, viral sequence/isolates etc.) to identify zoonotic risk factors and routes of exposure from specific wildlife or vector species. **In Aim 3, we will use syndromic surveillance of clinical cohorts to identify evidence of viral etiology for otherwise 'cryptic' outbreaks.** We will enroll and collect biological samples from patients in local clinics and hospitals who present with syndromes previously linked to known viral agents and live within communities at high risk for disease emergence. We will conduct molecular and follow-up serological diagnostic assays to identify likely viral etiology of syndromes that may represent otherwise cryptic events. Where viruses are identified, we will attempt to isolate and characterize them, and use survey data, ecological and phylogeographic analysis to identify likely reservoir hosts and inform intervention programs. Our US partner BSL-4 laboratory, NEIDL, will attempt isolation and characterize any viruses requiring high levels of containment (e.g. any novel filoviruses discovered).

3. Approach

Research team: The EID-SEARCH builds on long-term collaboration among world leaders in emerging virus research with proven experience collaborating internationally on field, lab and human surveillance research **(Fig. 2)**. Over the past two decades, our consortium partners have conducted high profile research on EIDs within the region and globally, including identifying the wildlife reservoirs for Nipah and Hendra virus, MERS- and SARS-CoVs (*20, 25, 57, 59, 63-65*), discovering SADS-CoV (*13*), and developing an array of serological, molecular, *in vitro* and *in vivo* approaches to characterizing high-risk CoVs, henipaviruses, and filoviruses (*60-62, 66-79*). Our team has substantial experience conducting human surveillance in the community and during outbreaks **(See sections 2.x.x, 3.x.x)**, including: USAID-PREDICT, and DTRA-funded viral surveillance of indigenous communities in Peninsular Malaysia and syndromic surveillance of hospital patients in Sabah (Co-I Hughes); collection of >2,000 serum samples from healthy blood donors and sanitation workers, febrile and influenza like illness (ILI), and diarrheal stool samples from children under 5 years of age (Co-I Kamruddin); collaboration on the MONKEYBAR project with the London School of Hygiene and Tropical Medicine to analyze risk and prevalence of the zoonotic malaria *Plasmodium knowlesi* (Co-Is William, Tock Hing); case control and cross-sectional studies in Sabah villages (~800, 10,000 participants resp.) (Co-Is GR, TYW); and community-based surveillance of hundreds of bat-exposed villagers in Thailand (Co-Is Wacharapluesedee and Hemachudha). As a proof-of-concept of how our team can rapidly scale up resources for diagnostics and surveillance in the face of a new EID, in 2016, we discovered a novel bat-origin HKU-2 CoV (*80, 81*) killing >20,000 pigs in S. China, designed PCR and LIPS serology tests, then surveyed pig farmers for evidence of exposure, and conducted experimental infections, pathogenesis and molecular studies, **all in the span of 3 months** (*13, 82*).

The administration of this center **(See section X)** will be led by PI Daszak, who has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5-20 yrs on NIAID- and USAID-funded research. He will oversee all activities in this EIDRC. Drs. Olival and Zambrana are leaders in analytical approaches to targeting surveillance and head the modeling and analytics work for USAID-PREDICT. Drs. Wang, Baric, Broder, Anderson, and Laing have developed a unique array of *in vitro* cell culture, serological and molecular reagents to identify and characterize emerging viruses in people and animals (henipaviruses and other paramyxoviruses, CoVs, filoviruses and many others). Dr. Wacharapluesedee, Hughes and collaborators have coordinated surveillance of people, wildlife, and livestock within Thailand and Malaysia for the past 10 years, in collaboration with Ministries of Health, Agriculture and Environment. Finally, this consortium has extensive external funding from NIH, DoD, USAID, DHS and other USG agencies that supports a range of laboratory, analytical and field studies directly related to the current proposal, and will bring substantial leverage to our EIDRC.

Commented [PD9]: Hongying – Figure needs edits to text: top panel, 'wildlife' should read "Aim 1: WILDLIFE", "AIM 2: COMMUNITY", "AIM 3: CLINIC". Right hand side: "Clinical symptoms" should be "Clinical history", "Occupational exposures" should be "Occupational exposure", "environment biosecurity" should be "environmental risk factors"

Middle panel: "Assay Development" and "Virus Characterization" should have "(Aim 1)" after them. Left hand side: should read "PCR diagnostics", "serology", "piloting diagnostics" and the last one is ok. Right hand side bullets should be "receptor binding characterization" "in vitro characterization" and "mouse models"

Bottom panel: all good

Commented [PD10]: PD will get stronger letter from Jerry Keusch: opportunity for visiting scholars to be certified for BSL-4 research, strong Ebola person at NEIDL to conduct characterization if we find filos.

Geographical focus: The three core (hub) countries for this proposed EIDRC are Thailand, Malaysia and Singapore, three contiguous EID hotspot countries that stretch through one of the most significant foci of EID origins globally. Our proposed work includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sarawak and Sabah, and thus contains overlapping wildlife biogeography with India, Bangladesh, Myanmar, SW China, Laos, Cambodia, Vietnam, Philippines, and Indonesia. It encompasses diverse cultural and socioeconomic backgrounds which have correspondingly diverse and intensive interactions with wildlife reservoirs and the global travel network. We have already begun sampling of wildlife hunters, livestock workers, wildlife farmers and market workers, rural villagers living close to high risk wildlife species, people otherwise highly exposed to animals in these countries, and clinical cohorts, as part of current/prior NIH, DTRA, and USAID funding. To develop a larger catchment area for emerging zoonoses, we will deploy our core member's extensive network of collaborators in clinics, research institutes and public health laboratories in almost every other Southeast Asian country. We will conduct regular meetings with key research, public health and community leaders, including >50 leading laboratories/institutions in ~~XX~~ 10 countries that are currently collaborating with core members of our consortium via other funded work (**Fig. 3**). We will use these, and our annual meetings, to share information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks across the region.

Fig.3 : Map of Southeast Asia indicating three hub countries for this proposed EIDRC (Red: Thailand, Singapore, Malaysia) and countries in which Key Personnel have collaborating partners (Green), indicating that EID-SEARCH provides access to key collaborators throughout the region.

Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife

1.1 Rationale/Innovation: The vast majority of emerging zoonotic pathogens are linked directly or indirectly to wildlife host, particularly from important mammalian groups like bats, rodents and non-human primates (*83*). Our previous work on wildlife origin viruses demonstrates that zoonotic viral emergence tends to occur in specific regions with high diversity of wildlife that harbor viruses of zoonotic potential, and human populations that have close contact to these wildlife **(Fig. 1)** (*2*). In Aim 1, we will strategically conduct EID surveillance by applying risk analytics to prioritize the regions, wildlife species, and human populations that have a particularly high risk of viral spillover. We will target high-risk zoonotic host reservoirs (bats, rodents and primates) in these regions and apply novel predictive models to estimate viral diversity and host range to identify wildlife species that have been ignored or under-sampled by previous zoonotic disease surveillance efforts. We will strategically collect specimens from these species and screen them, together with some of the tens of thousands of recently collected samples we have archived in freezers in our labs, for known and novel agents. We will begin screening using conserved PCR assays to identify known and novel virus in high risk RNA viral families, *Coronaviridae (CoV)*, *Paramyxoviridae (PMV)*, and *Filoviridae (FV)*. We will expand to other groups of pathogens, including arboviruses, pending available resources and priorities established with the EIDRC coordinating center (EIDRC-CC) and other regional EIDRCs. Using risk ranking algorithms and a series of ecological and virological risk factors, we will triage the wildlife viruses we discover for further genomic characterization and viral isolation. On this subset of viruses, we will then use *in vitro* and *in vivo* approaches (viral rescue, cell lines, gene edited humanized transgenic mice and the collaborative cross genetic reference mouse population) previously developed and widely used by our team to predict capacity of novel viruses to infect people and spillover (*61, 84-87*). These high-risk viruses and their close relatives will be targets for human community serosurveys and clinical sampling in Aim 2 and 3, respectively.

1.2 Preliminary data: 1.2.a. Geographic targeting: EcoHealth Alliance has led the field in conducting analyses to indicate where the highest risk of zoonotic spillover is across the globe, identifying SE Asia as a

Commented [KJO11]: Including those in green on map, plus Australia.

Commented [PD12]: Hongying – "EIDRC Program Countries" should be "EID-SEARCH core countries" and "External Partner Countries" should be "Collaborating Partners"

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Commented [PD15]: From Kevin - we need to specify in the methods below how many viruses (number of) we will have resources to do this on

major high risk location for pathogen emergence (*1, 2*). For Southeast Asia, these EID hotspots are sites where high wildlife biodiversity, human population density and rapid ecologic change collide **(Fig 1)**. Using host and ICTV viral data for all known mammalian viruses, and generalized additive models to correct for sampling bias, we are able to predict the relative number of yet-to-be-described viruses that a species harbors (*2*). Using these data to map unknown viral diversity demonstrates substantial undiscovered viral diversity in mammals across Southeast Asia, and particularly in regions of Thailand, Singapore and Malaysia **(Fig. 1)**. In a separate paper on risk of viruses emerging from bat hosts, we found that factors driving spillover (host diversity, climate) differ from those driving spread in human populations (population density, bushmeat hunting, livestock production) (*88*). We therefore analyze capacity for viral spread as a separate risk factor, using demographic and air travel data and flight model software we have produced with Dept Homeland Security funds (*89*). In the current proposed work, we will use all the above approaches to target wildlife sampling (archive and new field collections), and to inform sites for human sampling in Aim 2, to areas of high risk for spillover and spread.

1.2.b. Reservoir host species targeting: Our analysis of all known mammalian host-viral associations demonstrates that bats, rodents and primates represent the three most important reservoirs for zoonoses, responsible for the highest proportion of known and predicted viral diversity of zoonotic potential (*90*). Thus, the bulk of our proposed wildlife sampling and testing will consist of these three taxa with a focus on bats, because this group is the known or hypothesized reservoir for the majority of high-impact zoonotic CoVs, ~~henipaviruses and filovirusesPMVs and FVs~~ (*48, 91-93*). Bats also have the highest proportion of predicted zoonotic viruses in any mammalian group (*2*), and are exceedingly diverse in Southeast Asia (~500 species). To further prioritize taxonomic sampling targets within these diverse geographic hotspots, we have used a novel, Bayesian phylogeographic analysis to identify host taxa that are the most important centers of evolutionary diversity for a given viral group, e.g. bat genera for β-CoVs ~~using an extensive CoV sequence dataset from our NIH-funded research~~ **(Fig. 5)** (*49*). This approach will be extended to other mammal taxa and viral families of interest, allowing us to identify the ancestral wildlife hosts of specific zoonotic viral groups, where their diversity is likely highest.

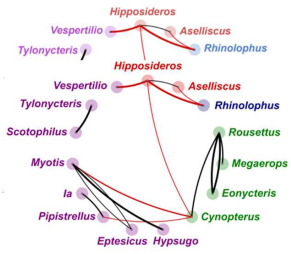

Fig 5: Preliminary analysis to identify the most important bat genera in Southeast Asia ~~as hosts~~ for beta-CoV evolutionary ~~diversitys~~ using ~~our previously-collected CoV~~ sequence data collected under our NIH-funded research. Line thickness is proportional to the probability of virus sharing between two genera, with~~,~~ in ~~In~~ter-family switches ~~are highlighted~~ in red. We will apply this Bayesian discrete phylogeographic model to identify taxonomic and geographic centers of viral diversification and sharing for relevant ~~paramyxovirus~~PMV, CoV ~~, coronavirus,~~ and other virus clades.

Finally, we have designed a strategy to estimate sampling gaps, using a viral 'mark-recapture' approach we previously published (*94, 95*) **(Fig. 6)**. Using prior data for bat, rodent and primate viral discovery vs. sampling rates, we can identify the likely total viral diversity for specific host groups in a region, and use this to estimate sample sizes to discovering the majority of unknown viruses within a specific family or genus. **In the current proposal,** we will use these analyses to estimate geographic and species-specific sampling targets **to more effectively identify new strains to support experimental infection studies and risk assessment.**

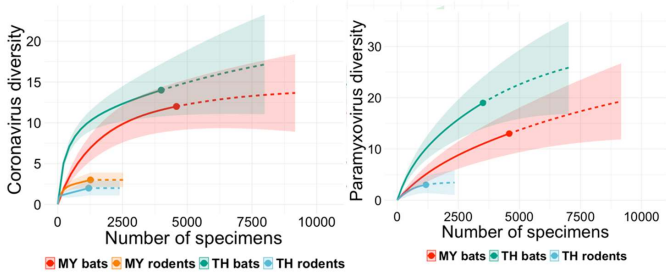

Fig. 6: Estimated ~~coronavirus~~ CoV **(left)** and ~~paramyxovirus~~ PMV **(right)** ~~putative~~ viral 'species' diversity in bats and rodents ~~for~~ from Thailand and Malaysia, using data from PCR screening and RdRp ~~gene~~ sequences ~~preliminary data~~ from >103,000 ~~PCR testsspecimens~~ in bats and 4,500 ~~testsin in~~ rodents. Bats in Thailand and Malaysia have 4X the number of viruses than rodent species, controlling for sampling effort. ~~CoV and paramyxovirus diversity estimates are comparable, but discovery has not yet saturated in any taxonomic group or location.~~ We estimate that additional collection

Commented [KJO16]: Wrong ref! Should be Olival et al. 2017.

Commented [KJO17]: This is currently in Aim 3.

Commented [KJO18]: Olival et al appears twice in refs, fix.

Commented [BRS19]: I would say we focus on these three species (plus vectors?). To demonstrate the capability of the team, this proposal will focus on bat surveillance (for a variety of reasons), noting that this approach will also be applied to other zoonotic vectors during the 5 year program throughout the region.

Commented [PD20]: Ralph – have we done this well enough now? If not, please add text/edit

Commented [PD21]: Kevin - Can you get rid of the shading on the background to this figure and change the colors so the circles and genus names are more visible (esp. the green). Colors should be darker and more contrasty

Commented [MOU22]: Revised figure inserted.

Commented [DA23]: PMV only appears in your paper to define paramyxovirus. I will just leave it as my opinion that I don't like it, but I am not going to change it back.

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of 5k-9k bat specimens ~~sampling of ~5,000 bats~~ and testing of our archived rodents specimens alone will identify >80% of remaining CoV and ~~paramyxovirus~~ PMV viral species in these key reservoirs, yielding >800 unique viral strains. ~~We will use this approach to define sampling targets for other wildlife taxa, and for viral *strain* diversity.~~

1.2.c. Sample testing to identify known and novel viruses: We have conducted extensive wildlife surveillance for novel viruses across Southeast Asia in prior NIAID funded work and as part of the USAID PREDICT project. Under the 10-year PREDICT program globally, we identified 912 novel and 210 known viruses, more than the total number of viruses previously recognized in mammals by the International Committee on Taxonomy of Viruses. This work included collecting nearly 300,000 individual mammal samples from 14 EHA-led PREDICT countries and PCR-screening more than 60,000 individual specimens(*51*). In southern China alone, this sampling lead to the identification of 178 β-CoVs, of which 172 were novel (52 novel SARSr-CoVs). Included were members of a new β-CoV clade, "lineage E" (41), diverse HKU3-related CoVs (179 sequences) within a 'sister' clade to the SARS-CoV lineage, and the wildlife origin of a new virus, SADS-CoV, responsible for killing >20,000 pigs in Guangdong Province (*13*). ~~Many of the identified bat species are found across the region.~~ We have collected 28,957 samples from bats, rodents and primates in Thailand and 51,373 in Malaysia, conducting 146,527 PCR tests on a large proportion of these specimens. We have **archived duplicate samples which are now available for use in this project**, including fecal, oral, urogenital and serum samples from bats, rodents, and non-human primates, and primate biopsies. In Thailand this screening has identified 100 novel viruses and 37 known viruses in wildlife reservoirs. In Malaysia this screening has identified 77 novel viruses and 23 known viruses in wildlife reservoirs (66 novel and 19 known in Sabah and 11 novel and 9 known from Peninsular Malaysia). ~~We used~~Using family level primers for ~~henipaviruses~~PMV, ~~filoviruses~~ FV, and CoVs, we ~~and~~ have already discovered 9 novel and 7 known CoVs and 26 novel and 2 known paramyxoviruses in Thailand; and . ~~This is in addition to~~ 13 novel and 5 known CoVs and 15 novel and 1 known paramyxovirus in Malaysia from wildlife. In the current proposed work, we will attempt to sequence, isolate and characterize those viruses that our analyses below suggest are most likely to be able to infect humans.

Our collaborative group has worked together on these projects, as well as other DTRA funded projects in Singapore/Thailand (Co-Is Broder, Wacharapleusadee, Wang) to design, cross-train, and use novel serological and molecular platforms to investigate virus infection dynamics in bats and examine potential spillover events with or without human disease (see Aim 2). For serology, Co-Is Wang and Anderson (Duke-NUS) will develop a phage-display method to screen antibodies for all known and related bat-borne viruses, focusing on henipaviruses, filoviruses and coronaviruses. Once various "trends" are discovered, additional platforms (including Luminex and LIPS) will be used for verification and expanded surveillance. For molecular investigation, we will combine targeted PCR approach with a discovery-driven enrichment NGS strategy to not only survey currently known viruses, but also to discover potential novel viruses. Both the phage-display serological and enrichment platforms can be rapidly updated to include new viruses identified. In Malaysia (Co-Is Hughes, Broder, Laing) we have transferred the Luminex serology platform to partner laboratories for screening of wildlife, livestock and human samples to investigate the spillover of henipaviruses and filoviruses at high risk interfaces. These interfaces include farms near the forest and indigenous communities with lots of subsistence hunting in Peninsular Malaysia, and preliminary screening has found serological evidence of past exposure to viruses antigenically-related to Nipah and Ebola in humans, bats and non-human primates (NHPs). In Thailand, Co-Is Broder, Laing and Wacharapluesadee have transferred a Luminex-based serology platform for detecting exposure to novel Asiatic filoviruses and known henipaviruses in wildlife and human samples, with testing underway and currently being validated in our Chulalongkorn University laboratory **(See also section 2.2.d)**.

1.2.d. In vitro & in vivo *characterization of viral potential for human infection:* Using Coronaviridae as an example, we have conducted *in vitro* and *in vivo* experiments to characterize the pathogenic potential of novel SARSr-CoVs from bats. We isolated three SARSr-CoVs from bat feces: WIV1, WIV16 and Rs4874, with S protein sequences that diverged from SARS-CoV by 3-7% (*20, 59, 96*). We conducted full-length genome sequencing of 12 other novel SARSr-CoVs, some highly similar to outbreak SARS-CoV in the most variable genes: N-terminal domain and receptor binding domain (RBD) of the S gene, ORF8 and ORF3 (*59*). Using our reverse genetics system, we constructed chimeric viruses with SARSr-CoV WIV1 backbone and the S gene of

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Commented [MOU27]: These are correct, based on EIDITH P1 + P2 for EHA countries.

Commented [KJO28]: Leave this out as it may look like we've already done a bulk of the work?

Commented [MOU29]: Think we need to abbreviate these viral families throughout, should Find/Replace. Also, henipa is not a viral family, so changing back to PMVs.

Commented [KJO30]: Move to Aim 2? I think it's good to have some flexibility to test wildlife sera using these assays, but not readily clear how this fits in with our PCR screening? We could do a broader survey of a smaller number of wildlife samples from more species, and those with high seroprev, we can follow up w molecular assays – though not sure this is necessary. All the serology in this section can prob go to Aim 2.

two different variants. All 3 SARSr-CoV isolates and the two chimeric viruses replicated efficiently in Vero E6 cells and in HeLa cells expressing hACE2, but not in those without ACE2 (*20, 59, 96*). We used the SARS-CoV reverse genetics system (*70*) to generate a chimeric virus with a mouse-adapted SARS-CoV backbone expressing SHC014 S protein with 10% sequence divergence from SARS-CoV S. This chimera replicated in primary human airway epithelium, using the human ACE2 receptor to enter into cells (*60*). Thus, **SARSr-CoVs with diverse variants of SL-CoV S protein without deletions in their RBD can use human ACE2 as receptor for cell entry.** The Baric lab has a well-established hACE2 and DPP4 transgenic mouse model that we will use this to characterize the capacity of specific SARSr-CoVs to infect (*84, 97*). We used this chimera approach to demonstrate that pathogenicity of bat SARSr-CoVs in humanized mice differs with divergent S proteins, **confirming the value of this model in assessing novel SARSr-CoV pathogenicity**. Infection of rWIV1-SHC014S caused mild SARS-like clinical signs in the transgenic hACE2 mouse model **that weren't reduced by immune-therapeutic monoclonals that attenuate SARS-CoV pathogenicity, nor after challenge following vaccination against SARS-CoV** (*60*). We repeated this virus characterization approach with chimeras using HKU3r-CoV S proteins that are ~25% divergent from SARS-CoV S, **and found that they are unable to use the ACE2 receptor**. Additionally, we were unable to culture HKU3r-CoVs in Vero E6 cells, or human cell lines.

A similar approach to the above pipeline for CoVs will be applied to novel henipaviruses and filoviruses we discover during our research. The broad mammalian tropism of Hendra virus and Nipah virus (*66*) is likely mediated by their usage of highly conserved ephrin ligands for cell entry (*15, 98*). A third isolated henipavirus, Cedar virus does not cause pathogenesis in animal models. Recently, **Co-Is Broder and Laing** have developed a reverse genetics system and rescued a recombinant CedV to test ephrin receptor usage/tropism and pathogenicity during henipavirus infections (*75*). CedV is unable to use the Ephrin-B3 receptor (*75, 99*) which is found in spinal cord and may underlie NiV encephalitis (*100*) and that pathogenesis also involves virulence factors V and W proteins. The putative henipaviruses, Ghana virus and Mojiang virus, predict V and W protein expression, with GhV able to bind to ephrin-B2, but not -B2 (*101*), and the receptor for MoJV remaining unknown (*102*) but is likely ephrin-B2 (*103*). Primary human lung endothelial cells are highly susceptible to Ebolavirus infection (*104*). Huh7 cells are good candidates for ebola in the liver, oftentimes used to isolate virus from clinical samples (*105*) Huh7 cells also offer advantages for reverse genetic recovery of novel filoviruses (*106*). Thus far, paramyxovirus attachment glycoproteins have been divided into three major groups: hemagglutinin (H), hemagglutinin-neuraminidase (HN), and attachment glycoproteins (G).

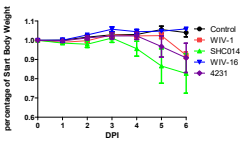

Fig. 7: Body weight change in hACE2 transgenic mice after SARSr-CoV infection, demonstrating that bat-origin SARSr-CoVs cause similar clinical presentation to outbreak strain (4231). From (*60, 61*).

Models of Outbred Populations. For novel viruses across all families, we will use the UNC collaborative cross mouse that captures 90% of the diversity within all mouse groups to assess the most suitable model for further experiments (*107*). We have used this model for CoV, ~~filo~~ FV (Ebola), Flaviviruses, and alphaviruses infections. ~~In subpopulations~~Clinical signs such as ~~it reproduces SARS~~ weight loss, hemorrhage, ~~in EBOV~~, encephalitis, and~~in WNV~~, acute or chronic ~~arthritis~~arthritis were recapitulated in these mice following SARS, EBOV, West Nile virus and~~in~~ Chikungunya infections, respectively (*85-87, 108-111*). *Bat Models*. Duke-NUS has developed two models for bat *in vivo* studies: a colony of *Eonycteris spelaea* (cave dwelling small fruit bat) and a bat mouse model, which contains the bat immune system in an immunodeficient mouse (*112*). In a proof of concept study, **Co-Is Wang and Anderson** infected *E. spelaea* bats with MERS-CoV to demonstrate their susceptibility and suitability for infection with a bat borne virus under BSL3 containment. These bats and the bat mouse model will serve to complement and expand studies the UNC collaborative cross mouse.

Commented [PD31]: Ralph/Danielle/Eric/Chris – need something substantial here on filovirus binding that shows we have a valid approach for novel filos

Commented [DA32]: From here and above, see my other version.

Commented [PD33]: Ralph – I think I mis-spoke in this fig. description – please correct.

Commented [DA34]: SARS weight loss? Meaning weight loss when infected with SARS-CoV?

Commented [PD35]: Ralph – please add data from the CC mouse re. filoviruses, to beef up the image of us as a filovirus group

Commented [PD36]: Linfa/Danielle – please draft a brief para explaining how we'll use these two models

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1.3. General Approach: We will conduct analyses to geographically- and taxonomically-target testing of samples from wild mammals that are most likely to harbor known high-profile zoonotic pathogens, or close relatives with potential to infect people. This includes selecting from tens of thousands of samples collected on recent projects and stored in freezers in our laboratories, and new sampling of wildlife in high risk locales. We will use viral family level PCR ~~screening~~ assays to identify and partially characterize viruses, then follow up sequencing of relevant receptor binding proteins to assess homology and predict binding affinity, then attempt to isolate and biologically characterize viruses that phylogenetic and other data suggest have high spillover and pandemic potential. We will follow this with *in vitro* binding assays and cell culture experiments, and *in vivo* animal model infections to assess their potential to infect people and cause disease **(Fig. 8)**. EHA will lead the study design, targeted sampling, and data analysis; Co-Is Hughes and Wacharapluesadee will lead field sampling and in-country testing; Duke-NUS, UNC, USUS will lead the serological and PCR diagnostic development and the *in vitro* and *in vivo* analyses, as well as cross-training and tech transfer to the hub labs in Thailand and Malaysia. This will include via support of one Malaysian Ph.D student, Mei-Ho Lee (CM Ltd.) who has worked with the EID-SEARCH collaborators for 9 years. The focus of her study will be infection potential of novel CoVs and paramyxoviruses already found in bats in Malaysia under preliminary data for this proposal.

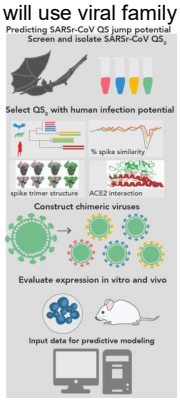

Fig. 8: Schematic showing EID-SEARCH approach to selection of sampling, viral testing, prioritization and characterization to identify novel, high zoonotic-risk viruses in wildlife.

Commented [PD37]: Hongying – this is a placeholder from DARPA proposal. We need a new one with Stages: 1) Geographic and taxonomic modeling to maximize discovery of potential zoonotic viruses; 2) PCR screening and partial RNA sequencing; 3) Full genome or RBD and Glycoprotien sequencing to model receptor binding based on sequence similarity; 4) Cell line infections (for prioritized viruses); 5) animal model infections (for prioritized); 6) High priority viruses ranked based on data (red, yellow, green) schematic.

1.4 Wildlife samples: 1.4.a. Geographic and taxonomic targeting for newly collected wildlife samples: We will prioritize sites within each country that rank highest for both the risk of zoonotic disease emergence (*113*) and the predicted number of 'missing' zoonotic viruses (*90*). Our preliminary analysis **(Fig. 1)** suggests priority areas include: the Kra Isthmus ~~of Kra~~ (S. Thailand and N. Peninsular Malaysia), NW Thailand (near the Myanmar-Laos border), and the lowland rainforests of Sarawak and Sabah. In each of these 'hottest of the hotspots' regions, we will identify 2-4 distinct sampling sites based on the geographic distribution of key hosts we prioritize for ~~intensive~~ additional wildlife sampling. We will priority rank bat, rodent, and primate species using ~~analysis of host trait data for the highest predicted number of viruses based~~host trait-based models (*90*). We will also identify species predicted to be centers of evolutionary diversity for Corona-, Paramyxo-, and Filoviridae using ancestral host reconstruction of available sequence data and Bayesian discrete phylogeographic models (*114-116*). ~~We will ensure wildlife sampling is complimentary to specimens archived in our extensive freezer banks. We will collect only the additional specimens projected from each prioritized taxa to achieve statistically robust sample sizes given predicted levels of viral richness and rates of discovery **(see 1.4.b)**.~~ We will leverage our strong collaborations with wildlife experts in Malaysia and Thailand to ground-truth geographic and taxonomic model outputs with local expert knowledge to ensure the sampling plan is realistic based on current species distributions, population size estimates, seasonality, and accessibility to sites.

Recognizing that most zoonotic viruses are multi-host, i.e. naturally harbored by multiple wildlife species, and that the phylogenetic relatedness of those hosts is predictable and varies across viral families (*90*) – we will use a combined network analysis and a phylogeographic model ~~also apply a generalizable phylogenetic and spatial modeling approach~~ to rapidly predict and prioritize new (unsampled) hosts for important, novel viruses we discover during our research. ~~We will use a combined network analysis and a phylogeographic model to estimate a viruses' host range and to~~We have shown this relatively simple model ~~prioritize additional wildlife species for sampling and testing for viruses we discover. Our recently developed model of viral sharing uses just two phylogeographic traits~~ (using just wildlife species range overlap and phylogenetic similarity between host species) is both generalizable to estimate host range for all mammalian and avian viruses *and* robust – accurately predicting hosts 98% of the time when cross-validated using data from known viruses ~~to successfully predict host species in the top 2% of all 4,200 possible mammal species~~ (*117*).

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Commented [PD38]: Kevin - That doesn't make clear sense straight away – please re-word better without increasing length

1.4.b. Sample size justifications for testing new and archived wildlife specimens: We calculate initial ~~We will~~sample sizes targets using ~~e~~ our preliminary data on average PCR prevalence from screening tens of thousands of bat, rodent and primate specimens for CoV and ~~Paramyxoviruses~~ PMVs under the USAID-PREDICT project in Malaysia and Thailand. We will refine these sample size targets, in real-time, over the course of our project using viral discovery curve analyses **(Fig. 6)** to either scale-up or scale-back new sampling and testing of archived specimens for each species depending on ~~their~~ viral ~~diversity~~capture rates. We will only collect additional specimens projected from each prioritized taxa to achieve statistically robust sample sizes and to complement our archived specimens. For example, ~~*Rousettus* spp. bats, known filovirus~~ reservoirs, were not adequately sampled under PREDICT research in Thailand. Preliminary analysis indicates we will require ~14,000 new samples to identify 80% of remaining viral species diversity in our study region, ~5,000 samples from Thailand and ~9,000 samples from Malaysia **(Fig. 9).** Viral strain ~~discovery~~versity from a sampling effort this size is expected to number in the hundreds of strains. For example, given ~6% prevalence of CoVs in bat species previously sampled under the PREDICT project, a target of 14,000 individuals would yield ~800 PCR positive individuals, representing, ~~conservatively,~~ an estimated ~~~200~~600 novel ~~CoV strains~~sequences/strains. Similarly, for paramyxoviruses, which have an average PCR detection prevalence of ~1.5%, sampling of 14,000 individuals would yield ~200 positives and an estimated ~~~50~~150 novel ~~paramyxovirus~~ strains. From each mammal, we will collect serum, whole blood, throat swabs, urine and fecal samples or rectal swab using our previously-validated ~~For example, given 5-12% prevalence of CoVs in the most common bat species we previously-sampled in Thailand and Malaysia, a target of XXX individuals per species would yield XX (± x) PCR positive individual bats, and an estimated ~xx novel strains. Similarly, for paramyxoviruses with an average PCR detection rate of X%, we would need XX individuals per species to detect 80% of predicted viral strain diversity with 80% power. Preliminary analysis indicates we will require XX,000 samples to identify 80% of remaining viral diversity **(Fig. 9).**~~ ~~All wildlife sampling will be~~ nondestructive methods (see Vertebrate Animals section) [REFS]. In the event that an animal is found dead or needs to be euthanized due to injury we will collect ~~biopsy~~ necropsy samples for screening.

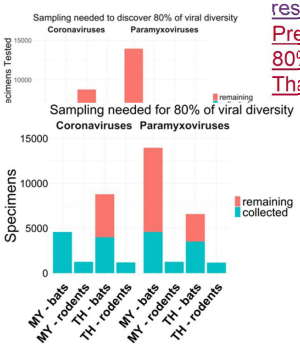

Fig. 9. Stacked bar plot showing number of currently-archived specimens **(blue)** and new specimens required **(orange)** to discover 80% of predicted number of CoVs and PMV species ~~s~~ from high-risk bat and rodent taxa in Malaysia and Thailand

We estimate the following sampling effort, broken down for each region: *Peninsular Malaysia:* Wildlife sampling will be led by CM Ltd / EHA in collaboration with PERHILITAN. Archive bat, rodent and NHP samples will be screened serologically using the BioPlex and other serology techniques identified through this project. New wildlife sampling will focus on bats but it is expected that rodents and NHPs will be sample as well. Wildlife sampling will be conducted around Orang Asli communities sampled as part of aim 2. Three new communities will be identified in the districts we have already sampled in. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife mainly bats will be sampled. Wildlife and domestic animal samples will include serum, whole blood, throat swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and tested at PERHILITAN's NWFL. *Sarawak, Malaysia:* Wildlife sampling will be led by CM Ltd / EHA in collaboration with UniMAS. Wildlife sampling will be conducted around Dyak communities sampled as part of aim 2. One community will be identified. Concurrent sampling trip will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife mainly bats will be sampled. Wildlife and domestic animal samples will include serum, whole blood, throat swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and tested at UniMAS or BMHRC. *Sabah, Malaysia:* Bat sampling will be led by CM Ltd / EHA in collaboration with SWD and WHU. Bat sampling will initially focus on 3 caves. Batu Supu Sabah low disturbance cave and surrounding area, Madai medium disturbance cave and surrounding area, Gomantong cave high disturbance cave and surrounding area. Each cave will be sampled

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Commented [KJO39]: Just assumed that 75% of detections would = unique sequences/strains. We can lower this to be more conservative.

Commented [MOU40]: Kevin will work with Noam and Evan to fill in these numbers.

Commented [KJO41]: PREDICT sampling protocols? Jon's FAO bat book manual, and other published for rodents and primates (from Bangladesh?)

Commented [PD42]: Hongying/KEvin Need less text on the figure, but larger font size, or crisper text to make it all visible at this size.

Commented [T43]: Will dig into budget tomorrow to confirm numbers.

Commented [T44]: With Dr Tan and possibly Dr Faisl – still waiting for Dr Faisl paper work and details of samples he has in storage will discuss with Dr Tan tomorrow.

Commented [T45]: Will dig into budget tomorrow to confirm numbers.

Commented [T46]: Discussing with Tam tomorrow.

twice once in wet season and once in dry season targeting 500 bats at each cave on each trip for a total of 3000 bats. Wildlife samples will include serum, whole blood, throat swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored at WHGFL, molecular testing will be conducted at WHGFL and serology screening at BMHRC. In addition at Madi cave the community that lives in the mouth of the cave will also be sampled targeting 150 people, while at Gomantong cave 25 workers involved in the bird next farming will be enrolled into the study.

Thailand: XXXXXXXXXX New bat specimens will be collected from bat species that wasere not collected during PREDICT2 study for example *Rousettus* bats (targeted species for Filovirus study), *Taphozous* bat (targeted species for MERS-CoV), *Rhinolophus* (targeted species for SARS-CoV) for serology and PCR assays (n=100 for each species).

~~*Thailand: XXXXXXXXXX*~~

1.4.c. Sample testing, viral isolation: Viral RNA will be extracted from bat fecal pellets/anal swabs ~~with High Pure Viral RNA Kit (Roche). RNA will be aliquoted, and stored at -80C.~~ PCR screening will be performed with pan-coronavirus, filovirus and paramyxovirus primers, in addition to virus specific primers where additional sensitivity is warranted in key viral reservoirs~~, i.e. Nipah virus specific primers [REF]~~. PCR products will be gel purified and sequenced. We will attempt isolation on samples that contain viruses of interest (determined in **1.4, below**), using Vero cells and primary cell culture from the wildlife taxonomic order (bat, rodent and primate cell lines). This includes the oOver ~~30~~ 70 bat cell lines are maintained at Duke-NUS from ~~four~~ five different bat species. These include primary lung, brain, bone marrow, heart, kidney, intestine, liver, lymph node, muscle, spleen, PBMC and ovary/testes from *Eonycteris spelaea, Cynopterus brachyotis, Rhinolophus Lepidus, Myotis muricola* and *Pteropus alecto* bats. In addition we have 8 immortalized cell lines, including those derived from lungs, therefore suitable for the isolation of respiratory viruses.

1.4.d. Moving beyond RNA viruses: To detect pathogens from other families, such as non-RNA viruses we will combine conserved PCR assays with NGS. We will perform either unbiased NGS, or use a pan-viral enrichment library to sequence full-length genomes. We will conduct this work via EID-CC research projects or as part of outbreak detection/investigation in the region from DNA viruses or other agents.

1.4.e. Sequencing Spike Glycoproteins: Based on earlier studies, our working hypothesis is that zoonotic coronaviruses, filoviruses and henipaviruses encode receptor binding glycoproteins that elicit efficient infection of primary human cells (e.g., lung, liver, etc.) (*12, 59, 60*). Characterization of these suggests SARSr-CoVs that are up to 10%, but not 25% different in the spike glycoprotein use human and bat ACE2 receptors for docking and entry. Uneven sampling gaps (e.g., no strains were found with 10-25% spike variation) prevent a thorough understanding of the transition point where the most divergent strains lose human ACE2 receptor usage. Our viral discovery curves **(Fig. 4)** suggest further sampling will reveal a rich diversity of as-yet-undiscovered SARSr-CoVs. In this aim we will use phylogenetic and viral discovery analysis to specifically target bat species and regions that are under sampled to allow sufficient power to identify and characterize missing SARSr-CoV strain diversity. Our previous work also suggests that SARSr-CoVs with S proteins that are ~10% divergent from SARS-CoV resist neutralization by therapeutic mAbs and escape SARS-CoV vaccines (*20, 60, 118*), suggesting that existing vaccines and immunotherapeutics could easily fail against future emerging SARS-like CoV. Moreover, viruses with 10-25% divergence in S proteins may bind to human cell receptors, but completely evade therapeutic and vaccine effects, and could therefore be a higher risk for public health. We will sequence the S proteins of novel SARSr-CoVs to prioritize viruses for experimental work in Aim 3 to test this hypothesis. Likewise, we will test the hypothesis that phylogenetic related zoonotic receptor binding protein genes of MERS-related, Ebola-related and ~~Nipah~~NiV/He~~N~~Vndra-related viruses will also program efficient entry via human ortholog receptors. For all novel virus strains, we will sequence the complete entry spike genes by amplifying overlapping fragments using degenerate primers as shown previously (*20, 59*). Full-length genomes of selected CoV strains (representative across subclades) will be sequenced using ~~NEBNext Ultra II DNA Library Prep Kit for Illumina and sequenced on a MiSeq sequencer~~NGS. ~~PCR or MinIon sequencing will be performed to fill gaps in the genome.~~ The full length spike gene sequences, including the amount of variation in the receptor binding residues that bind the appropriate human receptor will be used to select strains for Aim 3 experiments.

Commented [T47]: Will dig into budget tomorrow to confirm numbers.

Commented [PD48]: Kevin – who can write this bits?

Commented [KJO49]: I think all this could be deleted. Doesn't fit with the overall plan, or is redundant. We may want to call out a few specific sites Tom mentions (but won't that depend on the modeling!?), and you may want to keep the partner info Tom provided and call that out - maybe more in Aim 2 since its about people too?

Commented [MOU50]: Supaporn: From my DTRA study, specific Nipah primers and WHO 2C CoV primers is more sensitive than consensus PCR if we want to find the specific virus.

Commented [PD51]: Linfa – can you give a v. brief description of these please, e.g.: "This includes 13 primary lung, 5 primary gut epithelial, and 1 primary liver cell line. In addition we have 12 immortalized cell lines, 7 of which are intestinal, and therefore suitable for CoVs. Cell lines are derived from insectivorous and frugivorous bats (genera……)"

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1.5. Assessing risk for spillover. 1.5.a. Capacity to infect human cells: ~~We will use a series of cell cultures~~ Tto assess the capacity of target viruses to enter human cells~~. For CoVs, filoviruses and henipaviruses~~, we will use primary human ciliated airway epithelial cells (HAE)~~ ~~ and lung endothelial cell cultures from the lungs of transplant recipients represent highly differentiated human airway epithelium containing ciliated and non-ciliated epithelial and goblet cells, grown on an air-liquid interface for several weeks prior to use (*60, 61, 119*). We will prepare HAE and lung endothelial cultures from three different patient codes in triplicate in collaboration with the tissue procurement facility at the Cystic Fibrosis Center at UNC. Cultures will be inoculated with chimeric bat SARSr-CoVs or other ~~test~~ filoviruses or henipaviruses to assess efficient replication. At 72 hpi, cultures will be fixed for immunofluorescent staining using antisera to the SARS-CoV conserved nucleocapsid protein (N) or to structural genes of the other viruses (*62, 69*). SARSr-CoVs that differ significantly in S protein sequence (11-24%) from epidemic SARS-CoV yet replicate *in vitro*, will also be evaluated for sensitivity to neutralization in Vero cells by PRNT50 assays using broadly SARS-CoV cross reactive human mAbs S227.14, S230.15, S215.17, and S109.8 (*61, 120*) or monoclonal antibodies targeting the Ebola or Marburg E glycoprotein (Available through BEI), or NiVpah or HeVndra ~~viruses~~glycoproteins (*121*). As controls or if antisera is not available, the S genes of novel SARSr-CoV or spike entry genes of other novel viruses under investigation will be inserted into VEE 3526 replicon vectors (VRP3526-S), packaged and used to vaccinate mice (*122*). Polyclonal sera against SARS-related viruses will be harvested and tested for ability to cross neutralize SARS-CoV, GD03, WIV-1, SHC014, WIV-16, other novel SARSr-CoV and HKU3-SRBD by PRNT50 assay (*61, 123, 124*). Using PRNT50 titers with sera (n=4 each) among these viruses, antigenic cartography (*125*) will allow comparison of antigenic vs. phylogenetic distance, identifying the transition at which SARSr-CoV strains escape SARS-CoV based vaccines, informing immunotherapeutic and vaccine design strategies (*126-128*). Similar approaches will be applied to novel MERS-related viruses, other CoV, filoviruses or henipaviruses. While most wildlife-origin viruses with unknown transmission potential or pathogenicity in people are BSL-2 or -3, cell culture for potentially BSL-4 pathogens will be undertaken at collaborating institute NEIDL. To facilitate this, duplicate samples in culture medium from animals PCR +ve for filoviruses will be shipped to NEIDL **(See letter of support)** for culture, characterization and sharing with other approved agencies including NIH Rocky Mountain Laboratories ~~RML~~where our EID-SEARCH team has ongoing collaborations. When appropriate of feasible, training opportunities at NEIDL for in-country staff will be given.

1.5.b. Host ACE2, DPPR, Niemann-Pick C1 (NPC1) and EphrinB2/EphrinB3 receptors: We will sequence host ACE2 receptors of different bat species or different bat populations from a single species (e.g. *Rhinolophus sinicus*) to identify relative importance of different hosts of high risk SARSr-CoVs and the *intra*specific scale of host-CoV coevolution. Of particular interest is homology across 18 bat and human orthologue ACE2 contact interface residues that engage the SARS RBD as a potential indicator of SARSr-CoV cross species transmission potential and growth in human cells (*68*). ~~A lot of v~~Variation in the NPC1 receptor alters EBOV ability to infect in humans and bats, so this can be structurally mapped for prediction and downselection of strains (*129*). Signatures of positive selection in bat NPC1 were concentrated at the virus-receptor interface, with the strongest signal at the same residue that controls EBOV infection in *Eidolon helvum* cells. Thus NPC1 is a genetic determinant of filovirus susceptibility in bats, and some NPC1 variations likely reflect host adaptations to reduce filovirus replication and virulence (*130*). Nipah and Hendra use ephrin B2 or ephrin B3 as a receptor, and variation affects its ability to use human vs bat receptor molecules (*101, 131*).

1.5.c. Host-virus evolution and distribution: We will use Bayesian and Maximum Likelihood phylogenetic analyses of RNA-dependent RNA polymerase (RdRp) sequences from PCR screening ~~(or L genes)~~, receptor binding glycoproteins, and/or ~~ ~~full genome sequence data (when available) ~~data~~ to reconstruct the evolutionary history of the novel bat SARSr-CoVs, bat MERSr-CoV, filoviruses and henipaviruses that we identify. We will run phylogenetic and ancestral state reconstruction analyses **(Fig. 5)** to ~~reconstruct~~identify host taxa and geographic sites of ~~β-CoV, filovirus and henipavirus~~ evolutionary origins within bats, rodents and primates ~~using our expanded dataset and identify the host taxa and geographic regions~~ that together define hotspots of phylo-diversity for these viruses, allowing for more targeted surveillance. For receptor binding glycoprotein sequence data from characterized viruses, we will apply codon usage analyses and codon adaptation indices, as used recently on Henipaviruses (*132*), to assess the relative contributions of natural selection and mutation

Commented [BRS52]: Any antibodies available? If not we can synthesize them from published co-crystals.

Commented [DA53]: Chris has them

Commented [PD54]: Can someone answer Ralph's question please?

Commented [KJO55]: Make more specific about our plans to sequence NPC1 or Ephrin receptors, etc. Should we frame much of this as "add on projects" pending additional EIDRC-CC funding?

Commented [PD56]: Kevin/Alice – please edit this section to include relevance for filos and henipas as well (and other hosts)

pressure in shaping host adaptation and host range.~~We will rerun MCC analyses **(Fig. 3)** to reconstruct β-CoV evolutionary origins using our expanded dataset. Ecological niche models will be used to predict spatial distribution of PCR-positive species, identify target sites for additional bat sampling, and analyze overlap with people. We will use our viral discovery/accumulation curve approach **(Fig. 4)** to monitor progress towards discovery of >80% of predicted diversity of SARSr CoVs (lineage 1 & 2) or other virus families within species and sites, halting sampling when this target is reached, and freeing up resources for work other work~~ (*95, 133*).

1.5.d. Viral strain prioritization: Of the expected 100-200 novel SARSr-CoV strains, and approximately 600 total CoV strains, we will down-select to prioritize for further characterization based on S genes that are: i) different from SHC014, WIV1, SARS-CoV with diversity ranges of 10-25%; ii) have virus S RBD that could use human/bat receptors; iii) have recombinant chimeric spikes indicative of gene flow between clade I and II strains; iv) have bat ACE2 receptors that might select for spike RBDs that can use human receptors for entry (15/18 conserved residues in human/bat ACE2 molecules that bind SARSs-CoV S RBD domains are likely more efficient receptors than 3/18 conserved sites). Using structural models based on the SARS S glycoprotein, the extent and location of antigenic variation will be annotated onto the structure, further resolving the locations of highly evolving and conserved structural motifs/epitopes that function in protective immunity (*120, 134-136*). For novel henipaviruses, we will examine the receptor-binding proteins (e.g. envelope attachment (G) proteins) for sequence and structural homology with particular attention to conversation of amino acids that mediate ephrin receptor binding. We will prioritize strains that are predicted to interact with ephrin-B2 and expression V/W proteins to determine whether these viruses represent zoonotic threats. Using the recombinant Cedar virus (rCedV) platform, we will be able to rescue rCedV that express heterotypic G proteins to explore receptor utilization and tissue dissemination in animal models. We will assess ability to infect human cells using a continuous lung epithelial cell line, Calu3, that our group has previously used to generate data on NiV and HeV growth curves and a variety of host expression changes after infection (*137*). We will also use primary human airway cultures to assess human infection for Henipavirus and MERS- and SARS-related CoV (*138*) (*139*) . There is some evidence for Nipah and Hendra virus replication in mouse lung, low level titers in young animals, 10 fold higher titers in aged mice (*140, 141*). Thus it is likely these models could be improved in the collaborative cross mouse resource at UNC. We have identified CC lines that are highly vulnerable to wildtype SARS and others that develop hemorrhagic disease following mouse adapted Ebola infections (*85-87*).

1.5.e. Animal models: We will use transgenic mouse models and the UNC Collaborative Cross model to assess spillover potential of viruses. For work on CoVs, we will follow the protocols in the transgenic mouse model used by the Baric lab previously. Briefly, in the BSL3 lab, n=8 10- to 20-week old hACE2 or hDPP4 transgenic mice will be intranasally inoculated with 5 x 10^4 PFU of wildtype WIV-1 or chimeric bat SARSr-CoVs with different spike proteins or MERS-CoV and MERS-related CoV, respectively, then monitored daily for weight loss, morbidity, and clinical signs of disease. Mice will be euthanized at 2, 4, and 6 dpi (endpoint of 14 dpi), organs harvested and virus quantified by NP RT-PCR. After 7 days in formalin, tissues for histopathology will be removed from the BSL3 and stained with H&E, and for immunohistochemistry using polyclonal antibodies against the N protein. We will conduct limited evaluation of existing countermeasures using therapeutic monoclonal antibodies *in vitro* and *in vivo*. Existing SARSr-CoV or MERS-CoV mAbs (*142, 143*) will be diluted in DMEM starting at 1:20, and serial 2-fold dilutions mixed with an equal volume of 50 PFU of chimeric bat SARSr-CoVs with different spike proteins, then incubated on Vero E6 cells at 37°C for 1 h, then changed to a semi-solid medium (DMEM containing 1% methylcellulose and 2% FBS). Antibody neutralization titers will be evaluated by plaque quantification at 4-5dpi. hACE2 transgenic mice will be injected with SARS-CoV mAbs, and infected with chimeric bat SARSr-CoVs. Clinical signs and morbidity will be assessed and tissue pathology examined and compared with mice without treatment of mAbs to determine the therapeutic effect on SARSr-CoV infection, and protection of SARSr-CoV by wildtype SARS-S based vaccines assessed as described (*119, 144*). We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-CoV cross neutralization, synthetically reconstruct a small subset (1-2) and evaluate the ability of nucleoside analogues to inhibit growth in HAE cultures and/or *in vivo* (*61, 119*). For the Collaborative Cross model, we will….

Commented [KJO57]: I suggested we could get up to 600 CoV "strains" (all seqs, not just SARSr-CoVs) in section 1.4b, but we can reduce that.

Commented [PD58]: Ralph – please insert a couple of sentences on how we'll use the cc mouse and what we'd expect

Finally, for more detailed pathogenesis studies in the natural reservoir, we will use the bat models (natural and transgenic mouse model) at Duke-NUS. Both animal systems can be used to test susceptibility, pathogenesis and immune responses of any newly discovered viruses.

1.6. Potential problems/alternative approaches: **Challenges with logistics or obtaining permission ~~to~~ for wildlife sampling in sites we select~~sample bats in sites or provinces we select~~**. We have a >20-year track record of successful field work in Malaysia, ~~Thailand and~~ and >10 years in ThailandSingapore, and have ~~worked with~~strong established partnerships with local wildlife authorities and communities to ensure access. We have existing permissions in place which will be renewed at the start of this project. Due to the abundance of wildlife habitats in the region, if access to a site is withdrawn, we will rapidly identify a suitable, complementary site with similar bat species composition and abundance. Finally, we have already collected tens of thousands of wildlife samples from the region and will focus on these while waiting for permission. **We may not identify novel viruses in our sample wildlife species due to seasonality of viral shedding or other factors.** We have calculated sample sizes based ~~on a range of PCR data and are fairly conservative in our approach~~on realistic detection rates from our extensive preliminary data, and are confident that we will detect and identify large numbers of potentially zoonotic pathogens, particularly for CoVs and PMVs. ~~However, as a back-up, we already have identified dozens of novel viruses that are near-neighbors to known high-impact agents, and will continue characterizing these should discovery efforts yield few novel viruses~~We acknowledge that FV strain diversity may be harder to capture, given relatively low seroprevalence in bat populations – suggesting lower levels of viral circulations (*40, 41*). However, our team was the first and among the only groups to sequence FV RNA from Asian wildlife species(*44, 45, 55*), and we are confident that with our targeted sampling and testing strategy will identify additional strains of Ebola and related viruses. The sampling regions we have selected are subtropical, and our previous data, and even published studies in temperate regions (*145*), do not suggest a strong pattern of seasonality in CoV shedding, ~~although there are studies that suggest this~~and where seasonal patterns do occurs, i.e. for ~~in~~ henipaviruses ~~and filovirus (MARV?) shedding (REFS?)~~in Thailand (*146*) or can be predicted from wildlife serology data, we will be sure to ~~. Nonetheless to account for this we will~~ conduct sampling ~~evenly on quarterly basis within each province~~at different timepoints throughout the year to account for this.

Aim 2: Test evidence of viral spillover in high-risk communities using novel serological assays.

Commented [PD59]: Linfa/Danielle – could you put a couple of sentences in on what we might do with the models. Remember – this could be an additional short project from the EIDCC extra funding.

Commented [DA60]: This is added above. All the SARS parts are very detailed (with temperatures and dilutions etc). We can cut the detail out if we need to cut pages.

Commented [KJO61]: Emily and Noam reworking this section significantly, so not touching it much.

Country, site	Lead	# enrolled, focus	Findings
Peninsular Malaysia	Hughes	1,390 Orang Asli indigenous population, PCR/serol.	25+ novel CoVs, influenza, Nipah ab+ve, filovirus ab+ve
Malaysia Sabah	Kamruddin	1,283 serology	40% JE, 5% ZIKV ab+ve
Malaysia Sabah	William	10,800 zoonotic malaria	High burden of macaque-origin malaria

Malaysia Sabah	Hughes	150 bat cave workers	Ongoing
Malaysia Sarawak	Tan	500 Bidayuh and Iban people	8% PCR prevalence HPV in women
Malaysia PREDICT	Hughes	9,933 high zoonotic-risk communities, viral PCR, serology	Ongoing. Multiple known and novel CoVs, paramyxoviruses, others.
Thailand	Wacharaplu-esadee	1,000 bat guano harvesters/villagers	Novel HKU1-CoV assoc. clinical findings
Thailand PREDICT	Wacharaplu-esadee	9,269 high zoonotic-risk communities, viral PCR, serology	Ongoing. Multiple known and novel CoVs, paramyxoviruses, others.
Singapore	Wang	856, Melaka virus	7-11% ab+ve

2.1 Rationale/Innovation: Rapid response requires early detection. Thus, assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities that interface frequently with wildlife that harbor high risk emerging viral strains. To enhance the low statistical probability of identifying these rare events, we will target rural human communities inhabiting regions of high wildlife biodiversity, that also engage in practices that increase the risk of spillover (e.g. hunting and butchering wildlife). In Aim 2 we will build and expand on our preliminary biological sampling and enroll large cross-sectional samples in human populations with high behavioral risk of exposure to wildlife-origin viruses and that live close to sites identified in Aim 1 as having high viral diversity in wildlife. We will initially identify 2 sites in each of Thailand, Peninsular Malaysia, Sarawak, and Sabah for repeated biological sampling. We will design and deploy behavioral risk factor questionnaires, and specific and sensitive serological assays to identify the baseline frequency, and causes of, spillover of known and related novel viral pathogens in these populations. In participants where symptoms are reported in the last 10 days, paired serum specimens (acute and 21 days after fever) will be collected to determine titer of positive antibody. Samples will be marked for additional PCR-based assays to identify presence of known and novel CoVs, henipaviruses and filoviruses, and for isolation and biological characterization of potential pathogens, using the approach laid out in Aim 1.

2.2 Preliminary data on high-zoonotic risk human community surveillance: 2.2.a Biological sample collection in SE Asia: Our longterm collaboration in the region has included qualitative and quantitative survey data, and collecting large archives of banked human sera and other biological samples from high zoonotic-risk populations. For example, under the USAID-PREDICT project, EHA has collected 9,933 and 9,269 specimens in Malaysia and Thailand respectively from people living in zoonotic disease interfaces, all of which will be available for retrospective serological testing by EID-SEARCH. Our core group has collected and tested many thousand additional specimens from other important community cohorts **(Table 2)**, and some of these will continue under EID-SEARCH **(See section 2.4)**. Of particular interest is the access to indigenous communities in Peninsular Malaysia (Orang Asli) and Sarawak (Bidayuh, Iban people), and the bat guano harvesters in Thailand. These communities hunt, butcher and eat wildlife daily, and work in supremely high-risk zoonotic interfaces (e.g. bat caves). One of our indigenous community sites is experiencing an ongoing outbreak of unknown etiology that we are investigating currently **(See section 3.2.a)**.

Table 2: Current or recent biological sample collection from healthy populations conducted by members of our consortium in EID-SEARCH hub countries

2.2.b Human Contact Risk Factors: EHA is the lead organization across all 27 USAID-PREDICT project countries for collecting data on, and analyzing the human aspects of, zoonotic spillover risk (*147, 148*). We conducted exploratory studies using standardized one-on-one semi-structured interviews and site observation data with people living in hotspot areas or engaged in high-zoonotic spillover risk activities, using these data to assess local social and cultural norms underlying wildlife contact (*148*). We then developed a risk factor survey to accompany human biological sampling to evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. We conducted cross-sectional studies in high risk populations in 27 countries, and study participants provided biological samples (oral/oropharyngeal swab, nasal/nasopharyngeal swab, urine sample/urogenital swab, fecal sample/rectal swab, whole blood, serum, plasma). For example, survey and biological samples were collected from: 1,585 participants from 7 sites in Yunnan, Guangxi, and Guangdong provinces, China; 1,400 participants from 4 sites in Malaysia; and 678 participants from 4 sites in

Commented [KJO62]: Check these numbers, don't think we have 9000 people sampled, if this represents individuals! Per below, we say we have 9000 specimens, not people, so need to adjust.

Commented [KJO63]: Emily: Will we be sampling participants more than once?

Commented [PD64]: I think that would be good, depending on the sample size

Commented [KJO65]: Need to decide if we do PCR on community samples, we can add in the questionnaire if you've been sick in the last 10 days and if so, then maybe test only this subset.

Commented [PD66]: Kevin's comment makes sense to me

Commented [KJO67]: Don't match table numbers, need to change table header to # specimens, not # enrolled.

Thailand. Samples were tested with novel serological assays and results analyzed against risk factors from survey data (*12*). In China, CoV-seropositive individuals were mostly farmers/peasants (8/9), living in Yunnan province (7/9), 41-60 yrs old (7/9), with domestic contact with rodents (6/9), that are male (6/9). Although these results are preliminary and don't provide detailed information on routes of exposure, they identify key subpopulations to target that would likely increase the opportunity for identifying evidence of spillover for other CoVs, paramyxoviruses and filoviruses. Data from PREDICT surveys will be used to help identify target populations in Aim 2, identify strategies to better target at-risk people, and conduct focused **human animal contact surveys and serosurveys to produce statistically significant findings for henipaviruses, CoVs and filoviruses.** In Aim 2, we will use combined biological sampling and risk factor surveillance in targeted populations within the community to 1) identify risk factors correlated with seropositivity to henipaviruses, filoviruses and CoVs; 2) assess possible health effects of infection in people; and 3) where recent illness is reported, obtain clinical samples for PCR testing and potential viral isolation and characterization. Obtaining this information could be a significant step in understanding the likelihood of recent 'hidden' spillover events and their public health impacts, as well as the risk of future emergence of viral disease.

2.2.c. Risk factors for illness: Our preliminary data from PREDICT in Thailand, Malaysia and China revealed that frequent contact with wild (e.g. bats, rodents, non-human primates) and domestic animals (e.g. poultry, swine) among local communities was associated with a high percentage of self-reported severe/acute respiratory illness (SARI/ARI) and Influenza-like illness (ILI) symptoms. In addition, people involved in crop production, hunting and butchering of wildlife reported statistically significantly higher SARI and ILI symptoms (Li *et al.*, in prep.). **These preliminary data suggest a target population and sites for current proposal with the correct targets for serological surveillance and risk factor surveys in Aim 2 to produce statistically significant findings**. Coupled with further refining serological tools, we will be able to identify the likely routes of exposure to known or novel coronaviruses, henipaviruses, and filoviruses in the study region, as well as build data on the illnesses they cause in people.

2.2.d. Serological platform for community surveillance: One of the present challenges in emerging disease surveillance is that viremia can be short-lived, complicating viral-RNA detection (i.e. traditional PCR-based approaches), especially in zoonotic reservoir hosts, and requiring large samples sizes to understand infection patterns or host range. By contrast, antibodies are typically long-lived and can be used to assess prior virus exposure or infection with much smaller samples sizes (*149*). However, most serological assays only target a single protein, and it's not always clear which antigen is the most immunodominant during an adaptive humoral immune response – i.e. which is the best target for a serological assay. Henipaviruses, filovirus and CoVs express envelope receptor-binding proteins (e.g. CoV=spike (S) protein, filoviruses/henipaviruses=glycoprotein (GP/G)), which are the target of protective antibody responses. The Broder lab at USU, a leader in the expression and purification of native-like virus surface proteins for immunoassay application (*150*)?, developing monoclonal antibodies (*151, 152*) and as subunit vaccines (*153, 154*), is presently developing a novel, multiplex microsphere immunoassay (MMIA) allowing serum samples to be simultaneously tested for antibodies reactive with henipaviruses, filoviruses and coronaviruses. This MMIA uses a Luminex-based multiplex platform and custom designed virus receptor-binding proteins expressed in a mammalian cell-culture system and purified as native-like oligomeric proteins (*150*). These oligomeric antigens retain post-translational modifications and quaternary structures. This structural advantage over peptide antigens, allows the capture of conformational-dependent antibodies raised during a natural infection, which represent the majority of the protective humoral response.

Commented [KJO68]: Peter, sent over LASSO figure for ILI from Thailand and Malaysia for you to consider.

Commented [PD69]: Chris/Eric Is this the right ref?

Commented [KJO70]: See my comment in Aim 1 that some of that serology platform stuff can be moved here.

Serological platforms have been historically limited by the starting antigen, and cross-reactivity between known and unknown related viruses not included in the assay. The known diversity of henipaviruses includes five presently described viruses, NiV, HeV, CedV, GhV and MojV and this assay has been expanded to include all described species **(Fig. XXX)**. In the past, our collaborators have used NiV and HeV G protein antigens in a MMIA assay to identify a serological footprint of NiV-related African henipaviruses and exposure to virus(es) antigenically-related to NiV in livestock sampled in Bangladesh (*76, 77*). To strengthen serological data interpretation, the G protein from each virus is integrated into the MMIA so that virus-specific versus henipavirus cross-reactive antibodies can be detected and address specificity of past virus exposure. Extensive cross-reactivity exists within ebolaviruses and between ebolaviruses and marburgviruses, highlighted by studies examining antibody binding between heterologous viruses (*155-157*). Ongoing work is aimed at validating the MMIA pan-filovirus assay for specificity. The majority of ebolaviruses are endemic to Africa, however the discovery of Měnglà virus in Yunnan, China increased the further demonstrated that a diversity of Asiatic filoviruses with unknown pathogenicity and zoonotic potential exists. Our past work in collaboration with Duke-NUS demonstrated that three under-sampled fruit bat species had serological evidence of past exposure to a filovirus(es) most antigenically-related to ebolaviruses (e.g. Ebola virus, Bundibugyo virus), but unreactive with Reston virus GP, suggesting that novel filoviruses circulate within bat hosts (*41*).

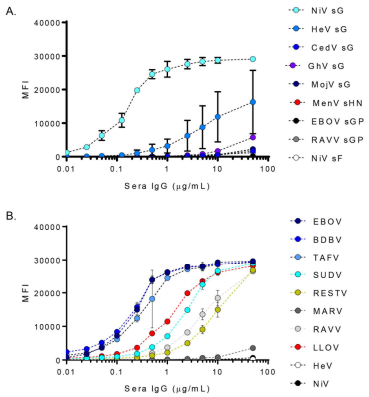

Fig. XXX: Validation of multiplex microsphere immunoassay (MMIA) specificity and identification of immunologically cross-reactive viruses for NiVpah (A) and EBOVbola (B).

2.3 General Approach/Innovation: We will use a dual study design to gain in depth understanding of exposure and risk factors for viral spillover **(Fig. XX)**. In Aim 2 we will conduct community-based surveillance using a brief, focused questionnaire to identify the prevalence and frequency of risk-factors for viral spillover in tandem with collection of high quality biological samples to determine the seroprevalence of spillover viruses in at-risk human populations. We will use generalized linear models to analyze correlation between biological results and risk-factors for viral spillover. In Aim 3, we will conduct clinic-based syndromic surveillance and biological sampling at clinics/hospitals that are the catchment healthcare facilities for the communities sampled in Aim 2. Both community-based and clinic-based syndromic surveillance programs are cross sectional case-control studies designed with the sample sizes necessary to statistically quantify (with a power of 80%) risk factors and health impacts for viral spillover, linked to serological status and PCR prevalence in symptomatic patients.

2.4 Target population & sample sizes: We will target populations in the same geographic regions as those identified in Aim 1 that harbor wildlife with high potential zoonotic viral diversity, that are EID hotspots, and that are well-connected through regional travel and trade hubs to the global travel network **(Fig X)**. We will target specific communities based on our analysis of previously-collected PREDICT behavioral questionnaire data, to assess key at-risk populations and occupations with high exposure to wildlife, as well as using other publicly available data (e.g. wildlife hunting licenses sold in a region, number of guano caves accessed for fertilizer etc.). We will identify populations at two sites in each of the following regions, building on our preliminary data **(Table 2)**: *Thailand (Co-I Wacharapluesadee):* We will continue surveillance in bat guano harvesters and their villages in Ratchaburi province where we have identified MERS-related CoV in bat guano and rectal swabs (*48, 158*) and serological evidence of α-CoVs, HKU1-CoV in people (Wacharapluesadee in prep.). Our second site will be in Chonburi province where we have found NiVpah virus (99% identity of whole genome sequence to NiV from a Bangladesh patient) in flying foxes but no outbreaks have yet been reported (REFThai MOPH, unpublished), and have found several novel paramyxoviruses and CoVs have been found in bat feces roosting there ((*159, 160*) Wacharapluesadee in prep.).

Commented [PD71]: Hongying -Need a new figure that lays out the community/clinical approach – small one with pretty images.

Commented [PD72]: Supaporn/Kevin/ -Need sample sizes for both populations and power calculations for serology for a typical virus from one of the three families we're targeting

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Sample size: Serological exposure likely low due to low risk of onward transmission. High risk occoupational groups vs. community sample at each site. Need to stratify sampling in villages based on

Thailand: 100 subjects of healthy high-risk community from 2 study sites (Chonburi and Ratchaburi) will enrolled on Year 2 and tested for antibody, and specimen from ill subject will be collected and teat by PCR and serology assays. Specimens from same subject will be collected on Y3 and Y4 for serology re-testing.

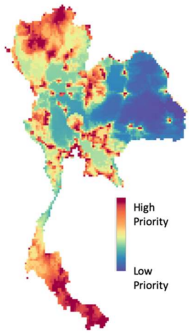
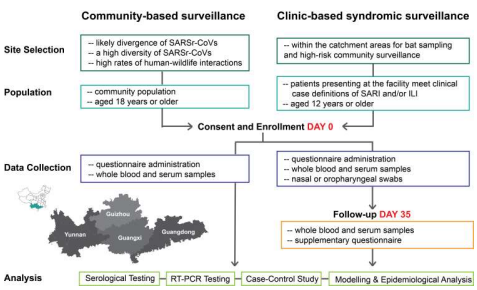


Fig. X: . Geographic analysis to target high-risk sites for human zoonotic disease surveillance in Thailand. Our spatial analysis from Aim 1 will be extended to optimize sites for human community surveillance in Thailand and Malaysia by ranking areas with high numbers of expected viruses in wildlife, greater spillover probability based on EID risk factors, and higher levels of connectivity (measured by road density data) that may facilitate person-person spread.

Peninsular Malaysia (Co-I Hughes, CM Ltd.): We will expand our sampling of the Orang Asli indigenous communities to include additional communities in the 3 Districts we have already sampled, and additional Districts in the States of Kelantan Perak, Pahang, and Kelantan. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife (mainly bats) will be sampled. Human samples will include serum, whole blood, throat swabs, nasal swabs, urine, and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and tested at NPHL. Participants who hunt, butcher or consume wildlife, or who rear animals will be considered suitable for enrolment. Participants will complete a consent form and questionnaire in addition to having samples collected. During these community trips the field team will conduct community meetings to help inform the Orang Asli about the public health risk of zoonoses. *Sarawak (Co-I Tan, UNIMAS):* We will expand our sampling of the Bidayuh and Iban indigenous people to include the Dayak and Orang Ulu ("people of the interior") because this group has a more intimate connection to remote wildlife-rich areas than the other two, albeit all are hunters. Human sampling will be led by UniMAS in collaboration with CM Ltd / EHA. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife (mainly bats) will be sampled, following the same protocols as for Peninsular Malaysia. *Sabah:* We will conduct sampling at the Madai bat and bird cave that has 200 permanent residents who harvest bird's nests for soup, and >1,000 during the harvesting season and at Gomantong cave – one of the largest bat and cave swiftlet nesting sites in the world. Protocols follow those for Peninsular Malaysia. *Singapore:* Active human surveillance will be not be conducted in Singapore, but archived human specimens e.g. from our Melaka virus serosurvey (*4*), will be made available to conduct retrospective serosurveys for specific pathogens that are identified as high interest to the EIDRC, NIH, and EID-CC.

Sample sizes: From our previous work we anticipate that 10-30% of the community population will have had exposure to wildlife allowing us to capture highly exposed and non-exposure individuals at each site. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock farming, transportation, selling, or slaughtering wildlife in the surveyed areas will be targeted so that they make up ≥30% of the sampled population in each community. We will stratify sampling to ensure appropriate representation of sex, demographic, and socio-economic factors at each community site. Estimating 5% overall seroprevalence in these high-exposure populations, these sample sizes are sufficient to estimate effect sizes of risk factors of 2X or greater with 80% power. For community-based surveillance, we will enroll xxxx individuals per county/province, pooled across two sites for each, allowing us to make county/province-level comparisons of differing effects.



Commented [S76]: I think the animal and human specimen collection should be in a same format for Thailand and Malaysia.

Commented [PD77]: Need this figure for Singapore, Pen. Malaysia, Sarawak, Sabah as well – all together is best

Commented [KJO78]: Sam is going to send a combined figure tomorrow AM.

Fig. XX: Human survey and sampling study design overview (Aim 2 and 3)

2.5 Data & sample collection: Following enrollment of eligible participants and completion of the consent procedure, biological specimens (5ml will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 2.5ml whole blood; two 500 µL serum samples) will be collected and a questionnaire will be administered by trained project staff that speak appropriate local language to all participants. Through the serological findings and the survey we will investigate the details of five risk factors related to high risk wildlife exposure, to maximize the power of the analyses, based on continuing analysis of our previous work, and will focus on: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With additional consent considerations for participants enrolled from clinical settings we will review clinical records to collect recent data on medical history, clinical syndromes, and patient etiology.

2.6: Laboratory analysis: 2.6.a Serological testing:

In a previous R01, we expressed his-tagged nucleocapsid protein (NP) of SARSr-CoV Rp3 in *E.coli* and developed a SARSr-CoV specific ELISA for serosurveillance using the purified Rp3 NP. The specificity of this assay was evaluated using polyclonal antibodies against HKU1, OC43, 229E, NL63, MERS-CoV and EBOV and no significant cross-activity detected (*12*). **This suggests that if we can expand our serology tests to cover other bat CoVs, and that doing so may identify many more seropositive individuals.** We will therefore use two serological testing approaches. First, we will expand test all human sera collected from both community- and clinic- based sampling for a panel of bat CoVs that will include SARS-CoV (outbreak strain), a range of lineage 2 bat SARSr-CoVs (including WIV1 and SHC014), lineage 1 HKU3r-CoVs, MERS-CoV, and the α-CoVs SADS-CoV and HKU10. We previously found serological evidence of human exposure to HKU10 (*12*), and HKU10 is known to jump from one host bat species to another (*161*) so is likely to have infected people more widely. It is possible that non-neutralizing cross reactive epitopes exist that afford an accurate measure of cross reactivity between clade1 and clade 2 strains which would allow us to target exposure to strains of 15-25% divergence from SARS-CoV, at a phylogenetic distance where therapeutic or vaccine efficacy may falter. Additionally, we will test samples for antibodies to common human CoVs (HCoV NL63, OC43 – see potential pitfalls/solutions below). Secondly, CoVs have a high propensity to recombine. To serologically target 'novel' recombinant virus exposure, we will conduct 1) ELISA screening with SARSr-CoV S or S1 (n-terminal domain); 2) confirm these results by Western blot; then 3) use NP based ELISA and LIPS assays with a diversity of SARSr-CoV NP. For NP ELISA, micro-titer plates will be coated with 100 ng/well of recombinant batCoV NP and incubated with human sera in duplicates followed by detection with HRP labeled goat anti-human IgG antibodies. For confirmation, all ELISA positive samples will be subjected to Western blot at a dilution of 1:100 by using batCoV-NP as antigen. We will use an S protein-based ELISA to distinguish the lineage of SARSr-CoV. As new SARSr-CoVs are discovered, we will rapidly design specific Luciferase immunoprecipitation system (LIPS) assays targeting the S1 genes of bat-CoV strains for follow-up serological surveillance, as per our previous work (*13*). Additionally, in Thailand, as an initial project on our EIDRC, we will develop serology assays to the novel CoVs found in bats at the bat guano site using LIPS assays and viral genomic data, and will screen archived specimens from 230 participants (2 years' collection in 2017-2018, a subset of the subjects have been sampled at multiple time points to allow us to assess seroconversion).

2.6.b RT-PCR testing. Specimens will be screened using RT-PCR for the RdRp gene (See section 1~~.3.b~~.4.c for details). Positive samples will be subjected to full genome sequencing and RT-PCR amplification of the ~~S~~ glycoprotein genes. Samples from the clinic-based syndromic surveillance will also be tested using RT-PCR for Influenza A & B, HCoV NL63, OC43, HKU1, SARS-CoV & 229E, NiV, HeV, Ebola (WHO PCR protocol) based on the symptom as rule-outs, and SADS-CoV and HKU10 as an opportunistic survey for potential spillover these CoVs as proposed above.

2.6.c Biological characterization of viruses identified: Novel viruses identified in humans will be recovered by cultivation or from full length genome sequences using reverse genetic approaches. Viruses will be characterized for growth in primary human cell lines and in mouse models, including the Collaborative Cross

Commented [PD79]: Hongying– this figure is from our CoV R01 renewal. Please modify to make relevant to SE Asia and henipas, filos and CoVs.

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Commented [DA82]: Can we cut out some of the very specific CoV details? Especially the recombination (which filo and paramyxo don't do).

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Mice or humanized mice expressing human receptors from related strains. The approaches are discussed in more detail under Aim 1.

2.7 Epidemiological analysis: We will conduct a cross sectional case-control study to identify risk factors for Henipavirus, Filoviruses, and CoVs spillover. "Cases" are defined as participants whose samples tested positive for Henipavirus, Filoviruses, or CoVs by serological tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative for Henipavirus, Filoviruses, or CoVs. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between biological results, serological/PCR status, and risk factors related to high risk wildlife exposure including: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) home or work proximity to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms lifetime and past 12mo. Additionally, we will use this procedure to determine how clinical presentation differs between Henipavirus, Filoviruses, or CoVs in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms.

2.8 Potential problems/alternative approaches: Rarity of spillover events means it may be difficult to identify sufficient seropositives to statistically analyze human risk profiles. First, we will be targeting our community-based surveillance to subpopulations with high-levels of wildlife exposure, at sites selected for diverse and prevalent viruses to increase likelihood of finding positive individuals. Second, our serology testing will include a panel of assays for a large diversity of viruses, increasing likelihood of positives. For example, in previous work our overall bat CoV PCR prevalence in the community was, 11.8%; β-CoV, 3.4%; α-CoV, 9.1%). Thus, using this broad serological panel to screen individuals prone to contact with species of higher zoonotic risk increases the potential for detecting spillover with enough power for statistical analyses, and will shed light on behaviors that predispose to spillover. Third, we will include common human CoVs, paramyxoviruses, and influenza viruses in our panel, so that even if low prevalence of wildlife viruses is found, we will be able to conduct a valuable cross-sectional study of the seroprevalence of more common human viruses. Finally, we will be able to assess relative measures of human-wildlife contact from our survey work. We will analyze intensity of contact with high-risk taxa against other risk factors to provide useful proxy information for spillover risk. **Serological testing may not match known viruses due to recombination events, particularly for CoVs.** We will use the three-tiered serological testing system outline in **2.6.a** to try to identify these 'novel' viruses, however, we will also remain flexible on interpretation of data to ensure we account for recombination.

Commented [KJO86]: Make this more general. There's a greater challenge of interpretation of serological data, esp. if we're using many platforms (each will have its own problems that our lab colleagues are probably more familiar with). Noam may be able to generally comment on a solution here from a statistical perspective.

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Commented [KJO88]: Noam is working on this section, so can review after/tomorrow.

Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts.

3.1 Rationale/Innovation: Our previous research **(Table 1)** and other data suggests that there are substantial numbers of outbreaks or clinical cases for which the etiologic agent is not diagnosed, e.g. (*162, 163*). To investigate this in SE. Asia, we will work directly with clinics and outbreak control organizations to identify the causes of some 'cryptic' outbreaks or cases in the region. Specifically, we will expand on current syndromic surveillance activities to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and who present at regional clinics or hospitals with symptoms typical of high impact zoonotic viruses. We will collect detailed survey data to assess their contact type and frequency with wildlife and livestock and conduct molecular and serological diagnostic assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. We conduct novel viral discovery assays on patients who present with symptoms indicative of a high-risk zoonotic virus. If novel pathogens are identified by PCR, we will attempt to isolate, obtain full genome sequence and/or biologically characterize the pathogen, using the *in vitro* and *in vivo* strategy laid out in Aim 1.

3.2 Preliminary data clinical surveillance: 3.2.a. Clinical surveys and outbreak investigations: Our longterm collaboration in the region has included the following activities investigating clinical syndromes: *Peninsular Malaysia*: **At the time of writing, there is an outbreak of suspected viral undiagnosed illness in the indigenous Batek Orang Asli ("people of the forest") community living at Gua Musang ("civet cat cave") district, in Kelantan, Malaysia, close to Taman Negara – a National Park containing the oldest known rainforest**. This community is one where we have conducted prior routine surveillance and biological

sampling, demonstrating proof-of-concept that our two-pronged high-risk community/clinic approach will work. Dozens of people are affected and the outbreak is currently being investigated by our collaborators at the Malaysian NPHL. The Orang Asli live in remote villages in the rainforest of Malaysia, with limited access to modern health care and extensive contact hunting, butchering, and eating wildlife as their primary source of protein. This outbreak, and others that will continue to emerge just like it, underscore the critical need for our EIDRC - to improve the technological tools, training, and human capacity for rapid EID surveillance, diagnosis, and response at the frontlines of disease emergence in SE Asia. ~~**Investigating this outbreak be a key priority if EID-SEARCH is funded.**~~ *Sabah*: Co-I Kamruddin (BMHRC) is leading an asymptomatic leptospirosis study on sanitation workers, an ILI study in a Kota Kinabalu clinic and diarrheal study in children under 5 yrs. Co-Is William, Tan and others have conducted clinical observational studies to determine the etiology of central nervous system infections and acute undifferentiated febrile illness in Sabah. Co-Is Tan, William have collaborated with Co-I Wang to enroll 90 adult and over 100 pediatric participants with central nervous system infections, negative tests for malaria and dengue from 3 district hospitals in Sabah to determine the various etiologies. While a high proportion tested positive for rickettsial agents, the majority had no clear diagnosis. *Sarawak:* Dr Tan (UNIMAS) is conducting a survey of high-risk female Kelabit indigenous people and has found 8% with HPV52 as the dominant strain. He is working on clinical cohorts of hand, foot and mouth disease and will recruit patients from that study in the current proposed work. Finally, he is conducting a longterm study of rotavirus and other viral etiologies of acute gastroenteritis in Kuching, Sarawak that will include swine herders and farmers. These are an important group in Malaysia because pig farms are shunned by the government and local villagers to they are placed far away from town centers, deep in the forest, and it is this scenario that led to the emergence of Nipah virus in 1998-9 (*18, 164*). *Thailand:* The Chulalongkorn Univ. TRC-EID laboratory works in coordination with the Thailand Ministry of Health to investigate outbreak etiology, including the first case of imported MERS-CoV in Thailand, **for which we produced sequence confirmation within 24 hours from acquiring the specimen** ~~**(Fig. X)**~~. Three imported MERS cases have been detected in Thailand since 2015, and all cases were confirmed at the TRC-EID. We worked during the Zika outbreak to identify > 500 PCR-positive Zika patients with sequence confirmation. We have also worked on returning and suspected Ebola travelers, syndromic surveillance for SARI, Encephalitis, Hand Foot and Mouth disease in children and Viral diarrhea. Working with the US CDC (Thailand), between 1998 and 2002 we enrolled a large cohort of children from Kamphaeng Phet Province to examine Dengue virus and JE epidemiology (*165, 166*). Among the 784/2,574 convalescent sera Henipavirus-specific antibodies were detected in 13 serum samples (1.7% seroprevalence), suggesting prior exposure to endemic henipaviruses or a related paramyxovirus in rural Thailand. *Singapore:* Duke-NUS has worked with the Ministry of Health to investigate Zika cases (*167*), and with EcoHealth and others, Duke-NUS has developed novel CoV assays for clinical surveillance, and for expedited analysis during outbreaks. Our pipeline of novel virus detection, reservoir host identification, serological assay development, and serological testing is as rapid as several months, with the development of novel serological assays from sequence data in 7 days (*13*). **For example, with the discovery of SADS-CoV, we identified and fully sequenced a new virus, screened archived wildlife specimens to identify the natural reservoir host species (*Rhinolophus* spp. bats), developed LIPS serological assays, and tested human and swine samples to assess seroprevelance in less than 3 months (*13*).**

3.2.b Analysis of self-reported illness: In addition to biological outcomes we have developed a standardized approach in PREDICT for analyzing data on self-reported symptoms related to targeted illnesses; of fever with cough and shortness of breath or difficulty breathing (severe acute respiratory illness - SARI), fever with muscle aches (fevers of unknown origin (FUO) and, cough or sore throat (influenza-like illness - ILI) from study participants. We used a least absolute shrinkage and selection operator (LASSO) regression to identify associations between participants who reported ILI and/or SARI symptoms and demographics, occupation, and contact with animals in the last year. Results have clear biological relevance. In Yunnan, China, salient associated variables or combination of variables were, in descending order of odds ratio: 1) having eaten raw or undercooked carnivores; 2) having slaughtered poultry and being a resident of Guangxi province; 3) having had contact with cats or bats in the house; and 4) being a male who has raised poultry **(Fig. 11).** We will expand this approach for all syndromes in Aim 3, and use more granular targeted questions designed to assess patient's exposure to wildlife in terms that are relevant to the specific country.

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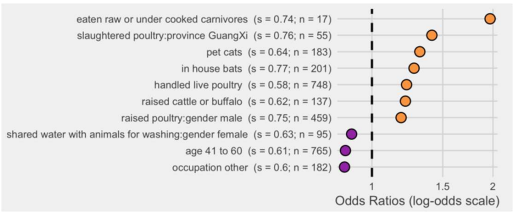
Fig. 11: Associated variables of self-reported ILI and/or SARI in prior 12 months (s = bootstrap support; n = number +ve out of 1,585 respondents). **Orange circles** = odds ratios > 1 (positively associated with the outcome); **purple** = odds ratios <1 (negatively associated with the outcome).



3.3 General Approach: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent is not identified. To capture these, we will expand on our current syndromic surveillance to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and present at regional clinics with influenza-like illness, severe respiratory illness, encephalitis, and other symptoms typical of high-impact emerging viruses. We will collect survey data tailored to the region to assess contact with wildlife, conduct molecular and serological assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. If novel pathogens are identified by PCR, we will isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work. We will then use behavioral survey data, ecological and phylogeographic analyses to assess the likely reservoir hosts to inform intervention programs.

3.4 Clinical cohorts. 3.4.a Cohort selection: We will initiate active clinic-based syndromic surveillance at 2 town-level level clinics and 2 provincial-level hospitals in Thailand, Peninsular Malaysia, Sabah and Sarawak. These all serve as the catchment healthcare facility for people in our community-based surveillance of Aim 2 and are inclusive of wildlife sampling sites in Aim 1. Patients ≥ 12 years old presenting at the health facility who meet the syndromic and clinical case definitions for SARI, ILI, encephalitis, fevers of unknown origin, or hemorrhagic fever will be recruited into the study. We will enroll a total of xxxx individuals for clinical syndromic surveillance, which accounts for up to 40% loss from follow-up. Study data will be pooled across country sites, as clinical patients are limited by the number of individuals presenting at hospitals. Sites: *Thailand:* Two new district hospitals at the same site of high-risk community will be included to the study to collect specimens (PREDICT sample protocol) from patients with respiratory and/or encephalitis symptom during 3 years of the study (Year 2 to Year 4), at least 100 patients from each hospital will be enrolled and laboratory tested by PCR and serology assays. The second blood after 21 days of fever will be collected for serology testing. ~~XXXXX~~ *Peninsular Malaysia:* Focusing immediately on the current outbreak in Orang Asli, we will work closely with the Malaysian Ministry of Health to support their investigation. We will also work with 6 Orang Asli clinics which focus solely on this indigenous community. *Sarawak:* XXXXXX *Sabah:* XXXXXXXXX. *Singapore:* ~~XXXXX~~ Given budget constraints we will not ~~do~~perform clinical cohorts, however in the ~~e~~event of a suspected outbreak we have close ~~relationshtips~~relationships with ~~xxxxx xxxxx~~all hospitals nationwide, and the central infectious disease reference lab.

3.4.b Behavioral and clinical interviews: 2.5 Clinic enrollment and follow-up: We will recruit inpatients and outpatients to participate in the study after initial screening to determine if they meet the clinical case definition of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever of unknown etiology; or 6) severe diarrhea. Once enrolled, biological samples will be collected and a questionnaire administered by trained hospital staff that speak appropriate local language. Samples will be taken concurrently when collecting samples for normative diagnostics. For inpatients, samples will be collected no more than 10 days of reported onset of illness to increase the chance of PCR detection of viruses (*168*). We will follow up 35 days after enrollment to collect an additional two 500 µL serum samples conduct a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period, and allow. ~~35 days gives~~ adequate time for development of IgG, ~~which occurs <28 days after onset of symptoms for SARS patients~~ (*169*). These samples may prove valuable to collaborators in that the PBMC could be used in production of therapeutic antibodies.

3.4.c Sampling:

Commented [KJO91]: Pasted in from Supaporn.

Commented [KJO92]: Make sure this matches our timeline and target numbers. Noam to calculate minimum samples size for detection assuming 1-3% ?? viral prev. Need some refs or data on detection rate for "novel" etiological agents.

Commented [PD93]: Tom, Kevin – we need information.

Commented [KJO94]: Linfa/Dani?

Commented [DA95]: Technically this is true, it is a small country and Linfa knows everyone in the field.

Commented [PD96]: Hongying Emily

Commented [PD97]: Need data for Nipah and filovirus patients

Commented [PD98]: Ralph: PBMC could be valuable for making therapeutic antibodies through collaborations?

Commented [DA99]: EBOV review PMID: 28820067 NiV PMID: 30869046

Following enrollment with signed consent form, biological specimens (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples) including two nasal or oropharyngeal swabs will be collected from all eligible participants, and a questionnaire will be administered. We will investigate five risk factors, to maximize the power of the analyses, all related to high risk wildlife exposure, based on continuing analysis of our previous work, and will include: 11) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With permission from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology.

3.5 Sample testing: PCR, Serol to link symptoms to etiologic agents

The standard syndromic diagnostic PCR assays for the common pathogens will be conducted and the results will be shared to with the hospital with 48 hours. Specimens will be further tested for 3 viral families; CoV, PmV and filovirus by consensus PCR. Appropriate specimen from deadNecropsy samples from deceased patients case will be further conducted characterized by NGS if the previous PCR tests are negative and unable to identify theied cause of infection. A VirScanThe serology panel assay will be conducted from using paired serum samples. This serological assay

Serology: Panel serology assay will be performed from paired serum to determine identifies antibodies with a the four- fold rise in titer in the convalescent sample as compared with the acute sample, thereby indicating a possible etiological agent.raising antibody. AThe- specific primer PCR offor the identified virus antibody positive case will be further tested fromin the acute specimens to confirm for infection.

3.5 Assessing potential for pandemic spread: Building off our spillover risk characterization in Aim 1, wWe will use statistical models built from collated biological, ecological, and genetic data to predict further assess the likelihood of human infection (or spillover) and also pathogenicity for a the range of diverse viruses we will characterize. The aim is to quickly identify and assess which viruses pose the most significant risk to human health and thus prioritize additional resources to more fully characterize, better understand pathogenicity, and invest in therapeutic development for those pathogens. We will combine two independent but related statistical models to predict pathogen spillover risk and pathogenicity. First, we will expand a simple generalized additive modeling approach (*90*) to assess spillover risk probability to a new virus that is currently not known to infect people, when we have only limited genetic data from initial PCR screening and sequencing. Additional data from receptor binding protein homology and cell line infection experiments can will be incorporated into the model framework as available. Second, given high likelihood to spillover and infect humans, or direct evidence of human infection from our clinical surveillance, we will then predict pathogenicity using nearest neighbor phylogenetic analyses as a first approximation and by incorporating more detailed data from genomic characterization and animal model experiments (Aim 1). For the pathogenicity model, hEuman epidemiological trait data for ~300 viral species known to infect people (e.g. case fatality rate, global estimates of number of human cases per year or decade, R_0, infectious period, and primary symptoms) will be aggregated including fromhave been collated by recent studies previous and will be used as reviews [Woolhouse ref] to use as predictor variables. Thirdly, we will build offapply tools already developed, and being refined by EHA under's previous DTRA and DHS supported research, to predict pandemic spread for viursesviruses we identify by integrating surveillance site data,using global flight models (*170*), as well as additional datasets (road networks, shipping routes, cell phone data) to measure on human movement and connectivity across Southeast Asia.

Commented [KJO100]: Blood just during 35 day follow up, or both? I guess both ideally, but weigh against IRB.

Commented [PD101]: This is v. weak – can someone strengthen it please!

Commented [KJO102]: Text from Supaporn

Commented [PD103]: This is v. weak – can someone strengthen it please!

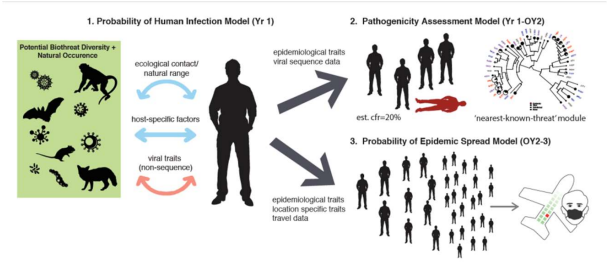
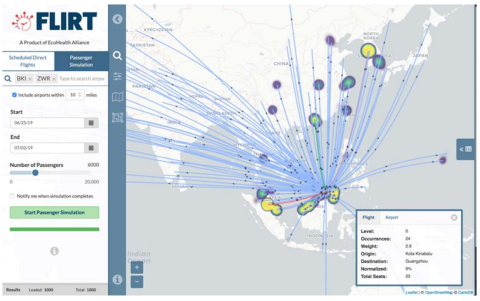


Fig. XXXXX: Conceptual framework highlighting 3 underlying models to estimate: **1)** probability of human infection, or spillover, the first necessary step to understand pathogenicity when status of human infection is unknown; **2)** pathogenicity, i.e. probability of causing clinical signs, case fatality rate estimates, and other metrics relevant to humans; and 3) probability of pathogen spread based on ecological, epidemiological, and airline connectivity and human migration data.

Commented [PD104]: Hongying – please work with Kevin to come up with something of relevance here. Also, make the text brief, bold and large so it's readable

Commented [KJO105]: See also FLIRT flight simulator figure below. Peter, let's decide if I should rework this figure to combine both, or how to proceed with this section.



3.7 Potential problems/alternative approaches: Patients visiting clinics may have cleared virus, but not yet developed IgG antibodies, reducing seropositive cases. Our 35 day follow-up sampling should avoid this because the maximum lag time between SARS infection and IgG development iwas ~28 days (*168*). We also expect that patients in rural communities will only visit clinics when symptoms have progressed, likely coinciding with late illness and onset of IgG. We will also have data from our community study, so won't be completely reliant on hospital data to identify PCR- or seropositives. Finally, the risk is outweighed by the potential public health importance of discovering active spillover of a new henipavirus, filovirus or CoV infection.

Commented [PD106]: Add data for Nipah and filo infections

Commented [DA107]: Add the same references I listed above

Additional Instructions – Specific to the EIDRC FOA:

Commented [PD108]: All – I've started putting a few notes in this section, but it will need substantial work. Please insert language from other proposals to fit these sections if you have them

4. Administrative Plan

In a clearly labeled section entitled **"Administrative Plan":**

- Describe the administrative and organizational structure of the EIDCRC Administrative and Leadership Team. Include the unique features of the organizational structure that serve to facilitate accomplishment of the long range goals and objectives.

Chula TRC-EID is National reference laboratory for diagnostic and confirmation of EIDs pathogen along with the National public health laboratory at the Department of Medical Science and the WHO Collaborating Centre for Research and Training on Viral Zoonoses. We have built a EID laboratory network in-country among three Ministry including Ministry of Public Health (Department of Medical Science and Department of Disease Control), Department of Livestock and Development, and Department of National Parks, Wildlife and Plant Conservation and Universities and among SEARO countries. Our clinicians provide case consultation for Thailand and SEARO countries. The laboratory and clinic training are regularly conducted.

Commented [PD109]: From Supaporn

For Malaysia –

NPHL – Molecular and serological screening (BioPlex) of PM Orang Asli samples, serological screening of syndromic samples from Sabah (already approved) and Sarawak (would need approval)

PERHILITAN molecular zoonosis laboratory, at the National Wildlife Forensic Laboratory - Molecular and serological screening (BioPlex) of PM wildlife samples

UPM Faculty of Veterinary Medicine - Molecular and serological screening (BioPlex) of PM livestock samples

KKPHL - Molecular of syndromic samples from Sabah (already done) and Sarawak (would need approval)

SWD WHGFL - Molecular screening of Sabah wildlife samples

BMHRC - Molecular and serological screening (BioPlex) of Sabah livestock samples, serological screening (BioPlex) of Sabah wildlife samples, Molecular and serological screening of syndromic samples from Sabah (already approved) and Sarawak (would need approval). BMHRC will need renovations and BioPlex to do this work – Menzies team might have funds for BioPlex that they would base here as part of DTRA project.

- Describe how communications will be planned, implemented, and provided to collaborators, teams, or sites. In addition, describe plans to achieve synergy and interaction within the EIDRC to ensure efficient cooperation, communication and coordination.
- Specifically address how the EIDRC Team will communicate with other EIDRCs, the EIDRC CC and NIAID and how the EIDRC will implement plans and guidance from the EIDRC CC to ensure coordination and collaboration across the Emerging Infectious Disease Program as a whole. Describe how the study site will provide information, if any, back to the local health authorities and the study subjects as needed. Note any adjustments or changes needed for rapid and flexible responses to outbreaks in emerging infectious diseases.

List out all of our involvement with outbreak investigations, ministries of health surveillance etc.
If possible the clean or summarized answers from the questionnaire should be shared to the hospital Infectious Control team and Bureau of Epidemiology for their information for controlling the outbreak.

Commented [PD110]: From Supaporn – do same for all countries

- Describe the overall management approach, including how resources will be managed, organized and prioritized, including pilot research projects.
- Highlight how the proposed staffing plan incorporates scientific and administrative expertise to fulfill the goal to develop the flexibility and capacity to respond rapidly and effectively to outbreaks in emerging infectious diseases in an international setting.

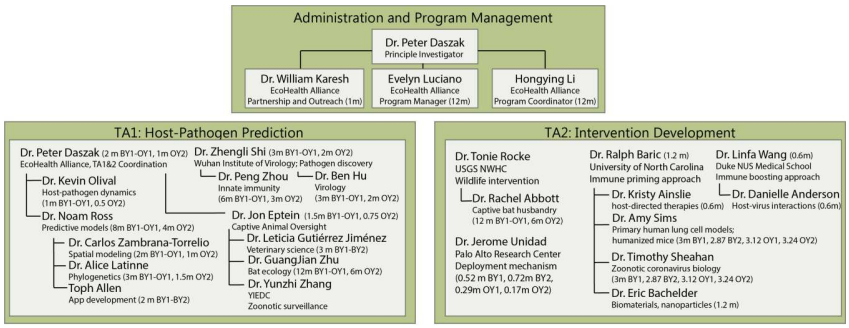
CM/EHA helped establish and manages with SWD the Wildlife Health, Genetic and Forensic Laboratory (certified as a BSL- 2 laboratory according to the US standard for laboratory specifications) that has all the equipment necessary to store samples, run extractions, PCR and analysis on biological samples for wildlife disease surveillance. The lab is used to conduct health checks on rescued and relocated wildlife before being released into new areas or sanctuaries, to screen samples for the PREDICT and Deep Forest Project and for genetic research and forensic investigations. CM/EHA also helped establish the new molecular zoonosis laboratories (certified as a BSL- 2 laboratory according to the US standard for laboratory specifications), at DWNP's National Wildlife Forensic Laboratory. The lab is used to screen samples for the PREDICT & DTRA projects. In addition CM/EHA also has access to NPHL and KKPHL for screening human samples and the Virology lab at FVMUPM for screening Livestock samples.

The Borneo Medical and Health Research Centre (BMHRC) is a centre of excellence that conducts teaching and scientific researches on communicable diseases, outbreak investigations, and ethnomedicine. BMHRC has offices space including a meeting room and seven laboratories which including preparation lab, parasitology lab, cell culture lab, bacteriology lab, natural product lab, virology lab, and molecular biology lab.

BMHRC is supported by an administration staff, medical lab technologist, and research assistants who are available to work on projects. Through this project we will help to establish a certified BSL-2 lab at the BMHRC. This lab can then be used for human testing to elevate pressure at KKPHL and QEH labs. It would also be an ideal location for a Luminex platform as human, wildlife and livestock samples could all be screened here. Co-Is Hughes and Kamruddin will be starting outbreak response training in August 2019 with a team from Sabah CDC, KKPHL and BMHRC. (Dr. Maria Suleiman now in PM But still collaborating with us, Dr. Muhammad Jikal current Director CDC working with us and Dato' Dr Cristina Rundi Director Sabah state health Dept is supportive) this collaborative group is working to develop a Sabah outbreak response team that will help support SSHD/ CDC outbreak team and also conduct sampling for disease surveillance to be based at BMHRC and we will develop this as part of this proposal. In addition the UMS hospital will come in line in 2021 providing additional opportunities for cohort studies and capacity building.

FROM DARPA grant:

Section 1.03 MANAGEMENT PLAN



Project DEFUSE lead institution is EcoHealth Alliance, New York, an international research organization focused on emerging zoonotic diseases. The PI, Dr. Daszak, has 25+ years' experience managing lab, field and modeling research projects on emerging zoonoses. Dr. Daszak will commit 2 months annually (one funded by DEFUSE, one funded by core EHA funds) to oversee and coordinate all project activities, with emphasis on modeling and fieldwork in TA1. Dr. Karesh has 40+ years' experience leading zoonotic and wildlife disease projects, and will commit 1 month annually to manage partnership activities and outreach. Dr. Epstein, with 20 years' experience working emerging bat zoonoses will coordinate animal trials across partners. Drs. Olival and Ross will manage modeling approaches for this project. Support staff includes a field surveillance team, modeling analysts, developers, data managers, and field consultants in Yunnan Province, China. The EHA team has worked extensively with other collaborators: Prof. Wang (15+ yrs); Dr. Shi (15+ yrs); Prof. Baric (5+ yrs) and Dr. Rocke (15+ yrs).

Subcontracts: #1 to Prof. Baric, UNC, to oversee reverse engineering of SARSr-CoVs, BSL-3 humanized mouse experimental infections, design and testing of targeted immune boosting treatments; **#2** to Prof. Wang, Duke-NUS, to oversee broadscale immune boosting, captive bat experiments, and analyze immunological and virological responses to immune modulators; **#3** to Dr. Shi, Wuhan Inst. Virol., to conduct PCR testing, viral discovery and isolation from bat samples collected in China, spike protein binding assays, humanized mouse work, and experimental trials on *Rhinolophus* bats; **#4** to Dr. Rocke, USGS NWHC, to refine delivery mechanisms for immune boosting treatments. Dr. Rocke will use a captive colony of bats at NWHC for initial trials, and oversee cave experiments in the US and China. **#5** to Dr. Unidad, PARC, to develop innovative aerosal platform into a field-deployable device for large-scale inoculation of the bats. Dr. Unidad will collaborate closely with Dr. Rocke in developing a field deployable prototype for both initial trails and cave experiments in China.

Collaborator Coordination: All key personnel will join regularly-scheduled web conferencing and conference calls in addition to frequent ad hoc email and telephone interactions between individuals and groups of investigators. Regular calls will include:

Commented [KJO111]: Figure like this would be good, but instead of breaking down by TA, maybe by country or Specific Aim. Per FOA, Need to also show how we'll link in with the Coordinating Center and other EIDRCs.

- Weekly meetings between the PI and Program Manager (on project and task status)
- Weekly web/phone meetings between Program Manager and subawardee admin. staff
- Monthly web/phone conferences between EHA PIs and all subawardee PIs.
- Monthly web conferences between key personnel (research presentations/coordination)
- Four in-person partner meetings annually with key personnel at EHA and two in-person partner meetings annually between subawardees.
- Annual in-person meeting among all key personnel

Evaluation metrics will include the generation of high-quality data, successful achievement of milestones and timelines, scientific interaction and collaboration, the generation of high quality publications, and effective budget management. The PI and subawardee PIs will attend a kickoff meeting and the PI will meet regularly with DAPRA at headquarters and at site visits.

Problem identification and resolution: Regular planning, monitoring, and evaluation meetings will be the primary mechanisms for problem identification. Minor problems (e.g. delays in sample availability or test results) will be dealt with internally through appropriate action and resolution monitored by Dr. Daszak and the Project Manager. In the event of significant problems, such as prolonged poor productivity, inadequate scientific collaboration, or major disputes regarding research direction or resource allocation, EHA will assist with resolving the problem through negotiation. Should a resolution not be forthcoming, consultation with our technical advisors and DARPA Program staff may be warranted.

Risk management: Maintaining a timeline and meeting milestones will require strict and continuous oversight of all project phases, frequent and regularly scheduled communication, and the ability to make decisions and implement strategies. A project of this nature requires a different mindset from that typically associated with basic research activities that move at an incremental pace with investigators gradually optimizing experimental systems, refining data, or waiting for additional data before moving ahead with an analysis approach. To maintain our timeline, we will continually evaluate these trade-offs to make decisions about when iteration is appropriate and when necessary to move forward with current information.

Biographies:

Dr. Peter Daszak is President and Chief Scientist of EcoHealth Alliance, Chair of the NASEM Forum on Microbial Threats, member of the Executive Committee and the EHA institutional lead for the $130 million USAID-EPT-PREDICT. His >300 scientific papers include the first global map of EID hotspots, estimates of unknown viral diversity, predictive models of virus-host relationships, and evidence of the bat origin of SARS-CoV and other emerging viruses.

Prof. Ralph Baric is a Professor in the UNC-Chapel Hill Dept. of Epidemiology and Dept. of Microbiology & Immunology. His work focuses on coronaviruses as models to study RNA virus transcription, replication, persistence, cross species transmission and pathogenesis. His group has developed a platform strategy to assess and evaluate countermeasures of the potential “pre-epidemic” risk associated with zoonotic virus cross species transmission.

Prof. Linfa Wang is the Emerging Infectious Diseases Programme Director at Duke-NUS Medical School. His research focuses on emerging bat viruses, including SARS-CoV, SADS-CoV, henipaviruses, and others and genetic work linking bat immunology, flight, and viral tolerance. A 2014 recipient of the Eureka Prize for Research in Infectious Diseases, he currently heads a Singapore National Research Foundation grant “Learning from bats” ($9.7M SGD).

Commented [PD112]: Some of references in this section are duplicates

Current list of contacts at key human (especially) and wildlife orgs in every SE Asian country from map above – please add to this if we’ve missed any

Indonesia – EHA: Eijkman (Dodi Safari), IPB (Imung Joko Pamungkas); **CM Ltd:** IPB (Imung Joko Pamungkas), Bogor Agricultural University (Diah Iskandriati)

SE China – EHA: Yunnan CDC (XX), Wuhan Inst. Virol. (Zhengli Shi);

Myanmar – Chulalongkorn (Thiravat and Supaporn) have collaborated extensively with Win Min Thit, MD (Yangoon General Hospital, Myanmar); Khin Yi Oo (National Health Laboratory, Myanmar) and also in training PREDICT Myanmar veterinarians in bat sampling under PREDICT.

Japan – **BMHRC**: Oita University (Hidekatsu Iha, Kiyoshi Aoyagi), Nagaskai University (Koichi Morita, Kazuhiko Moji)

Cambodia -
Laos - **CM Ltd:** Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit (Matthew Robinson)
Bangladesh – EHA: IEDCR (Meerjady Sabrina Flora, Ariful Islam), icddr,b (Mohammed Ziaur)

India – EHA: Jon's collaborators;

Vietnam – Wellcome CRU ? Liaison with Zhengli Shi for Serol assays etc.?

Philippines – EHA, CM Ltd: Biodiversity Management Bureau, Department of Environment and Natural Resources (Anson Tagtag), Bureau of Animal Industry (Davinio Catbagan), Research Institute of Tropical Medicine (Catalino Demetria).

Malaysia – **CM Ltd:** Borneo Medical and Health Research Center (Ahmed Kamruddin), Department of Wildlife and National Parks Peninsular Malaysia (Frankie Sitam, Jeffrine Japning, Rahmat Topani, Kadir Hashim, Hatta Musa), Hospital Universiti Malaysia Sabah (Helen Lasimbang), Faculty of Medicine, Universiti of Malaya (Sazaly Abu Bakar), Kota Kinabalu Public Health Laboratory (Jiloris Dony, Joel Jaimin), Ministry of Health (Chong Chee Kheong, Khebir Verasahib, Maria Suleiman), National Public Health Laboratory (Hani Mat Hussin, Noorliza Noordin, Yu Kie Chem), Queen Elizabeth Hospital (Giri Rajahram, Heng Gee Lee), Sabah State Health Department (Christina Rundi, Mohammad Jikal), Sabah Wildlife Department (Rosa Sipangkui, Senthilvel Nathan, Augustine Tuuga, Jumrafiah Sukor), Gleneagles Hospital Kota Kinabalu (Timothy Wiliam); **BMHRC:** CM Ltd (Tom Hughes), HQEII (Shahnaz Sabri), Kota Kinabalu Public Health Laboratory (Jiloris Dony), Sabah State Health Department (Mohammad Jikal)

Thailand – **CM Ltd:** Department of Disease Control, Ministry of Public Health (Rome Buathong, Pawinee Doungngern, Pongtorn Chartpituck), Department of National Park, Wildlife and Plant Conservation (Pattarapol Manee-On), Faculty of Forestry, Kasetsart University (Prateep Duengkae), Mahidol-Oxford Tropical Medicine Research Unit, (Stuart Blacksell, Richard Maude),Chulalongkorn University (Supaporn Wacharapluesadee).

Chulalongkorn lab is a WHO Collaborating Centre for Research and Training on Viral Zoonoses, we work closely with WHO SEARO and SEARO countries including Bangladesh, Bhutan, Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, (Thailand), Timor-Leste.
We had conducted the PREDICT lab training for Veterinary lab in the Southeast Asia region for FAO.
We conducted (transferred?) the bat sampling technique for scientists from Malaysia, Myanmar, the Philippines, and China.

5. Data Management Plan

In a clearly labeled section entitled **"Data Management Plan":**

- Describe internal and external data acquisition strategies to achieve harmonization of systems and procedures for data management, data quality, data analyses, and dissemination for all data and data-related materials generated by the research study with the EIDRC CC.
- Within the plan, indicate the extent to which dedicated systems or procedures will be utilized to harmonize the acquisition, curation, management, inventory and storage of data and samples. Describe

how training for the data and sample collection, in terms of the use of electronic data capture systems, will be provided to all staff including those at enrollment sites.

- Describe the quality control procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens.

This includes sample labeling etc. Get a bar-coding system, everyone has a scanner (cf. PREDICT) – Ralph has this for select agents and MERS. Also need a software system

Language from NIH CoV grant:

Data Sharing Plan: Data will be available to the public and researchers without cost or registration, and be released under a CC0 license, as soon as related publications are in press. Data will be deposited for in long-term public scientific repositories – all sequence data will be made publicly available via GenBank, species location data via the Knowledge Network for Biodiversity, and other data will be deposited in appropriate topic-specific or general repositories. Computer code for modeling and statistical analysis will be made available on a code-hosting site (GitHub), and archived in the Zenodo repository under an open-source, unrestrictive MIT license. Limited human survey and clinical data will be released following anonymization and aggregation per IRB requirements. Publications will be released as open-access via deposition to PubMed commons.

Viral isolates will remain at the Wuhan Institute of Virology initially. Isolates, reagents and any other products, should they be developed, will be made available to other NIH-funded researchers via applicable Wuhan Institute of Virology and EcoHealth Alliance Material Transfer Agreements and/or licensing agreements.

Sharing Model Organisms: We do not anticipate the development of any model organisms from this study. Should any be developed, they will be made available to other NIH-funded researchers via applicable Wuhan Institute of Virology and EcoHealth Alliance Material Transfer Agreements and/or licensing agreements.

Genomic Data Sharing: We anticipate obtaining genetic sequence data for 100s of novel coronavirus genotypes, including RNA-dependent RNA polymerase (RdRp) and Spike genes for all strains/genotypes. In addition, we will generate full viral genomes for a subset of the bat SARSr-CoVs that we identify. All sequence data will be deposited in the NIH genetic sequence database, GenBank. We will ensure that all meta-data associated with these sequences, including collection locality lat/long, species-level host identification, date of collection, and sequencing protocols will also submitted. The genotype data will be made publicly available no later than the date of initial publication or six months after the receipt of final sequencing data, whichever comes first. We anticipate sequence generation will occur over the 5 year proposed project period.

Genome Wide Association Studies (GWAS): Not applicable.

Commented [KJO113]: From CoV NIH grant

Thailand TRC-EID Chulalongkorn lab:Specimen management and BioBank: The software called PACS (Pathogen Asset Control System) supported by BTRP DTRA, is now used for managing the specimen from specimen registration (barcoding, and may move to use QR code system soon), aliquoting, tracking, short patient history record, data summary, searching, test reporting and etc. Our biobank system contains 28 freezers (-80C) and liquid N2 system. Its size is around 120 sq meters. There is a separate electric generator for our lab and biobank. There are CC TV cameras at the biobank and laboratory.

Chula TRC-EID has our own server for data management and analysis. We have the necessary software including Bioinformatic tools (Blast Standalone, SAMTOOL, GATK, AliVIEW, RaxML, FigTREE, MAFFT, Guppy, Megan, PoreChop, MEGA), Database (MySQL, MongoDB) Web, jQuery, Node.js, Express.js React,

API, PHP, Ruby, Python, Java, C#, HTML5, Bootstrap, CSS, Responsive, Cloud, Linux Command, PACS (sample management system), Virtualbox virtual machines, R programming languages, Perl scripts, Bash shell scripts, Open source software, Microsoft Office running on both Apple Mac OS X, Ubuntu, Linux, and Windows Operating Systems. Chula TRC-EID has a dedicated 16-core Ram 64GB Linux server with 4TB hard drives, dual quad-core Mac Pro Server with 2TB hard drives and another dedicated iMac Pro (Retina 4K, 21.5-inch, 2017) Processor 3.6GHz quad-core Intel Core i7 (Turbo Boost up to 4.2GHz) Ra, 16GB with 512GB hard drives. TRC-EID also has HPC Linux server CPU: 4 x 26cores Intel Xeon = 104 cores 2.1GHz with 52 TB HHD and 2x3.84TB SSD with GPU 2x Tesla v100 (CUDA core = 5120 cores/GPU, Tenser core = 640 cores/GPU). TRC-EID has a dedicated 10/100/1000 of LAN (Local Area Network), 2.4G / 5G Gigabit Wi-Fi 802.11ac.

Commented [KJO114]: From Supaporn for their lab, some may go in Facilities doc?

From DARPA:

Data Management and Sharing: EcoHealth Alliance will maintain a central database of data collected and generated via all project field, laboratory, and modeling work. The database will use secure cloud hosting services and enable export to archival and platform-independent formats. It will ensure data and metadata compatibility between project components, track data versioning and annotations, and enable compliance with DARPA data requests and disclosure requirements. All archived human sample data will be de-identified. Partners will provide raw and processed data to the central database throughout the course of the project. Project partners will have access to the data they generate in the database at all times, and maintain control over local copies. Release of any data from the database to non-DARPA outside parties or for public release or publication will occur only after consultations with all project partners. EHA has extensive experience in managing data for multi-partner partner projects (PREDICT, USAID IDEEAL, the Global Ranavirus Reporting System).

Commented [KJO115]: Text from DARPA Preempt

6. Clinical Management Plan

Clinical site selection

Our consortium partners have been conducting lab and human surveillance research, inclusive of human surveillance during outbreaks, for over the past two decades and in that time have developed strong relationships with local clinical facilities and processes. The plan for selection of clinical sites will begin in the same geographic regions as those identified in Aim 1. These are regions where we will sample wildlife due to the high potential of zoonotic viral diversity. Additionally, these will be clinical sites that serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2. In PREDICT we developed successful working relationships with the major healthcare facilities the hotspot areas in 6 different countries, including Thailand and Malaysia and we will continue to work with these sites going forward. Continuing to work with these hospitals means we will begin this project with successfully established partners and we can begin data collection quickly. This also reduces the number of additional sites needed to meet our goals within our geographic range. The capacity and capability requirements for clinical sites for this study is limited. All that is required of sites will be the ability to enroll patients that meet the clinical case definitions of interest, capacity to collect and temporarily store biological samples, and follow standards for data management and protection. We will be enrolling inpatient and outpatient participants within 10 days of the presentation of symptoms to increase the chance of PCR detection of viruses so we will not wait for advanced normative diagnostic tests, that are likely unavailable in the more remote sites, to be completed. We will work with clinical sites to determine the best process for project staff, in most cases supporting the time of currently site staff. These will be persons who are locally trained and certified to collect biological specimen from participants including blood draw technique, e.g. doctor or nurse. Last, the clinical site will need the capacity to securely store all personal health information and personally identifiable research data, this will include locked offices with locked filing cabinets to store all paper records and an encrypted computer. Training on project procedures will be provided for all local project staff by local country project management staff and partners from headquarters. The study at clinical sites will begin by developing relationships with local stakeholders at the hospitals and clinics in our geographic areas of interest that serve as catchment facilities for people of within the community surveillance study. We will recruit hospital staff that will be extensively trained for project procedures including enrollment of patients, sample types to be collected, storage of samples, administration of the questionnaire, and data

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management plans. During the Development and onboarding of new clinical sites we will assess the physical needs of the site and what supplies will be required for collection of data (e.g. locked filing cabinet, dry shipper, secure laptop). [Did not specifically address CONDUCT]. Oversight for clinical sites will be managed by the country coordinator for each country. This will be done with regular site visits to the clinical sites and annual visits by headquarter research staff. Site visits will include monitoring and evaluating the process for patient enrollment, collecting and storage of samples, administration of the questionnaire, and secure and data management procedures for secure personal health information and research data. During these visits any additional training will also be provided.

Standardized approach oversight and implementation

Management and oversite for all study sites will be managed by the local country coordinator who is supported by staff at headquarters. Implementation of a standardized approach across all study sites, community and clinical, will begin by extensive training of staff on procedures on requirements of the project, this includes: enrollment, data collection, and data management. Our research team has over 5 years of experience building capacity on human subjects' research and has developed training resources and materials for standardized implementation of community and clinical research. This includes SOPs for screening, enrollment, and retention of participants. In addition to yearly monitory reports the country coordinator will regularly visit each clinical site to ensure quality standards and compliance of procedures are being met. Through our work with clinical sites in PREDICT we have developed culturally appropriate screening measures for clinal sites that do not disrupt the flow or quality of medical treatment received by the patient. This was done by working collectively with clinical research staff to evaluate current procedures and patient flow at the site to determine the most efficient while minimal invasive inclusion of our study into the daily working of the clinal site. Most efficiently this was done by adding minimal basic screening questions to the current clinal intake forms, which allows the clinal research officer to quickly scan charts or logs for potential patients to enroll, allowing for minimal disruption and prevents potential enrollees from being overlooked if the research staff is not on duty. At clinical sites that do scheduled outpatient days we may have a trained member of the research staff with the intake nurse to monitor the patients coming to the clinic more rapidly to no delay screening or enrollment in such a fast pace environment. Using the screening tools, we will recruit and enroll patients who present with symptoms that meet the clinical case definitions of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever, of unknown etiology within the past 10 days. These patients will be enrolled, samples collected and survey conducted to assess the participants contact with wildlife through: 1) occupational exposures; 2) animal contact; 3) environment biosecurity. With permission from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology. The clinical cohort will be split into cases and controls. "Cases" are defined as participants whose samples tested positive for either coronaviruses, henipavirus, or filoviruses, serological tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between serological/PCR status and risk factors. In clinical study, we will use this procedure to determine how clinical presentation differs between virus-exposed and -unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. We will model the outcomes to analyze correlation between biological results and risk factors related to high risk wildlife exposure. Additionally, we will be following up with clinical participants to determine how clinical presentation differs between coronaviruses, henipavirus, or filoviruses in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. [How do these cohorts be leveraged for new emerging pathogens] Achieving the adequate numbers will be attained through duration of the as clinical participant enrollment. While patient's enrollment is limited by the number of individuals presenting at hospitals, during the PREDICT project we had an average of 105 patients enrolled per year, ranging from 77-244. The country coordinator will be continually monitoring the project database to be sure that we are on track to reach our target.

Clinical cohort setup, recruitment, enrollment

We will recruit inpatients and outpatients from clinical sites to participate in the study after initial screening to determine if they meet the clinical case definitions for 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever, of unknown etiology for symptoms reported onset of illness to increase the chance of PCR detection of viruses. Once enrolled, biological samples will be collected by trained hospital staff. When possible, samples will be taken concurrently with samples being collected for normative diagnostics. We will collect 2* 5mL of blood for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples and two nasal or oropharyngeal swabs will be collected. The cases are defined as participants whose samples tested positive for Henipaviruses, Filoviruses, or CoVs by PCR or serological tests. The controls will be selected from the pool of participants admitted to the studies and whose samples tested negative for coronaviruses, henipavirus, or filoviruses. We will follow up 35 days after enrollment to collect an additional 5mL blood draw for collection of two 500 µL serum samples and a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. Following up 35 days post initial collection gives adequate time for 1) development of IgG and IgM, which occurs <28 days after onset of symptoms for coronaviruses, henipavirus, or filoviruses infected patients to evaluate the immunological progression of disease and 2) further the risk assessment of the participants to monitor contact with wildlife, people, and to assess the likely reservoir hosts to collect information to inform potential intervention programs.

Utilization of collected data

Sampling of wildlife and the enrollment of community surveillance participants and a clinical cohort of participants give us the ability to assess the viruses the are circulating in each of the three populations in a similar geographic region. We will use phylogenetic analysis to compare the relationship between wildlife viruses found and viruses fond in human participants. Additionally, the questionnaire allows us to assess relative measures of human-wildlife contact from our survey work that we will analyze the intensity of contact with species known to be at a higher risk for spillover against other risk to provide useful proxy information for spillover risk.

The biological specimens to be collected (2*5mL whole blood for whole blood and serum analysis and nasal or oropharyngeal swabs) from all eligible participants and follow up specimen 35 days (3mL of whole blood for serum) after enrollment with the standardized questionnaire. Capacity for specimen is collection is not more than standard phlebotomy skills and we will collected by locally trained and certified personnel that are part of the research team in country. Staff we will trainline on ethical guidelines for conducing human subjects research and survey data collection methods. Collection of whole blood, serum, and nasal or oropharyngeal swabs allows us to test the samples for PCR to detect viruses in our priority viral families, serologically test for immunological response and historical exposure to zoonotic diseases of relevance, and the questionnaire will assess individuals evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. With this collective data set we aim to quantify and detect novel viruses likely to spillover regularly to people, are often unreported or misdiagnosed as their clinical manifestations are unknown to detect cryptic outbreaks causing previously ‘hidden’ clinical syndromes in people. Our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an ‘early warning system’ to conduct public health interventions and disrupt disease emergence.

Potential expansion

Expansion of the research project to other clinical sites or research areas could happen as new information is gathered, either through our research or an outbreak in the region. If expansion is required we could rapidly implement research activities in additional clinical or community sites through the same process we on-boarded the initial locations. First, by working with local stakeholders to create a working relationship and on-boarding new clinical research staff through advanced trainings of project aims, SOPs on data collection and storage, and ethical guidelines. The network of infectious disease researchers in the community and hospital

Commented [EH117]: Will these cohort fulfill the goals of the EIDRC.

Commented [EH118]: PREDICT we also make the stipulation that we will collective relatvive samples if avialble from treatmean collection, eg CSF if the MD is doing a spinal tap we will request an aliqot if possible

Commented [EH119]: Please add any information that might address: types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.

Commented [EH120]: Please add any information that might address: Describe methods that might be applied to elucidate a zoonotic source

settings would allow for an accelerated response to any potential infectious disease outbreaks as staff are well trained on ethical guidelines for working with human subjects, data and specimen collection, storage, and transpiration, project staff would be on the front lines ready for the call to action. Ideally, our testing for viruses in priority viral families may lead to the discovery cryptic outbreaks before it spreads in the general public. Biological results coupled with survey data on key risk factors will provided necessary information for granular understand of disease spread.

In a clearly labeled section entitled **"Clinical Management Plan":**

- Describe plans, processes, and procedures for the following activities: clinical site selection including feasibility, capacity, and capabilities, and study development, conduct, and oversight.
- Describe strategies for oversight and implementation of standardized approaches in the recruitment and clinical characterization of adequate numbers of relevant patient populations to ensure the prompt screening, enrollment, retention and completion of studies. Describe novel approaches and solutions to study enrollment. including the clinical meta-data that will be captured and describe how the staff at each enrollment/collection site will be trained and comply with the quality standards for data and sample collection and compliance with the standardized procedures. Provide a general description of the methods for establishing clinical cohorts and how cohorts may be leveraged for new emerging pathogens.
- Provide general approaches to identification of the types of clinical cohorts needed to fulfill the goals of the EIDRC. Discuss types of clinical cohorts and clinical assessments that will be utilized to study natural history and pathophysiology of disease, including characteristics of the cohort, site for recruitment, types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.
- Describe methods that might be applied to either elucidate a zoonotic source or a vector of transmission.

Our research team incredibly diverse and capable of working with a group of other viral families: Lay out all our additional vector experience incl. my WNV work, our work on Chik etc.
Baric: Flavi, Noro,
Linfa: …
EHA: …

- Describe capacity for and approaches to clinical, immunologic and biologic data and specimen collection, and how these data and specimens will address and accomplish the overall goals and objectives of the research study.

Ralph

1. Norovirus: collaboration with surveillance cohort CDC; first one to publish antigenic characterization of each new pandemic wave virus; and phase 2 and 3 trial of therapeutics
2. Tekada Sanofi Pasteur dengue
3. Nih tetravalent vaccine

- Provide plans for the potential addition of new sites and expanding scientific areas of research when needed for an accelerated response to an outbreak or newly emerging infectious disease.

Linfa – will launch VirCap platform on outbreak samples.

7. Statistical Analysis Plan

In a clearly labeled section within the Research Strategy entitled **"Statistical Analysis Plan":**

- Describe the overall plan for statistical analysis of preliminary research data, including observational

cohort data.

- Describe the overall staffing plan for biostatistical support and systems available for following functions: 1) preliminary data analyses, 2) estimates of power and sample size, 3) research study design and protocol development.
- Describe the quality control and security procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens. Describe any unique or innovative approaches to statistical analysis that may be utilized.

8. Project Milestones and Timelines

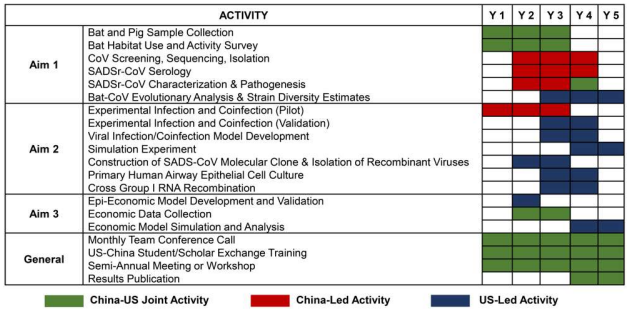
In a clearly labeled section entitled **"Project Milestones and Timelines":**

- Describe specific quantifiable milestones by annum and include annual timelines for the overall research study and for tracking progress from individual sites, collaborators, and Teams. Milestones must specify the outcome(s) for each activity. Milestones should be quantifiable and scientifically justified, and include the completion of major research study activities, including, for example, protocol development, case report forms, scheduled clinical visits, obtaining clearances study completion, and analysis of final data. Milestone criteria should not simply be a restatement of the specific aims. Using a Gantt chart or equivalent tool, describe the associated timelines and identified outcomes for the research study.

Show that for this proposal, we won't be doing the Specific Aims sequentially. We'll begin the human work (SA 2, 3) at the beginning of the project, along with SA1. We'll do the diagnostic assay development initially in Ralph's, Linfa's, Chris's lab, but then begin tech transfer in Y1….

Project Management & Timeline

PI Daszak will oversee all aspects of the project. Dr. Daszak and has two decades of experience managing international, multidisciplinary research in disease ecology, including successful multi-year projects funded by NSF, NIH and USAID. He will conduct monthly conference calls with all co-PIs, including the China team **(see Activity schedule below)**. He will be assisted by EHA Sr. Personnel Li, who is a US-based Chinese national with fluent Mandarin. Co-PI Olival will oversee Aim 1 working closely with China PI Tong, who will test samples. Co-PI Ma (China team) will coordinate and lead all Chinese experimental infections, with guidance from co-PI Saif, who will coordinate and lead all US experiments with co-PI Qiuhong Wang. Sr. Personnel Ross will conduct all modeling activities in Aim 2, working closely with co-PI Saif to parameterize the models. Co-PI Baric and Sr. Personnel Sims will lead infectious clone, recombinant viral and culture experiments. Consultant Linfa Wang will advise on serology, PCR and other assays. China team co-PIs Shi and Zhou will oversee viral characterization in Aim 1. Sr. Personnel Feferholtz will oversee the economic modeling in close collaboration with Ross.



Another Example of Milestones and specific tasks from a prev DHS (unfunded) proposal:

Expand existing databases to include predictor variables for pathogenicity risk model

Task 1: Collate available human clinical information from the literature for ~300 viral pathogens known to infect humans (Contract Award Date to 6 month)

Commented [KJO121]: Text and figure From NSF-NIH SADS grant. Peter will adapt

Task 2: Update EHA host-viral association database to include new data for mammalian and avian species from literature 2015-present (Contract Award Date to 3 month)
Task 3: Extract and curate full and partial genomic data from Genbank for 5-6 viral families with a high proportion of human-infectious pathogens for phylogenetic analyses – e.g. 'nearest-known-threat module'. (Month 2 to 6 month)

Develop underlying pathogenicity and spillover risk assessment models
Task 4: Fit generalized linear models in Stan for multiple response variables to measure pathogen impact (Month 7 to 10 month)
Task 5: Fit generalized linear models to estimate risk of human infection (spillover) based on host, ecological, and location specific traits (Month 4 to 8 month)
Task 6: Validate model effectiveness by simulating early-outbreak, limited-information scenarios from known pandemics to confirm model behavior (Month 8 to 10 month)

Commented [KJO122]: Just example, but probably just want to use a Gantt chart format per most of our grants, depending on space.

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From:	Danielle Anderson on behalf of Danielle Anderson <danielle.anderson@duke-nus.edu.sg>
To:	Kevin Olival; Peter Daszak
Cc:	Wang Linfa; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Eric Laing; Thomas Hughes; Supaporn Wacharapluesadee; Aleksei Avery Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross
Subject:	RE: EID-SEARCH v4
Date:	Wednesday, June 26, 2019 4:51:04 AM
Attachments:	EIDRC Southeast Asia v4_KJO_(DEA).docx

Hi Peter,

Here is the rest of my edits. I have to send this through now and go back to the other required documents to send back to Luke.

From: Kevin Olival [mailto:olival@ecohealthalliance.org]
Sent: Wednesday, 26 June, 2019 2:51 PM
To: Peter Daszak <daszak@ecohealthalliance.org>
Cc: Wang Linfa <linfa.wang@duke-nus.edu.sg>; Danielle Anderson <danielle.anderson@duke-nus.edu.sg>; Ralph S. Baric <rbaric@email.unc.edu>; Baric, Toni C <antoinette_baric@med.unc.edu>; Sims, Amy C <sims0018@email.unc.edu>; Chris Broder <christopher.broder@usuhs.edu>; Eric Laing <eric.laing@usuhs.edu>; Thomas Hughes <tom.hughes@ecohealthalliance.org>; Supaporn Wacharapluesadee <spwa@hotmail.com>; Aleksei Avery Chmura <chmura@ecohealthalliance.org>; Alison Andre <andre@ecohealthalliance.org>; Luke Hamel <hamel@ecohealthalliance.org>; Hongying Li <li@ecohealthalliance.org>; Emily Hagan <hagan@ecohealthalliance.org>; Noam Ross <ross@ecohealthalliance.org>
Subject: Re: EID-SEARCH v4
Importance: High

Peter, v4 with my edits, some revised figs, and additional comments.

Cheers,
Kevin

Kevin J. Olival, PhD
Vice President for Research

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On Jun 25, 2019, at 9:26 AM, Peter Daszak <daszak@ecohealthalliance.org> wrote:

Dear All,

Here's version 4. Apologies for the delay. It's in better shape now, but there are still lots of areas that need your attention. Please put in time today, and tomorrow to edit and firm up the text. I've put your names in some of the comment boxes where I need specific help but I also need you to read this critically now and edit and tighten it up. We have 30 pages of space, and our research plan is now 21 pages, so we need to lose maybe 3 or 4 pages. There are also some weak points, specifically on Filoviruses, on the details of where we'll do our clinical work, and on what we'll do with the animal models for henipas and filos.

Please get comments back with me before Wednesday 26th 4pm New York time, so that I have Wendesday evening and all day Thursday to incorporate them. In the meantime, I'll be working with folks here on better figures, and all the extra sections that come after the research plan. I'll need you all to help with those on Thursday while I'm finishing off the draft.

Thanks to all of you in advance and look forward to the next round.

Cheers,

Peter

Peter Daszak
President

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Twitter: @PeterDaszak

EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop

solutions that prevent pandemics and promote conservation.

From: Peter Daszak
Sent: Thursday, June 20, 2019 9:41 PM
To: Wang Linfa; Danielle Anderson (danielle.anderson@duke-nus.edu.sg); Ralph Baric (rbaric@email.unc.edu); Baric, Toni C; Sims, Amy C; Christopher Broder (christopher.broder@usuhs.edu); Eric Laing; 'Tom Hughes'
Cc: Aleksei Chmura (chmura@ecohealthalliance.org); Alison Andre; Luke Hamel (hamel@ecohealthalliance.org); Kevin Olival, PhD (olival@ecohealthalliance.org)
Subject: EIDRC-SEA v.3
Importance: High

Dear all,

Thanks to everyone for comments and text for the earlier draft at the beginning of the week. Kevin's spent the last couple of days pulling all the comments together into one draft and inserting his changes, and I've now attached a v3 of the draft. It's still rough, and now too long, but it's getting there. I wanted you to look at this rough version because I'm stuck in Liberia in meetings Friday, then flying back home Saturday. I'll continue editing this draft on the plane and will be working solely on this grant from home on Sunday, Monday, Tues, Wed, Thurs.

Please work on this rapidly if possible. If you can all push your comments through, using track changes, and get them back to me **by Sunday evening New York Time**, I will turn round a polished draft by Tuesday, giving us time for significant last go-over on Wed, Thurs. It's tight, but unfortunately, it is what it is, so please commit the time if you can!! As well as general edits (using track changes, with references in comment boxes), please do the following…

Linfa/Danielle – We especially need your input in section 1.1, 1.3, 1.4 and 2.5

Ralph/Amy - Please work your magic on this draft, inserting text, editing and reducing text, and making sure the technical details of the characterization are correct and fit the proposed work on CoVs, PMVs and Henipaviruses

Chris/Eric – Eric – congratulations on your (b) (6) and thanks for working on it at this time! Chris – please help and edit/finesse the language in the relevant sections for you

Tom – Your edits crossed over with Kevin's changes, so please insert all the bits that were new in the last version you sent to me, into this version. I think these are mainly the sections and comments from the other Malaysian colleagues, but please check and re-insert all key text. We especially need to be specific about which partner in Malaysia is doing what cohorts, and where, and about who's doing the testing

Thanks again all, and I'm looking forward to non-stop work on this as soon as I get on my flight in 24 hours…

Cheers,

Peter

Peter Daszak
President

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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

<EIDRC Southeast Asia v4.docx>

II. Research Strategy:

1. Significance:

Southeast Asia is a critical hotspot for emerging diseases due to its high biodiversity of wildlife and their viruses, and the presence of key ecological and socioeconomic drivers of emergence **(Fig. 1)** (*1*). These include dense human populations living in networks of rural and urban communities, with strong cultural and occupational connection to wildlife and livestock farming and trading, and intimate connection to global travel networks (*2*). The region is also undergoing rapid environmental and demographic change, both of which increase the risk of disease emergence and spread.

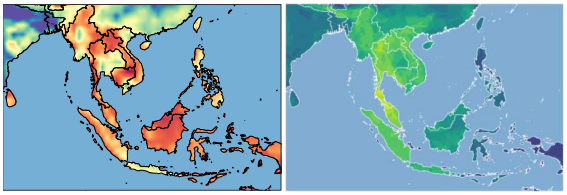

Fig. 1: Southeast Asia, and in particular, parts of Thailand and Malaysia are hotspots of risk for disease emergence **(left)**, and predicted high diversity of 'missing' or as-yet undiscovered viruses, yellow = highest diversity **(left)**. From (*1, 2*).

Not surprisingly a number of novel viruses, including near-neighbors of known agents, have emerged in the region leading to sometimes unusual clinical presentations **(Table 1)**.These factors are also behind a significant background burden of repeated Dengue virus outbreaks in the region (*16*), and over 30 known *Flavivirus* species circulating throughout S. and SE Asia (*17*). The events in **Table 1** are dominated by three viral families: coronaviruses, henipaviruses and filoviruses, which serve as initial examples of team research focus and capabilities. These viral groups have led to globally important emerging zoonoses (*18-29*). Their outbreaks have caused tens of thousands of deaths, and billions of dollars in economic damages (*30-32*). Furthermore, relatives and near-neighbors of known pathogens in these families have been reported throughout the region, including by members of our consortium: henipaviruses in bats in Thailand, Cambodia, Philippines, Laos, Indonesia, China, Malaysia, Bangladesh and India (*33-39*); a novel henipavirus, Mojiang virus, in wild rats in Yunnan (*10*); serological evidence of filoviruses in bats in Bangladesh (*40*) and Singapore (*41*); Nipah- and Ebola-like viruses in *Hipposideros*, *Cynopterus* and *Rhinolophus* species in Malaysia (Hughes *et al.*, in prep.); evidence of novel filoviruses in bats in China (*42-44*), including Měnglà virus that appears capable of infecting human cells (*45*); novel paramyxoviruses in bats and rodents consumed as ~~bushmeat~~bush meat in Vietnam (*46*); a lineage C β-CoV in bats in China that uses the same cell receptor as MERS-CoV and can infect human cells *in vitro* (*47*); MERSr-CoVs in dry bat guano being harvested as fertilizer in Thailand (*48*), and directly in bats (Wacharapluesadee *et al.*, in prep.);

Viral agent	Site, date	Impact	Novelty of event	Ref.
Melaka & Kampar virus	Malaysia 2006	SARI in family group, individual	1st disease due to bat-origin reoviruses, also Singapore, Vietnam etc.	(*3-6*)
Reston filovirus	Philippines 2008	Seropositive people killed pigs,	No prior findings of filoviruses in pigs	(*7*)
Thrombocytopenia Syndrome virus	E. Asia 2009	100s of deaths in people	Novel tick-borne zoonosis with large caseload	(*8*)
Reston filovirus	Shanghai 2011	PCR positive pigs	Further evidence of pig RESTV infection	(*9*)
Mojiang virus	Yunnan 2012	Death of 3 mineworkers	1st evidence of rodent origin henipavirus in people	(*10*)
Nipah-like virus	Philippines 2015	Killed horses	No prior horse infection for NiV, known for HeV	(*11*)
SARSr-CoV & HKU10-CoV	S. China 2015	Seropositive people	1st evidence human infection HKU10 & SARSr-CoV	(*12*)
SADS-CoV	China 2017	>25,000 pig deaths seronegative people	Novel emergence of bat-origin CoV	(*13*)
Nipah virus	Kerala 2018, 2019	Killed 17/19 people 2018, 1 infected 2019	1st outbreak of NiV outside Bangladesh, W. Bengal focus	(*14, 15*)

Table 1: Recent emergence events in SE Asia indicating potential for novel pathways of emergence, or unusual presentations for known or close relatives of known viruses.

172 novel β-CoVs (52 novel SARSr-CoVs) and a new β-CoV clade ("lineage E") in bat species in S. China that are also found throughout the region (*20, 47, 49, 50*); and 9 and 27 novel wildlife-origin CoVs and

paramyxoviruses in Thailand, ~~(~~respectively,~~)~~ and 9 novel CoVs in Malaysia identified as part our work under USAID-PREDICT funding (*49, 51*). These discoveries underscore the clear and present danger of future zoonotic events in the region. Furthermore, the wide diversity of potentially pathogenic viral strains has significant potential to help in the development of broadly active vaccines, immunotherapeutics and drugs (*47*).

Most high-impact zoonotic viruses originate in wildlife reservoirs, sometimes spilling over first into livestock 'amplifier' hosts, or directly into localized human populations with high levels of animal contact **(Fig. 2)**. Efforts to prevent emerging zoonoses have targeted these high-risk populations in regions prone to disease emergence (*29, 52*). However, these regions tend to be in the developing world, where nations often lack the resources to investigate novel zoonotic events, or wildlife reservoirs of zoonoses, and prioritize more immediate public health concerns such as Dengue virus or NTDs (*16*). Zoonotic disease surveillance and control is also hampered by inadequate information on basic disease ecology (e.g. range of wildlife reservoirs, risk factors for spillover) and pathogen biology (viral diversity, pathogenesis), and by logistical challenges sharing novel diagnostics, animal models, samples and reagents at sites where outbreaks begin (*53*). Countermeasure and vaccine development is challenged by the small number of isolates available, and the presence of closely-related strains, some with evidence of transmission to people but with unknown pathogenicity. For example, using NIH and other funding, we found serological evidence of exposure to African henipaviruses in 3-4% of a sample of people in Cameroon, with a significant risk factor being butchering of bats; and serological responses to bat SARSr-CoVs in 6/209 (2.87%) and bat HKU10-CoV in x/xx (x%) people living close to a bat cave in Yunnan, all novel viruses with unknown clinical impact (*12, 54*). We discovered two novel bat-borne reoviruses, Melaka and Pulau viruses, and showed that 14/109 (13%) of people living in close proximity to bat roosts on Tioman Island, Malaysia were exposed to these viruses (*3*) as well as 12/856 (1.4%) people screened in Singapore (*4*). Previous studies in the Philippines found famers and butchers who had contact with sick pigs tested positive for Reston ebolavirus antibodies but had not experienced illness (*7*), and it was our team that discovered Philippine bats as natural reservoir hosts for Reston virus (*55*). Preliminary screening in Malaysia with funding from DTRA for serological surveillance of ~~Henipaviruses~~ henipaviruses and ~~Filoviruses~~ filoviruses spillover found serological evidence of past exposure to Nipah (NiV) and Ebola antigenically-related viruses in non-human primates (NHPs) and ~~people~~ humans (Hughes, in prep.). Even when spillover leads to outbreaks of illness and mortality, they are often unreported, undiagnosed or misdiagnosed. Under a prior NIH R01, we initiated targeted syndromic surveillance of encephalitis patients in Bangladesh clinics and showed that NiV causes repeated annual outbreaks with an overall mortality rate of ~70% (*56*). NiV~~pah virus~~ has been reported in people in West Bengal, India, which borders Bangladesh (*26*), in bats in Central North India (*57*), and now in people in Kerala, South India in 2018 and 2019, raising the specter of future spillover at other sites in the region (*14, 58*).

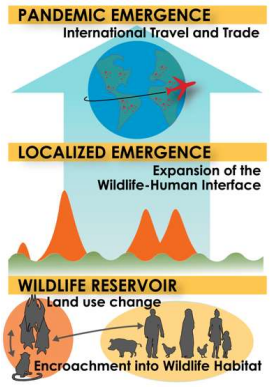

Fig. 2: Our Early Warning Approach: Most zoonotic viruses emerge from wildlife hosts, sometimes via livestock (Stage 1, lower panel), to cause small scale outbreaks (green) or larger chains (red) of human-to-human transmission (Stage 2, middle). In some cases, these spread more widely via air travel (Stage 3, upper). Our EIDRC proposal targets each stage: SA1 examines the diversity and spillover potential of viral threats in targeted SE Asian wildlife; SA2 seeks evidence of their spillover into focused high-risk human populations (stage 2); SA3 identifies their capacity to cause illness and to spread more widely (stage 3). Figure from (*52*).

Under a current NIAID R01, we have shown that we can use analytical approaches to target surveillance of wildlife and people and identify likely sites of spillover. We used phylogenetic analysis of bat hosts and their CoVs to identify a key cave complex in SW Yunnan Province that harbors all the genetic elements of SARS-CoV (*59*), as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics (*20, 47, 49, 50, 60-62*). We conducted behavioral risk surveys and biological sampling of human populations living close to this cave and found evidence of spillover (xx serology) in people highly exposed to wildlife. **This work provides proof-of-concept for an early warning approach that we will expand in this EIDRC to target key viral groups in**

Commented [PD1]: Peter will correct

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one of the world's most high-risk EID hotspots.

The overall premise ~~for of~~ this EIDRC proposal is that there is substantial evidence that: 1) diverse CoVs, henipaviruses and filoviruses related to known zoonotic pathogens are circulating in wildlife reservoirs in Southeast Asia; **2)** these viruses likely spillover regularly to people, are often unreported or misdiagnosed, and their clinical manifestations and potential to cause pandemics are unknown and underestimated; and **3)** our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence. To scale-up this approach, we propose to launch **EID-SEARCH** (the **E**merging **I**nfectious **D**iseases - **S**outh **E**ast **A**sia **R**esearch **C**ollaboration **H**ub), to better understand the risk of zoonotic viral emergence in Southeast Asia. We will initially focus on the diversity of known and novel CoVs, henipaviruses~~,~~ and filoviruses in wildlife reservoirs. We will use this work to build our center's interactive network throughout the region, bridge barriers, provide early focal point for basic and translational studies, develop sample handling and storage routines, communication networks, cohort management, infrastructure development, and cross-training in surveillance and novel diagnostic platforms. We will develop and transfer technology to test these viruses' capacity to infect human cells and mice~~ouse models~~, develop new specific and sensitive serological and molecular diagnostic tools, conduct surveillance of human communities with high risk of exposure to wildlife, and clinical cohorts to identify evidence of spillover of viruses causing previously 'cryptic' clinical syndromes in people. We will test the capacity of novel viruses ~~we isolate, or genetically characterize,~~ to infect human cells and/or mouse models, and thus asses potential for spillover and spread which will feed into our risk models. Our group also has extensive experience in other viruses, including vector-borne flaviviruses, and alphaviruses, and will use this experience to provide robust long-term surveillance, diagnostics, human and basic science opportunities. While the current proposal focuses its basic and applied research agendas on this group of viral EID, we note that the EID-SEARCH is poised to rapidly respond to any ~~new emerging~~ outbreak. Thus, filoviruses, henipaviruses and coronaviruses are examples of sentinel surveillance systems in place that can capture, identify and initiate a rapid response targeting any novel microbial pathogen that emerges in this region.

2. Innovation: Previous work by our consortium has developed strategies to target surveillance in wildlife and people to better anticipate and identify early spillover events and pre-empt outbreaks of emerging viruses. In the EID-SEARCH we scale up this approach in a central research "hub" made up of leading laboratories, clinics and surveillance sites in three critically high-risk EID hotspot countries in SE Asia. EID-SEARCH's reach is expanded through its regional network of collaborators that covers nearly all of the greater SE Asia region. The innovation of the EID-SEARCH is in: **1)** Its multidisciplinary approach that combines modeling to target geographic and taxonomic sampling targets with on-the-ground zoonotic disease surveillance in human and wildlife populations; **2)** the biological characterization approach that identifies how likely viruses are to be able to spillover into people, and enables evaluation of existing countermeasure technologies; an approach that is grounded in our successful work on SARSr-CoVs; and **3)** the strategic sampling of both high-risk communities with extensive animal contact at the front-lines of viral spillover *and* syndromic clinical cohorts that present with signs of novel viral emergence, to identify risk of viral spillover in an early warning system approach **(Fig. 2)**. **In Aim 1, we will target viruses in archived and newly collected wildlife samples, and characterize their risk of spillover to ~~people~~humans**. We will use spatial and phylogeographic analyses to identify the geography and species of wildlife to test samples from, and to further characterize viruses we have recently discovered. We will use *in silico* methods (inovel ecological-phylogenetic risk ranking and antigenic mapping of receptor binding domains), cell culture, and animal models to assess their potential for spillover into

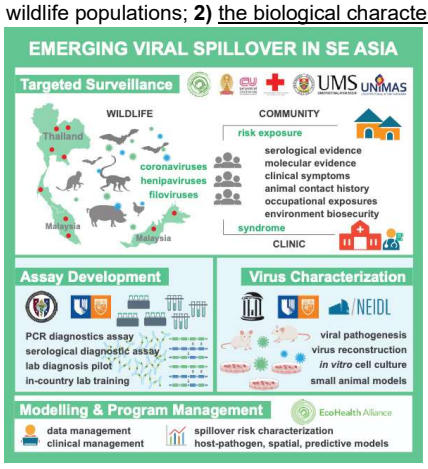

Fig. 2: EID-SEARCH approach, core members, and roles.

Commented [BRS3]: Statement should be in the aims. …Our center covers the worlds most high risk EID hostspot?

Commented [PD4]: Hongying – Figure needs edits to text: top panel, 'wildlife' should read "Aim 1: WILDLIFE", "AIM 2: COMMUNITY", "AIM 3: CLINIC". Right hand side: "Clinical symptoms" should be "Clinical history", "Occupational exposures" should be "Occupational exposure", "environment biosecurity" should be "environmental risk factors"

Middle panel: "Assay Development" and "Virus Characterization" should have "(Aim 1)" after them. Left hand side: should read "PCR diagnostics", "serology", "piloting diagnostics" and the last one is ok. Right hand side bullets should be "receptor binding characterization" "in vitro characterization" and "mouse models"

Bottom panel: all good

high-risk human populations. **In Aim 2, we will conduct focused, targeted surveys and sampling of human communities with high exposure to wildlife and other animals.** We will use serological tests targeting viruses identified in Aim 1 to identify the baseline spillover of known or novel CoVs, henipaviruses and filoviruses in these populations. Serological findings will be analyzed together with clinical metadata (including a simple animal-contact survey, location data, viral sequence/isolates etc.) to identify zoonotic risk factors and routes of exposure from specific wildlife or vector species. **In Aim 3, we will use syndromic surveillance of clinical cohorts to identify evidence of viral etiology for otherwise 'cryptic' outbreaks.** We will enroll and collect biological samples from patients in local clinics and hospitals who present with syndromes previously linked to known viral agents and live within communities at high risk for disease emergence. We will conduct molecular and serological diagnostic assays to identify likely viral etiology of syndromes that may represent otherwise cryptic events. Where viruses are identified, we will attempt to isolate and characterize them, and use survey data, ecological and phylogeographic analysis to identify likely reservoir hosts and inform intervention programs. Our US partner BSL-4 laboratory, NEIDL, ~~will attempt isolation and characterize~~ any viruses requiring high levels of containment (e.g. any novel filoviruses discovered).

Commented [PD5]: PD will get stronger letter from Jerry Keusch: opportunity for visiting scholars to be certified for BSL-4 research, strong Ebola person at NEIDL to conduct characterization if we find filos.

3. Approach

Research team: The EID-SEARCH builds on long-term collaboration among world leaders in emerging virus research with proven experience collaborating internationally on field, lab and human surveillance research **(Fig. 2)**. Over the past two decades, our consortium partners have conducted high profile research on EIDs within the region and globally, including identifying the wildlife reservoirs for NiV~~pah~~ and Hendra virus, MERS- and SARS-CoVs (*20, 25, 59, 63-66*), discovering SADS-CoV (*13*), and developing an array of serological, molecular, *in vitro* and *in vivo* approaches to characterizing high-risk CoVs, henipaviruses, and filoviruses (*60-62, 67-80*). Our team has substantial experience conducting human surveillance in the community and during outbreaks **(See sections 2.x.x, 3.x.x)**, including: USAID-PREDICT, and DTRA-funded viral surveillance of indigenous communities in Peninsular Malaysia and syndromic surveillance of hospital patients in Sabah (Co-I Hughes); collection of >2,000 serum samples from healthy blood donors and sanitation workers, febrile and influenza like illness (ILI), and diarrheal stool samples from children under 5 years of age (Co-I Kamruddin); collaboration on the MONKEYBAR project with the London School of Hygiene and Tropical Medicine to analyze risk and prevalence of the zoonotic malaria *Plasmodium knowlesi* (Co-Is William, Tock Hing); case control and cross-sectional studies in Sabah villages (~800, 10,000 participants resp.) (Co-Is GR, TYW). As a proof-of-concept of how our team can rapidly scale up resources for diagnostics and surveillance in the face of a new EID, in 2016, we discovered a novel bat-origin HKU-2 CoV (*81, 82*) killing >20,000 pigs in S. China, designed PCR and LIPS ~~serology~~ tests, ~~then~~ surveyed pig farmers for evidence of exposure, and conducted experimental infections, pathogenesis and molecular studies, **all in the span of 3 months** (*13, 83*).

The administration of this center **(See section X)** will be led by PI Daszak, who has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5- 20 ~~yrs~~ years on NIAID- and USAID-funded research. He will oversee all activities in this EIDRC. Drs. Olival and Zambrana are leaders in analytical approaches to targeting surveillance and head the modeling and analytics work for USAID-PREDICT. Drs. Wang, Baric, Broder, Anderson, and Laing have developed a unique array of *in vitro* cell culture, serological and molecular reagents to identify and characterize emerging viruses in people and animals (henipaviruses and other paramyxoviruses, CoVs, filoviruses and many others). Dr. Wacharapluesedee, Hughes and collaborators have coordinated surveillance of people, wildlife, and livestock within Thailand and Malaysia for the past 10 years, in collaboration with Ministries of Health, Agriculture and Environment. Finally, this consortium has extensive external funding from NIH, DoD, USAID, DHS and other USG agencies that supports a range of laboratory, analytical and field studies directly related to the current proposal, and will bring substantial leverage to our EIDRC.

Geographical focus: The three core (hub) countries for this proposed EIDRC are Thailand, Malaysia and Singapore, three contiguous EID hotspot countries that stretch through one of the most significant foci of EID origins globally. Our proposed work includes sampling wildlife and ~~people~~ humans in Thailand, Peninsular Malaysia, Sarawak and Sabah, and thus contains overlapping wildlife biogeography with India, Bangladesh,

Myanmar, SW China, Laos, Cambodia, Vietnam, Philippines, and Indonesia. It encompasses diverse cultural and socioeconomic backgrounds which have correspondingly diverse and intensive interactions with wildlife reservoirs and the global travel network. We have already begun sampling of wildlife hunters, livestock workers, wildlife farmers and market workers, rural villagers living close to high risk wildlife species, people otherwise highly exposed to animals in these countries, and clinical cohorts, as part of current/prior NIH, DTRA, and USAID funding. To develop a larger catchment area for emerging zoonoses, we will deploy our core member's extensive network of collaborators in clinics, research institutes and public health laboratories in almost every other Southeast Asian country. We will conduct regular meetings with key research, public health and community leaders, including >50 leading laboratories/institutions in XX countries that are currently collaborating with core members of our consortium via other funded work (**Fig. 3**). We will use these, and our annual meetings, to share information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks across the region.

Fig.3 : Map of Southeast Asia indicating three hub countries for this proposed EIDRC (Red: Thailand, Singapore, Malaysia) and countries in which Key Personnel have collaborating partners (Green), indicating that EID-SEARCH provides access to key collaborators throughout the region.

Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife

1.1 Rationale/Innovation: The vast majority of emerging zoonotic pathogens are linked directly or indirectly to wildlife host, particularly from important mammalian groups like bats, rodents and non-human primates (*84*). Our previous work on wildlife origin viruses demonstrates that zoonotic viral emergence tends to occur in specific regions with high diversity of wildlife that harbor viruses of zoonotic potential, and human populations that have close contact to these wildlife **(Fig. 1)** (*2*). In Aim 1, we will strategically conduct EID surveillance by applying risk analytics to prioritize the regions, wildlife species, and human populations that have a particularly high risk of viral spillover. We will target high-risk zoonotic host reservoirs (bats, rodents and primates) in these regions and apply novel predictive models to estimate viral diversity and host range to identify wildlife species that have been ignored or under-sampled by previous zoonotic disease surveillance efforts. We will strategically collect specimens from these species and screen them, together with some of the tens of thousands of recently collected samples we have archived ~~in freezers in our labs~~, for known and novel agents. We will begin screening using conserved PCR assays to identify known and novel viruses in high risk RNA viral families, *Coronaviridae*, *Paramyxoviridae*, and *Filoviridae*. We will expand to other groups of pathogens, pending available resources and priorities established with the EIDRC coordinating center (EIDRC-CC) and other regional EIDRCs. Using risk ranking algorithms and a series of ecological and virological risk factors, we will triage the wildlife viruses we discover for further genomic characterization and viral isolation. On this subset of viruses, we will then use *in vitro* and *in vivo* approaches (viral rescue, cell lines, gene edited humanized transgenic mice and the collaborative cross genetic reference mouse population) previously developed and widely used by our team to predict capacity of novel viruses to infect people and spillover (*61, 85-88*). These high-risk viruses and their close relatives will be targets for human community and clinical sampling in Aim 2 and 3, respectively.

1.2 Preliminary data: 1.2.a. Geographic targeting: EcoHealth Alliance has led the field in conducting analyses to indicate where the highest risk of zoonotic spillover is across the globe, identifying SE Asia as a major high risk location for pathogen emergence (*1, 2*). For Southeast Asia, these EID hotspots are sites where high wildlife biodiversity, human population density and rapid ecologic change collide **(Fig 1)**. Using host and ICTV viral data for all known mammalian viruses, and generalized additive models to correct for sampling bias, we are able to predict the relative number of yet-to-be-described viruses that a species harbors (*2*). Using these data to map unknown viral diversity demonstrates substantial undiscovered viral diversity in mammals

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across Southeast Asia, and particularly in regions of Thailand, Singapore and Malaysia **(Fig. 1)**. ~~In a~~ ~~sS~~eparate~~ly paper on risk of viruses emerging from bat hosts~~, we found that factors driving spillover (host diversity, climate) differ from those driving spread in human populations (population density, bush meat hunting, livestock production) (*89*). We therefore analyze capacity for viral spread as a separate risk factor, using demographic and air travel data and flight model software we have produced with Dept Homeland Security funds (*90*). In the current proposed work, we will use all the above approaches to target wildlife sampling (archive and new field collections), and to inform sites for human sampling in Aim 2, to areas of high risk for spillover and spread.

1.2.b. Reservoir host species targeting: Our analysis of all known mammalian host-viral associations demonstrates that bats, rodents and primates represent the three most important reservoirs for zoonoses, responsible for the highest proportion of known and predicted viral diversity of zoonotic potential (*91*). Thus, the bulk of our proposed wildlife sampling and testing will consist of these three taxa with a focus on bats, because this group is the known or hypothesized reservoir for the majority of high-impact zoonotic CoVs, henipaviruses and filoviruses (*48, 92-94*). Bats ~~also~~ have the highest proportion of predicted zoonotic viruses in any mammalian group (*2*), and are exceedingly diverse in Southeast Asia (~500 species). To further prioritize taxonomic sampling targets within these diverse geographic hotspots, we have used a novel, Bayesian phylogeographic analysis to identify host taxa that are the most important centers of evolutionary diversity for a given viral group, e.g. bat genera for β-CoVs using an extensive CoV sequence dataset from our NIH-funded research **(Fig. 5)** (*49*). This approach will be extended to other mammal taxa and viral families of interest, allowing us to identify the ancestral wildlife hosts of specific zoonotic viral groups, where their diversity is likely highest.

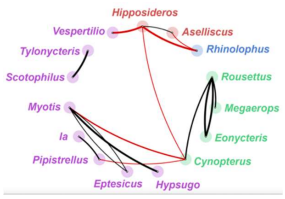

Fig 5: Preliminary analysis to identify the most important bat genera in Southeast Asia as hosts for beta-CoVs using our previously-collected CoV sequence data. Line thickness is proportional to the probability of virus sharing between two genera. Inter-family switches are highlighted in red. We will apply this Bayesian discrete phylogeographic model to identify taxonomic and geographic centers of viral diversification and sharing for relevant paramyxovirus, coronavirus, and other virus clades.

Finally, we have designed a strategy to estimate sampling gaps, using a viral 'mark-recapture' approach we previously published (*95, 96*) **(Fig. 6)**. Using prior data for bat, rodent and primate viral discovery vs. sampling rates, we can identify the likely total viral diversity for specific host groups in a region, and use this to estimate sample sizes to discovering the majority of unknown viruses within a specific family or genus. **In the current proposal,** we will use these analyses to estimate geographic and species-specific sampling targets **to more effectively identify new strains to support experimental infection studies and risk assessment.**

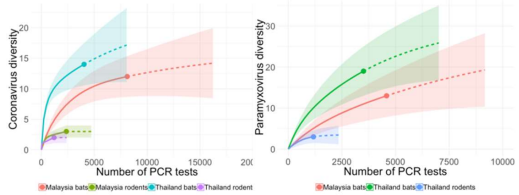

Fig. 6: Estimated coronavirus **(left)** and paramyxovirus **(right)** putative viral 'species' diversity in bats and rodents for Thailand and Malaysia, using RdRp gene sequence preliminary data from >13,000 PCR tests in bats and 4,500 in rodents. Bats in Thailand and Malaysia have 4X the number of viruses than rodent species, controlling for sampling effort. CoV and paramyxovirus diversity estimates are comparable, but discovery has not yet saturated in any taxonomic group or location. We estimate that additional sampling of ~5,000 bats and testing of our archived rodents specimens alone will identify >80% of remaining CoV and paramyxovirus viral species in these key reservoirs. We will use this approach to define sampling targets for other wildlife taxa, and for viral *strain* diversity.

1.2.c. Sample testing to identify known and novel viruses: We have conducted extensive wildlife surveillance for novel viruses across Southeast Asia in prior NIAID funded work and as part of the USAID PREDICT project. Under the 10-year PREDICT program globally, we identified 912 novel and 210 known viruses, more than the total number of viruses previously recognized in mammals by the I~~nternational Committee on Taxonomy of Viruses.~~ CTV. This work included collecting nearly 300,000 individual mammal samples from 14 EHA-led PREDICT countries and PCR-screening more than 60,000 individual specimens (*51*). In southern China alone, this sampling lead to the identification of 178 β-CoVs, of which 172 were novel

Commented [BRS14]: I would say we focus on these three species (plus vectors?). To demonstrate the capability of the team, this proposal will focus on bat surveillance (for a variety of reasons), noting that this approach will also be applied to other zoonotic vectors during the 5 year program throughout the region.

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Commented [PD16]: Kevin - Can you get rid of the shading on the background to this figure and change the colors so the circles and genus names are more visible (esp. the green). Colors should be darker and more contrasty

Commented [KJO17]: We can show just a single curve per country (aggregating all wildlife species) for simplicity, and could fit CoVs and PMVs on one graph.

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~~(52 novel SARSr-CoVs)~~. Included were members of a new β-CoV clade, "lineage E" (41), diverse HKU3-related CoVs (179 sequences) within a 'sister' clade to the SARS-CoV lineage, and the wildlife origin of a new virus, SADS-CoV, responsible for killing >20,000 pigs in Guangdong Province (*13*). Many of the identified bat species are found across the region. We have collected 28,957 samples from bats, rodents and primates in Thailand and 51,373 in Malaysia, conducting 146,527 PCR tests on a large proportion of these specimens. We have **archived duplicate samples which are now available for use in this project**, including fecal, oral, urogenital and serum samples from bats, rodents, and non-human primates, and primate biopsies. In Thailand this screening has identified 100 novel viruses and 37 known viruses in wildlife reservoirs. In Malaysia this screening has identified 77 novel viruses and 23 known viruses in wildlife reservoirs (66 novel and 19 known in Sabah and 11 novel and 9 known from Peninsular Malaysia). We used family level primers for henipaviruses, filoviruses and CoVs and have already discovered 9 novel and 7 known CoVs and 26 novel and 2 known paramyxoviruses in Thailand. This is in addition to 13 novel and 5 known CoVs and 15 novel and 1 known paramyxovirus in Malaysia from wildlife. In the current proposed work, we will attempt to sequence, isolate and characterize those viruses that our analyses below suggest are most likely to be able to infect humans.

Our collaborative group has worked together on these projects, as well as other DTRA funded projects in Singapore/Thailand (Co-Is Broder, Wacharapleusadee, Wang) to design, cross-train, and use novel serological and molecular platforms to investigate virus infection dynamics in bats and examine potential spillover events with or without human disease. For serology, Co-Is Wang and Anderson (Duke-NUS) will develop a phage-display method to screen antibodies for all known and related bat-borne viruses, focusing on henipaviruses, filoviruses and coronaviruses. Once various "trends" are discovered, additional platforms (including Luminex and LIPS) will be used for verification and expanded surveillance. For molecular investigation, we will combine a targeted PCR approach with a discovery-driven enrichment NGS strategy to not only survey currently known viruses, but also to discover potential novel viruses. Both the phage-display serological and enrichment platforms can be rapidly updated to include new viruses identified. In Malaysia (Co-Is Hughes, Broder, Laing) we have transferred the Luminex serology platform to partner laboratories for screening of wildlife, livestock and human samples to investigate the spillover of henipaviruses and filoviruses at high risk interfaces. These interfaces include farms near the forest and indigenous communities with lots of subsistence hunting in Peninsular Malaysia, and preliminary screening has found serological evidence of past exposure to viruses antigenically-related to Nipah and Ebola in humans, bats and ~~non-human primates (~~NHPs). In Thailand, Co-Is Broder, Laing and Wacharapluesadee have transferred a Luminex-based serology platform for detecting exposure to novel Asiatic filoviruses and known henipaviruses in wildlife and human samples, with testing underway and currently being validated in our Chulalongkorn University laboratory **(See also section 2.2.d)**.

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1.2.d. In vitro & in vivo *characterization of viral potential for human infection:* Using *Coronaviridae* as an example, we have conducted *in vitro* and *in vivo* experiments to characterize the pathogenic potential of novel SARSr-CoVs from bats. We isolated three SARSr-CoVs from bat feces: WIV1, WIV16 and Rs4874, with S protein sequences that diverged from SARS-CoV by 3-7% (*20, 59, 97*). We conducted full-length genome sequencing of 12 other novel SARSr-CoVs, some highly similar to outbreak SARS-CoV in the most variable genes: N-terminal domain and receptor binding domain (RBD) of the S gene, ORF8 and ORF3 (*59*). Using our reverse genetics system, we constructed chimeric viruses with SARSr-CoV WIV1 backbone and the S gene of two different variants. All 3 SARSr-CoV isolates and the two chimeric viruses replicated efficiently in Vero E6 cells and in HeLa cells expressing hACE2, but not in those without ACE2 (*20, 59, 97*). We used the SARS-CoV reverse genetics system (*71*) to generate a chimeric virus with a mouse-adapted SARS-CoV backbone expressing SHC014 S protein with 10% sequence divergence from SARS-CoV S. This chimera replicated in primary human airway epithelium, using the human ACE2 receptor to enter into cells (*60*). Thus, **SARSr-CoVs with diverse variants of SL-CoV S protein without deletions in their RBD can use human ACE2 as receptor for cell entry.** The Baric lab has a well-established hACE2 and DPP4 transgenic mouse model that we will use this to characterize the capacity of specific SARSr-CoVs to infect (*85, 98*). We used this chimera approach to demonstrate that pathogenicity of bat SARSr-CoVs in humanized mice differs with divergent S proteins, **confirming the value of this model in assessing novel SARSr-CoV pathogenicity**. Infection of rWIV1-SHC014S caused mild SARS-like clinical signs in the transgenic hACE2 mouse model **that weren't reduced by immune-therapeutic ~~monoclonals~~ antibodies that attenuate SARS-CoV pathogenicity, nor**

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after challenge following vaccination against SARS-CoV (*60*). We repeated this virus characterization approach with chimeras using HKU3r-CoV S proteins that are ~25% divergent from SARS-CoV S, **and found that they are unable to use the ACE2 receptor**. Additionally, we were unable to culture HKU3r-CoVs in Vero E6 cells, or human cell lines.

A similar approach to the above pipeline for CoVs will be applied to novel henipaviruses and filoviruses we discover ~~during our research~~. The broad mammalian tropism of HeV~~ndra virus~~ and NiV~~pah virus~~ (*67*) is likely mediated by their usage of highly conserved ephrin ligands for cell entry (*15, 99*). A third isolated henipavirus, Cedar virus (CedV), does not cause pathogenesis in animal models. Recently, **Co-Is Broder and Laing** have developed a reverse genetics system and rescued a recombinant CedV to test ephrin receptor usage/tropism and pathogenicity during henipavirus infections (*76*). CedV is unable to use the Ephrin-B3 receptor (*76, 100*) which is found in the spinal cord and may underlie NiV encephalitis (*101*) and that pathogenesis also involves virulence factors V and W proteins. The putative henipaviruses, Ghana virus and Mojiang virus, predict V and W protein expression, with GhV able to bind to ephrin-B2, but not ~~-B2~~ B3 (*102*), and the receptor for MoJV remaining unknown (*103*) but is likely ephrin-B2 (*104*). Primary human lung endothelial cells are highly susceptible to ~~Ebolavirus~~ EBOV infection (*105*). Huh7 cells are good candidates for ~~ebola~~ EBOV infection in the liver, oftentimes used to isolate virus from clinical samples (*106*) Huh7 cells also offer advantages for reverse genetic recovery of novel filoviruses (*107*). The three filovirus genera, *Ebolavirus*, *Marburgvirus* and *Cuevavirus*, use Niemann–Pick type C1 (NPC1) protein as cell entry receptor. **Co-Is Wang and Anderson** used cells originating from various mammalian species to determine spillover and/or zoonotic potential of MLAV, a newly discovered filovirus (REF). Despite the low amino acid sequence identity (22–39%) of the glycoprotein with other filoviruses, MLAV is capable of using NPC1 as entry receptor. Cells from humans, monkeys, dogs, hamsters and bats were able to be infected with a MLAV pseudovirus, implying a broad species cell tropism with a high risk of interspecies spillover transmission. ~~Thus far, paramyxovirus attachment glycoproteins have been divided into three major groups: hemagglutinin (H), hemagglutinin-neuraminidase (HN), and attachment glycoproteins (G).~~

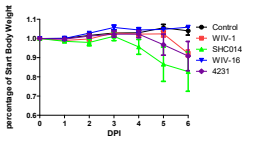

Fig. 7: Body weight change in hACE2 transgenic mice after SARSr-CoV infection, demonstrating that bat-origin SARSr-CoVs cause similar clinical presentation to outbreak strain (4231). From (*60, 61*).

Models of Outbred Populations. For novel viruses across all families, we will use the UNC collaborative cross mouse that captures 90% of the diversity within all mouse groups to assess the most suitable model for further experiments (*108*). We have used this model for CoV, filovirus ~~(Ebola)~~, f~~F~~lavirus~~es~~, and alphavirus~~es~~ infections. In subpopulations it reproduces SARS weight loss, hemorrhage in EBOV, enceph in WNV, acute or chronic arthiritis in Chikungunya infection (*86-88, 109-112*). *Bat Models.* Duke-NUS has developed two models for bat *in vivo* studies: a colony of *Eonycteris spelaea* (cave dwelling small fruit bat) and a bat mouse model, which contains the bat immune system in an immunodeficient mouse (*113*).

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1.3. General Approach: We will conduct analyses to geographically- and taxonomically-target testing of samples from wild mammals that are most likely to harbor known high-profile zoonotic pathogens, or close relatives with potential to infect people. This includes selecting from tens of thousands of samples collected on recent projects and stored in freezers in our laboratories, and new sampling of wildlife in high risk locales. We will use viral family level PCR screening to identify and partially characterize viruses, then follow up sequencing of relevant receptor binding proteins to assess homology and predict binding affinity, then attempt to isolate and biologically characterize viruses that phylogenetic and other data suggest have high spillover and pandemic potential. We will follow this with *in vitro* binding assays and cell culture experiments, and *in vivo* animal model infections to assess their potential to infect people and cause disease **(Fig. 8)**. EHA will lead the study design, targeted sampling, and data analysis; Co-Is Hughes and Wacharapluesadee will lead field sampling and in-country testing; Duke-NUS, UNC, USUS will lead the serological and PCR diagnostic development and the *in vitro* and *in vivo* analyses, as well as cross-training and tech transfer to the hub labs in Thailand and Malaysia. This will include via support of one Malaysian Ph.D student, Mei-Ho Lee (CM Ltd.) who has worked with the EID-SEARCH collaborators for 9 years. The focus of her study will be infection potential of novel CoVs and paramyxoviruses already found in bats in Malaysia under preliminary data for this proposal.

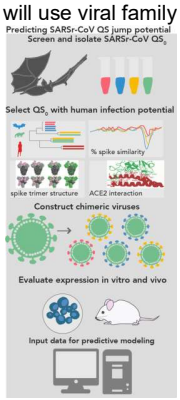

Fig. 8: Schematic showing EID-SEARCH approach to selection of sampling, viral testing, prioritization and characterization to identify novel, high zoonotic-risk viruses in wildlife.

Commented [PD27]: Hongying – this is a placeholder from DARPA proposal. We need a new one with Stages: 1) Geographic and taxonomic modeling to maximize discovery of potential zoonotic viruses; 2) PCR screening and partial RNA sequencing; 3) Full genome or RBD and Glycoprotien sequencing to model receptor binding based on sequence similarity; 4) Cell line infections (for prioritized viruses); 5) animal model infections (for prioritized); 6) High priority viruses ranked based on data (red, yellow, green) schematic.

1.4 Wildlife samples: 1.4.a. Geographic and taxonomic targeting for newly collected wildlife samples: We will prioritize sites within each country that rank highest for both the risk of zoonotic disease emergence (*114*) and the predicted number of 'missing' zoonotic viruses (*91*). Our preliminary analysis **(Fig. 1)** suggests priority areas include: the Isthmus of Kra (S. Thailand and N. Peninsular Malaysia), NW Thailand (near the Myanmar-Laos border), and the lowland rainforests of Sarawak and Sabah. In each of these 'hottest of the hotspots' regions, we will identify 2-4 distinct sampling sites based on the geographic distribution of key hosts we prioritize for intensive wildlife sampling. We will priority rank bat, rodent, and primate species using analysis of host trait data for the highest predicted number of viruses based (*91*). We will also identify species predicted to be centers of evolutionary diversity for Corona-, Paramyxo-, and Filoviridae using ancestral host reconstruction of available sequence data and Bayesian discrete phylogeographic models (*115-117*). We will ensure wildlife sampling is complimentary to specimens archived in our extensive freezer banks. We will collect only the additional specimens projected from each prioritized taxa to achieve statistically robust sample sizes given predicted levels of viral richness and rates of discovery **(see 1.4.b)**. We will leverage our strong collaborations with wildlife experts in Malaysia and Thailand to ground-truth geographic and taxonomic model outputs with local expert knowledge to ensure the sampling plan is realistic based on current species distributions, population size estimates, seasonality, and accessibility to sites.

Recognizing that most zoonotic viruses are multi-host, i.e. naturally harbored by multiple wildlife species, and that the phylogenetic relatedness of those hosts is predictable and varies across viral families (*91*) – we will also apply a generalizable phylogenetic and spatial modeling approach to rapidly predict new (unsampled) hosts for novel viruses we discover during our research. We will use a combined network analysis and a phylogeographic model to estimate a viruses' host range and to prioritize additional wildlife species for sampling and testing for viruses we discover. Our recently developed model of viral sharing uses just two phylogeographic traits (wildlife species range overlap and phylogenetic similarity between host species) to successfully predict host species in the top 2% of all 4,200 possible mammal species (*118*).

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1.4.b. Sample size justifications for testing new and archived wildlife specimens: We will use our preliminary data on average PCR prevalence from screening tens of thousands of bat, rodent and primate specimens for CoV and Paramyxoviruses under the USAID-PREDICT project in Malaysia and Thailand. We will refine these sample size targets, in real-time, over the course of our project using viral discovery curve analyses **(Fig. 6)** to either scale-up or scale-back sampling and testing of archived specimens for each species depending on their viral diversity. For example, given 5-12% prevalence of CoVs in the most common bat species we previously-sampled in Thailand and Malaysia, a target of XXX individuals per species would yield XX (± x) PCR positive individual bats, and an estimated ~xx novel strains. Similarly, for paramyxoviruses with an average PCR detection rate of X%, we would need XX individuals per species to detect 80% of predicted viral strain diversity with 80% power. Preliminary analysis indicates we will require XX,000 samples to identify 80% of remaining viral diversity **(Fig. 9)**. All wildlife sampling will be nondestructive. In the event that an animal is found dead or needs to be euthanized due to injury we will collect biopsy samples for screening.

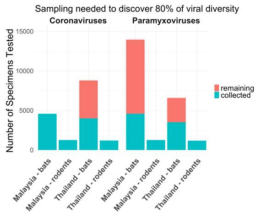

Fig. 9. Stacked bar plot showing number of currently-archived specimens **(blue)** and new specimens required **(orange)** to discover 80% of predicted number of CoVs and PMVs from high-risk bat and rodent taxa in Malaysia and Thailand

We estimate the following sampling effort, broken down for each region: *Peninsular Malaysia:* Wildlife sampling will be led by CM Ltd / EHA in collaboration with PERHILITAN. Archive bat, rodent and NHP samples will be screened serologically using the BioPlex and other serology techniques identified through this project. New wildlife sampling will focus on bats but it is expected that rodents and NHPs will be sample as well. Wildlife sampling will be conducted around Orang Asli communities sampled as part of aim 2. Three new communities will be identified in the districts we have already sampled in. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife mainly bats will be sampled. Wildlife and domestic animal samples will include serum, whole blood, throat swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and tested at PERHILITAN's NWFL. *Sarawak, Malaysia:* Wildlife sampling will be led by CM Ltd / EHA in collaboration with UniMAS. Wildlife sampling will be conducted around Dyak communities sampled as part of aim 2. One community will be identified. Concurrent sampling trip will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife mainly bats will be sampled. Wildlife and domestic animal samples will include serum, whole blood, throat swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and tested at UniMAS or BMHRC. *Sabah, Malaysia:* Bat sampling will be led by CM Ltd / EHA in collaboration with SWD and WHU. Bat sampling will initially focus on 3 caves. Batu Supu Sabah low disturbance cave and surrounding area, Madai medium disturbance cave and surrounding area, Gomantong cave high disturbance cave and surrounding area. Each cave will be sampled twice once in wet season and once in dry season targeting 500 bats at each cave on each trip for a total of 3000 bats. Wildlife samples will include serum, whole blood, throat swabs, urine sample of urogenital swab and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored at WHGFL, molecular testing will be conducted at WHGFL and serology screening at BMHRC. In addition at Madi cave the community that lives in the mouth of the cave will also be sampled targeting 150 people, while at Gomantong cave 25 workers involved in the bird next farming will be enrolled into the study. *Thailand:* XXXXXXXXX

1.4.c. Sample testing, viral isolation: Viral RNA will be extracted from bat fecal pellets/anal swabs with High Pure Viral RNA Kit (Roche). RNA will be aliquoted, and stored at -80C. PCR will be performed with pan-coronavirus, filovirus and paramyxovirus primers. PCR products will be gel purified and sequenced. We will attempt isolation on samples that contain viruses of interest (determined in **1.4, below**), using Vero cells and primary cell culture from the wildlife taxonomic order (bat, rodent and primate cell lines). This includes the over 30 bat cell lines maintained at Duke-NUS from four different bat species.

1.4.d. Moving beyond RNA viruses: To detect pathogens from other families, such as non-RNA viruses we will combine conserved PCR assays with NGS. We will perform either unbiased NGS, or use a pan-viral

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Commented [PD37]: Linfa – can you give a v. brief description of these please, e.g.: "This includes 13 primary lung, 5 primary gut epithelial, and 1 primary liver cell line. In addition we have 12 immortalized cell lines, 7 of which are intestinal, and therefore suitable for CoVs. Cell lines are derived from insectivorous and frugivorous bats (genera……)"

enrichment library to sequence full-length genomes. We will conduct this work via EID-CC research projects or as part of outbreak detection/investigation in the region from DNA viruses or other agents.

1.4.e. Sequencing Spike Glycoproteins: Based on earlier studies, our working hypothesis is that zoonotic coronaviruses, filoviruses and henipaviruses encode receptor binding glycoproteins that elicit efficient infection of primary human cells (e.g., lung, liver, etc.) (*12, 59, 60*). Characterization of these suggests SARSr-CoVs that are up to 10%, but not 25% different in the spike glycoprotein use human and bat ACE2 receptors for docking and entry. Uneven sampling gaps (e.g., no strains were found with 10-25% spike variation) prevent a thorough understanding of the transition point where the most divergent strains lose human ACE2 receptor usage. Our viral discovery curves **(Fig. 4)** suggest further sampling will reveal a rich diversity of as-yet-undiscovered SARSr-CoVs. In this aim we will use phylogenetic and viral discovery analysis to specifically target bat species and regions that are undersampled to allow sufficient power to identify and characterize missing SARSr-CoV strain diversity. Our previous work also suggests that SARSr-CoVs with S proteins that are ~10% divergent from SARS-CoV resist neutralization by therapeutic mAbs and escape SARS-CoV vaccines (*20, 60, 119*), suggesting that existing vaccines and immunotherapeutics could easily fail against future emerging SARS-like CoV. Moreover, viruses with 10-25% divergence in S proteins may bind to human cell receptors, but completely evade therapeutic and vaccine effects, and could therefore be a higher risk for public health. We will sequence the S proteins of novel SARSr-CoVs to prioritize viruses for experimental work in Aim 3 to test this hypothesis. Likewise, we will test the hypothesis that phylogenetic related zoonotic receptor binding protein genes of MERS-related, Ebola-related and Nipah/Hendra-related viruses will also program efficient entry via human ortholog receptors. For all novel virus strains, we will sequence the complete entry spike genes by amplifying overlapping fragments using degenerate primers as shown previously (*20, 59*). Full-length genomes of selected CoV strains (representative across subclades) will be sequenced using NEBNext Ultra II DNA Library Prep Kit for Illumina and sequenced on a MiSeq sequencer. PCR or MinIon sequencing will be performed to fill gaps in the genome. The full length spike gene sequences, including the amount of variation in the receptor binding residues that bind the appropriate human receptor will be used to select strains for Aim 3 experiments.

1.5. Assessing risk for spillover. 1.5.a. Capacity to infect human cells: We will use a series of cell cultures to assess the capacity of target viruses to enter human cells. For CoVs, filoviruses and henipaviruses, we will use primary human ciliated airway epithelial cells (HAE) and lung endothelial cell cultures from the lungs of transplant recipients represent highly differentiated human airway epithelium containing ciliated and non-ciliated epithelial and goblet cells, grown on an air-liquid interface for several weeks prior to use (*60, 61, 120*). We will prepare HAE and lung endothelial cultures from three different patient codes in triplicate in collaboration with the tissue procurement facility at the Cystic Fibrosis Center at UNC. Cultures will be inoculated with chimeric bat SARSr-CoVs or other test filoviruses or henipaviruses to assess efficient replication. At 72 hpi, cultures will be fixed for immunofluorescent staining using antisera to the SARS-CoV conserved nucleocapsid protein (N) or to structural genes of the other viruses (*62, 70*). SARSr-CoVs that differ significantly in S protein sequence (11-24%) from epidemic SARS-CoV yet replicate *in vitro*, will also be evaluated for sensitivity to neutralization in Vero cells by PRNT50 assays using broadly SARS-CoV cross reactive human mAbs S227.14, S230.15, S215.17, and S109.8 (*61, 121*) or monoclonal antibodies targeting the Ebola or Marburg E glycoprotein (Available through BEI), or Nipah or Hendra viruses (*122*). As controls or if antisera is not available, the S genes of novel SARSr-CoV or spike entry genes of other novel viruses under investigation will be inserted into VEE 3526 replicon vectors (VRP3526-S), packaged and used to vaccinate mice (*123*). Polyclonal sera against SARS-related viruses will be harvested and tested for ability to cross neutralize SARS-CoV, GD03, WIV-1, SHC014, WIV-16, other novel SARSr-CoV and HKU3-SRBD by PRNT50 assay (*61, 124, 125*). Using PRNT50 titers with sera (n=4 each) among these viruses, antigenic cartography (*126*) will allow comparison of antigenic vs. phylogenetic distance, identifying the transition at which SARSr-CoV strains escape SARS-CoV based vaccines, informing immunotherapeutic and vaccine design strategies (*127-129*). Similar approaches will be applied to novel MERS-related viruses, other CoV, filoviruses or henipaviruses. While most wildlife-origin viruses with unknown transmission potential or pathogenicity in people are BSL-2 or -3, cell culture for potentially BSL-4 pathogens will be undertaken at collaborating institute NEIDL. To facilitate this, duplicate samples in culture medium from animals PCR +ve for filoviruses will be

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shipped to NEIDL **(See letter of support)** for culture, characterization and sharing with other approved agencies including NIH RML. When appropriate of feasible, training opportunities at NEIDL for in-country staff will be given.

1.5.b. Host ACE2, DPPR, Niemann-Pick C1 (NPC1) and EphrinB2/EphrinB3 receptors: We will sequence host ACE2 receptors of different bat species or different bat populations from a single species (e.g. *Rhinolophus sinicus*) to identify relative importance of different hosts of high risk SARSr-CoVs and the *intra*specific scale of host-CoV coevolution. Of particular interest is homology across 18 bat and human orthologue ACE2 contact interface residues that engage the SARS RBD as a potential indicator of SARSr-CoV cross species transmission potential and growth in human cells (*69*). A lot of variation in the NPC1 receptor alters EBOV ability to infect in humans and bats, so this can be structurally mapped for prediction and downselection of strains (*130*). Signatures of positive selection in bat NPC1 were concentrated at the virus-receptor interface, with the strongest signal at the same residue that controls EBOV infection in *Eidolon helvum* cells. Thus NPC1 is a genetic determinant of filovirus susceptibility in bats, and some NPC1 variations likelyreflect host adaptations to reduce filovirus replication and virulence (*131*). Nipah and Hendra use ephrin B2 or ephrin B3 as a receptor, and variation affects its ability to use human vs bat receptor molecules (*102, 132*).

1.5.c. Host-virus evolution and distribution: We will use Bayesian and Maximum Likelihood phylogenetic analyses of RdRp (or L genes), receptor binding glycoproteins, and full genome sequence (when available) data to reconstruct the evolutionary history of the novel bat SARSr-CoVs, bat MERSr-CoV, filoviruses and henipaviruses that we identify. We will rerun MCC analyses **(Fig. 3)** to reconstruct β-CoV evolutionary origins using our expanded dataset. Ecological niche models will be used to predict spatial distribution of PCR-positive species, identify target sites for additional bat sampling, and analyze overlap with people. We will use our viral discovery/accumulation curve approach **(Fig. 4)** to monitor progress towards discovery of >80% of predicted diversity of SARSr-CoVs (lineage 1 & 2) or other virus families within species and sites, halting sampling when this target is reached, and freeing up resources for work other work (*96, 133*).

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1.5.d. Viral strain prioritization: Of the expected 100-200 novel SARSr-CoV strains, we will down-select to prioritize for further characterization based on S genes that are: i) different from SHC014, WIV1, SARS-CoV with diversity ranges of 10-25%; ii) have virus S RBD that could use human/bat receptors; iii) have recombinant chimeric spikes indicative of gene flow between clade I and II strains; iv) have bat ACE2 receptors that might select for spike RBDs that can use human receptors for entry (15/18 conserved residues in human/bat ACE2 molecules that bind SARSs-CoV S RBD domains are likely more efficient receptors than 3/18 conserved sites). Using structural models based on the SARS S glycoprotein, the extent and location of antigenic variation will be annotated onto the structure, further resolving the locations of highly evolving and conserved structural motifs/epitopes that function in protective immunity (*121, 134-136*). For novel henipaviruses, we will examine the receptor-binding proteins (e.g. envelope attachment (G) proteins) for sequence and structural homology with particular attention to conversation of amino acids that mediate ephrin receptor binding. We will prioritize strains that are predicted to interact with ephrin-B2 and expression V/W proteins to determine whether these viruses represent zoonotic threats. Using the recombinant Cedar virus (rCedV) platform, we will be able to rescue rCedV that express heterotypic G proteins to explore receptor utilization and tissue dissemination in animal models. We will assess ability to infect human cells using a continuous lung epithelial cell line, Calu3, that our group has previously used to generate data on NiV and HeV growth curves and a variety of host expression changes after infection (*137*). We will also use primary human airway cultures to assess human infection for Henipavirus and MERS- and SARS-related CoV (*138*) (*139*) . There is some evidence for Nipah and Hendra virus replication in mouse lung, low level titers in young animals, 10 fold higher titers in aged mice (*140, 141*). Thus it is likely these models could be improved in the collaborative cross mouse resource at UNC. We have identified CC lines that are highly vulnerable to wildtype SARS and others that develop hemorrhagic disease following mouse adapted Ebola infections (*86-88*).

1.5.e. Animal models: We will use transgenic mouse models and the UNC Collaborative Cross model to assess spillover potential of viruses. For work on CoVs, we will follow the protocols in the transgenic mouse model used by the Baric lab previously. Briefly, in the BSL3 lab, n=8 10- to 20-week old hACE2 or hDPP4

transgenic mice will be intranasally inoculated with 5 x 10^4 PFU of wildtype WIV-1 or chimeric bat SARSr-CoVs with different spike proteins or MERS-CoV and MERS-related CoV, respectively, then monitored daily for weight loss, morbidity, and clinical signs of disease. Mice will be euthanized at 2, 4, and 6 dpi (endpoint of 14 dpi), organs harvested and virus quantified by NP RT-PCR. After 7 days in formalin, tissues for histopathology will be removed from the BSL3 and stained with H&E, and for immunohistochemistry using polyclonal antibodies against the N protein. We will conduct limited evaluation of existing countermeasures using therapeutic monoclonal antibodies *in vitro* and *in vivo*. Existing SARSr-CoV or MERS-CoV mAbs (*142, 143*) will be diluted in DMEM starting at 1:20, and serial 2-fold dilutions mixed with an equal volume of 50 PFU of chimeric bat SARSr-CoVs with different spike proteins, then incubated on Vero E6 cells at 37°C for 1 h, then changed to a semi-solid medium (DMEM containing 1% methylcellulose and 2% FBS). Antibody neutralization titers will be evaluated by plaque quantification at 4-5dpi. hACE2 transgenic mice will be injected with SARS-CoV mAbs, and infected with chimeric bat SARSr-CoVs. Clinical signs and morbidity will be assessed and tissue pathology examined and compared with mice without treatment of mAbs to determine the therapeutic effect on SARSr-CoV infection, and protection of SARSr-CoV by wildtype SARS-S based vaccines assessed as described (*120, 144*). We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-CoV cross neutralization, synthetically reconstruct a small subset (1-2) and evaluate the ability of nucleoside analogues to inhibit growth in HAE cultures and/or *in vivo* (*61, 120*). For the Collaborative Cross model, we will….

Finally, for more detailed pathogenesis studies in the natural reservoir, we will use the bat models (natural and transgenic mouse model) at Duke-NUS. Both animal systems can be used to test susceptibility, pathogenesis and immune responses of any newly discovered viruses.

1.6. Potential problems/alternative approaches: to sample bats in sites or provinces we select. We have a >20-year track record of successful field work in Malaysia, Thailand and >10 years in Singapore, and have worked with local authorities and communities to ensure access. We have existing permissions in place which will be renewed at the start of this project. Due to the abundance of wildlife habitats in the region, if access to a site is withdrawn, we will rapidly identify a suitable, complementary site with similar bat species composition and abundance. Finally, we have already collected tens of thousands of wildlife samples from the region and will focus on these while waiting for permission. **We may not identify novel viruses in our sample wildlife species due to seasonality of viral shedding or other factors.** We have calculated sample sizes based on a range of PCR data and are fairly conservative in our approach. However, as a back-up, we already have identified dozens of novel viruses that are near-neighbors to known high-impact agents, and will continue characterizing these should discovery efforts yield few novel viruses. The sampling regions we have selected are subtropical, and our previous data, and even published studies in temperate regions (*145*), do not suggest a strong pattern of seasonality in CoV shedding, although there are studies that suggest this occurs in henipavirus and filovirus (MARV?) shedding (REFS?). Nonetheless to account for this we will conduct sampling evenly on quarterly basis within each province.

Aim 2: Test evidence of viral spillover in high-risk communities using novel serological assays.

Commented [PD41]: Ralph – please insert a couple of sentences on how we'll use the cc mouse and what we'd expect

Commented [PD42]: Linfa/Danielle – could you put a couple of sentences in on what we might do with the models. Remember – this could be an additional short project from the EIDCC extra funding.

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2.1 Rationale/Innovation: Rapid response requires early detection. Thus, assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities that interface frequently with wildlife that harbor high risk emerging viral strains. To enhance the low statistical probability of identifying these rare events, we will target rural human communities inhabiting regions of high wildlife biodiversity, that also engage in practices that increase the risk of spillover (e.g. hunting and butchering wildlife). In Aim 2 we will build and expand on our preliminary biological sampling and enroll large cross-sectional samples in human populations with high behavioral risk of exposure to wildlife-origin viruses and that live close to sites identified in Aim 1 as having high viral diversity in wildlife. We will initially identify 2 sites in each of Thailand, Peninsular Malaysia, Sarawak, and Sabah for repeated biological sampling. We will design and deploy behavioral risk factor questionnaires, and specific and sensitive serological assays to identify the baseline frequency, and causes of, spillover of known and related novel viral pathogens in these populations. In participants where symptoms are reported in the last 10 days, paired serum specimens (acute and 21 days after fever) will be collected to determine titer of positive antibody. Samples will be marked for additional PCR-based assays to identify presence of known and novel CoVs, henipaviruses and filoviruses, and for isolation and biological characterization of potential pathogens, using the approach laid out in Aim 1.

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Country, site	Lead	# enrolled, focus	Findings
Peninsular Malaysia	Hughes	1,390 Orang Asli indigenous population, PCR/serol.	25+ novel CoVs, influenza, Nipah ab+ve, filovirus ab+ve
Malaysia Sabah	Kamruddin	1,283 serology	40% JE, 5% ZIKV ab+ve
Malaysia Sabah	William	10,800 zoonotic malaria	High burden of macaque-origin malaria
Malaysia Sabah	Hughes	150 bat cave workers	Ongoing
Malaysia Sarawak	Tan	500 Bidayuh and Iban people	8% PCR prevalence HPV in women
Malaysia PREDICT	Hughes	9,933 high zoonotic-risk communities, viral PCR, serology	Ongoing. Multiple known and novel CoVs, paramyxoviruses, others.
Thailand	Wacharaplu-esadee	1,000 bat guano harvesters/villagers	Novel HKU1-CoV assoc. clinical findings
Thailand PREDICT	Wacharaplu-esadee	9,269 high zoonotic-risk communities, viral PCR, serology	Ongoing. Multiple known and novel CoVs, paramyxoviruses, others.
Singapore	Wang	856, Melaka virus	7-11% ab+ve

2.2 Preliminary data on high-zoonotic risk human community surveillance: 2.2.a Biological sample collection in SE Asia: Our longterm collaboration in the region has included qualitative and quantitative survey data, and collecting large archives of banked human sera and other biological samples from high zoonotic-risk populations. For example, under the USAID-PREDICT project, EHA has collected 9,933 and 9,269 specimens in Malaysia and Thailand respectively from people living in zoonotic disease interfaces, all of which will be available for retrospective serological testing by EID-SEARCH. Our core group has collected and tested many thousand additional specimens from other important community cohorts **(Table 2)**, and some of these will continue under EID-SEARCH **(See section 2.4)**. Of particular interest is the access to indigenous communities in Peninsular Malaysia (Orang Asli) and Sarawak (Bidayuh, Iban people), and the bat guano harvesters in Thailand. These communities hunt, butcher and eat wildlife daily, and work in supremely high-risk zoonotic interfaces (e.g. bat caves). One of our indigenous community sites is experiencing an ongoing outbreak of unknown etiology that we are investigating currently **(See section 3.2.a)**.

Table 2: Current or recent biological sample collection from healthy populations conducted by members of our consortium in EID-SEARCH hub countries

2.2.b Human Contact Risk Factors: EHA is the lead organization across all 27 USAID-PREDICT project countries for collecting data on, and analyzing the human aspects of, zoonotic spillover risk (*146, 147*). We conducted exploratory studies using standardized one-on-one semi-structured interviews and site observation data with people living in hotspot areas or engaged in high-zoonotic spillover risk activities, using these data to assess local social and cultural norms underlying wildlife contact (*147*). We then developed a risk factor survey to accompany human biological sampling to evaluate the type and frequency of animal contact, wildlife

exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. We conducted cross-sectional studies in high risk populations in 27 countries, and study participants provided biological samples (oral/oropharyngeal swab, nasal/nasopharyngeal swab, urine sample/urogenital swab, fecal sample/rectal swab, whole blood, serum, plasma). For example, survey and biological samples were collected from: 1,585 participants from 7 sites in Yunnan, Guangxi, and Guangdong provinces, China; 1,400 participants from 4 sites in Malaysia; and 678 participants from 4 sites in Thailand. Samples were tested with novel serological assays and results analyzed against risk factors from survey data (*12*). In China, CoV-seropositive individuals were mostly farmers/peasants (8/9), living in Yunnan province (7/9), 41-60 yrs old (7/9), with domestic contact with rodents (6/9), that are male (6/9). Although these results are preliminary and don't provide detailed information on routes of exposure, they identify key subpopulations to target that would likely increase the opportunity for identifying evidence of spillover for other CoVs, paramyxoviruses and filoviruses. Data from PREDICT surveys will be used to help identify target populations in Aim 2, identify strategies to better target at-risk people, and conduct focused **human animal contact surveys and serosurveys to produce statistically significant findings for henipaviruses, CoVs and filoviruses.** In Aim 2, we will use combined biological sampling and risk factor surveillance in targeted populations within the community to 1) identify risk factors correlated with seropositivity to henipaviruses, filoviruses and CoVs; 2) assess possible health effects of infection in people; and 3) where recent illness is reported, obtain clinical samples for PCR testing and potential viral isolation and characterization. Obtaining this information could be a significant step in understanding the likelihood of recent 'hidden' spillover events and their public health impacts, as well as the risk of future emergence of viral disease.

2.2.c. Risk factors for illness: Our preliminary data from PREDICT in Thailand, Malaysia and China revealed that frequent contact with wild (e.g. bats, rodents, non-human primates) and domestic animals (e.g. poultry, swine) among local communities was associated with a high percentage of self-reported severe/acute respiratory illness (SARI/ARI) and Influenza-like illness (ILI) symptoms. In addition, people involved in crop production, hunting and butchering of wildlife reported statistically significantly higher SARI and ILI symptoms (Li *et al.*, in prep.). **These preliminary data suggest a target population and sites for current proposal with the correct targets for serological surveillance and risk factor surveys in Aim 2 to produce statistically significant findings**. Coupled with further refining serological tools, we will be able to identify the likely routes of exposure to known or novel coronaviruses, henipaviruses, and filoviruses in the study region, as well as build data on the illnesses they cause in people.

2.2.d. Serological platform for community surveillance: One of the present challenges in emerging disease surveillance is that viremia can be short-lived, complicating viral-RNA detection (i.e. traditional PCR-based approaches), especially in zoonotic reservoir hosts, and requiring large samples sizes to understand infection patterns or host range. By contrast, antibodies are typically long-lived and can be used to assess prior virus exposure or infection with much smaller samples sizes (*148*). However, most serological assays only target a single protein, and it's not always clear which antigen is the most immunodominant during an adaptive humoral immune response – i.e. which is the best target for a serological assay. Henipaviruses, filovirus and CoVs express envelope receptor-binding proteins (e.g. CoV=spike (S) protein, filoviruses/henipaviruses=glycoprotein (GP/G)), which are the target of protective antibody responses. The Broder lab at USU, a leader in the expression and purification of native-like virus surface proteins for immunoassay application (*149*)?, developing monoclonal antibodies (*150, 151*) and as subunit vaccines (*152, 153*), is presently developing a novel, multiplex microsphere immunoassay (MMIA) allowing serum samples to be simultaneously tested for antibodies reactive with henipaviruses, filoviruses and coronaviruses. This MMIA uses a Luminex-based multiplex platform and custom designed virus receptor-binding proteins expressed in a mammalian cell-culture system and purified as native-like oligomeric proteins (*149*). These oligomeric antigens retain post-translational modifications and quaternary structures. This structural advantage over peptide antigens, allows the capture of conformational-dependent antibodies raised during a natural infection, which represent the majority of the protective humoral response.

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Serological platforms have been historically limited by the starting antigen, and cross-reactivity between known and unknown related viruses not included in the assay. The known diversity of henipaviruses includes five presently described viruses, NiV, HeV, CedV, GhV and MojV and this assay has been expanded to include all described species **(Fig. XXX)**. In the past, our collaborators have used NiV and HeV G protein antigens in a MMIA assay to identify a serological footprint of NiV-related African henipaviruses and exposure to virus(es) antigenically-related to NiV in livestock sampled in Bangladesh (*77, 78*). To strengthen serological data interpretation, the G protein from each virus is integrated into the MMIA so that virus-specific versus henipavirus cross-reactive antibodies can be detected and address specificity of past virus exposure. Extensive cross-reactivity exists within ebolaviruses and between ebolaviruses and marburgviruses, highlighted by studies examining antibody binding between heterologous viruses (*154-156*). Ongoing work is aimed at validating the MMIA pan-filovirus assay for specificity. The majority of ebolaviruses are endemic to Africa, however the discovery of Měnglà virus in Yunnan, China increased the further demonstrated that a diversity of Asiatic filoviruses with unknown pathogenicity and zoonotic potential exists. Our past work in collaboration with Duke-NUS demonstrated that three under-sampled fruit bat species had serological evidence of past exposure to a filovirus(es) most antigenically-related to ebolaviruses (e.g. Ebola virus, Bundibugyo virus), but unreactive with Reston virus GP, suggesting that novel filoviruses circulate within bat hosts (*41*).

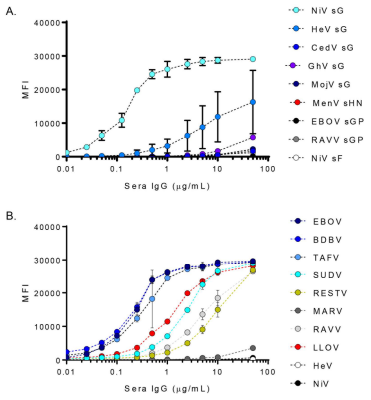

Fig. XXX: Validation of multiplex microsphere immunoassay (MMIA) specificity and identification of immunologically cross-reactive viruses for Nipah (A) and Ebola (B).

2.3 General Approach/Innovation: We will use a dual study design to gain in depth understanding of exposure and risk factors for viral spillover **(Fig. XX)**. In Aim 2 we will conduct community-based surveillance using a brief, focused questionnaire to identify the prevalence and frequency of risk-factors for viral spillover in tandem with collection of high quality biological samples to determine the seroprevalence of spillover viruses in at-risk human populations. We will use generalized linear models to analyze correlation between biological results and risk-factors for viral spillover. In Aim 3, we will conduct clinic-based syndromic surveillance and biological sampling at clinics/hospitals that are the catchment healthcare facilities for the communities sampled in Aim 2. Both community-based and clinic-based syndromic surveillance programs are cross sectional case-control studies designed with the sample sizes necessary to statistically quantify (with a power of 80%) risk factors and health impacts for viral spillover, linked to serological status and PCR prevalence in symptomatic patients.

2.4 Target population & sample sizes: We will target populations in the same geographic regions as those identified in Aim 1 that harbor wildlife with high potential zoonotic viral diversity, that are EID hotspots, and that are well-connected through regional travel and trade hubs to the global travel network **(Fig X)**. We will target specific communities based on our analysis of previously-collected PREDICT behavioral questionnaire data, to assess key at-risk populations and occupations with high exposure to wildlife, as well as using other publicly available data (e.g. wildlife hunting licenses sold in a region, number of guano caves accessed for fertilizer etc.). We will identify populations at two sites in each of the following regions, building on our preliminary data **(Table 2)**: *Thailand (Co-I Wacharapluesadee):* We will continue surveillance in bat guano harvesters and their villages in Ratchaburi province where we have identified MERS-related CoV in bat guano and rectal swabs (*48, 157*) and serological evidence of α-CoVs, HKU1-CoV in people (Wacharapluesadee in prep.). Our second site will be in Chonburi province where we have found Nipah virus (99% identity of whole genome sequence to NiV from a Bangladesh patient) in flying foxes but no outbreaks have yet been reported (REF), and have found several novel paramyxoviruses and CoVs have been found in bat feces roosting there (Wacharapluesadee in prep.).

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Commented [PD51]: Supaporn/Kevin/ -Need sample sizes for both populations and power calculations for serology for a typical virus from one of the three families we're targeting

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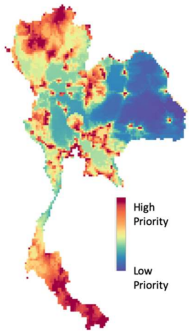

Fig. X: . Geographic analysis to target high-risk sites for human zoonotic disease surveillance in Thailand. Our spatial analysis from Aim 1 will be extended to optimize sites for human community surveillance in Thailand and Malaysia by ranking areas with high numbers of expected viruses in wildlife, greater spillover probability based on EID risk factors, and higher levels of connectivity (measured by road density data) that may facilitate person-person spread.

Peninsular Malaysia (Co-I Hughes, CM Ltd.): We will expand our sampling of the Orang Asli indigenous communities to include additional communities in the 3 Districts we have already sampled, and additional Districts in the States of Kelantan Perak, Pahang, and Kelantan. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife (mainly bats) will be sampled. Human samples will include serum, whole blood, throat swabs, nasal swabs, urine, and fecal sample or rectal swab. Samples will be collect in duplicate in VTM and Trizol. Samples will be stored and tested at NPHL. Participants who hunt, butcher or consume wildlife, or who rear animals will be considered suitable for enrolment. Participants will complete a consent form and questionnaire in addition to having samples collected. During these community trips the field team will conduct community meetings to help inform the Orang Asli about the public health risk of zoonoses. *Sarawak (Co-I Tan, UNIMAS):* We will expand our sampling of the Bidayuh and Iban indigenous people to include the Dayak and Orang Ulu ("people of the interior") because this group has a more intimate connection to remote wildlife-rich areas than the other two, albeit all are hunters. Human sampling will be led by UniMAS in collaboration with CM Ltd / EHA. Concurrent sampling trips will be conducted during which 150 members of the community, 30 hunting dogs or other domestic animals and 60 wildlife (mainly bats) will be sampled, following the same protocols as for Peninsular Malaysia. *Sabah:* We will conduct sampling at the Madai bat and bird cave that has 200 permanent residents who harvest bird's nests for soup, and >1,000 during the harvesting season and at Gomantong cave – one of the largest bat and cave swiftlet nesting sites in the world. Protocols follow those for Peninsular Malaysia. *Singapore:* Active human surveillance will be not be conducted in Singapore, but archived human specimens e.g. from our Melaka virus serosurvey (*4*), will be made available to conduct retrospective serosurveys for specific pathogens that are identified as high interest to the EIDRC, NIH, and EID-CC.

Sample sizes: From our previous work we anticipate that 10-30% of the community population will have had exposure to wildlife allowing us to capture highly exposed and non-exposure individuals at each site. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock farming, transportation, selling, or slaughtering wildlife in the surveyed areas will be targeted so that they make up ≥30% of the sampled population in each community. We will stratify sampling to ensure appropriate representation of sex, demographic, and socio-economic factors at each community site. Estimating 5% overall seroprevalence in these high-exposure populations, these sample sizes are sufficient to estimate effect sizes of risk factors of 2X or greater with 80% power. For community-based surveillance, we will enroll xxxx individuals per county/province, pooled across two sites for each, allowing us to make county/province-level comparisons of differing effects.

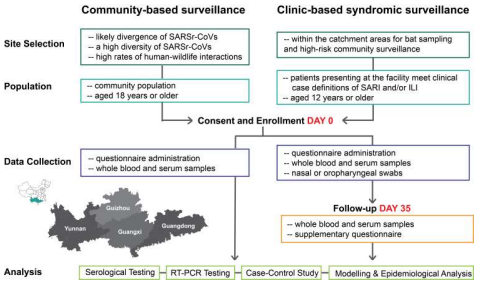

Fig. XX: Human survey and sampling study design overview (Aim 2 and 3)

2.5 Data & sample collection: Following enrollment of eligible participants and completion of the consent procedure, biological specimens (5ml will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 2.5ml whole blood; two 500 µL serum samples) will be collected and a questionnaire will be administered by trained project staff that speak appropriate local language to all participants. Through the serological findings and the survey we will investigate the details of five risk factors related to high risk wildlife exposure, to maximize the power of the analyses, based on continuing analysis of our previous work, and will focus on: 1) occupation and occupational exposures; 2) observed or reported

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Commented [PD54]: Hongying– this figure is from our CoV R01 renewal. Please modify to make relevant to SE Asia and henipas, filos and CoVs.

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interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With additional consent considerations for participants enrolled from clinical settings we will review clinical records to collect recent data on medical history, clinical syndromes, and patient etiology.

2.6: Laboratory analysis: 2.6.a Serological testing:

In a previous R01, we expressed his-tagged nucleocapsid protein (NP) of SARSr-CoV Rp3 in *E.coli* and developed a SARSr-CoV specific ELISA for serosurveillance using the purified Rp3 NP. The specificity of this assay was evaluated using polyclonal antibodies against HKU1, OC43, 229E, NL63, MERS-CoV and EBOV and no significant cross-activity detected (*12*). **This suggests that if we can expand our serology tests to cover other bat CoVs, and that doing so may identify many more seropositive individuals.** We will therefore use two serological testing approaches. First, we will expand test all human sera collected from both community- and clinic- based sampling for a panel of bat CoVs that will include SARS-CoV (outbreak strain), a range of lineage 2 bat SARSr-CoVs (including WIV1 and SHC014), lineage 1 HKU3r-CoVs, MERS-CoV, and the α-CoVs SADS-CoV and HKU10. We previously found serological evidence of human exposure to HKU10 (*12*), and HKU10 is known to jump from one host bat species to another (*158*) so is likely to have infected people more widely. It is possible that non-neutralizing cross reactive epitopes exist that afford an accurate measure of cross reactivity between clade1 and clade 2 strains which would allow us to target exposure to strains of 15-25% divergence from SARS-CoV, at a phylogenetic distance where therapeutic or vaccine efficacy may falter. Additionally, we will test samples for antibodies to common human CoVs (HCoV NL63, OC43 – see potential pitfalls/solutions below). Secondly, CoVs have a high propensity to recombine. To serologically target 'novel' recombinant virus exposure, we will conduct 1) ELISA screening with SARSr-CoV S or S1 (n-terminal domain); 2) confirm these results by Western blot; then 3) use NP based ELISA and LIPS assays with a diversity of SARSr-CoV NP. For NP ELISA, micro-titer plates will be coated with 100 ng/well of recombinant batCoV NP and incubated with human sera in duplicates followed by detection with HRP labeled goat anti-human IgG antibodies. For confirmation, all ELISA positive samples will be subjected to Western blot at a dilution of 1:100 by using batCoV-NP as antigen. We will use an S protein-based ELISA to distinguish the lineage of SARSr-CoV. As new SARSr-CoVs are discovered, we will rapidly design specific Luciferase immunoprecipitation system (LIPS) assays targeting the S1 genes of bat-CoV strains for follow-up serological surveillance, as per our previous work (*13*). Additionally, in Thailand, as an initial project on our EIDRC, we will develop serology assays to the novel CoVs found in bats at the bat guano site using LIPS assays and viral genomic data, and will screen archived specimens from 230 participants (2 years' collection in 2017-2018, a subset of the subjects have been sampled at multiple timepoints to allow us to assess seroconversion).

2.6.b RT-PCR testing. Specimens will be screened using RT-PCR for the RdRp gene (See section 1.3.b for details). Positive samples will be subjected to full genome sequencing and RT-PCR amplification of the S glycoprotein gene. Samples from the clinic-based syndromic surveillance will also be tested using RT-PCR for Influenza A & B, HCoV NL63, OC43, HKU1, SARS-CoV & 229E as rule-outs, and SADS-CoV and HKU10 as an opportunistic survey for potential spillover these CoVs as proposed above.

2.6.c Biological characterization of viruses identified: Novel viruses identified in humans will be recovered by cultivation or from full length genome sequences using reverse genetic approaches. Viruses will be characterized for growth in primary human cell lines and in mouse models, including the Collaborative Cross Mice or humanized mice expressing human receptors from related strains. The approaches are discussed in more detail under Aim 1.

2.7 Epidemiological analysis: We will conduct a cross sectional case-control study to identify risk factors for Henipavirus, Filoviruses, and CoVs spillover. "Cases" are defined as participants whose samples tested positive for Henipavirus, Filoviruses, or CoVs by serological tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative for Henipavirus, Filoviruses, or CoVs. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between biological results, serological/PCR status, and risk factors related to high risk wildlife exposure including: 1) occupation

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and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) home or work proximity to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms lifetime and past 12mo. Additionally, we will use this procedure to determine how clinical presentation differs between Henipavirus, Filoviruses, or CoVs in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms.

2.8 Potential problems/alternative approaches: Rarity of spillover events means it may be difficult to identify sufficient seropositives to statistically analyze human risk profiles. First, we will be targeting our community-based surveillance to subpopulations with high-levels of wildlife exposure, at sites selected for diverse and prevalent viruses to increase likelihood of finding positive individuals. Second, our serology testing will include a panel of assays for a large diversity of viruses, increasing likelihood of positives. For example, in previous work our overall bat CoV PCR prevalence in the community was, 11.8%; β-CoV, 3.4%; α-CoV, 9.1%). Thus, using this broad serological panel to screen individuals prone to contact with species of higher zoonotic risk increases the potential for detecting spillover with enough power for statistical analyses, and will shed light on behaviors that predispose to spillover. Third, we will include common human CoVs, paramyxoviruses, and influenza viruses in our panel, so that even if low prevalence of wildlife viruses is found, we will be able to conduct a valuable cross-sectional study of the seroprevalence of more common human viruses. Finally, we will be able to assess relative measures of human-wildlife contact from our survey work. We will analyze intensity of contact with high-risk taxa against other risk factors to provide useful proxy information for spillover risk. **Serological testing may not match known viruses due to recombination events, particularly for CoVs.** We will use the three-tiered serological testing system outline in **2.6.a** to try to identify these 'novel' viruses, however, we will also remain flexible on interpretation of data to ensure we account for recombination.

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Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts.

3.1 Rationale/Innovation: Our previous research **(Table 1)** and other data suggests that there are substantial numbers of outbreaks or clinical cases for which the etiologic agent is not diagnosed, e.g. (*159, 160*). To investigate this in SE. Asia, we will work directly with clinics and outbreak control organizations to identify the causes of some 'cryptic' outbreaks or cases in the region. Specifically, we will expand on current syndromic surveillance activities to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and who present at regional clinics or hospitals with symptoms typical of high impact zoonotic viruses. We will collect detailed survey data to assess their contact type and frequency with wildlife and livestock and conduct molecular and serological diagnostic assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. We conduct novel viral discovery assays on patients who present with symptoms indicative of a high-risk zoonotic virus. If novel pathogens are identified by PCR, we will attempt to isolate, obtain full genome sequence and/or biologically characterize the pathogen, using the *in vitro* and *in vivo* strategy laid out in Aim 1.

3.2 Preliminary data clinical surveillance: 3.2.a. Clinical surveys and outbreak investigations: Our longterm collaboration in the region has included the following activities investigating clinical syndromes: *Peninsular Malaysia*: **At the time of writing, there is an outbreak of suspected viral undiagnosed illness in the indigenous Batek Orang Asli ("people of the forest") community living at Gua Musang ("civet cat cave") district, in Kelantan, Malaysia, close to Taman Negara – a National Park containing the oldest known rainforest**. This community is one where we have conducted prior routine surveillance and biological sampling, demonstrating proof-of-concept that our two-pronged high-risk community/clinic approach will work. Dozens of people are affected and the outbreak is currently being investigated by our collaborators at the Malaysian NPHL. The Orang Asli live in remote villages in the rainforest of Malaysia, with limited access to modern health care and extensive contact hunting, butchering, and eating wildlife as their primary source of protein. This outbreak, and others that will continue to emerge just like it, underscore the critical need for our EIDRC - to improve the technological tools, training, and human capacity for rapid EID surveillance, diagnosis, and response at the frontlines of disease emergence in SE Asia. **Investigating this outbreak be a key priority if EID-SEARCH is funded.** *Sabah*: Co-I Kamruddin (BMHRC) is leading an asymptomatic leptospirosis study on sanitation workers, an ILI study in a Kota Kinabalu clinic and diarrheal study in children under 5 yrs. Co-Is William, Tan and others have conducted clinical observational studies to determine the

etiology of central nervous system infections and acute undifferentiated febrile illness in Sabah. Co-Is Tan, William have collaborated with Co-I Wang to enroll 90 adult and over 100 pediatric participants with central nervous system infections, negative tests for malaria and dengue from 3 district hospitals in Sabah to determine the various etiologies. While a high proportion tested positive for rickettsial agents, the majority had no clear diagnosis. *Sarawak:* Dr Tan (UNIMAS) is conducting a survey of high-risk female Kelabit indigenous people and has found 8% with HPV52 as the dominant strain. He is working on clinical cohorts of hand, foot and mouth disease and will recruit patients from that study in the current proposed work. Finally, he is conducting a longterm study of rotavirus and other viral etiologies of acute gastroenteritis in Kuching, Sarawak that will include swine herders and farmers. These are an important group in Malaysia because pig farms are shunned by the government and local villagers to they are placed far away from town centers, deep in the forest, and it is this scenario that led to the emergence of Nipah virus in 1998-9 (*18, 161*). *Thailand:* The Chulalongkorn Univ. TRC-EID laboratory works in coordination with the Thailand Ministry of Health to investigate outbreak etiology, including the first case of imported MERS-CoV in Thailand, **for which we produced sequence confirmation within 24 hours from acquiring the specimen (Fig. X)**. Three imported MERS cases have been detected in Thailand since 2015, and all cases were confirmed at the TRC-EID. We worked during the Zika outbreak to identify > 500 PCR-positive Zika patients with sequence confirmation. We have also worked on returning and suspected Ebola travelers, syndromic surveillance for SARI, Encephalitis, Hand Foot and Mouth disease in children and Viral diarrhea. Working with the US CDC (Thailand), between 1998 and 2002 we enrolled a large cohort of children from Kamphaeng Phet Province to examine Dengue virus and JE epidemiology (*162, 163*). Among the 784/2,574 convalescent sera Henipavirus-specific antibodies were detected in 13 serum samples (1.7% seroprevalence), suggesting prior exposure to endemic henipaviruses or a related paramyxovirus in rural Thailand. *Singapore:* Duke-NUS has worked with the Ministry of Health to investigate Zika cases (*164*), and with EcoHealth and others, Duke-NUS has developed novel CoV assays for clinical surveillance, and for expedited analysis during outbreaks. Our pipeline of novel virus detection, reservoir host identification, serological assay development, and serological testing is as rapid as several months, with the development of novel serological assays from sequence data in 7 days (*13*). **For example, with the discovery of SADS-CoV, we identified and fully sequenced a new virus, screened archived wildlife specimens to identify the natural reservoir host species (*Rhinolophus* spp. bats), developed LIPS serological assays, and tested human and swine samples to assess seroprevelance in less than 3 months (*13*).**

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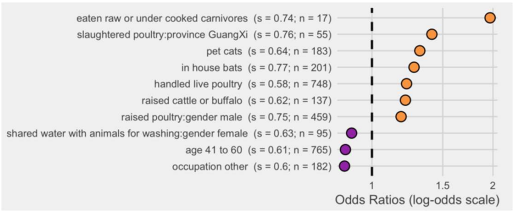
3.2.b Analysis of self-reported illness: In addition to biological outcomes we have developed a standardized approach in PREDICT for analyzing data on self-reported symptoms related to targeted illnesses; of fever with cough and shortness of breath or difficulty breathing (severe acute respiratory illness - SARI), fever with muscle aches (fevers of unknown origin (FUO) and, cough or sore throat (influenza-like illness - ILI) from study participants. We used a least absolute shrinkage and selection operator (LASSO) regression to identify associations between participants who reported ILI and/or SARI symptoms and demographics, occupation, and contact with animals in the last year. Results have clear biological relevance. In Yunnan, China, salient associated variables or combination of variables were, in descending order of odds ratio: 1) having eaten raw or undercooked carnivores; 2) having slaughtered poultry and being a resident of Guangxi province; 3) having had contact with cats or bats in the house; and 4) being a male who has raised poultry **(Fig. 11).** We will expand this approach for all syndromes in Aim 3, and use more granular targeted questions designed to assess patient's exposure to wildlife in terms that are relevant to the specific country.

Fig. 11: Associated variables of self-reported ILI and/or SARI in prior 12 months (s = bootstrap support; n = number +ve out of 1,585 respondents). **Orange circles** = odds ratios > 1 (positively associated with the outcome); **purple** = odds ratios <1 (negatively associated with the outcome).



3.3 General Approach: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent is not identified. To capture these, we will expand on our current syndromic surveillance to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and present at regional clinics with influenza-like illness, severe respiratory illness, encephalitis, and other symptoms typical of high-impact emerging viruses. We will collect survey data tailored to the region to assess contact with wildlife, conduct molecular and serological assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. If novel pathogens are identified by PCR, we will isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work. We will then use behavioral survey data, ecological and phylogeographic analyses to assess the likely reservoir hosts to inform intervention programs.

3.4 Clinical cohorts. 3.4.a Cohort selection: We will initiate active clinic-based syndromic surveillance at 2 town-level level clinics and 2 provincial-level hospitals in Thailand, Peninsular Malaysia, Sabah and Sarawak. These all serve as the catchment healthcare facility for people in our community-based surveillance of Aim 2 and are inclusive of wildlife sampling sites in Aim 1. Patients ≥ 12 years old presenting at the health facility who meet the syndromic and clinical case definitions for SARI, ILI, encephalitis, fevers of unknown origin, or hemorrhagic fever will be recruited into the study. We will enroll a total of xxxx individuals for clinical syndromic surveillance, which accounts for up to 40% loss from follow-up. Study data will be pooled across country sites, as clinical patients are limited by the number of individuals presenting at hospitals. Sites: *Thailand:* XXXXX *Peninsular Malaysia:* Focusing immediately on the current outbreak in Orang Asli, we will work closely with the Malaysian Ministry of Health to support their investigation. We will also work with 6 Orang Asli clinics which focus solely on this indigenous community. *Sarawak:* XXXXXX *Sabah:* XXXXXXXXX. *Singapore:* XXXXX

3.4.b Behavioral and clinical interviews: 2.5 Clinic enrollment and follow-up: We will recruit inpatients and outpatients to participate in the study after initial screening to determine if they meet the clinical case definition of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; 5) hemorrhagic fever of unknown etiology; or 6) severe diarrhea. Once enrolled, biological samples will be collected and a questionnaire administered by trained hospital staff that speak appropriate local language. Samples will be taken concurrently when collecting samples for normative diagnostics. For inpatients, samples will be collected no more than 10 days of reported onset of illness to increase the chance of PCR detection of viruses (*165*). We will follow up 35 days after enrollment to collect an additional two 500 µL serum samples conduct a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. 35 days gives adequate time for development of IgG, which occurs <28 days after onset of symptoms for SARS patients (*166*). These samples may prove valuable to collaborators in that the PBMC could be used in production of therapeutic antibodies.

3.4.c Sampling:

Following enrollment with signed consent form, biological specimens (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples) including two nasal or oropharyngeal swabs will be collected from all eligible participants, and a questionnaire will be administered. We will investigate five risk factors, to maximize the power of the analyses, all related to high risk wildlife exposure, based on continuing analysis of our previous work, and will include: 11) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With permission from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology.

3.5 Sample testing: PCR, Serol to link symptoms to etiologic agents

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The standard syndromic diagnostic PCR assay for the common pathogens will be conducted and the results will be shared to the hospital with 48 hours. Specimens will be further tested for 3 viral families; CoV, PmV and filovirus by consensus PCR. Appropriate specimen from dead case will be further conducted by NGS if the previous PCR tests are negative to identified cause of infection. The serology panel assay will be conducted from paired serum.

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3.5 Assessing potential for pandemic spread: We will use statistical models built from collated biological, ecological, and genetic data to predict the likelihood of human infection (or spillover) and pathogenicity for a the range of diverse viruses we will characterize. The aim is to quickly identify and assess which viruses pose the most significant risk to human health and thus prioritize additional resources to more fully characterize, better understand pathogenicity, and invest in therapeutic development for those pathogens. We will combine two independent but related statistical models to predict pathogen spillover risk and pathogenicity. First, we will expand a simple generalized additive modeling approach (*91*) to assess spillover risk probability to a new virus that is currently not known to infect people, when we have only limited genetic data from initial PCR screening and sequencing. Additional data from receptor binding protein homology and cell line infection experiments can be incorporated into the model framework as available. Second, given high likelihood to spillover and infect humans, or direct evidence of human infection from our clinical surveillance, we will then predict pathogenicity using nearest neighbor phylogenetic analyses as a first approximation and by incorporating more detailed data from genomic characterization and animal model experiments. For the pathogenicity model, human epidemiological data for ~300 viral species known to infect people (case fatality rate, global estimates of number of human cases per year or decade, R_0, infectious period, and primary symptoms) will be aggregated including from previous reviews [Woolhouse ref] to use as predictor variables. Thirdly, we will build off EHA's previous DTRA and DHS supported research to predict pandemic spread for viurses we identify by integrating surveillance site data, global flight models (*167*), as well as additional datasets (road networks, shipping routes, cell phone data) to measure human movement and connectivity across Southeast Asia.

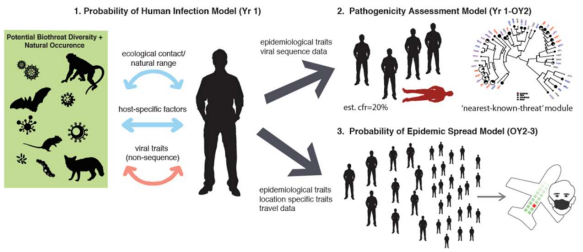

Fig. XXXXX: Conceptual framework highlighting 3 underlying models to estimate: **1)** probability of human infection, or spillover, the first necessary step to understand pathogenicity when status of human infection is unknown; **2)** pathogenicity, i.e. probability of causing clinical signs, case fatality rate estimates, and other metrics relevant to humans; and 3) probability of pathogen spread based on ecological, epidemiological, and airline connectivity and human migration data.

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3.7 Potential problems/alternative approaches: Patients visiting clinics may have cleared virus, but not yet developed IgG antibodies, reducing seropositive cases. Our 35 day follow-up sampling should avoid this because the maximum lag time between SARS infection and IgG development was ~28 days (*165*). We also expect that patients in rural communities will only visit clinics when symptoms have progressed, likely coinciding with late illness and onset of IgG. We will also have data from our community study, so won't be completely reliant on hospital data to identify PCR- or seropositives. Finally, the risk is outweighed by the potential public health importance of discovering active spillover of a new henipavirus, filovirus or CoV infection.

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Additional Instructions – Specific to the EIDRC FOA:

4. Administrative Plan

In a clearly labeled section entitled **"Administrative Plan":**

- Describe the administrative and organizational structure of the EIDCRC Administrative and Leadership Team. Include the unique features of the organizational structure that serve to facilitate accomplishment of the long range goals and objectives.

Chula TRC-EID is National reference laboratory for diagnostic and confirmation of EIDs pathogen along with the National public health laboratory at the Department of Medical Science and the WHO Collaborating Centre for Research and Training on Viral Zoonoses. We have built a EID laboratory network in-country among three Ministry including Ministry of Public Health (Department of Medical Science and Department of Disease Control), Department of Livestock and Development, and Department of National Parks, Wildlife and Plant Conservation and Universities and among SEARO countries. Our clinicians provide case consultation for Thailand and SEARO countries. The laboratory and clinic training are regularly conducted.

For Malaysia –
NPHL – Molecular and serological screening (BioPlex) of PM Orang Asli samples, serological screening of syndromic samples from Sabah (already approved) and Sarawak (would need approval)

PERHILITAN molecular zoonosis laboratory, at the National Wildlife Forensic Laboratory - Molecular and serological screening (BioPlex) of PM wildlife samples

UPM Faculty of Veterinary Medicine - Molecular and serological screening (BioPlex) of PM livestock samples

KKPHL - Molecular of syndromic samples from Sabah (already done) and Sarawak (would need approval)

SWD WHGFL - Molecular screening of Sabah wildlife samples

BMHRC - Molecular and serological screening (BioPlex) of Sabah livestock samples, serological screening (BioPlex) of Sabah wildlife samples, Molecular and serological screening of syndromic samples from Sabah (already approved) and Sarawak (would need approval). BMHRC will need renovations and BioPlex to do this work – Menzies team might have funds for BioPlex that they would base here as part of DTRA project.

- Describe how communications will be planned, implemented, and provided to collaborators, teams, or sites. In addition, describe plans to achieve synergy and interaction within the EIDRC to ensure efficient cooperation, communication and coordination.
- Specifically address how the EIDRC Team will communicate with other EIDRCs, the EIDRC CC and NIAID and how the EIDRC will implement plans and guidance from the EIDRC CC to ensure coordination and collaboration across the Emerging Infectious Disease Program as a whole. Describe how the study site will provide information, if any, back to the local health authorities and the study subjects as needed. Note any adjustments or changes needed for rapid and flexible responses to outbreaks in emerging infectious diseases.

List out all of our involvement with outbreak investigations, ministries of health surveillance etc.
If possible the clean or summarized answers from the questionnaire should be shared to the hospital Infectious Control team and Bureau of Epidemiology for their information for controlling the outbreak.

- Describe the overall management approach, including how resources will be managed, organized and prioritized, including pilot research projects.
- Highlight how the proposed staffing plan incorporates scientific and administrative expertise to fulfill the goal to develop the flexibility and capacity to respond rapidly and effectively to outbreaks in emerging

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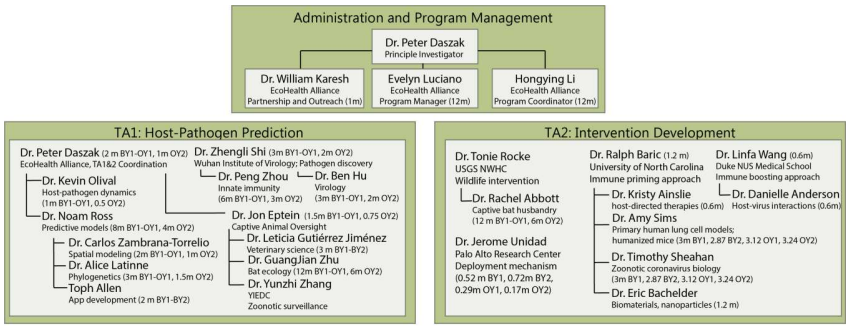
infectious diseases in an international setting.

CM/EHA helped establish and manages with SWD the Wildlife Health, Genetic and Forensic Laboratory (certified as a BSL- 2 laboratory according to the US standard for laboratory specifications) that has all the equipment necessary to store samples, run extractions, PCR and analysis on biological samples for wildlife disease surveillance. The lab is used to conduct health checks on rescued and relocated wildlife before being released into new areas or sanctuaries, to screen samples for the PREDICT and Deep Forest Project and for genetic research and forensic investigations. CM/EHA also helped establish the new molecular zoonosis laboratories (certified as a BSL- 2 laboratory according to the US standard for laboratory specifications), at DWNP's National Wildlife Forensic Laboratory. The lab is used to screen samples for the PREDICT & DTRA projects. In addition CM/EHA also has access to NPHL and KKPHL for screening human samples and the Virology lab at FVMUPM for screening Livestock samples.

The Borneo Medical and Health Research Centre (BMHRC) is a centre of excellence that conducts teaching and scientific researches on communicable diseases, outbreak investigations, and ethnomedicine. BMHRC has offices space including a meeting room and seven laboratories which including preparation lab, parasitology lab, cell culture lab, bacteriology lab, natural product lab, virology lab, and molecular biology lab. BMHRC is supported by an administration staff, medical lab technologist, and research assistants who are available to work on projects. Through this project we will help to establish a certified BSL-2 lab at the BMHRC. This lab can then be used for human testing to elevate pressure at KKPHL and QEH labs. It would also be an ideal location for a Luminex platform as human, wildlife and livestock samples could all be screened here. Co-Is Hughes and Kamruddin will be starting outbreak response training in August 2019 with a team from Sabah CDC, KKPHL and BMHRC. (Dr. Maria Suleiman now in PM But still collaborating with us, Dr. Muhammad Jikal current Director CDC working with us and Dato' Dr Cristina Rundi Director Sabah state health Dept is supportive) this collaborative group is working to develop a Sabah outbreak response team that will help support SSHD/ CDC outbreak team and also conduct sampling for disease surveillance to be based at BMHRC and we will develop this as part of this proposal. In addition the UMS hospital will come in line in 2021 providing additional opportunities for cohort studies and capacity building.

FROM DARPA grant:

Section 1.03 MANAGEMENT PLAN



Project DEFUSE lead institution is EcoHealth Alliance, New York, an international research organization focused on emerging zoonotic diseases. The PI, Dr. Daszak, has 25+ years' experience managing lab, field and modeling research projects on emerging zoonoses. Dr. Daszak will commit 2 months annually (one funded by DEFUSE, one funded by core EHA funds) to oversee and coordinate all project activities, with emphasis on modeling and fieldwork in TA1. Dr. Karesh has 40+ years' experience leading zoonotic and wildlife disease projects, and will commit 1 month annually to manage partnership activities and outreach. Dr. Epstein, with 20 years' experience working emerging bat zoonoses will coordinate animal trials across partners. Drs. Olival and Ross will manage modeling approaches for this project. Support staff includes

Commented [KJO72]: Figure like this would be good, but instead of breaking down by TA, maybe by country or Specific Aim. Per FOA, Need to also show how we'll link in with the Coordinating Center and other EIDRCs.

a field surveillance team, modeling analysts, developers, data managers, and field consultants in Yunnan Province, China. The EHA team has worked extensively with other collaborators: Prof. Wang (15+ yrs); Dr. Shi (15+ yrs); Prof. Baric (5+ yrs) and Dr. Rocke (15+ yrs).

Subcontracts: #1 to Prof. Baric, UNC, to oversee reverse engineering of SARSr-CoVs, BSL-3 humanized mouse experimental infections, design and testing of targeted immune boosting treatments; **#2** to Prof. Wang, Duke-NUS, to oversee broadscale immune boosting, captive bat experiments, and analyze immunological and virological responses to immune modulators; **#3** to Dr. Shi, Wuhan Inst. Virol., to conduct PCR testing, viral discovery and isolation from bat samples collected in China, spike protein binding assays, humanized mouse work, and experimental trials on *Rhinolophus* bats; **#4** to Dr. Rocke, USGS NWHC, to refine delivery mechanisms for immune boosting treatments. Dr. Rocke will use a captive colony of bats at NWHC for initial trials, and oversee cave experiments in the US and China. **#5** to Dr. Unidad, PARC, to develop innovative aerosal platform into a field-deployable device for large-scale inoculation of the bats. Dr. Unidad will collaborate closely with Dr. Rocke in developing a field deployable prototype for both initial trails and cave experiments in China.

Collaborator Coordination: All key personnel will join regularly-scheduled web conferencing and conference calls in addition to frequent ad hoc email and telephone interactions between individuals and groups of investigators. Regular calls will include:

- Weekly meetings between the PI and Program Manager (on project and task status)
- Weekly web/phone meetings between Program Manager and subawardee admin. staff
- Monthly web/phone conferences between EHA PIs and all subawardee PIs.
- Monthly web conferences between key personnel (research presentations/coordination)
- Four in-person partner meetings annually with key personnel at EHA and two in-person partner meetings annually between subawardees.
- Annual in-person meeting among all key personnel

Evaluation metrics will include the generation of high-quality data, successful achievement of milestones and timelines, scientific interaction and collaboration, the generation of high quality publications, and effective budget management. The PI and subawardee PIs will attend a kickoff meeting and the PI will meet regularly with DAPRA at headquarters and at site visits.

Problem identification and resolution: Regular planning, monitoring, and evaluation meetings will be the primary mechanisms for problem identification. Minor problems (e.g. delays in sample availability or test results) will be dealt with internally through appropriate action and resolution monitored by Dr. Daszak and the Project Manager. In the event of significant problems, such as prolonged poor productivity, inadequate scientific collaboration, or major disputes regarding research direction or resource allocation, EHA will assist with resolving the problem through negotiation. Should a resolution not be forthcoming, consultation with our technical advisors and DARPA Program staff may be warranted.

Risk management: Maintaining a timeline and meeting milestones will require strict and continuous oversight of all project phases, frequent and regularly scheduled communication, and the ability to make decisions and implement strategies. A project of this nature requires a different mindset from that typically associated with basic research activities that move at an incremental pace with investigators gradually optimizing experimental systems, refining data, or waiting for additional data before moving ahead with an analysis approach. To maintain our timeline, we will continually evaluate these trade-offs to make decisions about when iteration is appropriate and when necessary to move forward with current information.

Biographies:

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Dr. Peter Daszak is President and Chief Scientist of EcoHealth Alliance, Chair of the NASEM Forum on Microbial Threats, member of the Executive Committee and the EHA institutional lead for the $130 million USAID-EPT-PREDICT. His >300 scientific papers include the first global map of EID hotspots, estimates of unknown viral diversity, predictive models of virus-host relationships, and evidence of the bat origin of SARS-CoV and other emerging viruses.

Prof. Ralph Baric is a Professor in the UNC-Chapel Hill Dept. of Epidemiology and Dept. of Microbiology & Immunology. His work focuses on coronaviruses as models to study RNA virus transcription, replication,

persistence, cross species transmission and pathogenesis. His group has developed a platform strategy to assess and evaluate countermeasures of the potential “pre-epidemic” risk associated with zoonotic virus cross species transmission.
Prof. Linfa Wang is the Emerging Infectious Diseases Programme Director at Duke-NUS Medical School. His research focuses on emerging bat viruses, including SARS-CoV, SADS-CoV, henipaviruses, and others and genetic work linking bat immunology, flight, and viral tolerance. A 2014 recipient of the Eureka Prize for Research in Infectious Diseases, he currently heads a Singapore National Research Foundation grant “Learning from bats” ($9.7M SGD).

Current list of contacts at key human (especially) and wildlife orgs in every SE Asian country from map above – please add to this if we’ve missed any

Indonesia – EHA: Eijkman (Dodi Safari), IPB (Imung Joko Pamungkas); **CM Ltd:** IPB (Imung Joko Pamungkas), Bogor Agricultural University (Diah Iskandriati)

SE China – EHA: Yunnan CDC (XX), Wuhan Inst. Virol. (Zhengli Shi);

Myanmar – Chulalongkorn (Thiravat and Supaporn) have collaborated extensively with Win Min Thit, MD (Yangoon General Hospital, Myanmar); Khin Yi Oo (National Health Laboratory, Myanmar) and also in training PREDICT Myanmar veterinarians in bat sampling under PREDICT.

Japan – **BMHRC**: Oita University (Hidekatsu Iha, Kiyoshi Aoyagi), Nagaskai University (Koichi Morita, Kazuhiko Moji)

Cambodia -
Laos - **CM Ltd:** Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit (Matthew Robinson)
Bangladesh – EHA: IEDCR (Meerjady Sabrina Flora, Ariful Islam), icddr,b (Mohammed Ziaur)

India – EHA: Jon’s collaborators;

Vietnam – Wellcome CRU ? Liaison with Zhengli Shi for Serol assays etc.?

Philippines – EHA, CM Ltd: Biodiversity Management Bureau, Department of Environment and Natural Resources (Anson Tagtag), Bureau of Animal Industry (Davinio Catbagan), Research Institute of Tropical Medicine (Catalino Demetria).

Malaysia – **CM Ltd:** Borneo Medical and Health Research Center (Ahmed Kamruddin), Department of Wildlife and National Parks Peninsular Malaysia (Frankie Sitam, Jeffrine Japning, Rahmat Topani, Kadir Hashim, Hatta Musa), Hospital Universiti Malaysia Sabah (Helen Lasimbang), Faculty of Medicine, Universiti of Malaya (Sazaly Abu Bakar), Kota Kinabalu Public Health Laboratory (Jiloris Dony, Joel Jaimin), Ministry of Health (Chong Chee Kheong, Khebir Verasahib, Maria Suleiman), National Public Health Laboratory (Hani Mat Hussin, Noorliza Noordin, Yu Kie Chem), Queen Elizabeth Hospital (Giri Rajahram, Heng Gee Lee), Sabah State Health Department (Christina Rundi, Mohammad Jikal), Sabah Wildlife Department (Rosa Sipangkui, Senthilvel Nathan, Augustine Tuuga, Jumrafiah Sukor), Gleneagles Hospital Kota Kinabalu (Timothy Wiliam); **BMHRC:** CM Ltd (Tom Hughes), HQEII (Shahnaz Sabri), Kota Kinabalu Public Health Laboratory (Jiloris Dony), Sabah State Health Department (Mohammad Jikal)

Thailand – **CM Ltd:** Department of Disease Control, Ministry of Public Health (Rome Buathong, Pawinee Doungngern, Pongtorn Chartpituck), Department of National Park, Wildlife and Plant Conservation (Pattarapol Manee-On), Faculty of Forestry, Kasetsart University (Prateep Duengkae), Mahidol-Oxford Tropical Medicine Research Unit, (Stuart Blacksell, Richard Maude),Chulalongkorn University (Supaporn Wacharapluesadee).

Chulalongkorn lab is a WHO Collaborating Centre for Research and Training on Viral Zoonoses, we work closely with WHO SEARO and SEARO countries including Bangladesh, Bhutan, Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, (Thailand), Timor-Leste.
We had conducted the PREDICT lab training for Veterinary lab in the Southeast Asia region for FAO.
We conducted (transferred?) the bat sampling technique for scientists from Malaysia, Myanmar, the Philippines, and China.

5. Data Management Plan

In a clearly labeled section entitled **"Data Management Plan":**

- Describe internal and external data acquisition strategies to achieve harmonization of systems and procedures for data management, data quality, data analyses, and dissemination for all data and data-related materials generated by the research study with the EIDRC CC.
- Within the plan, indicate the extent to which dedicated systems or procedures will be utilized to harmonize the acquisition, curation, management, inventory and storage of data and samples. Describe how training for the data and sample collection, in terms of the use of electronic data capture systems, will be provided to all staff including those at enrollment sites.
- Describe the quality control procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens.

This includes sample labeling etc. Get a bar-coding system, everyone has a scanner (cf. PREDICT) – Ralph has this for select agents and MERS. Also need a software system

Language from NIH CoV grant:

Data Sharing Plan: Data will be available to the public and researchers without cost or registration, and be released under a CC0 license, as soon as related publications are in press. Data will be deposited for in long-term public scientific repositories – all sequence data will be made publicly available via GenBank, species location data via the Knowledge Network for Biodiversity, and other data will be deposited in appropriate topic-specific or general repositories. Computer code for modeling and statistical analysis will be made available on a code-hosting site (GitHub), and archived in the Zenodo repository under an open-source, unrestrictive MIT license. Limited human survey and clinical data will be released following anonymization and aggregation per IRB requirements. Publications will be released as open-access via deposition to PubMed commons.

Viral isolates will remain at the Wuhan Institute of Virology initially. Isolates, reagents and any other products, should they be developed, will be made available to other NIH-funded researchers via applicable Wuhan Institute of Virology and EcoHealth Alliance Material Transfer Agreements and/or licensing agreements.

Sharing Model Organisms: We do not anticipate the development of any model organisms from this study. Should any be developed, they will be made available to other NIH-funded researchers via applicable Wuhan Institute of Virology and EcoHealth Alliance Material Transfer Agreements and/or licensing agreements.

Genomic Data Sharing: We anticipate obtaining genetic sequence data for 100s of novel coronavirus genotypes, including RNA-dependent RNA polymerase (RdRp) and Spike genes for all strains/genotypes. In addition, we will generate full viral genomes for a subset of the bat SARSr-CoVs that we identify. All sequence data will be deposited in the NIH genetic sequence database, GenBank. We will ensure that all meta-data

associated with these sequences, including collection locality lat/long, species-level host identification, date of collection, and sequencing protocols will also submitted. The genotype data will be made publicly available no later than the date of initial publication or six months after the receipt of final sequencing data, whichever comes first. We anticipate sequence generation will occur over the 5 year proposed project period.

Genome Wide Association Studies (GWAS): Not applicable.

Commented [KJO74]: From CoV NIH grant

Thailand TRC-EID Chulalongkorn lab:Specimen management and BioBank: The software called PACS (Pathogen Asset Control System) supported by BTRP DTRA, is now used for managing the specimen from specimen registration (barcoding, and may move to use QR code system soon), aliquoting, tracking, short patient history record, data summary, searching, test reporting and etc. Our biobank system contains 28 freezers (-80C) and liquid N2 system. Its size is around 120 sq meters. There is a separate electric generator for our lab and biobank. There are CC TV cameras at the biobank and laboratory.

Chula TRC-EID has our own server for data management and analysis. We have the necessary software including Bioinformatic tools (Blast Standalone, SAMTOOL, GATK, AliVIEW, RaxML, FigTREE, MAFFT, Guppy, Megan, PoreChop, MEGA), Database (MySQL, MongoDB) Web, jQuery, Node.js, Express.js React, API, PHP, Ruby, Python, Java, C#, HTML5, Bootstrap, CSS, Responsive, Cloud, Linux Command, PACS (sample management system), Virtualbox virtual machines, R programming languages, Perl scripts, Bash shell scripts, Open source software, Microsoft Office running on both Apple Mac OS X, Ubuntu, Linux, and Windows Operating Systems. Chula TRC-EID has a dedicated 16-core Ram 64GB Linux server with 4TB hard drives, dual quad-core Mac Pro Server with 2TB hard drives and another dedicated iMac Pro (Retina 4K, 21.5-inch, 2017) Processor 3.6GHz quad-core Intel Core i7 (Turbo Boost up to 4.2GHz) Ra, 16GB with 512GB hard drives. TRC-EID also has HPC Linux server CPU: 4 x 26cores Intel Xeon = 104 cores 2.1GHz with 52 TB HHD and 2x3.84TB SSD with GPU 2x Tesla v100 (CUDA core = 5120 cores/GPU, Tenser core = 640 cores/GPU). TRC-EID has a dedicated 10/100/1000 of LAN (Local Area Network), 2.4G / 5G Gigabit Wi-Fi 802.11ac.

Commented [KJO75]: From Supaporn for their lab, some may go in Facilities doc?

From DARPA:

Data Management and Sharing: EcoHealth Alliance will maintain a central database of data collected and generated via all project field, laboratory, and modeling work. The database will use secure cloud hosting services and enable export to archival and platform-independent formats. It will ensure data and metadata compatibility between project components, track data versioning and annotations, and enable compliance with DARPA data requests and disclosure requirements. All archived human sample data will be de-identified. Partners will provide raw and processed data to the central database throughout the course of the project. Project partners will have access to the data they generate in the database at all times, and maintain control over local copies. Release of any data from the database to non-DARPA outside parties or for public release or publication will occur only after consultations with all project partners. EHA has extensive experience in managing data for multi-partner partner projects (PREDICT, USAID IDEEAL, the Global Ranavirus Reporting System).

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6. Clinical Management Plan

Clinical site selection

Our consortium partners have been conducting lab and human surveillance research, inclusive of human surveillance during outbreaks, for over the past two decades and in that time have developed strong relationships with local clinical facilities and processes. The plan for selection of clinical sites will begin in the same geographic regions as those identified in Aim 1. These are regions where we will sample wildlife due to the high potential of zoonotic viral diversity. Additionally, these will be clinical sites that serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2. In PREDICT we developed successful working relationships with the major healthcare facilities the hotspot areas in 6 different countries, including Thailand and Malaysia and we will continue to work with these sites going forward. Continuing to

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work with these hospitals means we will begin this project with successfully established partners and we can begin data collection quickly. This also reduces the number of additional sites needed to meet our goals within our geographic range. The capacity and capability requirements for clinical sites for this study is limited. All that is required of sites will be the ability to enroll patients that meet the clinical case definitions of interest, capacity to collect and temporarily store biological samples, and follow standards for data management and protection. We will be enrolling inpatient and outpatient participants within 10 days of the presentation of symptoms to increase the chance of PCR detection of viruses so we will not wait for advanced normative diagnostic tests, that are likely unavailable in the more remote sites, to be completed. We will work with clinical sites to determine the best process for project staff, in most cases supporting the time of currently site staff. These will be persons who are locally trained and certified to collect biological specimen from participants including blood draw technique, e.g. doctor or nurse. Last, the clinical site will need the capacity to securely store all personal health information and personally identifiable research data, this will include locked offices with locked filing cabinets to store all paper records and an encrypted computer. Training on project procedures will be provided for all local project staff by local country project management staff and partners from headquarters. The study at clinical sites will begin by developing relationships with local stakeholders at the hospitals and clinics in our geographic areas of interest that serve as catchment facilities for people of within the community surveillance study. We will recruit hospital staff that will be extensively trained for project procedures including enrollment of patients, sample types to be collected, storage of samples, administration of the questionnaire, and data management plans. During the Development and onboarding of new clinical sites we will assess the physical needs of the site and what supplies will be required for collection of data (e.g. locked filing cabinet, dry shipper, secure laptop). [Did not specifically address CONDUCT]. Oversight for clinical sites will be managed by the country coordinator for each country. This will be done with regular site visits to the clinical sites and annual visits by headquarter research staff. Site visits will include monitoring and evaluating the process for patient enrollment, collecting and storage of samples, administration of the questionnaire, and secure and data management procedures for secure personal health information and research data. During these visits any additional training will also be provided.

Standardized approach oversight and implementation

Management and oversite for all study sites will be managed by the local country coordinator who is supported by staff at headquarters. Implementation of a standardized approach across all study sites, community and clinical, will begin by extensive training of staff on procedures on requirements of the project, this includes: enrollment, data collection, and data management. Our research team has over 5 years of experience building capacity on human subjects' research and has developed training resources and materials for standardized implementation of community and clinical research. This includes SOPs for screening, enrollment, and retention of participants. In addition to yearly monitory reports the country coordinator will regularly visit each clinical site to ensure quality standards and compliance of procedures are being met. Through our work with clinical sites in PREDICT we have developed culturally appropriate screening measures for clinal sites that do not disrupt the flow or quality of medical treatment received by the patient. This was done by working collectively with clinical research staff to evaluate current procedures and patient flow at the site to determine the most efficient while minimal invasive inclusion of our study into the daily working of the clinal site. Most efficiently this was done by adding minimal basic screening questions to the current clinal intake forms, which allows the clinal research officer to quickly scan charts or logs for potential patients to enroll, allowing for minimal disruption and prevents potential enrollees from being overlooked if the research staff is not on duty. At clinical sites that do scheduled outpatient days we may have a trained member of the research staff with the intake nurse to monitor the patients coming to the clinic more rapidly to no delay screening or enrollment in such a fast pace environment. Using the screening tools, we will recruit and enroll patients who present with symptoms that meet the clinical case definitions of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever, of unknown etiology within the past 10 days. These patients will be enrolled, samples collected and survey conducted to assess the participants contact with wildlife through: 1) occupational exposures; 2) animal contact; 3) environment biosecurity. With permission from each clinic, and consent from participants, we will review clinical records to

collect data on medical history, clinical syndromes, and patient etiology. The clinical cohort will be split into cases and controls. “Cases” are defined as participants whose samples tested positive for either coronaviruses, henipavirus, or filoviruses, serological tests. “Controls” will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between serological/PCR status and risk factors. In clinical study, we will use this procedure to determine how clinical presentation differs between virus-exposed and -unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. We will model the outcomes to analyze correlation between biological results and risk factors related to high risk wildlife exposure. Additionally, we will be following up with clinical participants to determine how clinical presentation differs between coronaviruses, henipavirus, or filoviruses in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. [How do these cohorts be leveraged for new emerging pathogens] Achieving the adequate numbers will be attained through duration of the as clinical participant enrollment. While patient’s enrollment is limited by the number of individuals presenting at hospitals, during the PREDICT project we had an average of 105 patients enrolled per year, ranging from 77-244. The country coordinator will be continually monitoring the project database to be sure that we are on track to reach our target.

Clinical cohort setup, recruitment, enrollment

We will recruit inpatients and outpatients from clinical sites to participate in the study after initial screening to determine if they meet the clinical case definitions for 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever, of unknown etiology for symptoms reported onset of illness to increase the chance of PCR detection of viruses. Once enrolled, biological samples will be collected by trained hospital staff. When possible, samples will be taken concurrently with samples being collected for normative diagnostics. We will collect 2* 5mL of blood for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples and two nasal or oropharyngeal swabs will be collected. The cases are defined as participants whose samples tested positive for Henipaviruses, Filoviruses, or CoVs by PCR or serological tests. The controls will be selected from the pool of participants admitted to the studies and whose samples tested negative for coronaviruses, henipavirus, or filoviruses. We will follow up 35 days after enrollment to collect an additional 5mL blood draw for collection of two 500 µL serum samples and a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. Following up 35 days post initial collection gives adequate time for 1) development of IgG and IgM, which occurs <28 days after onset of symptoms for coronaviruses, henipavirus, or filoviruses infected patients to evaluate the immunological progression of disease and 2) further the risk assessment of the participants to monitor contact with wildlife, people, and to assess the likely reservoir hosts to collect information to inform potential intervention programs.

Utilization of collected data

Sampling of wildlife and the enrollment of community surveillance participants and a clinical cohort of participants give us the ability to assess the viruses the are circulating in each of the three populations in a similar geographic region. We will use phylogenetic analysis to compare the relationship between wildlife viruses found and viruses fond in human participants. Additionally, the questionnaire allows us to assess relative measures of human-wildlife contact from our survey work that we will analyze the intensity of contact with species known to be at a higher risk for spillover against other risk to provide useful proxy information for spillover risk.

The biological specimens to be collected (2*5mL whole blood for whole blood and serum analysis and nasal or oropharyngeal swabs) from all eligible participants and follow up specimen 35 days (3mL of whole blood for serum) after enrollment with the standardized questionnaire. Capacity for specimen is collection is not more than standard phlebotomy skills and we will collected by locally trained and certified personnel that are part of the research team in country. Staff we will trainline on ethical guidelines for conducing human subjects

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Commented [EH79]: PREDICT we also make the stipulation that we will collective relatvive samples if avialble from treatmean collection, eg CSF if the MD is doing a spinal tap we will request an aliqot if possible

Commented [EH80]: Please add any information that might address: types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.

Commented [EH81]: Please add any information that might address: Describe methods that might be applied to elucidate a zoonotic source

research and survey data collection methods. Collection of whole blood, serum, and nasal or oropharyngeal swabs allows us to test the samples for PCR to detect viruses in our priority viral families, serologically test for immunological response and historical exposure to zoonotic diseases of relevance, and the questionnaire will assess individuals evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. With this collective data set we aim to quantify and detect novel viruses likely to spillover regularly to people, are often unreported or misdiagnosed as their clinical manifestations are unknown to detect cryptic outbreaks causing previously 'hidden' clinical syndromes in people. Our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence.

Potential expansion
Expansion of the research project to other clinical sites or research areas could happen as new information is gathered, either through our research or an outbreak in the region. If expansion is required we could rapidly implement research activities in additional clinical or community sites through the same process we on-boarded the initial locations. First, by working with local stakeholders to create a working relationship and on-boarding new clinical research staff through advanced trainings of project aims, SOPs on data collection and storage, and ethical guidelines. The network of infectious disease researchers in the community and hospital settings would allow for an accelerated response to any potential infectious disease outbreaks as staff are well trained on ethical guidelines for working with human subjects, data and specimen collection, storage, and transpiration, project staff would be on the front lines ready for the call to action. Ideally, our testing for viruses in priority viral families may lead to the discovery cryptic outbreaks before it spreads in the general public. Biological results coupled with survey data on key risk factors will provided necessary information for granular understand of disease spread.

In a clearly labeled section entitled **"Clinical Management Plan":**

- Describe plans, processes, and procedures for the following activities: clinical site selection including feasibility, capacity, and capabilities, and study development, conduct, and oversight.
- Describe strategies for oversight and implementation of standardized approaches in the recruitment and clinical characterization of adequate numbers of relevant patient populations to ensure the prompt screening, enrollment, retention and completion of studies. Describe novel approaches and solutions to study enrollment. including the clinical meta-data that will be captured and describe how the staff at each enrollment/collection site will be trained and comply with the quality standards for data and sample collection and compliance with the standardized procedures. Provide a general description of the methods for establishing clinical cohorts and how cohorts may be leveraged for new emerging pathogens.
- Provide general approaches to identification of the types of clinical cohorts needed to fulfill the goals of the EIDRC. Discuss types of clinical cohorts and clinical assessments that will be utilized to study natural history and pathophysiology of disease, including characteristics of the cohort, site for recruitment, types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.
- Describe methods that might be applied to either elucidate a zoonotic source or a vector of transmission.

Our research team incredibly diverse and capable of working with a group of other viral families: Lay out all our additional vector experience incl. my WNV work, our work on Chik etc.
Baric: Flavi, Noro,
Linfa: …
EHA: …

- Describe capacity for and approaches to clinical, immunologic and biologic data and specimen

collection, and how these data and specimens will address and accomplish the overall goals and objectives of the research study.

Ralph

1. Norovirus: collaboration with surveillance cohort CDC; first one to publish antigenic characterization of each new pandemic wave virus; and phase 2 and 3 trial of therapeutics
2. Tekada Sanofi Pasteur dengue
3. Nih tetravalent vaccine

- Provide plans for the potential addition of new sites and expanding scientific areas of research when needed for an accelerated response to an outbreak or newly emerging infectious disease.

Linfa – will launch VirCap platform on outbreak samples.

7. Statistical Analysis Plan

In a clearly labeled section within the Research Strategy entitled **"Statistical Analysis Plan":**

- Describe the overall plan for statistical analysis of preliminary research data, including observational cohort data.
- Describe the overall staffing plan for biostatistical support and systems available for following functions: 1) preliminary data analyses, 2) estimates of power and sample size, 3) research study design and protocol development.
- Describe the quality control and security procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens. Describe any unique or innovative approaches to statistical analysis that may be utilized.

8. Project Milestones and Timelines

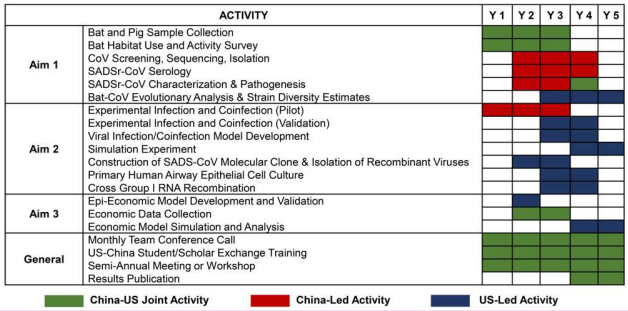
In a clearly labeled section entitled **"Project Milestones and Timelines":**

- Describe specific quantifiable milestones by annum and include annual timelines for the overall research study and for tracking progress from individual sites, collaborators, and Teams. Milestones must specify the outcome(s) for each activity. Milestones should be quantifiable and scientifically justified, and include the completion of major research study activities, including, for example, protocol development, case report forms, scheduled clinical visits, obtaining clearances study completion, and analysis of final data. Milestone criteria should not simply be a restatement of the specific aims. Using a Gantt chart or equivalent tool, describe the associated timelines and identified outcomes for the research study.

Show that for this proposal, we won't be doing the Specific Aims sequentially. We'll begin the human work (SA 2, 3) at the beginning of the project, along with SA1. We'll do the diagnostic assay development initially in Ralph's, Linfa's, Chris's lab, but then begin tech transfer in Y1….

Project Management & Timeline

PI Daszak will oversee all aspects of the project. Dr. Daszak and has two decades of experience managing international, multidisciplinary research in disease ecology, including successful multi-year projects funded by NSF, NIH and USAID. He will conduct monthly conference calls with all co-PIs, including the China team **(see Activity schedule below)**. He will be assisted by EHA Sr. Personnel Li, who is a US-based Chinese national with fluent Mandarin. Co-PI Olival will oversee Aim 1 working closely with China PI Tong, who will test samples. Co-PI Ma (China team) will coordinate and lead all Chinese experimental infections, with guidance from co-PI Saif, who will coordinate and lead all US experiments with co-PI Qiuhong Wang. Sr. Personnel Ross will conduct all modeling activities in Aim 2, working closely with co-PI Saif to parameterize the models. Co-PI Baric and Sr. Personnel Sims will lead infectious clone, recombinant viral and culture experiments. Consultant Linfa Wang will advise on serology, PCR and other assays. China team co-PIs Shi and Zhou will oversee viral characterization in Aim 1. Sr. Personnel Feferholtz will oversee the economic modeling in close collaboration with Ross.



Commented [KJO82]: Text and figure From NSF-NIH SADS grant. Peter will adapt

Another Example of Milestones and specific tasks from a prev DHS (unfunded) proposal:

Expand existing databases to include predictor variables for pathogenicity risk model

Task 1: Collate available human clinical information from the literature for ~300 viral pathogens known to infect humans (Contract Award Date to 6 month)

Task 2: Update EHA host-viral association database to include new data for mammalian and avian species from literature 2015-present (Contract Award Date to 3 month)

Task 3: Extract and curate full and partial genomic data from Genbank for 5-6 viral families with a high proportion of human-infectious pathogens for phylogenetic analyses – e.g. 'nearest-known-threat module'. (Month 2 to 6 month)

Develop underlying pathogenicity and spillover risk assessment models

Task 4: Fit generalized linear models in Stan for multiple response variables to measure pathogen impact (Month 7 to 10 month)

Task 5: Fit generalized linear models to estimate risk of human infection (spillover) based on host, ecological, and location specific traits (Month 4 to 8 month)

Task 6: Validate model effectiveness by simulating early-outbreak, limited-information scenarios from known pandemics to confirm model behavior (Month 8 to 10 month)

Commented [KJO83]: Just example, but probably just want to use a Gantt chart format per most of our grants, depending on space.

REFERENCES

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From:	Danielle Anderson on behalf of Danielle Anderson <danielle.anderson@duke-nus.edu.sg>
To:	Kevin Olival; Peter Daszak
Cc:	Wang Linfa; Ralph S. Baric; Baric, Toni C; Sims, Amy C; Chris Broder; Eric Laing; Thomas Hughes; Supaporn Wacharapluesadee; Aleksei Avery Chmura; Alison Andre; Luke Hamel; Hongying Li; Emily Hagan; Noam Ross
Subject:	RE: EID-SEARCH v4
Date:	Wednesday, June 26, 2019 2:58:21 AM
Attachments:	EIDRC Southeast Asia v4 (DA).docx

I was working on this at the same time as Kevin, so I will stop here on this version.
I will continue from where I left off using Kevins new version

From: Kevin Olival [mailto:olival@ecohealthalliance.org]
Sent: Wednesday, 26 June, 2019 2:51 PM
To: Peter Daszak <daszak@ecohealthalliance.org>
Cc: Wang Linfa <linfa.wang@duke-nus.edu.sg>; Danielle Anderson <danielle.anderson@duke-nus.edu.sg>; Ralph S. Baric <rbaric@email.unc.edu>; Baric, Toni C <antoinette_baric@med.unc.edu>; Sims, Amy C <sims0018@email.unc.edu>; Chris Broder <christopher.broder@usuhs.edu>; Eric Laing <eric.laing@usuhs.edu>; Thomas Hughes <tom.hughes@ecohealthalliance.org>; Supaporn Wacharapluesadee <spwa@hotmail.com>; Aleksei Avery Chmura <chmura@ecohealthalliance.org>; Alison Andre <andre@ecohealthalliance.org>; Luke Hamel <hamel@ecohealthalliance.org>; Hongying Li <li@ecohealthalliance.org>; Emily Hagan <hagan@ecohealthalliance.org>; Noam Ross <ross@ecohealthalliance.org>
Subject: Re: EID-SEARCH v4
Importance: High

Peter, v4 with my edits, some revised figs, and additional comments.

Cheers,
Kevin

Kevin J. Olival, PhD
Vice President for Research

EcoHealth Alliance
460 West 34th Street, Suite 1701
New York, NY 10001

1.212.380.4478 (direct)
1.917.856.3900 (mobile)
1.212.380.4465 (fax)
www.ecohealthalliance.org

> On Jun 25, 2019, at 9:26 AM, Peter Daszak <daszak@ecohealthalliance.org> wrote:

Dear All,

Here's version 4. Apologies for the delay. It's in better shape now, but there are still lots of areas that need your attention. Please put in time today, and tomorrow to edit and firm up the text. I've put your names in some of the comment boxes where I need specific help but I also need you to read this critically now and edit and tighten it up. We have 30 pages of space, and our research plan is now 21 pages, so we need to lose maybe 3 or 4 pages. There are also some weak points, specifically on Filoviruses, on the details of where we'll do our clinical work, and on what we'll do with the animal models for henipas and filos.

Please get comments back with me before Wednesday 26th 4pm New York time, so that I have Wendesday evening and all day Thursday to incorporate them. In the meantime, I'll be working with folks here on better figures, and all the extra sections that come after the research plan. I'll need you all to help with those on Thursday while I'm finishing off the draft.

Thanks to all of you in advance and look forward to the next round.

Cheers,

Peter

Peter Daszak
President

EcoHealth Alliance
460 West 34th Street – 17th Floor
New York, NY 10001

Tel. +1 212-380-4474
Website: www.ecohealthalliance.org
Twitter: @PeterDaszak

From: Peter Daszak
Sent: Thursday, June 20, 2019 9:41 PM
To: Wang Linfa; Danielle Anderson (danielle.anderson@duke-nus.edu.sg); Ralph Baric (rbaric@email.unc.edu); Baric, Toni C; Sims, Amy C; Christopher Broder (christopher.broder@usuhs.edu); Eric Laing; 'Tom Hughes'
Cc: Aleksei Chmura (chmura@ecohealthalliance.org); Alison Andre; Luke Hamel (hamel@ecohealthalliance.org); Kevin Olival, PhD (olival@ecohealthalliance.org)
Subject: EIDRC-SEA v.3
Importance: High

Dear all,

Thanks to everyone for comments and text for the earlier draft at the beginning of the week. Kevin's spent the last couple of days pulling all the comments together into one draft and inserting his changes, and I've now attached a v3 of the draft. It's still rough, and now too long, but it's getting there. I wanted you to look at this rough version because I'm stuck in Liberia in meetings Friday, then flying back home Saturday. I'll continue editing this draft on the plane and will be working solely on this grant from home on Sunday, Monday, Tues, Wed, Thurs.

Please work on this rapidly if possible. If you can all push your comments through, using track changes, and get them back to me **by Sunday evening New York Time**, I will turn round a polished draft by Tuesday, giving us time for significant last go-over on Wed, Thurs. It's tight, but unfortunately, it is what it is, so please commit the time if you can!! As well as general edits (using track changes, with references in comment boxes), please do the following…

Linfa/Danielle – We especially need your input in section 1.1, 1.3, 1.4 and 2.5

Ralph/Amy - Please work your magic on this draft, inserting text, editing and reducing text, and making sure the technical details of the characterization are correct and fit the proposed work on CoVs, PMVs and Henipaviruses

Chris/Eric – Eric – congratulations on your (b) (6) and thanks for working on it at this time! Chris – please help and edit/finesse the language in the relevant sections for you

Tom – Your edits crossed over with Kevin's changes, so please insert all the bits that were new in the last version you sent to me, into this version. I think these are mainly the sections and comments from the other Malaysian colleagues, but please check and re-insert all key text. We especially need to be specific about which partner in Malaysia is doing what cohorts, and where, and about who's doing the testing

Thanks again all, and I'm looking forward to non-stop work on this as soon as I get on my flight in 24 hours…

Cheers,

Peter

Peter Daszak
President

EcoHealth Alliance
460 West 34th Street – 17th Floor
New York, NY 10001

Tel. +1 212-380-4474
Website: www.ecohealthalliance.org
Twitter: @PeterDaszak

EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

<EIDRC Southeast Asia v4.docx>

Understanding risk of zoonotic virus emergence in EID hotspots of Southeast Asia
II. Research Strategy:

1. Significance:

Southeast Asia is a well-defined hotspot for emerging diseases due to its high biodiversity of wildlife and their viruses, and the presence of critical ecological and socioeconomic drivers of disease emergence (*1*). These include dense human populations living in networks of rural and urban communities, with strong cultural and occupational connection to wildlife and livestock farming and trading, and intimate connection to global travel networks (*2*). The region is also undergoing rapid environmental and demographic change, both of which increase the risk of disease emergence and spread. It is therefore not surprising that a number of recent discoveries have identified novel viruses, including near-neighbors of known agents spilling over to livestock and people through often novel pathways, leading to sometimes unusual clinical presentations **(Table 1)**.These factors are also behind a significant background burden of repeated Dengue virus outbreaks in the region (*3*), and over 30 known *Flavivirus* species in South and Southeast Asia (*4*).

Viral agent	Site, date	Impact	Novelty of event	Ref.
Melaka virus & Kampar virus	Malaysia, 2006	Caused SARI in family group, individual	1st disease due to bat-origin reoviruses (other reports Singapore, Vietnam etc.)	(*5-8*)
Reston filovirus	Philippines 2008	Seropositive people killed pigs,	No prior findings of filoviruses in pigs	(*9*)
Thrombocytopenia Syndrome virus	China 2009	Domestic animals, illness in people	Novel syndrome with large caseload	(*10*)
Reston filovirus	Shanghai 2011	PCR positive pigs	Further evidence of pig RESTV infection	(*11*)
Mojiang virus	Yunnan 2012	Implicated in death of 3 mineworkers exposed to rats	1st evidence of rodent origin henipavirus in people	(*12*)
Nipah-like virus	Philippines 2015	Killed horses	No prior horse infection for NiV, known for HeV	(*13*)
SADS-CoV (HKU2)	China 2017	>25,000 pig deaths seronegative people	Novel emergence of bat-origin CoV	(*14*)
SARSr-CoV & HKU10-CoV	Yunnan, Guangxi 2015	Seropositive people	1st evidence human infection HKU10 & SARSr-CoV	(*15*)
Nipah virus	Kerala, 2018, 2019	19 people infected/ 17 dead 2018, 1 infected 2019	1st outbreak of NiV outside Bangladesh, W. Bengal focus	(*16, 17*)

Table 1: Recent reports from SE Asia indicating potential for novel pathways of emergence, or unusual presentations for known or close relatives of known viruses.

These events are dominated by three viral families: coronaviruses, henipaviruses and filoviruses, which will act as the key research viral groups in this proposal. These viral groups have led to some of the most important recent emerging zoonoses (*18-29*). Their outbreaks have caused tens of thousands of deaths, and billions of dollars in economic damages (*30-32*). In addition to the events in **Table 1**, relatives and near-neighbors of known pathogens in these families have been reported throughout the region, including by members of our consortium including: henipaviruses in frugivorious bats in Thailand, Cambodia, Philippines, Laos, Indonesia, China, Malaysia ,Bangladesh and India (*33-39*); a novel henipavirus, Mojiang virus, in wild rats in Yunnan (*12*); serological evidence of filoviruses in bats in Bangladesh (*40*) and Nipah- and Ebola-like viruses in *Hipposideros*, *Cynopterus* and *Rhinolophus* species in Malaysia (EHA, unpublished data); evidence of novel filoviruses in bats in Singapore (*41*) and China (*42-44*), including Měnglà virus that appears capable of infecting human cells (*45*); novel paramyxoviruses in bats and rodents consumed as bushmeat in Vietnam (*46*), a lineage C β-CoV in bats in China that uses the same cell receptor as MERS-CoV and can infect human cells *in vitro* (*47*); MERSr-CoVs found in dry bat guano being harvested as fertilizer in Khao Chong Pran cave, Ratchaburi, Thailand (*48*) and later directly in bats (CU, unpublished); 52 novel SARSr-CoVs in 9 bat species in southern China (172 novel β-CoVs from >16,000 sampled bats) that are also found throughout the region (*20, 47, 49, 50*), a new β-CoV clade ("lineage E") in bats (*50*); and 9 and 27 novel wildlife-origin CoVs and paramyxoviruses in Thailand (respectively) and 9 novel CoVs in Malaysia identified as part our work under USAID-PREDICT funding (*49, 51*). These discoveries underscore the genetic diversity of potentially

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pathogenic viral strains in wildlife in the region, and their potential to help in the development of broadly active vaccines, immunotherapeutics and drugs (*52*).

Most high-impact zoonotic viruses originate in wildlife reservoirs, sometimes spilling over first into livestock 'amplifier' hosts, or directly into localized human populations with high levels of animal contact **(Fig. 1)**. Efforts to prevent emerging zoonoses have targeted these high-risk populations in regions prone to disease emergence (*29, 53*). However, surveillance and control is hampered by inadequate information on basic disease ecology (e.g. range of wildlife reservoirs, risk factors for spillover) and pathogen biology (viral diversity, pathogenesis), and by logistical challenges sharing novel diagnostics, animal models, samples and reagents at sites where outbreaks begin (*54*). Countermeasure and vaccine development is challenged by the small number of isolates available, and the presence of closely-related strains, some with evidence of transmission to people but with unknown pathogenicity. For example, using NIH and other funding, we found serological evidence of exposure to African henipaviruses in 3-4% of a sample of people in Cameroon, with a significant risk factor being butchering of bats; and serological responses to bat SARSr-CoVs in 6/209 (2.87%) and bat HKU10-CoV in x/xx (x%) people living close to a bat cave in Yunnan, all novel viruses with unknown clinical impact (*15, 55*). We discovered two novel bat-borne reoviruses, Melaka and Pulau viruses, and showed that 14/109 (13%) of people living in close proximity to bat roosts on Tioman Island, Malaysia were exposed to these viruses (*5*) as well as 12/856 (1.4%) people screened in Singapore (*6*). Previous studies in the Philippines found famers and butchers who had contact with sick pigs tested positive for Reston ebolavirus antibodies but had not experienced illness (*9*), and it was our team that discovered Philippine bats as natural reservoir hosts for Reston virus (*56*). Preliminary screening in Malaysia with funding from DTRA for serological surveillance of Henipaviruses and Filoviruses spillover found serological evidence of past exposure to Nipah and Ebola antigenically-related viruses in non-human primates (NHPs) and people (Hughes, unpublished data). Even when spillover leads to outbreaks of illness and mortality, they are often unreported, undiagnosed or misdiagnosed. Under a prior NIH R01, we initiated targeted syndromic surveillance of encephalitis patients in Bangladesh clinics and showed that NiV causes repeated annual outbreaks with an overall mortality rate of ~70%, and that it is also present in bats in India (REF). Nipah virus was originally misdiagnosed as 'aberrant measles' in West Bengal, India (REF), has now emerged repeatedly in North India, and in Kerala, South India in 2018 and 2019 (ongoing), raising the specter of future spillover at other sites across the region (*16, 57*).

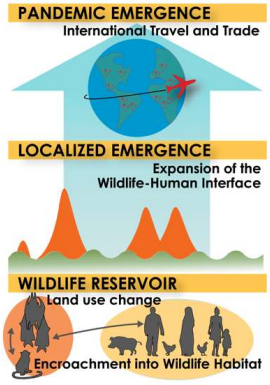

Fig. 1: Our Early Warning Approach: Most zoonotic viruses emerge from wildlife hosts, sometimes via livestock (Stage 1, lower panel), to cause small scale outbreaks (green) or larger chains (red) of human-to-human transmission (Stage 2, middle). In some cases, these spread more widely via air travel (Stage 3, upper). Our EIDRC proposal targets each stage: SA1 examines the diversity and spillover potential of viral threats in targeted SE Asian wildlife; SA2 seeks evidence of their spillover into focused high-risk human populations (stage 2); SA3 identifies their capacity to cause illness and to spread more widely (stage 3). Figure from (*53*).

Under a current NIAID R01, we have shown that we can use analytical approaches to target surveillance of wildlife and people and identify likely sites of spillover. We used phylogenetic analysis of bat hosts and their CoVs to identify a key cave complex in SW Yunnan Province that harbors all the genetic elements of SARS-CoV (*58*), as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics (*20, 47, 49, 50, 59-61*). We conducted behavioral risk surveys and biological sampling of human populations living close to this cave and found evidence of spillover (xx serology) in people highly exposed to wildlife. **This work provides proof-of-concept for an early warning approach that we will expand in this EIDRC to target key viral groups in one of the world's most high-risk EID hotspots.**

The overall rationale for this EIDRC proposal is that there is substantial evidence that: 1) diverse CoVs, henipaviruses and filoviruses related to known zoonotic pathogens are circulating in wildlife reservoirs in Southeast Asia; **2)** these viruses likely spillover regularly to people, are often unreported or misdiagnosed, and

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their clinical manifestations and potential to cause pandemics are unknown and underestimated; and **3)** our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence. The overarching goal of this proposal is to launch the **EIDRC-SEA** (**S**outh**E**ast **A**sia) to better understand the risk of zoonotic viral emergence in Southeast Asia. We will initially focus on the diversity of known and closely related CoVs, henipaviruses, and filoviruses in wildlife reservoirs. We will use this work to build our center's interactive network throughout the region, bridge barriers, provide early focal point for basic and translational studies, develop sample handling and storage routines, communication networks, cohort management, infrastructure development and such. We will test these viruses' capacity to infect human cells and mouse models, develop new specific and sensitive serological and molecular diagnostic tools, and conduct surveillance of human populations with high risk of exposure to wildlife, and clinical cohorts to identify evidence of spillover and pandemic potential, and of viruses causing previously 'cryptic' clinical syndromes in people. Our group also has extensive experience in other viral pathogens, including vector-borne flaviviruses, and will use this experience to provide robust long-term ~~surveillance~~surveillance, diagnostics, human and basic science opportunities. While the current proposal focuses its basic and applied research agendas on this group of viral EID, we note that the EIDRC-SEA is poised to rapidly respond to any new emerging outbreak. Thus, filoviruses, henipaviruses and coronaviruses are examples of sentinel ~~surveillance~~surveillance systems in place that can capture, identify and initiate a rapid response targeting any novel microbial pathogen that emerges in this region.

2. Innovation: Previous work by our consortium has developed a system to target surveillance in wildlife and people to better anticipate and identify early spillover events and pre-empt outbreaks of emerging viruses (REF). The EIDRC-SEA we take this approach and scale ~~it~~ up to cover three critically high-risk EID hotspot countries in Southeast Asia, within a regional network of collaborators. The innovation of the EIDRC-SEA is in: 1) its multidisciplinary approach that combines modeling to target the geography for wildlife and human sampling, novel phylogenetic and *in vitro* and animal model approaches to obtain precise biological risk assessments of viral spillover potential into people, the development and transfer of novel serological and molecular diagnostics capabilities throughout the region, and use of parallel large scale cohorts to identify spillover and illness due to known and novel viruses; 2) the biological characterization approach that identifies how likely viruses are to be able to spillover into people, the evaluation of existing countermeasure technologies; technologies that are grounded on our successful work on SARSr-CoVs; and 3) the combination of geographically targeted human populations with high risk of animal contact and syndromic clinical cohorts that present with signs of novel viral emergence, to identify risk of viral spillover in an early warning system approach. **In Aim 1, we will target viruses in new and archived wildlife samples, and characterize their risk of spillover to people**. We will use spatial and phylogeographic analyses to identify the geography and species of wildlife to test samples from, as well as further characterize viruses we have recently discovered. We will use *in silico* methods (i.e. novel ecological-phylogenetic risk ranking and antigenic mapping of receptor binding domains), cell culture, and animal models to assess their potential for spillover into high-risk human populations. **In Aim 2, we will conduct focused, targeted surveys and sampling of human communities with high exposure to wildlife and other animals.** We will use serological tests targeting viruses identified in Aim 1 to identify the baseline spillover of known or novel CoVs, henipaviruses and filoviruses in these populations. Serological findings will be analyzed together with clinical meta-data (including a simple animal-contact survey, location data, etc) to identify zoonotic risk factors and routes of exposure from specific wildlife or vector species. **In Aim 3, we will use syndromic surveillance of clinical cohorts to identify evidence of viral etiology for otherwise 'cryptic' outbreaks.** We will enroll and collect biological samples from patients in local clinics and hospitals who present with syndromes previously linked to known viral agents and live within communities at high risk for disease emergence. We will conduct molecular and serological diagnostic assays to identify likely viral etiology of syndromes that may represent otherwise cryptic events. Where viruses are identified, we will attempt to isolate and characterize them, and use survey data, ecological and phylogeographic analysis to identify likely reservoir hosts and inform intervention programs. Our US partner BSL-4 laboratory, NEIDL, will attempt isolation and characterize any viruses ~~requring~~requiring high levels of containment (e.g. any novel ~~Filoviruses~~ filoviruses discovered).

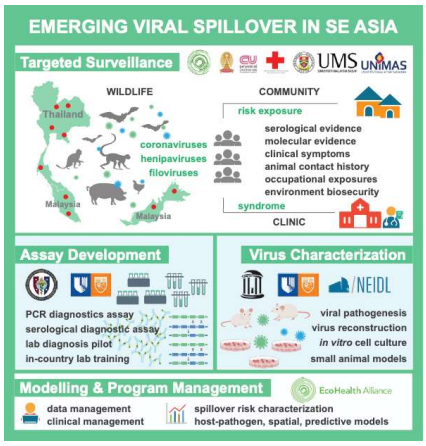
Commented [BRS3]: Number one innovation is the nuclear core sites that make up the EIDRC and its expanded network of long-term collaborators that saturation the most biodiverse EID host spot in the world, 2) extensive sample collections from wildlife, people in close proximity to EID hotspots, and samples from cohorts in individuals seeking medical care at our participating centers? (major goal of proposal if I remember correctly is to set up surveillance network for rapid response and interface wit health care facilities to collect large sample sets and/or develop clinical cohorts for testing intervention technologies (in the future).

Commented [KJO4]: From MeiHo: Another innovation from EIDRC is the quick response if an outbreak happens in the region when the team has been developed and trained under this initiative.

Commented [KJO5]: List out what other labs will do too? Or just rely on our Org chart figure for all of this?

3. Approach

Research team: The EIDRC-SEA builds on long-term collaboration among world leaders in emerging virus research with proven experience collaborating internationally on field, lab and human surveillance research **(Fig. 2)**. Over the past two decades, our consortium partners have collaborated together within the region and globally to conduct high profile research on EIDs. This work includes identifying the wildlife reservoirs for Nipah and Hendra virus, MERS- and SARS-CoVs (*20, 25, 58, 62-65*), discovering SADS-CoV (*14*), and developing an array of serological, molecular, *in vitro* and *in vivo* approaches to characterizing high-risk CoVs, henipaviruses, and filoviruses (*59-61, 66-79*). Our team has substantial experience conducting human surveillance during outbreaks (e.g– The ongoing viral surveillance using molecular and serological techniques with the indigenous communities of Peninsular Malaysia and syndromic surveillance of hospital patients in Sabah with undiagnosed syndromes through the USAID-PREDICT program and the DTRA funded serological surveillance of Henipaviruses and Filoviruses spillover. - Please insert examples that you've been involved in and REFS), and as part of longitudinal efforts to pre-empt pandemics (*80, 81*).



In 2016, our collaborative team, together with partners in China, discovered an HKU2-clade α-CoV(*82, 83*) in 5 pig farms in Guangdong Province affected by fatal diarrheal disease. We used PCR, serology, pathology, phylogenetic analysis, and infection experiments to show that this novel virus, SADS-CoV, originated in *Rhinolophus* spp. bats and killed > 20,000 pigs at these farms – all in the span of three months (*14, 84*)

Fig. 2: Interdisciplinary team & roles in the proposed EIDRC-SEA.

PI Daszak has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5- 20 yrs on NIAID- and USAID-funded research. He will oversee all activities in this EIDRC. Drs. Olival, Zambrana, and Ross are leaders in analytical approaches to targeting surveillance and head the modeling and analytics work for USAID-PREDICT. Drs. Wang, Baric, Broder, Anderson, and Laing have developed a unique array of *in vitro* cell culture, serological and molecular reagents to identify and characterize emerging viruses in people and animals (henipaviruses and other paramyxoviruses, CoVs, filoviruses and many others). Dr. Wacharapluesedee, Hughes and collaborators have coordinated surveillance of people, wildlife, and livestock within Thailand and Malaysia for the past 10 years, in collaboration with Ministries of Health, Agriculture and Environment. Finally, this consortium has extensive external funding from DoD, USAID, NIH, DHS and other USG agencies that supports a range of laboratory, analytical and field studies directly related to the current proposal, and will bring substantial leverage to the EIDRC **(See Section XX)**.

Fig.3 : Map of Southeast Asia indicating the three core countries for this proposed EIDRC (White: Thai, Sing, Mal) and those that Key Personnel are actively collaborating with (Green: field sites and collaborating labs indicated with asterisk).

Geographical focus: The three core countries for this proposed EIDRC are Thailand, Malaysia and Singapore, three contiguous EID hotspot countries that stretch through one of the most significant foci of EID origins globally. Our proposed work includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sarawak and Sabah, and thus contains overlapping wildlife biogeography with India, Bangladesh, Myanmar, SW China, Laos, Cambodia, Vietnam, Philippines, and Indonesia. It encompasses

Commented [KJO6]: From Tom: **Tim, Giri, Tsin Wen – Please can you add in details for MonkeyBar**

Tsin Wen – Please can you add details here on your Univ N. Sumatra malaria/fever patients to find malaria

Tim and Tsin Wen – Please can you add in details of your encephalitis study

Dr Tan – Please can you add in details of your work with Dayak and other human studies you have done

Dr Lee – Please can you add in details of any hospital based or other human surveillance you have been involved with.

Prof Kamruddin – Please can you add in details of your blood donor, Garbage collector, Febrile and diarrhea

Please add any other examples you have.

diverse cultural and socioeconomic backgrounds which have correspondingly diverse and intensive interactions with wildlife reservoirs and the global travel network. We have already begun sampling of wildlife hunters, livestock workers, wildlife farmers and market workers, rural villagers living close to high risk wildlife species, people otherwise highly exposed to animals in these countries, and clinical cohorts, as part of current/prior NIH and USAID funding. To develop a larger catchment area for emerging zoonoses, we will deploy our consortium's extensive network of collaborators in clinics, research institutes and public health laboratories in every other Southeast Asian country. We will conduct regular meetings with key research, public health and community leaders, including >50 leading laboratories/institutions in XX countries that are currently collaborating with core members of our consortium via other funded work (**Fig. 3**). We will use these, and our annual meetings, to share information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks across the region.

Current list of contacts at key human (especially) and wildlife orgs in every SE Asian country from map above – please add to this if we've missed any

Indonesia – EHA: Eijkman (Dodi Safari), IPB (Imung Joko Pamungkas);
SE China – EHA: Yunnan CDC (XX), Wuhan Inst. Virol. (Zhengli Shi);

Myanmar – Chulalongkorn (Thiravat and Supaporn) have collaborated extensively with Win Min Thit, MD (Yangoon General Hospital, Myanmar); Khin Yi Oo (National Health Laboratory, Myanmar) and also in training PREDICT Myanmar veterinarians in bat sampling under PREDICT.

Cambodia -
Laos - **CM Ltd:** Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit (Matthew Robinson)
Bangladesh – EHA: IEDCR (Meerjady Sabrina Flora, Ariful Islam), icddr,b (Mohammed Ziaur)

India – EHA: Jon's collaborators;
Vietnam – Wellcome CRU ? Liaison with Zhengli Shi for Serol assays etc.?
Philippines – EHA, **CM Ltd:** Biodiversity Management Bureau, Department of Environment and Natural Resources (Anson Tagtag),

Malaysia – **CM Ltd:** Borneo Medical and Health Research Center (Ahmed Kamruddin), Department of Wildlife and National Parks Peninsular Malaysia (Frankie Sitam, Jeffrine Japning, Rahmat Topani, Kadir Hashim, Hatta Musa), Hospital Universiti Malaysia Sabah (Helen Lasimbang), Faculty of Medicine, Universiti of Malaya (Sazaly Abu Bakar), Kota Kinabalu Public Health Laboratory (Jiloris Dony, Joel Jaimin), Ministry of Health (Chong Chee Kheong, Khebir Verasahib, Maria Suleiman), National Public Health Laboratory (Hani Mat Hussin, Noorliza Noordin, Yu Kie Chem), Queen Elizabeth Hospital (Giri Rajahram, Heng Gee Lee), Sabah State Health Department (Christina Rundi), Sabah Wildlife Department (Rosa Sipangkui, Senthilvel Nathan, Augustine Tuuga, Jumrafiah Sukor),

Thailand – **CM Ltd:** Mahidol-Oxford Tropical Medicine Research Unit, (Stuart Blacksell, Richard Maude), Ministry of Public Health (Prayuth Sudathip, Hinjoy Soawapak), Faculty of Forestry, Kasetsart University (Prateep Duengkae), Chulalongkorn University (Supaporn Wacharapluesadee).

Chulalongkorn lab is a WHO Collaborating Centre for Research and Training on Viral Zoonoses, we work closely with WHO SEARO and SEARO countries including Bangladesh, Bhutan, Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, (Thailand), Timor-Leste.
We had conducted the PREDICT lab training for Veterinary lab in the Southeast Asia region for FAO.
We conducted (transferred?) the bat sampling technique for scientists from Malaysia, Myanmar, the Philippines, and China.

Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife

Commented [BRS7]: Show this somehow in the picture. Primary sites, collaborative linkages that extend the breadth of the EIDRC.

Commented [KJO8]: From Supaporn

Commented [PD9]: Remember to correct specific aims with same title

1.1 Rationale/Innovation: The vast majority of emerging zoonotic pathogens are linked directly or indirectly to wildlife host, particularly from important mammalian groups like bats, rodents and non-human primates (*2*). Our previous work on wildlife origin viruses demonstrates that zoonotic viral emergence tends to occur in specific regions with high diversity of wildlife that harbor viruses of zoonotic potential, and human populations that have close contact to these wildlife (Fig 4) (*2*). In Aim 1, we will strategically conduct EID surveillance by applying risk analytics to prioritize the regions, wildlife species, and human populations that have a particularly high risk of viral spillover. We will target high-risk zoonotic host reservoirs (bats, rodents and primates) in these regions and apply novel predictive models to estimate viral host range and identify new host species that have been previously ignored by EID surveillance systems. We will strategically collect specimens from under- or not-yet-sampled species of wildlife and screen these together with some of the tens of thousands of recently collected samples we have archived in freezers in our labs for known and novel agents. We will begin screening using conserved PCR assays to identify known and novel virus in high risk RNA viral families, *Coronaviridae*, *Paramyxoviridae*, and *Filoviridae*. We will expand to other groups of pathogens, pending available resources, after priority setting with the EIDRC coordinating center (EIDRC-CC) and other regional EIDRCs. Using risk ranking algorithms and a series of ecological and virological risk factors, we will triage the wildlife viruses we discover for further genomic characterization and viral isolation attempts. For the subset of highly-ranked and genetically characterized viruses, we will then use *in vitro* and *in vivo* approaches (viral rescue, cell lines, and mouse models) previously developed and widely use by our team [REFS?] to predict capacity of novel viruses to ~~]~~ infect people and spillover. These ~~high risk~~high-risk viruses will be targets for human community and clinical sampling in Aim 2 and 3, respectively~~fully~~. This approach is built on substantial previous proof-of concept preliminary data:

Geographic targeting: EcoHealth Alliance has led the field in conducting analyses to indicate where the highest risk of zoonotic spillover is across the globe, identifying SE Asia as a particular high risk location for pathogen emergence (*1, 2*). For Southeast Asia, these EID hotspots are sites where high wildlife biodiversity, human population density and rapid ecologic change collide **(Fig 4a)**. Using host and ICTV viral data for all known mammalian viruses, and generalized additive models to correct for sampling bias, we are able to predict the relative number of yet-to-be-described viruses that a species harbors (*2*). Using these data to map unknown viral diversity demonstrates substantial undiscovered viral diversity in mammals across Southeast Asia, and particularly in regions of Thailand, Singapore and Malaysia **(Fig. 4b)**. In a separate paper on risk of viruses emerging from bat hosts, we found that factors driving spillover (host diversity, climate) differ from those driving spread in human populations (population density, bushmeat hunting, livestock production) (*85*). We therefore analyze capacity for viral spread as a separate risk factor, using demographic and air travel data and flight model software we have produced with Dept Homeland Security funds (*86*). In the current proposed work, we will use all the above approaches to target wildlife sampling (archive and new field collections), and to inform sites for human sampling in Aim 2, to areas of high risk for spillover and spread.

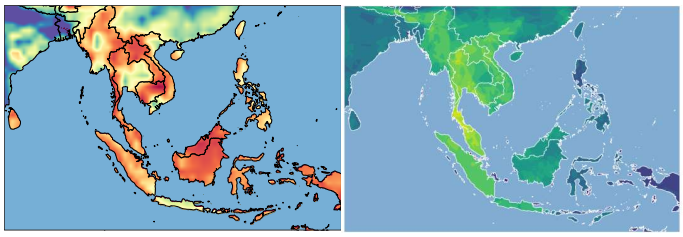


Fig. 4: Our previous work has shown proof-of-concept in geographically targeting sampling in 'hotspot' areas of highest risk for wildlife-to-human spillover and disease emergence **(Fig. 4a)**, and regions with high diversity of predicted 'missing' or as-yet undiscovered viruses, yellow = highest diversity **(Fig 4b)**. From (*1, 2*).

Reservoir host species targeting:

Our analysis of all known mammalian host-viral associations demonstrates that bats, rodents and primates represent the three most important reservoirs for zoonoses, responsible for the highest proportion of known and predicted viral diversity of zoonotic potential (*2*). The bulk of our sampling and sample testing will consist of bats, because this group is the known or hypothesized reservoir for the majority of high-impact zoonotic CoVs, henipaviruses and filoviruses (*48, 87-89*) ~~(ADD REFS)~~. Bats also have the highest proportion of predicted zoonotic viruses in any mammalian group (*2*). We have used a novel phylogeographic analysis,

Commented [KJO10]: Will we? If so, make sure we mention primates more and include in vert animal sections.

Commented [KJO11]: Do you think we need to add in or mention vectors (arthropods)? We don't have big banks of these as far as I know, so maybe best not too?

Commented [DA12R11]: We are not focusing on arboviruses, so I would leave it out

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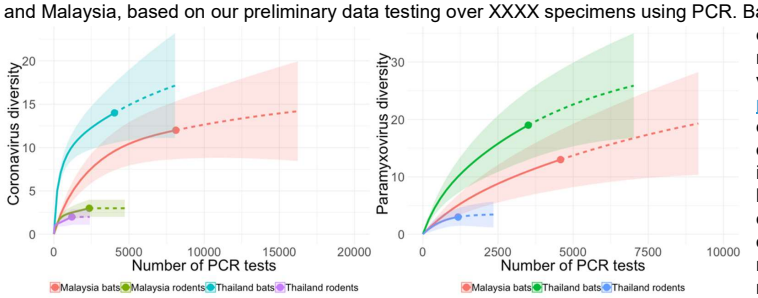
Commented [KJO14]: Peter, consider showing this figure earlier on considering Ralph's point about highlighting the EID rich geography of SE Asia first.

Commented [PD15]: Kevin to edit

Commented [BRS16]: I would say we focus on these three species (plus vectors?). To demonstrate the capability of the team, this proposal will focus on bat surveillance (for a variety of reasons), noting that this approach will also be applied to other zoonotic vectors during the 5 year program throughout the region.

Maximum Clade Credibility (MCC) tree, to reconstruct the geographic areas of evolutionary origin for β-CoVs that we sequenced in bats (*49*). This approach allows us to identify the ancestral home of specific zoonotic viral groups in wildlife, where their diversity is likely highest. Finally, we have designed a strategy to estimate sampling gaps, using a viral 'mark-recapture' approach we previously published (*90, 91*) **(Fig. 5)**. Using prior data for bat, rodent and primate viral discovery vs. sampling rates, we can identify the likely total viral diversity for specific host groups in a region, and use this to estimate sample sizes to discovering the majority of unknown viruses within a specific family or genus. **In the current proposal,** we will use these analyses to estimate geographic and species-specific sampling targets **to more effectively identify new strains to support experimental infection studies and risk assessment.**

Fig. 5: Estimates of CoV (left) and PMV paramyxoviruses (right) viral species diversity in bats and rodents for Thailand and Malaysia, based on our preliminary data testing over XXXX specimens using PCR. Bats in Thailand and Malaysia are estimated to have 4X the number of novel viruses in these viral families. CoV and ~~PMV~~ paramyxovirus diversity estimates are comparable, but discovery has not yet saturated in any taxonomic group or location. We used this to estimate that additional sampling of X,000 bats and X,XXX rodents will identify >80% of remaining CoV and ~~PMV~~ paramyxovirus viral species from key wildlife species in these regions. We will apply this approach to our target host and viral taxa, for Thailand and Malaysia to calculate sampling targets to capture maximal viral species diversity (shown), but also for estimating targets for capturing viral strain diversity.



Sample testing to identify known and novel viruses: We have conducted extensive wildlife surveillance for novel viruses across Southeast Asia in prior NIAID funded work and as part of the USAID PREDICT project. ~~.~~In the first 5 years of the PREDICT program globally (2009-2014), we found more viruses (815 novel, 169 known) than the total number of viruses previously recognized in mammals by the International Committee on Taxonomy of Viruses. This work include~~d~~s collecting samples from nearly 300,000 individual mammal specimens from 14 EHA-led PREDICT countries and PCR-screening more than 60,000 individual specimens(*51*). In southern China alone, this sampling lead to the identification of ~~.~~178 β-CoVs, of which 172 were novel (52 novel SARSr-CoVs). ~~This i~~Included were~~s~~ members of a new β-CoV clade, "lineage E" (41), diverse HKU3-related CoVs (179 sequences) within a 'sister' clade to the SARS-CoV lineage, and the wildlife origin of a new virus, ~~.~~(SADS-CoV, responsible for)~~ killing >20,000 pigs in Guangdong Province (*14*). Many of the identified~~se~~ bat species are found across the region. We have collected 28,760 samples from bats, rodents and primates in Thailand and 62,012 in Malaysia under PREDICT, conducting 146,503 PCR tests on a large proportion of these specimens, and archiving duplicates which are now available for use in this project(*92*). In Thailand this screening has identified XX. In Malaysia this screening has identified 77 novel viruses and 23 known viruses in wildlife reservoirs (66 novel and 19 known in Sabah and 11 novel and 9 known from Peninsular Malaysia). We used family level primers for henipaviruses, filoviruses and CoVs and have already discovered XX in Thailand and 13 novel and 5 known CoVs and 15 novel and 1 known ~~PMV~~ paramyxovirus in Malaysia from wildlife. In the current proposed work, we will attempt to sequence, isolate and characterize ~~the full genomes for~~ those viruses that our analyses below suggest are most likely to be able to infect humans.

Our collaborative group has worked together on these projects, as well as ~~under~~ other DTRA funded projects~~ing~~ in Singapore/Thailand (Co-Is Wacharapleusadee, Wang) to design, cross-train, and use novel serological and molecular platforms to investigate virus infection dynamics in bats and examine potential spillover events with or without human disease. For serology, our lab at Duke-NUS will ~~be~~ develop~~ing~~ a phage-display method to screen antibodies for all known and related bat-borne viruses, focusing on henipaviruses, filoviruses and coronaviruses. Once various "trends" are discovered, additional platforms (including Luminex

Commented [KJO17]: We can show just a single curve per country (aggregating all wildlife species) for simplicity, and could fit CoVs and PMVs on one graph.

Commented [DA18]: PMV is not a common abbreviation for paramyxovirus (I have not seen this in a paper apart from here). I will remove the abbreviations.

Commented [PD19]: All to edit

Commented [KJO20]: More?

Commented [KJO21]: Latest numbers from EIDITH for P1 and P2

Commented [PD22]: Kevin – need the PREDICT numbers here please

Commented [T23]: Do we want to mention other viruses we found of public health concern that are not part of the 3 viral families we are focusing on in this proposal?

and LIPS) will be used for verification and expanded surveillance. For molecular investigation, we will combine targeted PCR approach with a discovery-driven enrichment NGS strategy to not only survey currently known viruses, but also to discover potential novel viruses. Both the phage-display serological and enrichment platforms can be rapidly updated to include new viruses identified. In Malaysia (Co-Is Hughes, Broder, Laing) ~~the~~ we have ~~transfer~~transferred ~~of~~ the Luminex serology platform to partner laboratories for screening of wildlife, livestock and human samples ~~screening~~ to investigate the spillover of ~~Henipaviruses~~ henipaviruses and ~~Filoviruses~~ filoviruses at high risk interfaces. These interfaces includ~~e~~ing farms near the forest and indigenous communities with lots of subsistence hunting in Peninsular Malaysia, and preliminary screening has found serological evidence of past exposure to ~~Nipah and Ebola~~viruses antigenically-related ~~viruses~~ to Nipah and Ebola in humans, bats and, ~~NHPs~~ non-human primates (NHPs)~~and people~~. In Thailand (Co-Is Broder, Laing and Wacharapluesadee) tech transfer of Luminex-based serology platform for detecting exposure to novel Asiatic filoviruses and known henipaviruses in wildlife and human samples is underway and currently being validated in our Chulalongkorn University laboratory. This is part of a planned DoD regional center of excellence for training in Thailand (details please?).

We have found 13 novel CoVs and 15 novel ~~PMVs~~ paramyxoviruses and have begun using Illumina sequencing and virome capture sequencing to further characterize 6 ~~of these~~ novel CoVs and 10 ~~of these~~ novel paramyxoviruses~~PMVs~~. To date, we have completed the full genome sequencing for two novel CoVs that were part of the PREDICT Deep Forest project in Sabah – PREDICT_CoV-51 found in *Hipposideros cervinus* and PREDICT-CoV-52 (HKU10-like) found in *Hipposideros diadema*. As part of our PREDICT further characterization work, we will investigate the zoonotic potential of these viruses (i.e., their ability to infect human cells). We will reverse engineer the viral RNA from the genome sequence ~~we obtained~~, recover ~~the~~ infectious virus, and test its ability to infect a range of human cells. This involves generating a very large synthetic construct of the viral genome, which is extremely difficult and time consuming. Therefore, we use two strategies to investigate the risk of PREDICT CoVs and ~~PMV~~paramyxoviruses: 1) We use surrogate experimental systems to evaluate ~~just the~~ important ~~parts~~ components of the virus. In this case, we focus only on the CoV receptor binding domain (RBD) in the spike protein and glycoproteins of paramyxoviruses and filoviruses, rather than the whole virus, and ~~test whether it is able to~~determine if these regions can mediate entry into human cells. The advantage of this approach is that it can be done quickly and ~~give us~~provide a ~~good (but~~ preliminary~~)~~ assessment of the zoonotic risk. This work ~~is~~ will be done with our partners at the NIH Rocky Mountain. 2) ~~While this work is ongoing~~Concurrently, we will~~start to~~ reverse engineer the ~~full~~ complete virus ~~to, so we can~~ investigate cell-entry in the context of authentic virus. This work ~~is~~ will be done at the Center for Infection and Immunity, Mailman School of Public Health, Columbia University. ~~This can take 6-12 months to generate a construct and test it which is why we use both approaches.~~

For Peninsular Malaysia we have 1438 serum samples from Orang Asli (1390 from current study and 48 archived samples), 560 serum samples from bats, 167 serum samples from rodents and 1022 serum samples from NHPs, 301 bat biopsy samples, 123 rodent biopsy samples and 3845 NHPs biopsy samples. For Sabah we have 10 serum samples from syndromic surveillance patients, 1179 serum samples from bats, 485 serum samples from rodents and 83 serum samples from NHPs, 294 bat biopsy samples, 114 rodent biopsy samples and 62 NHP~~s~~ biopsy samples. Biopsy samples include brain, heart, lung~~s~~, liver, kidney, spleen, large and small intestine.

Commented [KJO24]: From Supaporn: I do not have this information yet, but it is possible.

Commented [L25]: Not me as I am not involved

Commented [PD26]: I think this is something that Linfa, Supaporn or Chris mentioned?

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Commented [T27]: Waiting to hear from Simon that same technique for Cov and PMV

Commented [KJO28]: More from Tom. With this comment: **Malaysian colleagues please can you add details here of any archived samples you have that could potentially be included in this proposal using similar level of detail to the above.**

In vitro & in vivo *characterization viral potential for human infection for Coronaviruses:* We have used *in vitro* and *in vivo* experiments to characterize the pathogenic potential of novel SARSr-CoVs from bats. We isolated three SARSr-CoVs from bat feces: WIV1, WIV16 and Rs4874, with S protein sequences that diverged from SARS-CoV by 3~~% to~~ 7% (*20, 58, 93*). We conducted full-length genome sequencing of 12 other novel SARSr-CoVs from a specific cave site in Yunnan China, some highly similar to outbreak SARS-CoV in the most variable genes: N-terminal domain and receptor binding domain (RBD) of the S gene, ORF8 and ORF3 (*58*). Using our reverse genetics system, we constructed chimeric viruses with SARSr-CoV WIV1 backbone and the S gene of different variants, including WIV1-Rs4231S and WIV1-Rs7327S. All 3 SARSr-CoV isolates and the two chimeric viruses replicated efficiently in Vero E6 cells and in HeLa cells expressing hACE2, but not in HeLa cells that don't express ACE2 (*20, 58, 93*) **(Fig. 5a)**. We used the SARS-CoV reverse genetics system (*70*) to generate a chimeric virus with a mouse-adapted SARS-CoV backbone expressing SHC014 S protein with 10% sequence divergence from SARS-CoV S. This chimera replicated in primary human airway epithelium, using the human ACE2 receptor to enter into cells (*59*) (**Fig. 5b**). Thus, **SARSr-CoVs with diverse variants of SL-CoV S protein without deletions in their RBD can use human ACE2 as receptor for cell entry.**

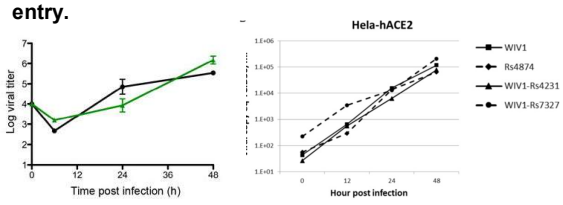

Fig. 5a (left): RT-PCR shows that bat SARSr-CoVs WIV1, Rs4874, and chimeras WIV1-Rs4231S, WIV1-Rs7327S grow in HeLa cells expressing human ACE2. **Fig. 5b (right):** Viral replication of SARS-CoV Urbani (black) and SARS-SHC014S (green) primary air-liquid interface human airway epithelial cell cultures at an MOI of 0.01.

We infected transgenic mice expressing hACE2 with 10^5 pfu of full-length recombinant WIV1 and three chimeric viruses (WIV1 backbone with SHC014S, WIV16S and Rs4231S). hACE2 transgenic mice challenged with rWIV1-SHC014S experienced ~20% body weight loss by 6dpi; rWIV1 and rWIV-4231S produced less body weight loss, and rWIV1-WIV16S led to no body weight loss **(Fig. 6a)**. At 2 and 4 dpi, viral loads in lung tissues of mice challenged with all three chimeras reached > 10^6 genome copies/g, significantly higher than rWIV1 infection **(Fig. 6b)**. This demonstrates that pathogenicity of SARSr-CoVs in humanized mice differs with divergent S proteins, **confirming the value of this model in assessing novel SARSr-CoV pathogenicity**.

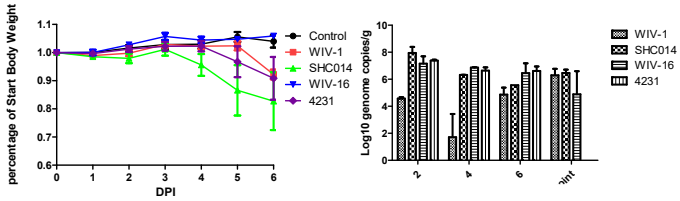

Fig. 6: *In vivo* infection of SARSr-CoVs in hACE2 transgenic mice. **6a (left)** Body weight change after infection; **6b (right)** Viral load in lung tissues.

Infection of rWIV1-SHC014S caused mild SARS-like clinical signs in the transgenic hACE2 mouse model **that weren't reduced by immune-therapeutic monoclonals that attenuate SARS-CoV ~~pathogenecity~~pathogenicity.** Vaccination against SARS-CoV did not reduce severity of clinical signs in mice subsequently infected with rSARS-SHC014S (*59*). We found 2/4 broad human mAbs against SARS-CoV RBD cross-neutralized WIV1, but none could efficiently neutralize SHC014 which is less similar to SARS-CoV in the RBD (*94*). We repeated this virus characterization approach with chimeras using HKU3r-CoV S proteins that are ~25% divergent from SARS-CoV S, **and found that they are unable to use the ACE2 receptor**. Additionally, we were unable to culture HKU3r-CoVs in Vero E6 cells, or human cell lines. **The ability of HKU3r-CoVs to infect people, and their receptor binding target, remain unknown.**

All – what can we say about Nipah and filoviruses in this section – what is the rationale for a similar approach (e.g. we're looking for viruses somewhere between Cedar and Nipah in the Henipaviruses, and trying to assess the likelihood of some of the novel filoviruses infecting human cells).

Commented [KJO29]: Think we need to shorten the CoV part of this from the previous NIH. I've added in some basic info and refs on Henipas and Filos in a separate section below (for now), but Ralph/Linfa/Dani/Chris/Eric should check and ideally we integrate into one section. WE need to specify what labs are doing what!

Commented [PD30]: Ralph, Amy – this is directly from our CoV R01 renewal – please reduce the length of this section and adapt for this proposal. We prob don't need all these figures – just one or two would do.

Commented [PD31]: Ralph, Amy, Linfa, Danielle, Chris, Eric – need details for what approach we'll use for henipas and filos

Are we just going to use cell culture for henipas and filos, or can we look at spike protein diversity?

What about using bat cell lines and batized mice from Linfa?

In vitro & in vivo *characterization viral potential for human infection for Henipaviruses and Filoviruses*
A similar approach to the above pipeline for CoVs will be applied to novel ~~Henipaviruses~~ henipaviruses and ~~Filoviruses~~ filoviruses we discover during our research.

Henipaviruses

Hendra virus and Nipah virus have a broad mammalian tropism with natural infection recorded in bats, horses, pigs, humans, cattle, goats and dogs. This broad species tropism is likely mediated by heniapvirus receptor usage of highly conserved ephrins ligands (e.g. ephrin-B2 and -B3) for cell entry. Ephrins are critical in evolutionary developmental process such as cell migration, axonal guidance and angiogenesis (REF), and eprin tissue distribution correlates with the multi-systematic cellular pathology, vasculitis and encephalitis disease presentation during HeV and NiV infection. The third isolated henipavirus is Cedar virus, yet unlike NiV/HeV, CedV does not cause pathogenesis in animal models. Recently, Dr. Broder and Laing have developed a reverse genetics system for Cedar henipavirus and have rescued a recombinant Cedar virus that is used as a model henipavirus tool to understand how ephrin receptor usage/tropism contributes to pathogenicity during henipavirus infections. Ephrin-B3 is distributed in the spinal cord, and usage of ephrin-B3 has been postulated to underlie the enhanced encephalitis seen during NiV infection. Further dissimilar from HeV/NiV, CedV is unable to utilize ephrin-B3 as a receptor (Marsh 2012; Laing 2018) nor does it express the interferon antagonizing virus factors: V and W proteins (Marsh 2012). However, Dr. Broder and Laing have discovered that CedV has a promiscuous/broad ephrin receptor usage and in addition to ephrin-B2 is able to utilize ephrins-B1, -A2 and -A5 for cell entry (Can include microscopy image/column graph for unpublished figure). Additionally, it was discovered that CedV can utilize mouse ephrin-A1, which differs from human ephrin-A1 by one amino acid residue in the key binding pocket, demonstrating the first evidence of henipavirus species-specific receptor use (Can include unpublished figure). Like ephrin-B3, ephrin-B1 is widely distributed throughout spinal cord tissues, however, CedV is non-pathogenic. Pathogenicity of henipaviruses is likely mediated by a contribution of both utility of ephrin receptors and expression of virulence factors V and W proteins. The full genome assembles of putative henipaviruses, Ghana virus and Mojiang virus, predict expression of V and W proteins. GhV is able to bind to ephrin-B2, but not -B2 (Lee – African emergent henipavirus B2 crystal) and the receptor for MoJV remains unknwon (Lee, idiosyncratic 2017), but is unlikely an ephrin. Thus, when we identify novel henipaviruses or paramyxoviruses, we will examine the receptor-binding proteins (e.g. envelope attachment (G) proteins) for sequence and structural homology with particular attention to conversation of amino acids that mediate ephrin receptor binding. We will prioritize molecular experimentation with novel henipaviruses that are predicted to interact with ephrin-B2 and expression V/W proteins to determine whether these viruses represent zoonotic threats. Using the recombinant Cedar virus (rCedV) platform, we will be able to rescue rCedV that express heterotypic G proteins to explore receptor utilization and tissue dissemination in animal models.

For novel Henipaviruses we will assess ability to infect human cells using a continuous lung epithelial cell line, Calu3, that our group has previously used to generate data on NiV and HeV growth curves (*95*). We will also use primary human airway cultures to assess human infection for Henipavirus and MERS- and SARS-related CoV (*96*) (*97*) . There is some evidence for Nipah and Hendra virus replication in mouse lung, low level titers in young animals, 10 fold higher titers in aged mice (*98, 99*). Thus it is likely these models could be improved in the collaborative cross mouse resource at UNC. We have identified CC lines that are highly vulnerable to wildtype SARS and others that develop hemorhaggic disease following mouse adapted ebola infections.

Am J Pathol. 2003 Dec;163(6):2371-82 Primary human lung endothelial cells are highly susceptible to EBOLA virus infection, so we can use these cells. Huh7 cells are good candidates for ebola in the liver, oftentimes used to isolate virus from clinical samples (Viruses. 2019 Feb 16;11(2). pii: E161.) Huh7 cells also ofers advantages for reverse genetic recovery of novel filoviruses (J Infect Dis. 2015 Oct 1;212 Suppl 2:S129-37.)

Commented [KJO32]: Think we need to shorten the CoV part above and still incorporate more for Henipas and Filos. For now some info below, but Ralph/Linfa/Dani/Chris/Eric should check and try to integrate into one section with additional info one the Cedar virus work, Mojiang, etc.. WE also need to specify what labs are doing what!

Commented [KJO33]: General question, do we want to limit to Henipas, or keep more general to Paramyxoviruses? Do we care about Rubulaviruses, etc.

Commented [KJO34]: Ralph: Am J Pathol. 2003 Dec;163(6):2371-82 Primary human lung endothelial cells are highly susceptible to EBOLA virus infection, so we can use these cells. Huh7 cells are good candidates for ebola in the liver, oftentimes used to isolate virus from clinical samples (Viruses. 2019 Feb 16;11(2). pii: E161.) Huh7 cells also ofers advantages for reverse genetic recovery of novel filoviruses (J Infect Dis. 2015 Oct 1;212 Suppl 2:S129-37.)

We have growth curves of Nipha and Hendra in Calu3 cells, a continuous lung epithelial cell, including a variety of host expression changes after infection. Likely grow in primary human airway cultures as well as they infect ciliated and secretory cells (J Gen Virol. 2016 May;97(5):1077-86.). Nipha and hendra also infect primary human microvascular endothelial cells, like mers and ebola (PMC3477106, ... [1]

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Attachment Glycoprotein has greater genetic diversity
Thus far, paramyxovirus attachment glycoproteins have been divided into three major groups: hemagglutinin (H), hemagglutinin-neuraminidase (HN), and attachment glycoproteins (G)

pon mapping sequence conservation between GhV-G and MojPV-G onto the GhV-G-ephrinB2 co-crystal structure, it becomes apparent that MojPV is unlikely to utilize ephrinB2 receptor (*100*)

Cedar uses Ephrin B, but doesn't cause disease. reverse genetics of Henipas - would need to be done in BSL4, pseudovirus can do in BSL 2, but no pathogenesis. Benhur Lee has done similar work to what Ralph has done, structural comparison w diff receptors for Cedar, Henipas, and ferret model. Doesn't express G protein and is therefore less pathogenic.

Commented [DA41]: This is not correct. Cedar expresses G. The gene responsible for the difference in pathogenesis is P. If we were using the assays described above, we would assume cedar was most likely pathogenic. I don't know how to incorporate more details until we delete some of the SARS stuff- or just leave that general paragraph above that says "the same will be don't for filo/paramyxo'

Mojiang diff cell binding: https://www.nature.com/articles/ncomms16060
" Furthermore, we find that MojV-G is antigenically distinct, indicating that MojV would less likely be detected in existing large-scale serological screening studies focused on well-established HNVs. "

1.2 General Approach:
We will conduct analyses to geographically- and taxonomically-target testing of samples from wild mammals that are most likely to harbor known high-profile zoonotic pathogens, or close relatives with potential to infect people. This includes selecting from tens of thousands of samples collected on recent projects and stored in freezers in our laboratories, and new collection from wildlife in high risk locales. We will use serological & PCR testing, to identify viruses, then attempt to isolate and biologically characterize viruses that phylogenetic and other data suggest have high spillover and pandemic potential. We will then conduct *in vitro* binding assays and cell culture experiments, and *in vivo* animal model infections to assess their potential to infect people and cause disease. EHA will lead the study design, targeted sampling, and data analysis; Co-Is Hughes and Wacharapluesadee will lead field sampling and in-country testing; Duke-NUS, UNC, USUS will lead the serological and PCR diagnostic development and the *in vitro* and *in vivo* analyses.

A key goal of this work will be technology transfer to the region, including cell culture, animal models, serological and PCR diagnostics, as well as knowledge transfer by embedding staff in partner labs and by regular meetings. This will leverage substantially from the other funding available to consortium partners.
A key goal of this work will be technology transfer to the region, including cell culture, animal models, serological and PCR diagnostics, as well as knowledge transfer by maintaining our current team members and embedding additional staff in partner labs, supporting one Malaysian PhD student and by regular meetings. This will leverage substantially from the other funding available to consortium partners. The PhD student Mei Ho Lee is part of the CM Ltd team and has worked with the collaborators on this project for 9 years. Mei Ho will enroll at the Faculty of Tropical Medicine at Mahidol University in Bangkok. The focus of her study will be to further investigate the infection potential of some of novel ~~Cov~~ CoVs and PMVs ~~viruses~~ found in Malaysia through PREDICT.

CM/EHA helped establish and manages with SWD the Wildlife Health, Genetic and Forensic Laboratory (certified as a BSL- 2 laboratory according to the US standard for laboratory specifications) that has all the equipment necessary to store samples, run extractions, PCR and analysis on biological samples for wildlife disease surveillance. The lab is used to conduct health checks on rescued and relocated wildlife before being released into new areas or sanctuaries, to screen samples for the PREDICT and Deep Forest Project and for genetic research and forensic investigations. CM/EHA also helped establish the new molecular zoonosis laboratories (certified as a BSL- 2 laboratory according to the US standard for laboratory specifications), at DWNP's National Wildlife Forensic Laboratory. The lab is used to screen samples for the PREDICT & DTRA

projects. In addition CM/EHA also has access to NPHL and KKPHL for screening human samples and the Virology lab at FVMUPM for screening Livestock samples.

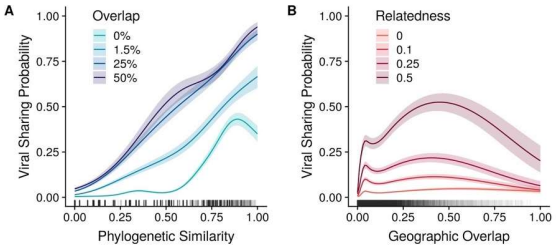
Through this project we will help to establish a certified BSL-2 lab at the BMHRC. This lab can then be used for human testing to elevate pressure at KKPHL and QEH labs. It would also be an ideal location for a Luminex platform as human, wildlife and livestock samples could all be screened here. Co-Is Hughes and Kamruddin will be starting outbreak response training in August 2019 with a team from Sabah CDC, KKPHL and BMHRC. (Dr. Maria Suleiman now in PM But still collaborating with us, Dr. Muhammad Jikal current Director CDC working with us and Dato' Dr Cristina Rundi Director Sabah state health Dept is supportive) this collaborative group is working to develop a Sabah outbreak response team that will help support SSHD/ CDC outbreak team and also conduct sampling for disease surveillance to be based at BMHRC and we will develop this as part of this proposal. In addition the UMS hospital will come in line in 2021 providing additional opportunities for cohort studies and capacity building.

1.3 Wildlife samples: 1.3.a Geographic and taxonomic targeting for newly collected wildlife samples

Adapt text from the rationale/innovation above and make brief statements about the work we'll do:

- Geographic targeting: we will use refined hotspot, 'missing viruses', and FLIRT analyses
- Targeting of host species/viral discovery targets: We will use phylogenetic MCC analyses and viral discovery curves
- Additional approaches: mapping of cave sites,

Using network analyses and a phylogeographic model of host range we recently developed [REF- Greg], we will prioritize additional wildlife species to be sampled and tested for the known and novel viruses we discover. Applying a generalizable model that includes just two phylogeographic traits (wildlife species range overlap and phylogenetic similarity between host species, Fig X.), we are able model the viral sharing network for 4200 mammal species and successfully predicted known hosts above 98% of other mammal species.



1.3.b **Sample size justifications for testing new and archived wildlife specimens**

To calculate sample sizes, we will use our preliminary data on average prevelance from screening bat, rodent and primate specimens for CoVs, PMVs, and FVs under PREDICT in Malaysia and Thailand, previously published data, and our viral discovery curve analyses (Fig 5) to determine the required samples sizes to maximize discovery of wildlife viruses with zoonotic potential.

Commented [KJO42]: From Tom.

Commented [DA43R42]: Delete the duplicate text

Commented [KJO44]: Peter, I'll clean ups this section a little more and flesh it out while you are working on this draft.

Commented [KJO45]: From Tom: We should consider wildlife sampling at –
- Danum Valley, Sabah
- Tabin Forest Reserve, Sabah
- Around Orang Asli settlements PM (focus on Kedah, Kelantan, Pahang and Perlis),
- Around Dayak settlements in Sarawak
- Kubah National Park, Sarawak
- Batu Supu Sabah low disturbance cave and surrounding area
- Madai medium disturbance cave and surrounding area
- Gomantong high disturbance cave and surrounding area
- Caves in Peninsular Malaysia

Commented [PD46]: Please check section 1.4.b and see if parts of that should be here..

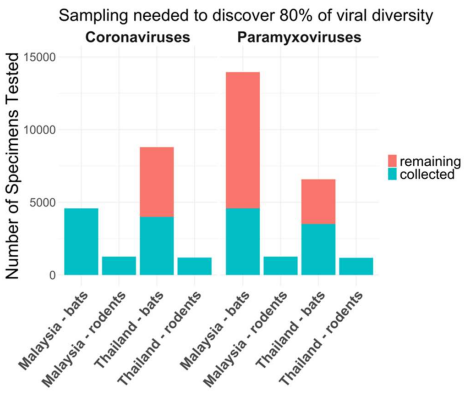


Figure X. Stacked bar plot showing number of currently-archived specimens (blue) and new specimens required (orange) to discover 80% of predicted number of CoVs and PMVs from high-risk bat and rodent taxa in Malaysia and Thailand. Estimates of predicted viral richness calculated from viral discover curve analysis (Fig 5) using data from thousands of wildlife specimens that we have previously tested and sequenced.

In Y1 we will use our bat host and viral trait modeling, phylogeographic analyses of ~~RdRp and S~~polymerase and receptor binding ~~P~~protein ~~p~~protein sequences, and geographic and host species-based viral discovery curve analyses to identify ~~SARSr-CoV~~virus diversity hotspot regions for bat sampling. We will sample at 8 new sites in four provinces. We will use cave site data (*101*), and demographic information to identify two sites in each of Yunnan, Guangxi, Guangdong, and Guizhou where humans likely have contact with bats. In Yunnan, we will identify two unsampled caves close to, but distinct from, the Jinning cave (*58*). ~~This will provide adequate coverage of lineage 1 and 2 SARSr-CoVs, including a rich source of new HKU3r-CoVs, which have unknown potential for zoonotic spillover. Sampling will begin towards the end of Y1.~~ We will use survey data from our previous R01 and host-specific viral accumulation curve data to target an additional 10 under-sampled *Rhinolophus* spp., 5 that were SARSr-CoV negative in our study, and a small number of related bat genera (including *Hipposideros* spp. and *Aselliscus* spp.) we previously found PCR positive for SARSr-CoVs **(Table 1)**. We will sample at least 5,000 bats from these 4 provinces (~1250 per province). Given ~5-12% prevalence of SARSr-CoVs in *Rhinolophus* spp. at our previous sites, **this sample size would give us 425 (±175) positive individual bats, and ~125 novel strains.**

1.3c Sample testing, viral isolation: Viral RNA will be extracted from bat fecal pellets/anal swabs with High Pure Viral RNA Kit (Roche). RNA will be aliquoted, and stored at -80C. ~~One-step hemi-nested RT-~~PCR will be performed with pan-coronavirus, filovirus and paramyxovirus primers~~(Invitrogen) will be used to detect the presence of CoV sequences using primers that target a 440-nt fragment in the RNA-dependent RNA polymerase gene (RdRp) of all known α- and β-CoVs (*102*).~~. PCR products will be gel purified and sequenced~~. with an ABI Prism 3730 DNA analyzer~~.

We will attempt isolation on samples that contain viruses of interest (determined in **1.4, below**), using Vero ~~E6~~ cells and primary cell culture from the wildlife taxonomic order (bat, rodent and primate cell lines). This includes the over ~~XX~~ 30 bat cell lines maintained at Duke-NUS from four different bat species.

1.3d Moving beyond RNA viruses: ~~Show how our platform is generziable to other~~ To detect pathogens from other families, such as ~~groups and~~ non-RNA viruses we will combine~~i.e. using~~ conserved PCR assays with NGS. We will perform either unbiased NGS, or use a pan-viral enrichment library to sequence full-length genomes. ~~sy and vircapseq~~. If and when depending necessary on EID-CC research projects and outbreaks detected in the region from DNA viruses or other agents]

1.3.e Sequencing ~~S~~ receptor binding proteins: *Our working hypothesis is that ~~many~~ zoonotic ~~SARS-like, MERS-like,~~ coronaviruses, filoviruses and henipaviruses encode ~~spike~~ receptor binding glycoproteins that ~~program~~ elicit efficient infection of primary human cells (e.g., lung, liver, etc.).* For example, our previous R01 work identified diverse SARSr-CoVs with high propensity for human infection (*15, 58, 59*). Characterization of these suggests SARSr-CoVs that are up to 10%, but not 25% different in the spike glycoprotein use human and bat ACE2 receptors for docking and entry. Uneven sampling gaps (e.g., no strains were found with 10-25% spike variation) prevent a thorough understanding of the transition point where the most divergent strains

Commented [BRS47]: CoV specific, ebola and nipha/Hendra have different spike glycoprotein designations and polymerase designations.

Commented [L48]: More generic terms to use: "Viral polymerase and receptor binding protein sequences"

Commented [BRS49]: Chinese locations? Do we have equivalent cool bat caves in malasia, Thailand, etc? to chase bat viruses in?

Commented [BRS50]: Don't forget about th eovel mers-like cov discovered in the region (PMC6002729) and that can use the human receptor (don't be so SARS centric).

Commented [DA51R50]: This section and the numbers make it too CoV specific.

Commented [KJO52]: I still need to rework this.

Commented [PD53]: Ralph, Amy, Linfa, Danielle, Chris, Eric - This is from our CoV R01. Please insert details that apply also to henipa and filoviruses.

Commented [KJO54]: Should we just rely on PREDICT PCR protocols for PMVs and Filos? We can add in methods from PREDICT protocols. Unless there are better screening assays to use?

Commented [PD55]: Brief description of these please

Commented [KJO56]: Added this b/c noted on call w Linfa.

Commented [PD57]: Ralph et al. – I'm assuming this will still be of value for the CoVs, so a reduced version of this could be in here, but what about spike proteins of filos and henipas – is that not of any use for assessing their capacity to infect human cells. Can we do similar work with filovirus pseudotypes from sequencing the spike proteins, for example, to assess binding to human receptors? If so, please draft some text and point to some references

lose human ACE2 receptor usage. Our viral discovery curves **(Fig. 4)** suggest further sampling will reveal a rich diversity of as-yet-undiscovered SARSr-CoVs. In this aim we will use phylogenetic and viral discovery analysis to specifically target bat species and regions that are undersampled to allow sufficient power to identify and characterize missing SARSr-CoV strain diversity. Our previous work also suggests that SARSr-CoVs with S proteins that are ~10% divergent from SARS-CoV resist neutralization by therapeutic mAbs and escape SARS-CoV vaccines (*20, 59, 103*), suggesting that. However, viruses with 10-25% divergence in S proteins may bind to human cell receptors, but completely evade therapeutic and vaccine effects, and could therefore be a higher risk for public health. We will sequence the S proteins of novel SARSr-CoVs to prioritize viruses for experimental work in Aim 3 to test this hypothesis. Likewise, we will test the hypothesis that phylogenetic related zoonotic spike receptor binding protein genes of MERS-relatedlike, Ebola-relatedlike and Nipha/Hendra-relatedlike viruses will also program efficient entry via human ortholog receptors.

For all novel SARSr-CoV strains, we will sequence the complete S gene by amplifying overlapping fragments using degenerate primers as shown previously (*20, 58*). Full-length genomes of selected SARSr-CoV strains (representative across subclades) will be sequenced via high throughput sequencing method followed by genome walking. The sequencing libraries will be constructed using NEBNext Ultra II DNA Library Prep Kit for Illumina and sequenced on a MiSeq sequencer. PCR will be performed to fill gaps in the genome. The full length S gene sequences, including the amount of variation in the S receptor binding residues that bind the ACE2 receptor, will be used to select strains for Aim 3 experiments.

1.4. Assessing risk for spillover. 1.4.a Capacity to infect human cells: We will use a series of cell cultures to assess the capacity of target viruses to enter human cells. For CoVs, Filovirusesfiloviruses and *Henipaviruses*henipaviruses, we will use primary human ciliated airway epithelial cells (HAE) and lung endothelial cell cultures from the lungs of transplant recipients represent highly differentiated human airway epithelium containing ciliated and non-ciliated epithelial and goblet cells, grown on an air-liquid interface for several weeks prior to use (*59, 60, 104*). We will prepare HAE and lung endothelial cultures from three different patient codes in triplicate in collaboration with the tissue procurement facility at the Cystic Fibrosis Center at UNC. Cultures will be inoculated with chimeric bat SARSr-CoVs to assess efficient replication. At 72 hpi, cultures will be fixed for immunofluorescent staining using antisera to the SARS-CoV conserved nucleocapsid protein (N) or to structural genes of the other virus strains (*61, 69*). SARSr-CoVs that differ significantly in S protein sequence (11-24%) from epidemic SARS-CoV yet replicate *in vitro*, will also be evaluated for sensitivity to neutralization in Vero cells by PRNT50 assays using broadly SARS-CoV cross reactive human mAbs S227.14, S230.15, S215.17, and S109.8 (*60, 92*). As controls or if antisera is not available, the S genes of novel SARSr-CoV or structural genes of other novel viruses under investigation will be inserted into VEE 3526 replicon vectors (VRP3526-S), packaged and used to vaccinate mice (*105*). Polyclonal sera against SARS-like viruses will be harvested and tested for ability to cross neutralize SARS-CoV, GD03, WIV-1, SHC014, WIV-16, other novel SARSr-CoV and HKU3-SRBD by PRNT50 assay (*60, 106, 107*). Using PRNT50 titers with sera (n=4 each) among these viruses, antigenic cartography (*108*) will allow comparison of antigenic vs. phylogenetic distance, identifying the transition at which SARSr-CoV strains escape SARS-CoV based vaccines, informing immunotherapeutic and vaccine design strategies (*109-111*). Similar approaches will be applied to MERS-like viruses, other CoV, filoviruses or henipaviruses

Filoviruses (primary endothelial cells – ralph has primary lung endos – see if anyone has liver primary hepatocytes. Other option are monocytes) – where will we do this? BSL4? (NEIDL). Duplicate samples in culture medium from animals PCR +ve for filoviruses will be shipped to NEIDL (See letter of support) for culture and sharing with other agencies. Check on this re. wildlife filos…See if can do training opportunities so they can work with the NEIDL – visiting scholar appointments

1.4.xx Host ACE2, DPPR, Niemann-Pick C1 (NPC1) and EphrinB2/EphrinB3 receptors: We will sequence host ACE2 receptors of different bat species or different bat populations from a single species (e.g. *Rhinolophus sinicus*) to identify relative importance of different hosts of high risk SARSr-CoVs and the *intra*specific scale of host-CoV coevolution. Of particular interest is homology across 18 bat and human

Commented [L58]: Please use XXX-related instead of XXX-like as the field does like the XXX-like term anymore although it was used ten years ago!

Commented [BRS59]: Way to specific. We should do spike genes of all novel CoV, including searching for elusive group 1 or group 2 CoV (group 2d, 2e, etc.) that have the potential to replicate in human cells (intestinal CaCO2 or lung HAE….noting that bat cov are enteric pathogens so human caco2 may be likely target). Same with filoviruses and nipha/Hendra like strains.

Commented [L60]: No live Mengla virus yet. The WIV team is trying to rescue now

Commented [BRS61]: Can we get our hands on the live Chinese ebola like strains? We could try to use reverse genetics to recover, although I personally have no documented success with filovirus reverse genetics. Am familiar with approach and the bsl4 in boston should allow recovery.

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Commented [PD62]: Ralph, Linfa, Chris – please expand and draft – I think this could be a good role for NEIDL, but don't know what the rules are…

Commented [BRS63]: Check my comments above, I covered this completely and the situation is good.

orthologue ACE2 contact interface residues that engage the SARS RBD as a potential indicator of SARSr-CoV cross species transmission potential and growth in human cells (*68*).

1.4.b Host-virus evolution and distribution: We will use Bayesian and Maximum Likelihood phylogenetic analyses of RdRp (or L genes), ~~Spike~~ receptor binding glycoproteins, and full genome sequence (when available) data to reconstruct the evolutionary history of the novel bat SARSr-CoVs, bat MERSr-CoV, filoviruses and henipaviruses that we identify. We will rerun MCC analyses **(Fig. 3)** to reconstruct β-CoV evolutionary origins using our expanded dataset. Ecological niche models will be used to predict spatial distribution of PCR-positive species, identify target sites for additional bat sampling, and analyze overlap with people. We will use our viral discovery/accumulation curve approach **(Fig. 4)** to monitor progress towards discovery of >80% of predicted diversity of SARSr-CoVs (lineage 1 & 2) or other virus families within species and sites, halting sampling when this target is reached, and freeing up resources for work other work (*91, 112*).

1.4.c Viral strain prioritization: Of the expected 100-200 novel SARSr-CoV strains, we will down-select to prioritize for further characterization based on S genes that are: i) different from SHC014, WIV1, SARS-CoV with diversity ranges of 10-25%; ii) have virus S RBD that could use human/bat receptors; iii) have recombinant chimeric spikes indicative of gene flow between clade I and II strains; iv) have bat ACE2 receptors that might select for spike RBDs that can use human receptors for entry (15/18 conserved residues in human/bat ACE2 molecules that bind SARSs-CoV S RBD domains are likely more efficient receptors than 3/18 conserved sites). Using structural models based on the SARS S glycoprotein, the extent and location of antigenic variation will be annotated onto the structure, further resolving the locations of highly evolving and conserved structural motifs/epitopes that function in protective immunity (*92, 113-115*).

1.4.d Animal models: Transgenic Mice. We will use a series of mouse models to assess spillover potential of viruses. First, the Baric lab has a well-established hACE2 transgenic mouse model that we will use this to characterize the capacity of specific SARSr-CoVs to infect. This group also has transgenic mice expressing hDPP4 receptors (PMC5578707, PMC5165197). Briefly, in BSL3, n=5 10- to 20-week old hACE2 or hDPP4 transgenic mice will be intranasally inoculated with 1 x 10^4 PFU of wildtype WIV-1 or chimeric bat SARSr-CoVs with different spike proteins or MERS-CoV and MERS-like CoV, respectively, then monitored daily for weight loss, morbidity, and clinical signs of disease. Mice will be euthanized at 2, 4, and 6 dpi (endpoint of 14 dpi), organs harvested and virus quantified by NP RT-PCR. After 7 days in formalin, tissues for histopathology will be removed from the BSL3 and stained with H&E, and for immunohistochemistry using polyclonal antibodies against the N protein. We will conduct limited evaluation of existing countermeasures using therapeutic monoclonal antibodies *in vitro* and *in vivo*. Existing SARSr-CoV or MERS-CoV mAbs (PMC4769911, PMC4547275) will be diluted in DMEM starting at 1:20, and serial 2-fold dilutions mixed with an equal volume of 50 PFU of chimeric bat SARSr-CoVs with different spike proteins, then incubated on Vero E6 cells at 37°C for 1 h, then changed to a semi-solid medium (DMEM containing 1% methylcellulose and 2% FBS). Antibody neutralization titers will be evaluated by plaque quantification at 4-5dpi. hACE2 transgenic mice will be injected with SARS-CoV mAbs, and infected with chimeric bat SARSr-CoVs. Clinical signs and morbidity will be assessed and tissue pathology examined and compared with mice without treatment of mAbs to determine the therapeutic effect on SARSr-CoV infection, and protection of SARSr-CoV by wildtype SARS-S based vaccines assessed as described (*104, 116*). We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-CoV cross neutralization, synthetically reconstruct a small subset (1-2) and evaluate the ability of nucleoside analogues to inhibit growth in HAE cultures and/or *in vivo* (*60, 104*).

Models of Outbred Populations. For novel viruses across all families, we will use the UNC collaborative cross mouse that captures 90% of the diversity within all mouse groups to assess the most suitable model for further experiments. We have used this model for CoV, filo (Ebola), Flaviviruses, and alphaviruses infections. In subpopulations it reproduces SARS weight loss, hemorrhage in EBOV, enceph in WNV, acute or chronic arthiritis in Chikungunya infection ().

Duke-NUS has developed two models for bat *in vivo* studies: a colony of *Eonycteris spelaea* (cave dwelling small fruit bat) and a bat mouse model, which contains the bat immune system in an immunodeficient mouse (*117*). Both animal systems can be used to test susceptibility, pathogenesis and immune responses of any

Commented [BRS64]: A lot of variation in the NPC1 receptor alters EBOV ability to infect in humans and bats, so this can be structurally mapped for prediction and downselection of strains (J Infect Dis. 2018 Nov 22;218(suppl_5):S397-S402.) PMC4709267
Second paper says: *We found signatures of positive selection in bat NPC1 concentrated at the virus-receptor interface, with the strongest signal at the same residue that controls EBOV infection in Eidolon helvum cells. Our work identifies NPC1 as a genetic determinant of filovirus susceptibility in bats, and suggests that some NPC1 variations reflect host adaptations to reduce filovirus replication and virulence.*

Nipha and Hendra use ephrin B2 or ephrin B3 as a receptor, and variation effects its ability to use human vs bat receptor molecules (PMC4418902, PMC2045465)

Commented [PD65]: Kevin/Ralph – I don't think this is relevant for our EIDRC – please let me know and delete if correct.

Commented [PD66]: KEvin/Alice – please modify this section for filos and henipas as well (and other hosts) - or does it belong in 1.3.a above?

Commented [BRS67]: Way to sars centric

Commented [PD68]: Ralph – can you reduce this section to a feasible amount of work for this project, and remove a bunch of text

Commented [BRS69]: We need to do this with novel Nipha/Hendra and EBoV as well.

Commented [PD70]: Ralph – please draft a brief para explaining what we'll do with this mouse model

Commented [BRS71]: Trends Genet. 2018 Oct;34(10):777-789.

Commented [BRS72]: G3 (Bethesda). 2018 Feb 2;8(2):427-445.
G3 (Bethesda). 2017 Jun 7;7(6):1653-1663.
Genom Data. 2016 Oct 14;10:137-140.
Genom Data. 2016 Oct 14;10:114-117.
PLoS Genet. 2015 Oct 9;11(10):e1005504.
Science. 2014 Nov 21;346(6212):987-91.
PLoS Pathog. 2013 Feb;9(2):e1003196.

Commented [PD73]: Linfa/Danielle – please draft a brief para explaining how we'll use either or both…

newly discovered viruses.

1.5 Potential problems/alternative approaches: Permission to sample bats in sites or provinces we select. We have a >20-year track record of successful field work in Malaysia, Thailand and >10 years in Singapore, and have worked with local authorities and communities to ensure access. We have existing permissions in place which will be renewed at the start of this project. Due to the abundance of wildlife habitats in the region, if access to a site is withdrawn, we will rapidly identify a suitable, complementary site with similar bat species composition and abundance. Finally, we have already collected tens of thousands of wildlife samples from the region and will focus on these while waiting for permission. **We may not identify novel viruses in our sample wildlife species due to seasonality of viral shedding or other factors**. We have calculated sample sizes based on a range of PCR data and are fairly conservative in our approach. However, as a back-up, we already have identified dozens of novel viruses that are near-neighbors to known high-impact agents, and will continue characterizing these should discovery efforts yield few novel viruses. The sampling regions we have selected are subtropical, and our previous data, and even published studies in temperate regions (*118*), do not suggest a strong pattern of seasonality in CoV shedding, although there are studies that suggest this occurs in henipavirus and filovirus (MARV?) shedding (REFS?). Nonetheless to account for this we will conduct sampling evenly on quarterly basis within each province.

Commented [PD74]: Kevin – please edit/check

Commented [PD75]: Kevin – please check the veracity of these comments

Aim 2: Test evidence of viral spillover in high-risk communities using novel serological assays.

Commented [KJO76]: We can make this aim all about human serology, and 3rd aim more about molecular characterization of human pathogens and outbreak etiologies.

2.1 Rationale/Innovation: Assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities that are the first to get infected. To enhance low statistical probability of identifying these rare events, populations will be targeted that both live in rural locations close to high wildlife biodiversity, and also engage in practices that enhance the risk of spillover (e.g. hunting and butchering wildlife). In Aim 2 we will expand from our current work in the region to identify and enroll large cross-sectional samples from human populations that have a high behavioral risk of exposure to wildlife origin viruses and live close to sites identified in Aim 1 as having high viral diversity in wildlife. We will initially identify X-X sites in each Thailand, Peninsular Malaysia, Sarawak, and Sabah for repeated biological sampling of high-risk human communities, design and deploy specific and sensitive serological assays to identify the baseline spillover of known or novel viral pathogens in these populations. In participants where symptoms are reported samples will be marked for additional PCR-based assays to identify presence of known and novel CoVs, henipaviruses and filoviruses, attempt to isolate and biologically characterize the pathogen, using the collaborative cross mouse (Aim 1) to identify an appropriate animal model to conduct preliminary pathogenesis work.

Commented [KJO77]: Emily: Will we be sampling participants more than once?

Commented [KJO78]: Need to decide if we do PCR on community samples, we can add in the questionnaire if you've been sick in the last 10 days and if so, then maybe test only this subset.

At the time of writing, a currently undiagnosed outbreak in the Batek Orang Asli (indigenious) population living in Gua Musang ("civet cat cave") district, in Kelantan, Malaysia has infected dozens of people and is currently being investigated by our colleagues at NPHL. The Orang Asli live in remote villages in the rainforest of Malaysia, with limited access to modern health care and extensive contact hunting, butchering, and eating wildlife as their primary source of protein. This outbreak, and others that will continue to emerge just like it, underscore the critical need for our EIDRC -- to improve the technological tools, training, and human capacity for rapid EID surveillance, diagnosis, and response at the frontlines of disease emergence in SE Asia.

Commented [KJO79]: https://en.wikipedia.org/wiki/Gua_Musang_District

Commented [KJO80]: From Tom email.

Preliminary data human biological sampling: Our longterm collaboration in the region has included identification of key at-risk human populations using previously collected qualitative and quantitative survey data, and developing and gaining access to large archives of banked human sera and other biological samples. In the last 5 years under the USAID-PREDICT project, EHA has collected 9933 and 9269 human specimens in Malaysia and Thailand, respectively, which will be available for retrospective serological testing under our proposed work. Other studies from our research team have included the collection and testing of many thousand more specimens from community cohorts. Preliminary findings and details from these data and other research from are listed below:

Commented [PD81]: All – please read and share with collaborators to flesh out and correct details here.

Peninsular Malaysia: Data from 1390 Orang Asli samples, screened by PCR for 5 viral families found: 4 known CoVs in 16 people and one known Influenza virus in one person. Serology screening is ongoing but preliminary screening as part of training government partner to use the Luminex serology platform found serological evidence of past exposure to Nipah and Ebola antigenically-related viruses in people.

Sabah: Data from 10 Syndromic Surveillance samples, screened by PCR for 5 viral families found: 1 known virus - Human Parainfluenzavirus 2 (GenBank Accession No.AF533011) in the family Paramxyoviruses. Serology screening is yet to start.

Kamruddin (UMS Borneo Medical Health Research Center) has identified some high risk communities.

Tim, Giri, Tsin Wen has archived samples from Kudat Monkey bar project – 2,000 human sera already screened for bacteria and parasites, we've been reached out to about this. Data on macaque tracking data/human tracking data also. Encephalitis study etc Dr. Yeo Tisn Wen, DUKE-NUS, Singapore – Has worked with Giri and tim on knowlesi work with communities – 2000 selected villagers 500micros looking for asymptomatic parasitemias – led by King Fonese from LSTMH – 10,000 people – archived serum samples (prob 2-3,000 – in London, KK)

Sarawak: Dr Tan (Cheng Sieng) Center for Tropical and Emerging Diseases, Faculty of Medicine at UNIMAS – high risk Dayak communities that he's identified. We will do further sampling, questionnaires etc. Has Prelim data on HPV work in Dayak communities in remote Sarawak – we can work with those communities and can reach many by car, others by 4WD or boats. Has also Enterovirus 71 paper and is interested in CoVs. Has access to caves and guano. Will look into what other archived samples he has. Will give us access to Sarawak Forestry and Health Depts.

Thailand: We have conducted longitudinal community-based surveillance for the last 5 years in two villages (Chonburi and Ratchaburi provinces) at high risk of infection from bats. In Chonburi province we sampled and tested over 300 villagers living around the largest population of flying foxes (Pteropus lylei), at a temple (Wat) roosting site where we have seasonal detections of Nipah virus from bat urine each year (with viral shedding peaking in May-July. Serological screening of monks and We also have sampled and tested over 300 villages from Ratchaburi province, including a population of bat guano miners, and workers from Department of National Parks – both of whom have extensive contact with wildlife in the area. We previously detected a MERS-like CoV in the bat guano being harvested (*48*). Molecular screening of guano miners identified human CoV, HKU1 strain, with high viral load in one healthy bat guano miner and our team was able to obtain the whole genome sequence from a clinical specimen from this individual (*119*). No evidence of novel bat CoVs have yet been detected in these populations.

US CDC (Thailand): A large cohort of children from the Kamphaeng Phet Province, Thailand were enrolled as part of a multi-year study (1998-2002) to examine the epidemiology of dengue virus (DENV) and Japanese encephalitis virus (JEV) (*120, 121*). From 2,574 paired serum samples, 784 (30%) convalescent sera were selected at random among samples drawn between 1998-2002. Among the 784 samples tested, subjects ranged in age from 6 to16 years (mean 9 years) and 51% were male. Henipavirus-specific antibodies were detected in 13 serum samples (1.7% seroprevalence) by Enzyme-linked Immunosorbent Assay (ELISA) using a soluble highly-native recombinant G glycoprotein (sG_{NiV} and sG_{HeV}) capture antigens. The ELISA endpoint titers for sG_{NiV} reactive samples ranged from 1:20 to 1:1280, with six of the thirteen samples exhibiting a titer of 1:640 or greater. Although none of the samples inhibited NiV or HeV infection *in* vitro, 3 of the 13 ELISA sero-positive patient serum samples reacted with denatured G antigen in Western blot. The presence of henipavirus-specific antibody responses suggests prior exposure to endemic henipaviruses or a henipa-like virus in rural Thailand. The

Commented [T82]: Unpublished

Commented [KJO83]: Previous text from PD: We have ~25 new CoVs in Sabah. Simon is using HTS to further characterize and Tom will get the data for this proposal from Simon

Commented [T84]: Malaysian colleagues please can you add details here of any human sampling and findings from those studies that are relevant to this proposal

Commented [T85]: These are from wildlife so I have moved to "Identify Known and novel" section above

Commented [KJO86]: From PD: Kamruddin (UMS Borneo Medical Health Research Center) has identified some high risk communities. We can help with them and build out the BSL-2 lab, as well as work on outbreak response within DHRU (in collab with Sabah CDC?).

Commented [KJO87]: From Tom

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presence of pre-existing henipavirus antibodies among these children in Thailand suggest that ~~"background"~~ henipavirus infections do occur and were self-limited illness without serious health impacts.

Singapore: ~~No a~~Active human surveillance will be not be conducted in Singapore, but archived human specimens (~~REFS,~~ e.g. Melaka virus serosurvey) will be made available to conduct retrospective serosurveys for specific pathogens that are identified as high interest to the EIDRC, NIH, and EID-CC.

Human Contact Risk Factors: EHA is the lead global organization in the USAID-PREDICT project for characterizing the human aspects of zoonotic spillover risk. The approach began by conducting exploratory studies using standardized one-on-one semi-structured interviews and site observation data with people living in hotspot areas or engaged in high-zoonotic spillover risk activities (e.g. we interviewed 88 people involved in trading wild bats in China – REF). This allowed us to assess the local social and cultural norms and individual attitudes underlying wildlife contact. We used these study findings to develop a risk survey to accompany human biological samples to evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. We conducted cross-sectional studies in high risk populations in >15 countries during the last 5 years, and study participants provided biological samples (oral/oropharyngeal swab, nasal/nasopharyngeal swab, urine sample/urogenital swab, fecal sample/rectal swab, whole blood, serum, plasma). Survey and biological samples were collected from 1,585 participants from 7 sites in China in Yunnan, Guangxi, and Guangdong provinces, 1,400 participants from 4 sites in Malaysia and 678 participants from 4 sites in Thailand. During this study serological assays were developed for implementation in China, and survey responses and demographic data suggest specific risk factors included type of occupation, keeping of pets and visiting wet markets. Seropositive individuals were mostly farmers/peasants (8/9), living in Yunnan province (7/9), 41-60 yrs old (7/9), with domestic contact with rodents (6/9), and are male (6/9). However, these characteristics were common and occurred in ~40% of survey respondents. Although these results are preliminary and don't provide detailed information on routes of exposure, they suggest that further refining of tools for serological tests coupled with survey data will identify likely routes of exposure to CoVs, novel CoVs, Henipavirus, and Filoviruses in our expanded study. The results of these surveys will be used in Aim 2 of this EIDRC proposal to better identify target populations and in Aim 2, we identify strategies to better target at-risk people, and conduct focused **human animal contact surveys and serosurveys to produce statistically significant findings for henipaviruses, CoVs and filoviruses.**

~~*Behavioral risk*~~*: Clinical meta-data to assess human-animal contact:* EHA is the global lead organization in the USAID-PREDICT project for assessing the risk of zoonotic spillover, including extensive behavioral surveys in high-risk populations under this award. Our general approach has been to conduct qualitative exploratory studies using standardized one-on-one semi-structured ethnographic interviews and observational data in among people engaged in clearly high-zoonotic risk activities (e.g. we interviewed 88 people involved in trading wild bats in China – REF). This allows us to assess local social and cultural norms and individual attitudes underlying wildlife contact. We used qualitative study findings to develop a human behavioral risk questionnaire on the type and frequency of animal contact, wildlife observed in daily life, and unusual illnesses reported over the past 12 months. Under our USAID-PREDICT project, we conducted cross-sectional studies in high risk populations in >15 countries during the last 5 years. In total, we have enrolled XX,XXX participants who have provided serum, nasopharyngeal, oral, and fecal samples and each completed behavioral risk questionnaires to assess animal contact. Questionnaires and biological samples were collected from 1390 participants from 9 sites in the Districts of Gua Musang, Kuala Lipis and Kuala Kangsar Malaysia, 10 syndromic surveillance patients at the Queen Elizabeth Hospital in Sabah, and 673 people from 3 sites in Thailand. The results of these surveys will be used in Aim 2 to better identify target populations.

Serological Evidence of Exposure: In China, we developed serological assays for HKU9 CoVs (β), SARSr-CoV Rp3 (β), HKU10 CoV (α), and MERS-CoV (β) and used ELISA and Western blot to test serum samples collected in 2016/17. **We found 7 individuals (7/733, 0.95%) living within a 6 km radius of the Jinning Cave, and 6/209 people (2.87%) at one site, with evidence of exposure to bat SARSr-CoVs.** We found

Commented [PD88]: Linfa, Danielle – are we going to do human sampling in high risk communities in Singapore? If so, what would you propose and do you already have some prelim work you've done. Note these are high risk populations, i.e. high exposure to wildlife. Clinical cohorts are in Aim 3

Commented [DA89]: PMID: 30411364

Commented [PD90]: Hongying/Emily to edit

Commented [EH91]: For the survey and biological data collection we worked with 27 countries an managed 11

For Qualitative work we oversaw 13 countries

Commented [PD92]: Hongying/Emily – any information from Thailand or Malaysian surveys?

Commented [KJO93]: Edits from Emily.

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Commented [KJO95]: Make sure to delete most reference to "Behavioral" research throughout, but okay to do some surveys to collect "clinical meta-data".

Commented [KJO96]: Sum up for ALL countries?

Commented [KJO97]: Same as paragraph above, but including my and Tom's edits.

evidence among human populations in Guangxi Province of people with prior exposure to the bat α-CoV HKU10 (2/412, 0.48%). This is of potential public health interest because HKU10 is known to be able to jump host species within bats, and therefore may have high propensity for emergence (*122*). However, the low seroprevalence (0.6%-2.7% at positive sites) suggests we need a larger sample size, and a focused, targeted study to maximize the likelihood of identifying seropositive cases. In Aim 2, we will use combined biological-behavioral risk surveillance in targeted populations within the community and clinical settings to 1) identify risk factors correlated with seropositivity (exposure to) and PCR positive status (infection with) henipaviruses, filoviruses and CoVs; and 2) assess possible health effects of infection in people. Obtaining this information could be a significant step in understanding the likelihood of recent 'hidden' spillover events and their public health impacts, as well as the risk of future emergence of viral disease.

Risk Factors: In China, questionnaire response and demographic data suggest specific risk factors included type of occupation, keeping of pets and visiting wet markets. Seropositive individuals were mostly farmers/peasants (8/9), living in Yunnan province (7/9), 41-60 yrs old (7/9), with domestic contact with rodents (6/9), and are male (6/9). However, these characteristics were common and occurred in ~40% of survey respondents. Although these results are preliminary and don't provide detailed information on routes of exposure, they suggest that further refining use serological tests coupled with qualitative and questionnaire data will identify likely routes of exposure to novel CoVs in China. In Aim 2 of this EIDRC proposal, we identify strategies to better target at-risk people, and conduct focused **questionnaires and serosurveys to produce statistically significant findings for henipaviruses, CoVs and filoviruses.**

Commented [PD98]: Hongying/Emily – any information from Thailand or Malaysian surveys?

2.2 General Approach/Innovation: We will use a dual study design to gain in depth understanding of exposure and risk factors for viral spillover **(Fig. XX)**. In Aim 2 we will conduct community-based surveillance with a focused questionnaire to identify the prevalence and frequency of risk-factors for viral spillover in these communities in tandem with biological sampling to determine the seroprevalence of spillover viruses in at-risk human populations. We will use generalized linear models to analyze correlation between biological results and risk-factors for viral spillover. In Aim 3, we will conduct clinic-based syndromic surveillance and biological sampling at clinics/hospitals that are the catchment healthcare facilities for the community-based surveillance sites of Aim 2 (details in Aim 3). Both community-based and clinic-based syndromic surveillance programs are cross sectional case-control studies designed with the sample sizes necessary to statistically quantify (with a power of 80%) risk factors and health impacts for viral spillover, linked to serological/PCR status and symptoms.

Commented [KJO99]: ?

2.3 Target population & sample sizes: We will target sites in the same geographic regions as those identified in Aim 1 that harbor wildlife with high potential zoonotic viral diversity, in regions that are EID hotspots, and are well-connected to regional travel and trade hubs and the global travel network (Fig X). We will target specific communities based on our analysis of the previously collected PREDICT behavioral questionnaire data to assess key at-risk populations and occupations with high exposure to wildlife, as well as using other publicly available data (e.g. wildlife hunting licenses sold in a region, number of guano caves accessed for fertilizer etc.). We will also expand on work currently underway in the following particularly high exposure populations:

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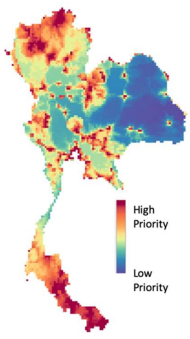


Fig X. *Preliminary analysis of geographic sites to target for human zoonotic disease surveillance in Thailand. Novel spatial analysis optimizes site selection by ranking areas with high numbers of expected viruses in wildlife and greater human access to these sites using road density data.*

Commented [KJO101]: Think we need to be strategic and just pick a couple sites per country, so it doesn't look too ambitious given budget.

Thailand: In 2018 We have conducted behavioral risk surveys and biological sampling in 117 guano miners and the communities in Ratchaburi province where MERS like CoV has been found from dry bat guano and bat rectal swab during the PREDICT study. These workers visit bat caves and dig fecal material to use as fertilizer, and are therefore highly exposed to bats and rodents in particular. At Khao Chong Pran cave in Ratchaburi we have detected and characterized novel Alpha CoVs, HKU1, and MERS like CoV (short sequence)

((*48, 119*) and Chulalongkorn, unpublished). As an initial project on our EIDRC, we will develop serology assays to the novel CoVs found in bats at this site using LIPS assays and viral genomic data, and screen archived specimens from 230 participants (2 years' collection in 2017-2018, a subset of the subjects have been sampled at multiple timepoints to allow us to assess seroconversion). Clinical syndromic surveillance at the district hospital will be conducted from the patient with respiratory symptom, the known viral and bacterial pathogens will be tested by real-time PCR commercial kit (the test result will be provided to the hospital within 48 hours for treatment), along with PCR for 3 viral family and serology for CoV found from bat in this area.

The second site is at Chonburi province where Nipah virus (99% identity of whole genome sequence to NiV from Bangladesh patient) has been found from Lylei flying foxes but no infected patient been reported. Several novel Paramyxovirus and CoV have been found from bat feces roosting here. Screening using serology assays for a panel of Henipaviruses is ongoing via our existing collaborations with USUS's Luminex platform, and will be expanded under our EIDRC. The clinical syndromic surveillance at the district hospital will be conducted from the patient with encephalitis and respiratory symptom, the known viral and bacterial pathogens will be tested by real-time PCR commercial kit (the test result will be provided to the hospital within 48 hours for treatment), along with PCR for 3 viral family and serology for NiV, novel CoV, novel PmV found from bat in this area.

The serology platform based on the sequence from bat need to be develop and used as an antigen for serology testing. HKU1 full genome sequence from guano miner (*119*)

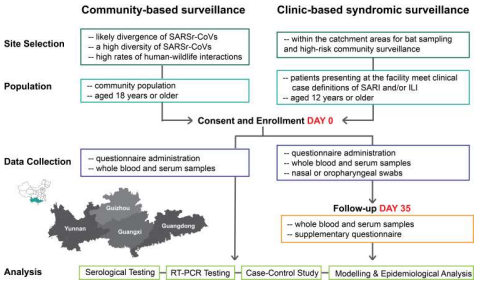
Peninsular Malaysia: We have sampled Orang Asli populations during 2016 – 2018 in 3 districts, enrolling 1390 people. We will expand this to include additional communities in the Districts we have already sampled, additional Districts in the States of Kelantan Perak and Pahang and also Districts in the State of Kelantan. The Ministry of Health, Department of Wildlife and National Parks Peninsular Malaysia and Department of Veterinary Services are all supportive of this expansion and the continued surveillance and capacity building that this will entail.

Sarawak: Dr Tan (Cheng Sieng) Center for Tropical and Emerging Diseases, Faculty of Medicine at UNIMAS – high risk Dayak communities that he's identified. We will do further sampling, questionnaires etc. Has Prelim data on HPV work in Dayak communities in remote Sarawak – we can work with those communities and can reach many by car, others by 4WD or boats. Has also Enterovirus 71 paper and is interested in CoVs. Has access to caves and guano. Will look into what other archived samples he has. Will give us access to Sarawak Forestry and Health Depts.

Sabah: Queen Elizabeth hospital syndromic surveillance, UMS Hospital syndromic surveillance, BMHRC human sampling outbreak response

Singapore: No human sampling

Community-based surveillance will be conducted at XX sites in each country, a total of xx sites. From our previous work we anticipate that 10-30% of the community population will have had exposure to wildlife allowing us to capture highly exposed and non-exposure individuals at each site. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock farming, transportation, selling, or slaughtering wildlife in the surveyed areas will be targeted so that they make up ≥30% of the sampled population in each community. We will stratify sampling to ensure appropriate representation of sex, demographic, and socio-economic factors at each community site. Estimating 5% overall seroprevalence in these high-exposure populations, these sample sizes are sufficient to estimate effect sizes of risk factors of 2X or greater with 80% power. For community-based surveillance, we will enroll xxxx individuals per



Commented [KJO102]: LIPS?

Commented [S103]: planned clinical surveillance work at 2 sites

Commented [KJO104]: From Supaponr, should this be moved to Aim 3?

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Commented [PD107]: Tom – please start writing some plans for what we csould do.

county/province, pooled across two sites for each, allowing us to make county/province-level comparisons of differing effects.

Fig. XX: Human survey and sampling study design overview (Aim 2 and 3)

2.4 Data & sample collection: Following enrollment of eligible participants and completion of the consent procedure, biological specimens (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples) will be collected and a questionnaire will be administered by trained project staff that speak appropriate local language to all participants. Through the serological findings and the survey we will investigate the details of five risk factors related to high risk wildlife exposure, to maximize the power of the analyses, based on continuing analysis of our previous work, and will focus on: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With additional consent considerations for participants enrolled from clinical settings we will review clinical records to collect recent data on medical history, clinical syndromes, and patient etiology.

2.5: Laboratory analysis: 2.5.a Serological testing:

One of the present challenges in emerging disease surveillance is that viremia can be short-lived, complicating viral-RNA detection (i.e. traditional PCR-based approaches), especially in zoonotic reservoir hosts, and requiring large samples sizes to understand infection patterns or host range. In contrast, antibodies are typically long-lived and can be used to assess prior virus exposure or infection with much smaller samples sizes . However, most serological assays only target a single protein, and it's not always clear which antigen is the most immunodominant during an adaptive humoral immune response – i.e. which is the best target for a serological assay. Henipaviruses, filovirus and CoVs express envelope receptor-binding proteins (e.g. CoV=spike (S) protein, filoviruses/henipaviruses=glycoprotein (GP/G)), which are the target of protective antibody responses. The Broder lab at USU, a leader in the expression and purification of native-like virus surface proteins for immunoassay application (Bossart 2008), developing monoclonal antibodies (Zhu 2008, Bossart 2009) and as subunit vaccines (Bossart 2012, Mire 2014), is presently developing a novel, multiplex microsphere immunoassay (MMIA) allowing serum samples to be simultaneously tested for antibodies reactive with henipaviruses, filoviruses and coronaviruses (Table XX).

This MMIA uses a Luminex-based multiplex platform and custom designed virus receptor-binding proteins expressed in a mammalian cell-culture system and purified as native-like oligomeric proteins (Bossart 2008, Chan YP 2009). These oligomeric antigens retain post-translational modifications and quaternary structures. This structural advantage over peptide antigens, allows the capture of conformational-dependent antibodies raised during a natural infection, which represent the majority of the protective humoral response.

Serological platforms have been historically limited by the starting antigen, and cross-reactivity between known and unknown related viruses not included in the assay. The known diversity of henipaviruses includes five presently described viruses, NiV, HeV, CedV, GhV and MojV and this assay has been expanded to include all described species (Preliminary Figure A). In the past, our collaborators have used NiV and HeV G protein antigens in a MMIA assay to identify a serological footprint of NiV-like African henipaviruses and exposure to virus(es) antigenically-related to NiV in livestock sampled in Bangladesh (Peel 2012, Chowdury 2014). To strengthen serological data interpretation, the G protein from each virus is integrated into the MMIA so that virus-specific versus henipavirus cross-reactive antibodies can be detected and address specificity of past virus exposure. Extensive cross-reactivity exists within ebolaviruses and between ebolaviruses and marburgviruses, highlighted by studies examining antibody binding between heterologous viruses (Shuh 2019, MacNeill 2011, Natesan 2016). Ongoing work is aimed at validating the MMIA pan-filovirus assay for specificity (Preliminary Figure B). The majority of ebolaviruses are endemic to Africa, however the discovery of Mengla virus in Yunnan, China increased the further demonstrated that a diversity of Asiatic filoviruses with unknown

Commented [PD108]: All – not sure if we should have one figure for community and clinical cohort surveillance (aim 2 and 3 respectively) or if we should have two separate ones?

Commented [PD109]: Hongying– this figure is from our CoV R01 renewal. Please modify to make relevant to SE Asia and henipas, filos and CoVs.

Commented [PD110]: Emily/hongying

Commented [EH111]: We will need to take more than this from people as there are not vacutainers that small. Also do we want to use a serum separator tube specifically?

For 1mL of serum we will need 2.5mL of blood plus whole blood.

Commented [PD112]: Chris, Eric, Linfa, Dani etc. please draft some language here…

Commented [EL113]: Nations, F. a. A. O. o. t. U. in *FAO Animal Production and Health Manual* Vol. No. 12 (ed H.E. Field S.H. Newman, C.E. de Jong and J.H. Epstein.) (Rome, 2011).

pathogenicity and zoonotic potential exists. Our past work in collaboration with Dr. Smith and Mendenhall at Duke-NUS demonstrated that three under sampled fruit bat species had serological evidence of past exposure to a filovirus(es) most antigenically-related to ebolaviruses (e.g. Ebola virus, Bundibugyo virus), but unreactive with Reston virus GP, suggesting that novel filoviruses circulate within bat hosts (Laing 2018).

The below is from our previous CoV proposal:

In our previous R01, we expressed his-tagged nucleocapsid protein (NP) of SARSr-CoV Rp3 in *E.coli* and developed a SARSr-CoV specific ELISA for serosurveillance using the purified Rp3 NP. The specificity of this assay was evaluated using polyclonal antibodies against HKU1, OC43, 229E, NL63, MERS-CoV and EBOV and no significant cross-activity was detected (*15*). **While this shows it is a specific test for Rp3, it suggests that if we can expand our serology tests to cover other bat CoVs, we may identify many more seropositive individuals.** In this renewal, we will therefore use two serological testing approaches. First, we will expand test all human sera collected from both community- and clinic- based sampling for a panel of bat CoVs that will include SARS-CoV (outbreak strain), a range of lineage 2 SARSr-CoVs (including WIV1 and SHC014), lineage 1 HKU3r-CoVs, MERS-CoV, and the α-CoVs SADS-CoV and HKU10. We previously found serological evidence of human exposure to HKU10 (*15*), but HKU10 is known to jump from one host bat species to another (*122*) and is therefore likely to have infected people more widely. Incoporating serological testing for SHC014 is also likely to yield higher seroprevalence because it is readily divergent from SARS-CoV wildtype and therefore unlikely to have been picked up in our earlier testing. It is possible that non-neutralizing cross reactive epitopes exist that afford an accurate measure of cross reactivity between clade1 and clade 2 strains which would allow us to target exposure to strains of 15-25% divergence from SARS-CoV. Additionally, we will test samples for antibodies to common human CoVs (HCoV NL63, OC43 – see potential pitfalls/solutions below). Secondly, we recognize that CoVs have a high propensity to recombine. To serologically target 'novel' recombinant virus exposure, we will conduct 1) ELISA screening with SARSr-CoV S or RBD; 2) confirm these results by Western blot; then 3) use NP based ELISA and LIPS assays with a diversity of SARSr-CoV NP. For NP ELISA, micro-titer plates will be coated with 100 ng/well of recombinant batCoV NP and incubated with human sera in duplicates followed by detection with HRP labeled goat anti-human IgG antibodies. For confirmation, all ELISA positive samples will be subjected to Western blot at a dilution of 1:100 by using batCoV-NP as antigen. We will use an S protein-based ELISA to distinguish the lineage of SARSr-CoV. As new SARSr-CoVs are discovered, we will rapidly design specific Luciferase immunoprecipitation system (LIPS) assays targeting the S1 genes of bat-CoV strains for follow-up serological surveillance, as per our previous work (*14*).

2.5.b RT-PCR testing. Specimens will be screened using RT-PCR for the RdRp gene (See section 1.3.b for details). Positive samples will be subjected to full genome sequencing and RT-PCR amplification of the S glycoprotein gene. Samples from the clinic-based syndromic surveillance will also be tested using RT-PCR for Influenza A & B, HCoV NL63, OC43, HKU1, SARS-CoV & 229E as rule-outs, and SADS-CoV and HKU10 as an opportunistic survey for potential spillover these CoVs as proposed above.

2.6 Epidemiological analysis: We will conduct a cross sectional case-control study to identify risk factors for Henipavirus, Filoviruses, and CoVs spillover. "Cases" are defined as participants whose samples tested positive for Henipavirus, Filoviruses, or CoVs by serological tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative for Henipavirus, Filoviruses, or CoVs. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between biological results, serological/PCR status, and risk factors related to high risk wildlife exposure including: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) home or work proximity to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms lifetime and past 12mo. Additionally, we will use this procedure to determine how clinical presentation differs between Henipavirus, Filoviruses, or CoVs in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms.

2.2.2 Behavioral risks among population: Qualitative and quantitative human study have conducted among

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29260678

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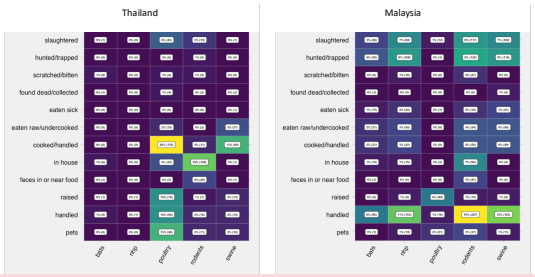
xxxx residents at xxxx community sites in Thailand and Malaysia, revealing the frequent contact with both wild (e.g., bats, rodents, non-human primates) and domestic animals (e.g., poultry, swine) among local communities (**Figure X**), which are associated with a high percentage of self-reported severe/acute respiratory illness (SARI/ARI) and Influenza-like illness (ILI) symptoms.



Figures of LASSO ILI/SARI analysis

Figure X High-risk human animal contact among community residents in Thailand and Malaysia

In addition, people making a living on crops production, engage in practices that enhance the risk of spillover (e.g. hunting and butchering wildlife) are at the risk of exposure and reported SARI and ILI symptoms (**Figure X – need a figure for this**). **These preliminary data suggest a target population and sites for current proposal with the correct targets for serological surveillance and risk factor surveys in Aim 2 to produce statistically significant findings**. Coupled with further refining serological tools, we will identify the likely routes of exposure to known or novel coronaviruses, henipaviruses, and filoviruses in the study region.

2.7 Biological characterization of viruses identified: XXX

2.8 Potential problems/alternative approaches: Rarity of spillover events means it may be difficult to identify sufficient seropositives to statistically analyze human risk profiles. First, we will be targeting our community-based surveillance to subpopulations with high-levels of wildlife exposure, at sites selected for diverse and prevalent viruses to increase likelihood of finding positive individuals. Second, our serology testing will include a panel of assays for a large diversity of viruses, increasing likelihood of positives. For example, in previous work our overall bat CoV PCR prevalence in the community was, 11.8%; β-CoV, 3.4%; α-CoV, 9.1%). Thus, using this broad serological panel to screen individuals prone to contact with species of higher zoonotic risk increases the potential for detecting spillover with enough power for statistical analyses, and will shed light on behaviors that predispose to spillover. Third, we will include common human CoVs, paramyxoviruses in our panel, so that even if low prevalence of wildlife viruses is found, we will be able to conduct a valuable cross-sectional study of the seroprevalence of more common human viruses. Finally, we will be able to assess relative measures of human-wildlife contact from our survey work. We will analyze intensity of contact with high-risk taxa against other risk factors to provide useful proxy information for spillover risk. **Serological testing may not match known CoVs due to recombination events.** We will use the three-tiered serological testing system outline in **2.6.a** to try to identify these 'novel' CoVs, however, we will also remain flexible on interpretation of data to ensure we account for recombination.

Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts.

3.1 Rationale/Innovation: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent is not diagnosed. By working directly with individuals reporting to clinics with symptoms similar to known high-impact viral agents, we may be able to capture novel emerging diseases in at-risk communities before they spread into the general population and risk becoming pandemic. This will have clear value for public health in the region, and potentially wider. To do this, we will expand on current syndromic surveillance activities to enroll, and collect high quality biological samples from patients who live in

Commented [KJO117]: From Hongying and Emily, may go in diff section for prelim behavioral data. Showing freq and type of animal contact based on Thailand and Malayisa P2 survery data.

Commented [PD118]: All –Not sure if we should weigh too heavily on this for the community survey – or should it just be serology for Aim 2. If you want include this section, please modify and insert language from Aim 1 here.

Commented [KJO119]: I think Aim 2 should just be serology, esp. since developing new LIPS assays or applying some of the serochip stuff from Linfa and Luminex from USU is already a lot.

Commented [KJO120]: Make this more general. There's a greater challenge of interpretation of serological data, esp. if we're using many platforms (each will have its own problems that our lab colleagues are probably more familiar with). Noam may be able to generally comment on a solution here from a statistical perspective.

rural communities linked to Aim 2 and present at regional clinics or hospitals with symptoms typical of high impact zoonotic viruses. We will collect detailed survey data to assess their contact type and frequency with wildlife and livestock and conduct molecular and serological diagnostic assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. We will enroll and conduct novel viral discovery assays on patients who present with unique clinical signs or relevant symptomatic cases. If novel pathogens are identified by PCR, we will attempt to isolate, obtain full genome sequence and/or biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work.

Preliminary data clinical surveillance: Our longterm collaboration in the region has included the following activities investigating clinical syndromes:

Singapore: In collaboration with EcoHealth Alliance and colleagues from the Wuhan ~~Virological~~ Institute of Virology in China, we have ~~been~~ developeding novel CoV assays for clinical surveillance. Our pipeline of novel virus detection, reservoir host identification, serological assay development, and serological testing has been optimized and streamlined to be as rapid as several months, with the development of novel serological assays from sequence data in 7 days [REF]. For example, with the discovery of SADS-CoV, we identified and ~~and~~ fully ~~genome~~ sequenced a new virus, screened archived wildlife specimens to identify the natural reservoir host species (Rhinolophus spp. bats), developed LIPS serological assays, and tested human and swine samples to assess seroprevelance in less than 3 months (*14*). [Maybe a timeline figure too]?

~~ADD Lancet ID paper on Zika sequencing by Dani and Duke-NUS team.~~

Following the detection of Zika virus in Singapore in 2016, we reported the first comprehensive analysis of the outbreak . Clinical and epidemiological data were collected from patients with confirmed Zika virus infection and mosquitoes were captured from areas with Zika disease clusters to assess which species were present, their breeding numbers, and to test for Zika virus. Mosquito virus strains were compared with human strains through phylogenetic analysis after full genome sequencing using our enrichment NGS protocol . We extended our studies into non-human primate models to assess the impact of flavivirus cross-reactivity in assays utilized to detect Zika virus, an important factor during outbreak of surveillance.

Thailand: The TRC-EID laboratory is a leader in rapid detection of human clinical specimens using broadly reactive and specific assays and in-house sequencing technology. For example, in coordination with the Thailand Ministry of Health, we identified the first case of imported MERS-CoV in Thailand using sequence confirmation within 24 hours from acquiring the specimen (Fig X).

Three imported MERS cases ~~were~~ have been detected in Thailand since 2015, and all cases were confirmed at Chula lab. More than 500 Zika patients were tested positive by PCR and sequence confirmation. The specimens from suspected Ebola travelers were tested negative, PREDICT filovirus protocol has been used as an additional protocol further investigation. The syndromic surveillance has been conducted and tested at Chula lab including SARI, Encephalitis, Hand Foot and Mouth disease in children and Viral diarrhea. These are research studies supported by PREDICT, US DoD and Thai government grant and the routine surveillance by the Thai Bureau of Epidemiology.

Commented [KJO121]: ?

Commented [PD122]: All – please read and share with collaborators to flesh out and correct details here.

Commented [KJO123]: Per Linfa's comments, we should highlight the work from SADS-COV from new virus detection, to assay development, to serological testing in X months.

Commented [KJO124]: From Linfa and Dani, ref – SADS?

Commented [DA125R124]: PMID: 29618817 (same as ref 14 below)

Commented [KJO126]: Hongying? Similar to the revised Thialand one?

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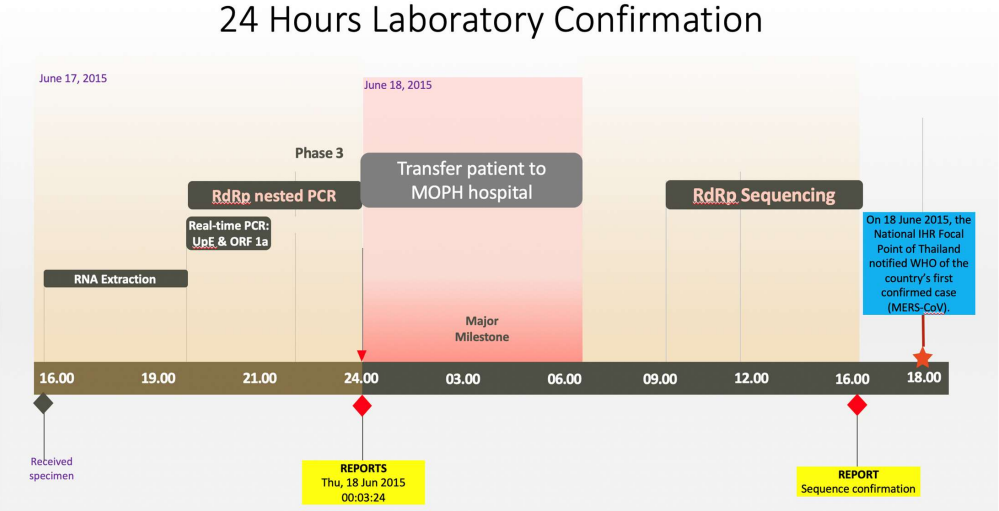
PMID: 31022183

Commented [DA129]: PMID: 30155852

Commented [KJO130]: Hongying was going to work on a very narrow, horizontal version of this if we want to include it.

Commented [DA131]: This part isn't clear. Is it a list of the different assays?



Peninsular Malaysia:

Sabah: Linfa has worked with Timothy Williams on an encephalitis cohort from Queen Elizabeth Hospital 1 & 2 (poss has worked with Linfa on these) and this is another possibility. He's doing outbreak response training in August with team from Sabah CDC (Dr. Maria now in Pen. But still collaborating with us, Jikal current director working with us and Dato Cristina dir. Sabah state health Dept and is supportive) this collaborative group is working to develop a Sabah outbreak response team – we will support that – to be based at BMHRC and we will develop this as part of this proposal. Prof. Kamruddin will summarize the serum samples he has.
POCs: Sabah State Health Dept (e.g. Dr. Giri Shan Rajahram), Queen Elizabeth Hospital (Dr. LEE Heng-Gee)

Sarawak: From Linfa – Dr. Ooi used to work with Jayne Cardosa at UNIMAS and has access to samples of patients with unusual presentation

Strategy for analysis of self-reported illness: In addition to biological outcomes we have developed a standardized approach in PREDICT for analyzing data on self-reported symptoms related to targeted illnesses; of fever with cough and shortness of breath or difficulty breathing (severe acute respiratory illness - SARI), fever with muscle aches (fevers of unknown origin (FUO) and, cough or sore throat (influenza-like illness - ILI) from study participants. We used a least absolute shrinkage and selection operator (LASSO) regression to identify associations between participants who reported ILI-like and/or SARI symptoms and demographics, occupation, and contact with animals in the last year. Results have clear biological relevance. In Yunnan, China, salient associated variables or combination of variables were, in descending order of odds ratio: 1) having eaten raw or undercooked carnivores; 2) having slaughtered poultry and being a resident of Guangxi province; 3) having had contact with cats or bats in the house; and 4) being a male who has raised poultry **(Fig. 11).** We will expand this approach for all syndromes in Aim 3, and use more granular targeted questions

Commented [PD132]: Tom to add – Queen Elizabeth Hospital?

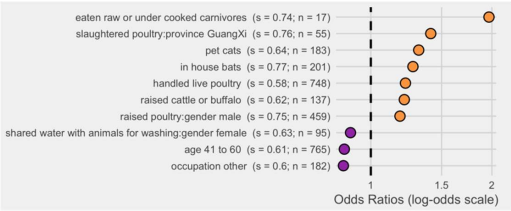
Commented [KJO133]: Comment from Tom: **Sabah team please add details of any cohort work you have done**

Commented [PD134]: Tom

Commented [KJO135]: Comment from Tom: **Dr Tan have you done any clinical cohort work in Sarawak?**

Commented [PD136]: Emily/Hongying

Commented [KJO137]: Emily: Will we do this in the community as well. We could also add animal contact as the outcome. We have data and figures for that as well



designed to assess patient's exposure to wildlife in terms that are relevant to the specific country.**Fig. 11:** Associated variables of self-reported ILI and/or SARI in prior 12 months (s = bootstrap support; n = number +ve out of 1,585 respondents). Orange circles = odds ratios > 1 (positively associated with the outcome); purple = odds ratios <1 (negatively associated with the outcome).

3.2 General Approach: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent is not identified. To capture these, we will expand on our current syndromic surveillance to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and present at regional clinics with influenza-like illness, severe respiratory illness, encephalitis, and other symptoms typical of high-impact emerging viruses. We will collect survey data tailored to the region to assess contact with wildlife, conduct molecular and serological assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. If novel pathogens are identified by PCR, we will isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work. We will then use behavioral survey data, ecological and phylogeographic analyses to assess the likely reservoir hosts to inform intervention programs.

3.3 Clinical cohorts. 3.3.a Cohort selection: We will initiate active clinic-based syndromic surveillance at XX town-level level clinics and xx provincial-level hospitals in each country, in total xx clinical/hospital sites, that all serve as the catchment healthcare facility for people in our community-based surveillance of Aim 2 and are inclusive of wildlife sampling sites in Aim 1. Patients ≥ 12 years old presenting at the health facility who meet the syndromic and clinical case definitions for SARI, ILI, encephalitis, fevers of unknown origin, or hemorrhagic fever will be recruited into the study. We will enroll a total of xxxx individuals for clinical syndromic surveillance, which accounts for up to 40% loss from follow-up. Study data will be pooled across country sites, as clinical patients are limited by the number of individuals presenting at hospitals.

3.3.b Behavioral and clinical interviews: 2.5 Clinic enrollment and follow-up: We will recruit inpatients and outpatients to participate in the study after initial screening to determine if they meet the clinical case definition of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever of unknown etiology. Once enrolled, biological samples will be collected and a questionnaire administered by trained hospital staff that speak appropriate local language. Samples will be taken concurrently when collecting samples for normative diagnostics. For inpatients, samples will be collected no more than 10 days of reported onset of illness to increase the chance of PCR detection of viruses (*123*). We will follow up 35 days after enrollment to collect an additional two 500 µL serum samples conduct a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. 35 days gives adequate time for development of IgG, which occurs <28 days after onset of symptoms for SARS patients (*124*).

3.3.c Sampling:

Following enrollment with signed consent form, biological specimens (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples) including two nasal or oropharyngeal swabs will be collected from all eligible participants, and a questionnaire will be administered. We will investigate five risk factors, to maximize the power of the analyses, all related to high risk wildlife exposure, based on continuing analysis of our previous work, and will include: 11) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With permission from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology.

3.4 Sample testing: PCR, Serol to link symptoms to etiologic agents

Commented [PD138]: Hongying/Emily

Commented [KJO139]: MeiHo: Just QEL in Sabah

Commented [PD140]: Hongying Emily

Commented [EH141]: Both inpatient and outpatient

Commented [PD142]: Need data for Nipah and filovirus patients

3.5 Assessing potential for pandemic spread: We will…leverage EHA's work with DHS to develop FLIRT (Flight Risk Tracker) that tracks the probable pathways for spread for viruses that are able to be transmitted among people….

IBIS mines and collates data regarding on-going bioevents from biosurveillance networks and uses data on commercial air traffic to assess their risk of and likely routes of ingress into the United States. In contrast, BAT can use relatively rudimentary, high-level data, such as might be available for a newly discovered biothreat that has not yet caused a bioevent or is not subject to intense biosurveillance, in order to assess the agent's inherent pathogenicity. Thus, BAT and IBIS address distinct, but highly complementary, use cases.

Traditional approaches to pathogen assessment focus only on internal functional components of pathogens and often fail to account for the broader characteristics of host-pathogen interactions now recognized as critical for risk determination. Initial development will focus on characterization of viral pathogens, which constitute the majority of emerging diseases, especially those with pandemic potential. will use statistical models built from collated biological, ecological, and genetic data to predict the likelihood of human infection (or spillover) and pathogenicity for a diverse range of biothreats. The overarching aim of the proposed technology is to improve overall situational awareness of existing and novel infectious agents around the world, allowing DHS to more quickly identify and assess which threats pose the most significant risk to human health and thus either more rapidly deploy resources to respond to and mitigate their impact when necessary.

Lab-based virology and bacteriology studies are necessary but not sufficient because they are narrowly-focused and do not consider the broader suite of ecological, evolutionary, and host-specific factors that may affect an infectious agent's pathogenicity and transmissibility.

The initial phase of our work will build upon the existing, curated global-level pathogen and host databases currently housed at EHA. Specifically, we will employ systematic, targeted literature reviews to extend and customize these databases to include viral ecological, epidemiological, and biochemical traits to fit our statistical models and target pathogenicity predictions for the BAT. Predictor variables will span a range of data granularity, and vary according to the four information levels. For example for Level 1, variables may include location of detection (to integrate underlying EID hotspot models based on demographic and geographic factors), and non-human host range and taxonomy. For level 2, coarse-grained viral traits that are independent of sequence data may include: viral family, nucleic acid type, mode of transmission, and phylogenetic host breadth for virus (see Figure 2). For levels 3 and 4 viral sequence data will be integrated by including viral phylogenetic distance and 'nearest-known-threat' analyses (see Figure 3). For the pathogenicity model, human epidemiological data for ~300 viral species known to infect people (case fatality rate, global estimates of number of human cases per year or decade, R_0, infectious period, and primary symptoms) will be aggregated to use as predictor variables.

For ease of interpretation and for applicability across a broad range of pathogens, we propose to use two independent but related statistical models that will be used to predict pathogen spillover risk and pathogenicity, respectively. First, a model will be developed using spillover as the relevant outcome. In this case, spillover will be defined as either a binary variable, with "0" indicating a virus that is not known to spillover into human populations and establish infection whereas "1" will indicate a virus that is capable of spillover (or is solely a human pathogen). This model will enable us to assign a spillover risk probability to a new biothreat agent that is currently not known to infect people. Given the capacity to spillover and infect humans, we would then use a second model to predict pathogenicity of the novel pathogen. We will model pathogenicity in human hosts on a discrete scale from 1 to 10, with 1 representing a pathogen having no discernible negative effect on human health (i.e., asymptomatic infection) and 10 representing the most severely pathogenic bioagents with high case fatality rates. Additional functionality for the BAT (i.e., Option Years 2-3) may include integration of models to assess pathogen spread via travel and travel networks.

Commented [PD143]: KEvin to draft

Commented [KJO144]: Pasted in a bunch of stuff, but still need to clean this up.

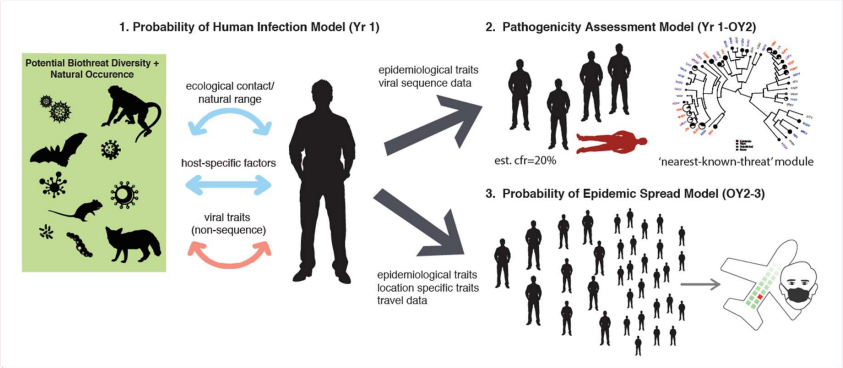


Figure 1. Conceptual framework highlighting 3 underlying models of the Biothreat Assessment Tool (BAT) to estimate: **1)** probability of human infection, or spillover, the first necessary step to understand pathogenicity when status of human infection is unknown (Year 1); **2)** pathogenicity, i.e. probability of causing clinical signs, case fatality rate estimates, and other metrics relevant to humans (or domestic animal species) (Year 1 – Option Year 2); 3) probability of pathogen spread based on ecological, epidemiological, and trade data (Option Years 2-3).

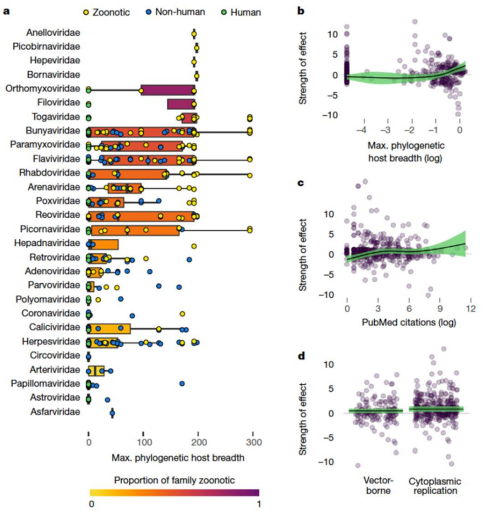


Figure 2. Example of analysis using Level 2 information (limited pathogen characterization) to assess likelihood of human infection (zoonotic potential) for a virus. Figure from Olival et al. 2017, analysis of ~500 unique mammalian viruses. Similar analyses will be developed to assess level of pathogenicity.

From CoV renewal: use data from **3.3** to identify rank SARSr-CoV strains most likely to infect people and evade therapeutic and vaccine modalities. We will use bat survey and zoological data (*101*) to build species distribution models (*125*) and predict the distribution of bat species that harbor low, medium and high risk viral strains. Stacking these modeled distributions for the ~20-30 *Rhinolophus* and related species that occur in the

Commented [KJO145]: From our unfunded DHS proposal a couple of years ago, to modify if we want to include this.

region will allow estimates of SARSr-CoV diversity for a given locality. We will use machine learning models (boosted regression trees) and spatial 'hotspot' mapping approaches to identify the ecological, socio-economic and other correlates of SARSr-CoV diversity and spillover (from serosurveys) (*1, 2, 85*). We will include data from our human behavioral surveys and sampling to give a direct measure of where risk of spillover to people is likely to be highest in the region.

Potential problems/alternative approaches: Patients visiting clinics may have cleared virus, but not yet developed IgG antibodies, reducing seropositive cases. Our 35 day follow-up sampling should avoid this because the maximum lag time between SARS infection and IgG development was ~28 days (*123*). We also expect that patients in rural communities will only visit clinics when symptoms have progressed, likely coinciding with late illness and onset of IgG. We will also have data from our community study, so won't be completely reliant on hospital data to identify PCR- or seropositives. Finally, the risk is outweighed by the potential public health importance of discovering active spillover of a new henipavirus, filovirus or CoV infection.

ALL – the sections below are going to be critical to demonstrate our capacity to do this work. I've not yet started drafting, but please don't hesitate to insert whole sections from other proposals you've done if they're relevant here – we'll need to be creative and get all the language we can from others…

Additional Instructions – Specific to the EIDRC FOA:

Administrative Plan

In a clearly labeled section entitled **"Administrative Plan":**

- Describe the administrative and organizational structure of the EIDCRC Administrative and Leadership Team. Include the unique features of the organizational structure that serve to facilitate accomplishment of the long range goals and objectives.
- Describe how communications will be planned, implemented, and provided to collaborators, teams, or sites. In addition, describe plans to achieve synergy and interaction within the EIDRC to ensure efficient cooperation, communication and coordination.
- Specifically address how the EIDRC Team will communicate with other EIDRCs, the EIDRC CC and NIAID and how the EIDRC will implement plans and guidance from the EIDRC CC to ensure coordination and collaboration across the Emerging Infectious Disease Program as a whole. Describe how the study site will provide information, if any, back to the local health authorities and the study subjects as needed. Note any adjustments or changes needed for rapid and flexible responses to outbreaks in emerging infectious diseases.

List out all of our involvement with outbreak investigations, ministries of health surveillance etc.

- Describe the overall management approach, including how resources will be managed, organized and prioritized, including pilot research projects.
- Highlight how the proposed staffing plan incorporates scientific and administrative expertise to fulfill the goal to develop the flexibility and capacity to respond rapidly and effectively to outbreaks in emerging infectious diseases in an international setting.

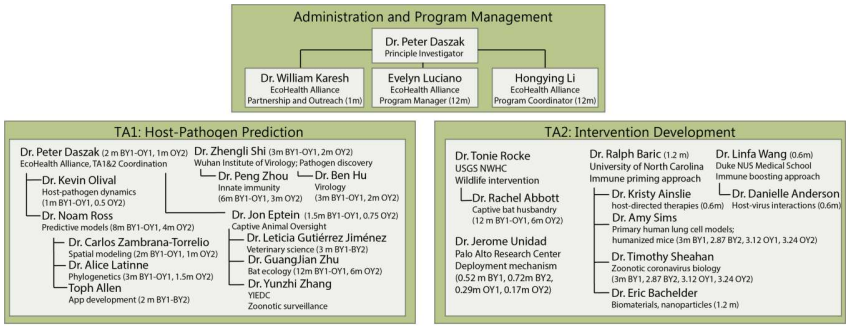
FROM DARPA grant:

Commented [PD146]: Add data for Nipah and filo infections

Commented [PD147]: All – I've started putting a few notes in this section, but it will need substantial work. Please insert language from other proposals to fit these sections if you have them

Commented [KJO148]: Peter, I've added in some examples from other grants

Section 1.03 MANAGEMENT PLAN



Project DEFUSE lead institution is EcoHealth Alliance, New York, an international research organization focused on emerging zoonotic diseases. The PI, Dr. Daszak, has 25+ years' experience managing lab, field and modeling research projects on emerging zoonoses. Dr. Daszak will commit 2 months annually (one funded by DEFUSE, one funded by core EHA funds) to oversee and coordinate all project activities, with emphasis on modeling and fieldwork in TA1. Dr. Karesh has 40+ years' experience leading zoonotic and wildlife disease projects, and will commit 1 month annually to manage partnership activities and outreach. Dr. Epstein, with 20 years' experience working emerging bat zoonoses will coordinate animal trials across partners. Drs. Olival and Ross will manage modeling approaches for this project. Support staff includes a field surveillance team, modeling analysts, developers, data managers, and field consultants in Yunnan Province, China. The EHA team has worked extensively with other collaborators: Prof. Wang (15+ yrs); Dr. Shi (15+ yrs); Prof. Baric (5+ yrs) and Dr. Rocke (15+ yrs).

Subcontracts: #1 to Prof. Baric, UNC, to oversee reverse engineering of SARSr-CoVs, BSL-3 humanized mouse experimental infections, design and testing of targeted immune boosting treatments; **#2** to Prof. Wang, Duke-NUS, to oversee broadscale immune boosting, captive bat experiments, and analyze immunological and virological responses to immune modulators; **#3** to Dr. Shi, Wuhan Inst. Virol., to conduct PCR testing, viral discovery and isolation from bat samples collected in China, spike protein binding assays, humanized mouse work, and experimental trials on *Rhinolophus* bats; **#4** to Dr. Rocke, USGS NWHC, to refine delivery mechanisms for immune boosting treatments. Dr. Rocke will use a captive colony of bats at NWHC for initial trials, and oversee cave experiments in the US and China. **#5** to Dr. Unidad, PARC, to develop innovative aerosal platform into a field-deployable device for large-scale inoculation of the bats. Dr. Unidad will collaborate closely with Dr. Rocke in developing a field deployable prototype for both initial trails and cave experiments in China.

Collaborator Coordination: All key personnel will join regularly-scheduled web conferencing and conference calls in addition to frequent ad hoc email and telephone interactions between individuals and groups of investigators. Regular calls will include:

- Weekly meetings between the PI and Program Manager (on project and task status)
- Weekly web/phone meetings between Program Manager and subawardee admin. staff
- Monthly web/phone conferences between EHA PIs and all subawardee PIs.
- Monthly web conferences between key personnel (research presentations/coordination)
- Four in-person partner meetings annually with key personnel at EHA and two in-person partner meetings annually between subawardees.
- Annual in-person meeting among all key personnel

Evaluation metrics will include the generation of high-quality data, successful achievement of milestones and timelines, scientific interaction and collaboration, the generation of high quality publications, and effective budget management. The PI and subawardee PIs will attend a kickoff meeting and the PI will meet regularly with DAPRA at headquarters and at site visits.

Commented [KJO149]: Figure like this would be good, but instead of breaking down by TA, maybe by country or Specific Aim. Per FOA, Need to also show how we'll link in with the Coordinating Center and other EIDRCs.

Problem identification and resolution: Regular planning, monitoring, and evaluation meetings will be the primary mechanisms for problem identification. Minor problems (e.g. delays in sample availability or test results) will be dealt with internally through appropriate action and resolution monitored by Dr. Daszak and the Project Manager. In the event of significant problems, such as prolonged poor productivity, inadequate scientific collaboration, or major disputes regarding research direction or resource allocation, EHA will assist with resolving the problem through negotiation. Should a resolution not be forthcoming, consultation with our technical advisors and DARPA Program staff may be warranted.

Risk management: Maintaining a timeline and meeting milestones will require strict and continuous oversight of all project phases, frequent and regularly scheduled communication, and the ability to make decisions and implement strategies. A project of this nature requires a different mindset from that typically associated with basic research activities that move at an incremental pace with investigators gradually optimizing experimental systems, refining data, or waiting for additional data before moving ahead with an analysis approach. To maintain our timeline, we will continually evaluate these trade-offs to make decisions about when iteration is appropriate and when necessary to move forward with current information.

Biographies:

Dr. Peter Daszak is President and Chief Scientist of EcoHealth Alliance, Chair of the NASEM Forum on Microbial Threats, member of the Executive Committee and the EHA institutional lead for the $130 million USAID-EPT-PREDICT. His >300 scientific papers include the first global map of EID hotspots(*1, 126*), estimates of unknown viral diversity(*127*), predictive models of virus-host relationships(*2*), and evidence of the bat origin of SARS-CoV(*20*) and other emerging viruses (*40, 128-130*).

Prof. Ralph Baric is a Professor in the UNC-Chapel Hill Dept. of Epidemiology and Dept. of Microbiology & Immunology. His work focuses on coronaviruses as models to study RNA virus transcription, replication, persistence, cross species transmission and pathogenesis. His group has developed a platform strategy to assess and evaluate countermeasures of the potential "pre-epidemic" risk associated with zoonotic virus cross species transmission(*60, 70, 131-134*).

Prof. Linfa Wang is the Emerging Infectious Diseases Programme Director at Duke-NUS Medical School. His research focuses on emerging bat viruses, including SARS-CoV, SADS-CoV, henipaviruses, and others (*20, 135-138*) and genetic work linking bat immunology, flight, and viral tolerance (*139-142*). A 2014 recipient of the Eureka Prize for Research in Infectious Diseases, he currently heads a Singapore National Research Foundation grant "Learning from bats" ($9.7M SGD).

Data Management Plan

In a clearly labeled section entitled **"Data Management Plan":**

- Describe internal and external data acquisition strategies to achieve harmonization of systems and procedures for data management, data quality, data analyses, and dissemination for all data and data-related materials generated by the research study with the EIDRC CC.
- Within the plan, indicate the extent to which dedicated systems or procedures will be utilized to harmonize the acquisition, curation, management, inventory and storage of data and samples. Describe how training for the data and sample collection, in terms of the use of electronic data capture systems, will be provided to all staff including those at enrollment sites.
- Describe the quality control procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens.

This includes sample labeling etc. Get a bar-coding system, everyone has a scanner (cf. PREDICT) – Ralph has this for select agents and MERS. Also need a software system

Language from NIH CoV grant:

Data Sharing Plan: Data will be available to the public and researchers without cost or registration, and be released under a CC0 license, as soon as related publications are in press. Data will be deposited for in long-

term public scientific repositories – all sequence data will be made publicly available via GenBank, species location data via the Knowledge Network for Biodiversity, and other data will be deposited in appropriate topic-specific or general repositories. Computer code for modeling and statistical analysis will be made available on a code-hosting site (GitHub), and archived in the Zenodo repository under an open-source, unrestrictive MIT license. Limited human survey and clinical data will be released following anonymization and aggregation per IRB requirements. Publications will be released as open-access via deposition to PubMed commons.

Viral isolates will remain at the Wuhan Institute of Virology initially. Isolates, reagents and any other products, should they be developed, will be made available to other NIH-funded researchers via applicable Wuhan Institute of Virology and EcoHealth Alliance Material Transfer Agreements and/or licensing agreements.

Sharing Model Organisms: We do not anticipate the development of any model organisms from this study. Should any be developed, they will be made available to other NIH-funded researchers via applicable Wuhan Institute of Virology and EcoHealth Alliance Material Transfer Agreements and/or licensing agreements.

Genomic Data Sharing: We anticipate obtaining genetic sequence data for 100s of novel coronavirus genotypes, including RNA-dependent RNA polymerase (RdRp) and Spike genes for all strains/genotypes. In addition, we will generate full viral genomes for a subset of the bat SARSr-CoVs that we identify. All sequence data will be deposited in the NIH genetic sequence database, GenBank. We will ensure that all meta-data associated with these sequences, including collection locality lat/long, species-level host identification, date of collection, and sequencing protocols will also submitted. The genotype data will be made publicly available no later than the date of initial publication or six months after the receipt of final sequencing data, whichever comes first. We anticipate sequence generation will occur over the 5 year proposed project period.

Genome Wide Association Studies (GWAS): Not applicable.

Commented [KJO150]: From CoV NIH grant

Thailand TRC-EID Chulalongkorn lab:Specimen management and BioBank: The software called PACS (Pathogen Asset Control System) supported by BTRP DTRA, is now used for managing the specimen from specimen registration (barcoding, and may move to use QR code system soon), aliquoting, tracking, short patient history record, data summary, searching, test reporting and etc. Our biobank system contains 28 freezers (-80C) and liquid N2 system. Its size is around 120 sq meters. There is a separate electric generator for our lab and biobank. There are CC TV cameras at the biobank and laboratory.

Chula TRC-EID has our own server for data management and analysis. We have the necessary software including Bioinformatic tools (Blast Standalone, SAMTOOL, GATK, AliVIEW, RaxML, FigTREE, MAFFT, Guppy, Megan, PoreChop, MEGA), Database (MySQL, MongoDB) Web, jQuery, Node.js, Express.js React, API, PHP, Ruby, Python, Java, C#, HTML5, Bootstrap, CSS, Responsive, Cloud, Linux Command, PACS (sample management system), Virtualbox virtual machines, R programming languages, Perl scripts, Bash shell scripts, Open source software, Microsoft Office running on both Apple Mac OS X, Ubuntu, Linux, and Windows Operating Systems. Chula TRC-EID has a dedicated 16-core Ram 64GB Linux server with 4TB hard drives, dual quad-core Mac Pro Server with 2TB hard drives and another dedicated iMac Pro (Retina 4K, 21.5-inch, 2017) Processor 3.6GHz quad-core Intel Core i7 (Turbo Boost up to 4.2GHz) Ra, 16GB with 512GB hard drives. TRC-EID also has HPC Linux server CPU: 4 x 26cores Intel Xeon = 104 cores 2.1GHz with 52 TB HHD and 2x3.84TB SSD with GPU 2x Tesla v100 (CUDA core = 5120 cores/GPU, Tenser core = 640 cores/GPU). TRC-EID has a dedicated 10/100/1000 of LAN (Local Area Network), 2.4G / 5G Gigabit Wi-Fi 802.11ac.

Commented [KJO151]: From Supaporn for their lab, some may go in Facilities doc?

From DARPA:

Data Management and Sharing: EcoHealth Alliance will maintain a central database of data collected and generated via all project field, laboratory, and modeling work. The database will use secure cloud hosting services and enable export to archival and platform-independent formats. It will ensure data and metadata compatibility between project components, track data versioning and annotations, and enable compliance with DARPA data requests and disclosure requirements. All archived human sample data will be de-identified. Partners will provide raw and processed data to the central database throughout the course of the project. Project partners will have access to the data they generate in the database at all times, and maintain control over local copies. Release of any data from the database to non-DARPA outside parties or for public release or publication will occur only after consultations with all project partners. EHA has extensive experience in managing data for multi-partner partner projects (PREDICT, USAID IDEEAL, the Global Ranavirus Reporting System).

Commented [KJO152]: Text from DARPA Preempt

Clinical Management Plan
Clinical site selection

Our consortium partners have been conducting lab and human surveillance research, inclusive of human surveillance during outbreaks, for over the past two decades and in that time have developed strong relationships with local clinical facilities and processes. The plan for selection of clinical sites will begin in the same geographic regions as those identified in Aim 1. These are regions where we will sample wildlife due to the high potential of zoonotic viral diversity. Additionally, these will be clinical sites that serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2. In PREDICT we developed successful working relationships with the major healthcare facilities the hotspot areas in 6 different countries, including Thailand and Malaysia and we will continue to work with these sites going forward. Continuing to work with these hospitals means we will begin this project with successfully established partners and we can begin data collection quickly. This also reduces the number of additional sites needed to meet our goals within our geographic range. The capacity and capability requirements for clinical sites for this study is limited. All that is required of sites will be the ability to enroll patients that meet the clinical case definitions of interest, capacity to collect and temporarily store biological samples, and follow standards for data management and protection. We will be enrolling inpatient and outpatient participants within 10 days of the presentation of symptoms to increase the chance of PCR detection of viruses so we will not wait for advanced normative diagnostic tests, that are likely unavailable in the more remote sites, to be completed. We will work with clinical sites to determine the best process for project staff, in most cases supporting the time of currently site staff. These will be persons who are locally trained and certified to collect biological specimen from participants including blood draw technique, e.g. doctor or nurse. Last, the clinical site will need the capacity to securely store all personal health information and personally identifiable research data, this will include locked offices with locked filing cabinets to store all paper records and an encrypted computer. Training on project procedures will be provided for all local project staff by local country project management staff and partners from headquarters. The study at clinical sites will begin by developing relationships with local stakeholders at the hospitals and clinics in our geographic areas of interest that serve as catchment facilities for people of within the community surveillance study. We will recruit hospital staff that will be extensively trained for project procedures including enrollment of patients, sample types to be collected, storage of samples, administration of the questionnaire, and data management plans. During the Development and onboarding of new clinical sites we will assess the physical needs of the site and what supplies will be required for collection of data (e.g. locked filing cabinet, dry shipper, secure laptop). [Did not specifically address CONDUCT]. Oversight for clinical sites will be managed by the country coordinator for each country. This will be done with regular site visits to the clinical sites and annual visits by headquarter research staff. Site visits will include monitoring and evaluating the process for patient enrollment, collecting and storage of samples, administration of the questionnaire, and secure and data management procedures for secure personal health information and research data. During these visits any additional training will also be provided.

Commented [EH153]: Is that true

Standardized approach oversight and implementation

Management and oversite for all study sites will be managed by the local country coordinator who is supported by staff at headquarters. Implementation of a standardized approach across all study sites, community and clinical, will begin by extensive training of staff on procedures on requirements of the project, this includes: enrollment, data collection, and data management. Our research team has over 5 years of experience building capacity on human subjects' research and has developed training resources and materials for standardized implementation of community and clinical research. This includes SOPs for screening, enrollment, and retention of participants. In addition to yearly monitory reports the country coordinator will regularly visit each clinical site to ensure quality standards and compliance of procedures are being met. Through our work with clinical sites in PREDICT we have developed culturally appropriate screening measures for clinal sites that do not disrupt the flow or quality of medical treatment received by the patient. This was done by working collectively with clinical research staff to evaluate current procedures and patient flow at the site to determine the most efficient while minimal invasive inclusion of our study into the daily working of the clinal site. Most efficiently this was done by adding minimal basic screening questions to the current clinal intake forms, which allows the clinal research officer to quickly scan charts or logs for potential patients to enroll, allowing for minimal disruption and prevents potential enrollees from being overlooked if the research staff is not on duty. At clinical sites that do scheduled outpatient days we may have a trained member of the research staff with the intake nurse to monitor the patients coming to the clinic more rapidly to no delay screening or enrollment in such a fast pace environment. Using the screening tools, we will recruit and enroll patients who present with symptoms that meet the clinical case definitions of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever, of unknown etiology within the past 10 days. These patients will be enrolled, samples collected and survey conducted to assess the participants contact with wildlife through: 1) occupational exposures; 2) animal contact; 3) environment biosecurity. With permission from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology. The clinical cohort will be split into cases and controls. "Cases" are defined as participants whose samples tested positive for either coronaviruses, henipavirus, or filoviruses, serological tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between serological/PCR status and risk factors. In clinical study, we will use this procedure to determine how clinical presentation differs between virus-exposed and -unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. We will model the outcomes to analyze correlation between biological results and risk factors related to high risk wildlife exposure. Additionally, we will be following up with clinical participants to determine how clinical presentation differs between coronaviruses, henipavirus, or filoviruses in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. [How do these cohorts be leveraged for new emerging pathogens] Achieving the adequate numbers will be attained through duration of the as clinical participant enrollment. While patient's enrollment is limited by the number of individuals presenting at hospitals, during the PREDICT project we had an average of 105 patients enrolled per year, ranging from 77-244. The country coordinator will be continually monitoring the project database to be sure that we are on track to reach our target.

Clinical cohort setup, recruitment, enrollment

We will recruit inpatients and outpatients from clinical sites to participate in the study after initial screening to determine if they meet the clinical case definitions for 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever, of unknown etiology for symptoms reported onset of illness to increase the chance of PCR detection of viruses. Once enrolled, biological samples will be collected by trained hospital staff. When possible, samples will be taken concurrently with samples being collected for normative diagnostics. We will collect 2* 5mL of blood for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL

Commented [EH154]: Will these cohort fulfill the goals of the EIDRC.

serum samples and two nasal or oropharyngeal swabs will be collected. The cases are defined as participants whose samples tested positive for Henipaviruses, Filoviruses, or CoVs by PCR or serological tests. The controls will be selected from the pool of participants admitted to the studies and whose samples tested negative for coronaviruses, henipavirus, or filoviruses-. We will follow up 35 days after enrollment to collect an additional 5mL blood draw for collection of two 500 µL serum samples and a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. Following up 35 days post initial collection gives adequate time for 1) development of IgG and IgM, which occurs <28 days after onset of symptoms for coronaviruses, henipavirus, or filoviruses infected patients to evaluate the immunological progression of disease and 2) further the risk assessment of the participants to monitor contact with wildlife, people, and to assess the likely reservoir hosts to collect information to inform potential intervention programs.

Commented [EH155]: PREDICT we also make the stipulation that we will collective relatvive samples if avialble from treatmean collection, eg CSF if the MD is doing a spinal tap we will request an aliqot if possible

Commented [EH156]: Please add any information that might address: types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.

Utilization of collected data

Sampling of wildlife and the enrollment of community surveillance participants and a clinical cohort of participants give us the ability to assess the viruses the are circulating in each of the three populations in a similar geographic region. We will use phylogenetic analysis to compare the relationship between wildlife viruses found and viruses fond in human participants. Additionally, the questionnaire allows us to assess relative measures of human-wildlife contact from our survey work that we will analyze the intensity of contact with species known to be at a higher risk for spillover against other risk to provide useful proxy information for spillover risk.

Commented [EH157]: Please add any information that might address: Describe methods that might be applied to elucidate a zoonotic source

The biological specimens to be collected (2*5mL whole blood for whole blood and serum analysis and nasal or oropharyngeal swabs) from all eligible participants and follow up specimen 35 days (3mL of whole blood for serum) after enrollment with the standardized questionnaire. Capacity for specimen is collection is not more than standard phlebotomy skills and we will collected by locally trained and certified personnel that are part of the research team in country. Staff we will trainline on ethical guidelines for conducing human subjects research and survey data collection methods. Collection of whole blood, serum, and nasal or oropharyngeal swabs allows us to test the samples for PCR to detect viruses in our priority viral families, serologically test for immunological response and historical exposure to zoonotic diseases of relevance, and the questionnaire will assess individuals evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. With this collective data set we aim to quantify and detect novel viruses likely to spillover regularly to people, are often unreported or misdiagnosed as their clinical manifestations are unknown to detect cryptic outbreaks causing previously ‘hidden’ clinical syndromes in people. Our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an ‘early warning system’ to conduct public health interventions and disrupt disease emergence.

Potential expansion

Expansion of the research project to other clinical sites or research areas could happen as new information is gathered, either through our research or an outbreak in the region. If expansion is required we could rapidly implement research activities in additional clinical or community sites through the same process we on-boarded the initial locations. First, by working with local stakeholders to create a working relationship and on-boarding new clinical research staff through advanced trainings of project aims, SOPs on data collection and storage, and ethical guidelines. The network of infectious disease researchers in the community and hospital settings would allow for an accelerated response to any potential infectious disease outbreaks as staff are well trained on ethical guidelines for working with human subjects, data and specimen collection, storage, and transpiration, project staff would be on the front lines ready for the call to action. Ideally, our testing for viruses in priority viral families may lead to the discovery cryptic outbreaks before it spreads in the general public. Biological results coupled with survey data on key risk factors will provided necessary information for granular understand of disease spread.

In a clearly labeled section entitled **"Clinical Management Plan":**

- Describe plans, processes, and procedures for the following activities: clinical site selection including feasibility, capacity, and capabilities, and study development, conduct, and oversight.
- Describe strategies for oversight and implementation of standardized approaches in the recruitment and clinical characterization of adequate numbers of relevant patient populations to ensure the prompt screening, enrollment, retention and completion of studies. Describe novel approaches and solutions to study enrollment. including the clinical meta-data that will be captured and describe how the staff at each enrollment/collection site will be trained and comply with the quality standards for data and sample collection and compliance with the standardized procedures. Provide a general description of the methods for establishing clinical cohorts and how cohorts may be leveraged for new emerging pathogens.
- Provide general approaches to identification of the types of clinical cohorts needed to fulfill the goals of the EIDRC. Discuss types of clinical cohorts and clinical assessments that will be utilized to study natural history and pathophysiology of disease, including characteristics of the cohort, site for recruitment, types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.
- Describe methods that might be applied to either elucidate a zoonotic source or a vector of transmission.

Our research team incredibly diverse and capable of working with a group of other viral families: Lay out all our additional vector experience incl. my WNV work, our work on Chik etc.
Baric: Flavi, Noro,
Linfa: …
EHA: …

- Describe capacity for and approaches to clinical, immunologic and biologic data and specimen collection, and how these data and specimens will address and accomplish the overall goals and objectives of the research study.

Ralph

1. Norovirus: collaboration with surveillance cohort CDC; first one to publish antigenic characterization of each new pandemic wave virus; and phase 2 and 3 trial of therapeutics
2. Tekada Sanofi Pasteur dengue
3. Nih tetravalent vaccine

- Provide plans for the potential addition of new sites and expanding scientific areas of research when needed for an accelerated response to an outbreak or newly emerging infectious disease.

Linfa – will launch VirCap platform on outbreak samples.

Statistical Analysis Plan

In a clearly labeled section within the Research Strategy entitled **"Statistical Analysis Plan":**

- Describe the overall plan for statistical analysis of preliminary research data, including observational cohort data.
- Describe the overall staffing plan for biostatistical support and systems available for following functions: 1) preliminary data analyses, 2) estimates of power and sample size, 3) research study design and protocol development.
- Describe the quality control and security procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens. Describe any unique or innovative approaches to statistical analysis that may be utilized.

Project Milestones and Timelines

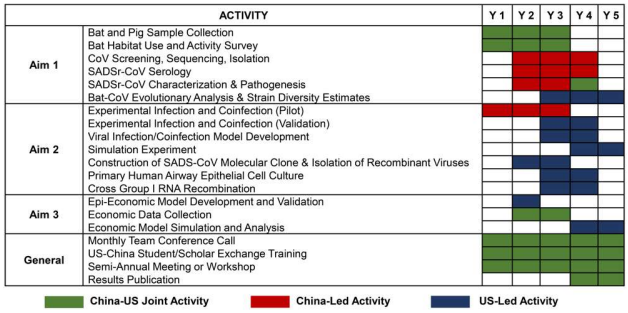
In a clearly labeled section entitled **"Project Milestones and Timelines":**

- Describe specific quantifiable milestones by annum and include annual timelines for the overall research study and for tracking progress from individual sites, collaborators, and Teams. Milestones must specify the outcome(s) for each activity. Milestones should be quantifiable and scientifically justified, and include the completion of major research study activities, including, for example, protocol development, case report forms, scheduled clinical visits, obtaining clearances study completion, and analysis of final data. Milestone criteria should not simply be a restatement of the specific aims. Using a Gantt chart or equivalent tool, describe the associated timelines and identified outcomes for the research study.

Show that for this proposal, we won't be doing the Specific Aims sequentially. We'll begin the human work (SA 2, 3) at the beginning of the project, along with SA1. We'll do the diagnostic assay development initially in Ralph's, Linfa's, Chris's lab, but then begin tech transfer in Y1….

Project Management & Timeline

PI Daszak will oversee all aspects of the project. Dr. Daszak and has two decades of experience managing international, multidisciplinary research in disease ecology, including successful multi-year projects funded by NSF, NIH and USAID. He will conduct monthly conference calls with all co-PIs, including the China team **(see Activity schedule below)**. He will be assisted by EHA Sr. Personnel Li, who is a US-based Chinese national with fluent Mandarin. Co-PI Olival will oversee Aim 1 working closely with China PI Tong, who will test samples. Co-PI Ma (China team) will coordinate and lead all Chinese experimental infections, with guidance from co-PI Saif, who will coordinate and lead all US experiments with co-PI Qiuhong Wang. Sr. Personnel Ross will conduct all modeling activities in Aim 2, working closely with co-PI Saif to parameterize the models. Co-PI Baric and Sr. Personnel Sims will lead infectious clone, recombinant viral and culture experiments. Consultant Linfa Wang will advise on serology, PCR and other assays. China team co-PIs Shi and Zhou will oversee viral characterization in Aim 1. Sr. Personnel Feferholtz will oversee the economic modeling in close collaboration with Ross.



Commented [KJO158]: Text and figure From NSF-NIH SADS grant. Peter will adapt

Another Example of Milestones and specific tasks from a prev DHS (unfunded) proposal:

Expand existing databases to include predictor variables for pathogenicity risk model

Task 1: Collate available human clinical information from the literature for ~300 viral pathogens known to infect humans (Contract Award Date to 6 month)

Task 2: Update EHA host-viral association database to include new data for mammalian and avian species from literature 2015-present (Contract Award Date to 3 month)

Task 3: Extract and curate full and partial genomic data from Genbank for 5-6 viral families with a high proportion of human-infectious pathogens for phylogenetic analyses – e.g. 'nearest-known-threat module'. (Month 2 to 6 month)

Develop underlying pathogenicity and spillover risk assessment models

Task 4: Fit generalized linear models in Stan for multiple response variables to measure pathogen impact (Month 7 to 10 month)
Task 5: Fit generalized linear models to estimate risk of human infection (spillover) based on host, ecological, and location specific traits (Month 4 to 8 month)
Task 6: Validate model effectiveness by simulating early-outbreak, limited-information scenarios from known pandemics to confirm model behavior (Month 8 to 10 month)

Commented [KJO159]: Just example, but probably just want to use a Gantt chart format per most of our grants, depending on space.

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Page 10: [1] Commented [KJO34] **Kevin J. Olival** **6/20/2019 4:36:00 PM**

Ralph: Am J Pathol. 2003 Dec;163(6):2371-82 Primary human lung endothelial cells are highly susceptible to EBOLA virus infection, so we can use these cells. Huh7 cells are good candidates for ebola in the liver, oftentimes used to isolate virus from clinical samples (Viruses. 2019 Feb 16;11(2). pii: E161.) Huh7 cells also ofers advantages for reverse genetic recovery of novel filoviruses (J Infect Dis. 2015 Oct 1;212 Suppl 2:S129-37.)

We have growth curves of Nipha and Hendra in Calu3 cells, a continuous lung epithelial cell, including a variety of host expression changes after infection. Likely grow in primary human airway cultures as well as they infect ciliated and secretory cells (J Gen Virol. 2016 May;97(5):1077-86.). Nipha and hendra also infect primary human microvascular endothelial cells, like mers and ebola (PMC3477106, PMC3791741). I'm less sure what kind of neural cell should be used for Nipha and Hendra.

Some evidence for nipha and Hendra replication in mouse lung, low level titers in young animals, 10 fold higher titers in aged mice (PMC4057804, PMC3393746). Thus it is likely these models could be improved in the collaborative cross mouse resource here at UNC. We have identified CC lines that are highly vulnerable to wildtype SARS and others that develop hemorhaggic disease following mouse adapted ebola infections.

Understanding risk of zoonotic virus emergence in EID hotspots of Southeast Asia
II. Research Strategy:

1. Significance:

Southeast Asia is a well-defined hotspot for emerging diseases due to its high biodiversity of wildlife and their viruses, and the presence of critical ecological and socioeconomic drivers of disease emergence (*1*). These include dense human populations living in networks of rural and urban communities, with strong cultural and occupational connection to wildlife and livestock farming and trading, and intimate connection to global travel networks (*2*). The region is also undergoing rapid environmental and demographic change, both of which increase the risk of disease emergence and spread. It is therefore not surprising that a number of recent discoveries have identified novel viruses, including near-neighbors of known agents spilling over to livestock and people through often novel pathways, leading to sometimes unusual clinical presentations **(Table 1)**.These factors are also behind a significant background burden of repeated Dengue virus outbreaks in the region (*3*), and over 30 known *Flavivirus* species in South and Southeast Asia (*4*).

Viral agent	Site, date	Impact	Novelty of event	Ref.
Melaka virus & Kampar virus	Malaysia, 2006	Caused SARI in family group, individual	1st disease due to bat-origin reoviruses (other reports Singapore, Vietnam etc.)	(*5-8*)
Reston filovirus	Philippines 2008	Seropositive people killed pigs,	No prior findings of filoviruses in pigs	(*9*)
Thrombocytopenia Syndrome virus	China 2009	Domestic animals, illness in people	Novel syndrome with large caseload	(*10*)
Reston filovirus	Shanghai 2011	PCR positive pigs	Further evidence of pig RESTV infection	(*11*)
Mojiang virus	Yunnan 2012	Implicated in death of 3 mineworkers exposed to rats	1st evidence of rodent origin henipavirus in people	(*12*)
Nipah-like virus	Philippines 2015	Killed horses	No prior horse infection for NiV, known for HeV	(*13*)
SADS-CoV (HKU2)	China 2017	>25,000 pig deaths seronegative people	Novel emergence of bat-origin CoV	(*14*)
SARSr-CoV & HKU10-CoV	Yunnan, Guangxi 2015	Seropositive people	1st evidence human infection HKU10 & SARSr-CoV	(*15*)
Nipah virus	Kerala, 2018, 2019	19 people infected/ 17 dead 2018, 1 infected 2019	1st outbreak of NiV outside Bangladesh, W. Bengal focus	(*16, 17*)

Table 1: Recent reports from SE Asia indicating potential for novel pathways of emergence, or unusual presentations for known or close relatives of known viruses.

These events are dominated by three viral families: coronaviruses, henipaviruses and filoviruses, which will act as the key research viral groups in this proposal. These viral groups have led to some of the most important recent emerging zoonoses (*18-29*). Their outbreaks have caused tens of thousands of deaths, and billions of dollars in economic damages (*30-32*). In addition to the events in **Table 1**, relatives and near-neighbors of known pathogens in these families have been reported throughout the region, including by members of our consortium including: henipaviruses in frugivorious bats in Thailand, Cambodia, Philippines, Laos, Indonesia, China, Malaysia ,Bangladesh and India (*33-39*); a novel henipavirus, Mojiang virus, in wild rats in Yunnan (*12*); serological evidence of filoviruses in bats in Bangladesh (*40*) and Nipah- and Ebola-like viruses in *Hipposideros*, *Cynopterus* and *Rhinolophus* species in Malaysia (EHA, unpublished data); evidence of novel filoviruses in bats in Singapore (*41*) and China (*42-44*), including Měnglà virus that appears capable of infecting human cells (*45*); novel paramyxoviruses in bats and rodents consumed as bushmeat in Vietnam (*46*), a lineage C β-CoV in bats in China that uses the same cell receptor as MERS-CoV and can infect human cells *in vitro* (*47*); MERSr-CoVs found in dry bat guano being harvested as fertilizer in Khao Chong Pran cave, Ratchaburi, Thailand (*48*) and later directly in bats (CU, unpublished); 52 novel SARSr-CoVs in 9 bat species in southern China (172 novel β-CoVs from >16,000 sampled bats) that are also found throughout the region (*20, 47, 49, 50*), a new β-CoV clade ("lineage E") in bats (*50*); and 9 and 27 novel wildlife-origin CoVs and paramyxoviruses in Thailand (respectively) and 9 novel CoVs in Malaysia identified as part our work under USAID-PREDICT funding (*49, 51*). These discoveries underscore the genetic diversity of potentially pathogenic viral strains in wildlife in the region, and their potential to help in the development of broadly active vaccines, immunotherapeutics and drugs (*52*).

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Most high-impact zoonotic viruses originate in wildlife reservoirs, sometimes spilling over first into livestock 'amplifier' hosts, or directly into localized human populations with high levels of animal contact **(Fig. 1)**. Efforts to prevent emerging zoonoses have targeted these high-risk populations in regions prone to disease emergence (*29, 53*). However, surveillance and control is hampered by inadequate information on basic disease ecology (e.g. range of wildlife reservoirs, risk factors for spillover) and pathogen biology (viral diversity, pathogenesis), and by logistical challenges sharing novel diagnostics, animal models, samples and reagents at sites where outbreaks begin (*54*). Countermeasure and vaccine development is challenged by the small number of isolates available, and the presence of closely-related strains, some with evidence of transmission to people but with unknown pathogenicity. For example, using NIH and other funding, we found serological evidence of exposure to African henipaviruses in 3-4% of a sample of people in Cameroon, with a significant risk factor being butchering of bats; and serological responses to bat SARSr-CoVs in 6/209 (2.87%) and bat HKU10-CoV in x/xx (x%) people living close to a bat cave in Yunnan, all novel viruses with unknown clinical impact (*15, 55*). We discovered two novel bat-borne reoviruses, Melaka and Pulau viruses, and showed that 14/109 (13%) of people living in close proximity to bat roosts on Tioman Island, Malaysia were exposed to these viruses (*5*) as well as 12/856 (1.4%) people screened in Singapore (*6*). Previous studies in the Philippines found famers and butchers who had contact with sick pigs tested positive for Reston ebolavirus antibodies but had not experienced illness (*9*), and it was our team that discovered Philippine bats as natural reservoir hosts for Reston virus (*56*). Preliminary screening in Malaysia with funding from DTRA for serological surveillance of Henipaviruses and Filoviruses spillover found serological evidence of past exposure to Nipah and Ebola antigenically-related viruses in non-human primates (NHPs) and people (Hughes, unpublished data). Even when spillover leads to outbreaks of illness and mortality, they are often unreported, undiagnosed or misdiagnosed. Under a prior NIH R01, we initiated targeted syndromic surveillance of encephalitis patients in Bangladesh clinics and showed that NiV causes repeated annual outbreaks with an overall mortality rate of ~70%, and that it is also present in bats in India (REF). Nipah virus was originally misdiagnosed as 'aberrant measles' in West Bengal, India (REF), has now emerged repeatedly in North India, and in Kerala, South India in 2018 and 2019 (ongoing), raising the specter of future spillover at other sites across the region (*16, 57*).

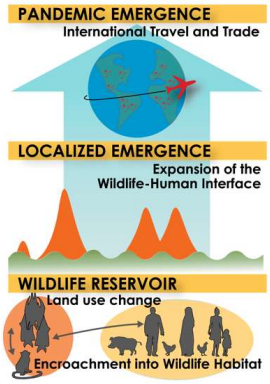

Fig. 1: Our Early Warning Approach: Most zoonotic viruses emerge from wildlife hosts, sometimes via livestock (Stage 1, lower panel), to cause small scale outbreaks (green) or larger chains (red) of human-to-human transmission (Stage 2, middle). In some cases, these spread more widely via air travel (Stage 3, upper). Our EIDRC proposal targets each stage: SA1 examines the diversity and spillover potential of viral threats in targeted SE Asian wildlife; SA2 seeks evidence of their spillover into focused high-risk human populations (stage 2); SA3 identifies their capacity to cause illness and to spread more widely (stage 3). Figure from (*53*).

Under a current NIAID R01, we have shown that we can use analytical approaches to target surveillance of wildlife and people and identify likely sites of spillover. We used phylogenetic analysis of bat hosts and their CoVs to identify a key cave complex in SW Yunnan Province that harbors all the genetic elements of SARS-CoV (*58*), as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics (*20, 47, 49, 50, 59-61*). We conducted behavioral risk surveys and biological sampling of human populations living close to this cave and found evidence of spillover (xx serology) in people highly exposed to wildlife. **This work provides proof-of-concept for an early warning approach that we will expand in this EIDRC to target key viral groups in one of the world's most high-risk EID hotspots.**

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The overall rationale for this EIDRC proposal is that there is substantial evidence that: 1) diverse CoVs, henipaviruses and filoviruses related to known zoonotic pathogens are circulating in wildlife reservoirs in Southeast Asia; **2)** these viruses likely spillover regularly to people, are often unreported or misdiagnosed, and their clinical manifestations and potential to cause pandemics are unknown and underestimated; and **3)** our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be

used as an 'early warning system' to conduct public health interventions and disrupt disease emergence. The overarching goal of this proposal is to launch the **EIDRC-SEA** (**S**outh**E**ast **A**sia) to better understand the risk of zoonotic viral emergence in Southeast Asia. We will initially focus on the diversity of known and closely related CoVs, henipaviruses, and filoviruses in wildlife reservoirs. We will use this work to build our center's interactive network throughout the region, bridge barriers, provide early focal point for basic and translational studies, develop sample handling and storage routines, communication networks, cohort management, infrastructure development and such. We will test these viruses' capacity to infect human cells and mouse models, develop new specific and sensitive serological and molecular diagnostic tools, and conduct surveillance of human populations with high risk of exposure to wildlife, and clinical cohorts to identify evidence of spillover and pandemic potential, and of viruses causing previously 'cryptic' clinical syndromes in people. Our group also has extensive experience in other viral pathogens, including vector-borne flaviviruses, and will use this experience to provide robust long-term surveillance, diagnostics, human and basic science opportunities. While the current proposal focuses its basic and applied research agendas on this group of viral EID, we note that the EIDRC-SEA is poised to rapidly respond to any new emerging outbreak. Thus, filoviruses, henipaviruses and coronaviruses are examples of sentinel surveillance systems in place that can capture, identify and initiate a rapid response targeting any novel microbial pathogen that emerges in this region.

2. Innovation: Previous work by our consortium has developed a system to target surveillance in wildlife and people to better anticipate and identify early spillover events and pre-empt outbreaks of emerging viruses (REF). The EIDRC-SEA we take this approach and scale it up to cover three critically high-risk EID hotspot countries in Southeast Asia, within a regional network of collaborators. The innovation of the EIDRC-SEA is in: 1) its multidisciplinary approach that combines modeling to target the geography for wildlife and human sampling, novel phylogenetic and *in vitro* and animal model approaches to obtain precise biological risk assessments of viral spillover potential into people, the development and transfer of novel serological and molecular diagnostics capabilities throughout the region, and use of parallel large scale cohorts to identify spillover and illness due to known and novel viruses; 2) the biological characterization approach that identifies how likely viruses are to be able to spillover into people, the evaluation of existing countermeasure technologies; technologies that are grounded on our successful work on SARSr-CoVs; and 3) the combination of geographically targeted human populations with high risk of animal contact and syndromic clinical cohorts that present with signs of novel viral emergence, to identify risk of viral spillover in an early warning system approach. **In Aim 1, we will target viruses in new and archived wildlife samples, and characterize their risk of spillover to people**. We will use spatial and phylogeographic analyses to identify the geography and species of wildlife to test samples from, as well as further characterize viruses we have recently discovered. We will use *in silico* methods (i.e. novel ecological-phylogenetic risk ranking and antigenic mapping of receptor binding domains), cell culture, and animal models to assess their potential for spillover into high-risk human populations. **In Aim 2, we will conduct focused, targeted surveys and sampling of human communities with high exposure to wildlife and other animals.** We will use serological tests targeting viruses identified in Aim 1 to identify the baseline spillover of known or novel CoVs, henipaviruses and filoviruses in these populations. Serological findings will be analyzed together with clinical meta-data (including a simple animal-contact survey, location data, etc) to identify zoonotic risk factors and routes of exposure from specific wildlife or vector species. **In Aim 3, we will use syndromic surveillance of clinical cohorts to identify evidence of viral etiology for otherwise 'cryptic' outbreaks.** We will enroll and collect biological samples from patients in local clinics and hospitals who present with syndromes previously linked to known viral agents and live within communities at high risk for disease emergence. We will conduct molecular and serological diagnostic assays to identify likely viral etiology of syndromes that may represent otherwise cryptic events. Where viruses are identified, we will attempt to isolate and characterize them, and use survey data, ecological and phylogeographic analysis to identify likely reservoir hosts and inform intervention programs. Our US partner BSL-4 laboratory, NEIDL, will attempt isolation and characterize any viruses requring high levels of containment (e.g. any novel Filoviruses discovered).

3. Approach

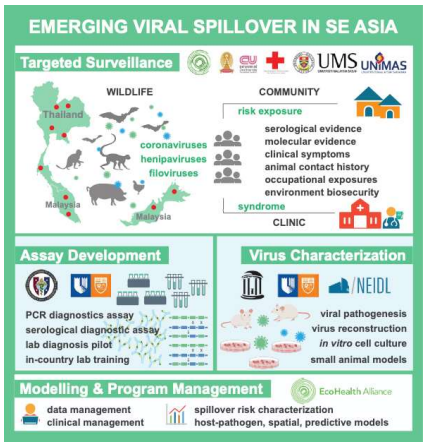
Research team: The EIDRC-SEA builds on long-term collaboration among world leaders in emerging virus

Commented [BRS3]: Number one innovation is the nuclear core sites that make up the EIDRC and its expanded network of long-term collaborators that saturation the most biodiverse EID host spot in the world, 2) extensive sample collections from wildlife, people in close proximity to EID hotspots, and samples from cohorts in individuals seeking medical care at our participating centers? (major goal of proposal if I remember correctly is to set up surveillance network for rapid response and interface wit health care facilities to collect large sample sets and/or develop clinical cohorts for testing intervention technologies (in the future).

Commented [KJO4]: From MeiHo: Another innovation from EIDRC is the quick response if an outbreak happens in the region when the team has been developed and trained under this initiative.

Commented [KJO5]: List out what other labs will do too? Or just rely on our Org chart figure for all of this?

research with proven experience collaborating internationally on field, lab and human surveillance research **(Fig. 2)**. Over the past two decades, our consortium partners have collaborated together within the region and globally to conduct high profile research on EIDs. This work includes identifying the wildlife reservoirs for Nipah and Hendra virus, MERS- and SARS-CoVs (*20, 25, 58, 62-65*), discovering SADS-CoV (*14*), and developing an array of serological, molecular, *in vitro* and *in vivo* approaches to characterizing high-risk CoVs, henipaviruses, and filoviruses (*59-61, 66-79*). Our team has substantial experience conducting human surveillance during outbreaks (e.g– The ongoing viral surveillance using molecular and serological techniques with the indigenous communities of Peninsular Malaysia and syndromic surveillance of hospital patients in Sabah with undiagnosed syndromes through the USAID-PREDICT program and the DTRA funded serological surveillance of Henipaviruses and Filoviruses spillover. - Please insert examples that you've been involved in and REFS), and as part of longitudinal efforts to pre-empt pandemics (*80, 81*).



In 2016, our collaborative team, together with partners in China, discovered an HKU2-clade α-CoV(*82, 83*) in 5 pig farms in Guangdong Province affected by fatal diarrheal disease. We used PCR, serology, pathology, phylogenetic analysis, and infection experiments to show that this novel virus, SADS-CoV, originated in *Rhinolophus* spp. bats and killed > 20,000 pigs at these farms – all in the span of three months (*14, 84*)

Fig. 2: Interdisciplinary team & roles in the proposed EIDRC-SEA.

PI Daszak has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5- 20 yrs on NIAID- and USAID-funded research. He will oversee all activities in this EIDRC. Drs. Olival, Zambrana, and Ross are leaders in analytical approaches to targeting surveillance and head the modeling and analytics work for USAID-PREDICT. Drs. Wang, Baric, Broder, Anderson, and Laing have developed a unique array of *in vitro* cell culture, serological and molecular reagents to identify and characterize emerging viruses in people and animals (henipaviruses and other paramyxoviruses, CoVs, filoviruses and many others). Dr. Wacharapluesedee, Hughes and collaborators have coordinated surveillance of people, wildlife, and livestock within Thailand and Malaysia for the past 10 years, in collaboration with Ministries of Health, Agriculture and Environment. Finally, this consortium has extensive external funding from DoD, USAID, NIH, DHS and other USG agencies that supports a range of laboratory, analytical and field studies directly related to the current proposal, and will bring substantial leverage to the EIDRC **(See Section XX)**.

Fig.3 : Map of Southeast Asia indicating the three core countries for this proposed EIDRC (White: Thai, Sing, Mal) and those that Key Personnel are actively collaborating with (Green: field sites and collaborating labs indicated with asterisk).

Geographical focus: The three core countries for this proposed EIDRC are Thailand, Malaysia and Singapore, three contiguous EID hotspot countries that stretch through one of the most significant foci of EID origins globally. Our proposed work includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sarawak and Sabah, and thus contains overlapping wildlife biogeography with India, Bangladesh, Myanmar, SW China, Laos, Cambodia, Vietnam, Philippines, and Indonesia. It encompasses diverse cultural and socioeconomic backgrounds which have correspondingly diverse and intensive interactions with wildlife reservoirs and the global travel network. We have already begun sampling of wildlife hunters, livestock workers, wildlife farmers and market workers, rural villagers living close to high risk wildlife

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Tsin Wen – Please can you add details here on your Univ N. Sumatra malaria/fever patients to find malaria

Tim and Tsin Wen – Please can you add in details of your encephalitis study

Dr Tan – Please can you add in details of your work with Dayak and other human studies you have done

Dr Lee – Please can you add in details of any hospital based or other human surveillance you have been involved with.

Prof Kamruddin – Please can you add in details of your blood donor, Garbage collector, Febrile and diarrhea

Please add any other examples you have.

species, people otherwise highly exposed to animals in these countries, and clinical cohorts, as part of current/prior NIH and USAID funding. To develop a larger catchment area for emerging zoonoses, we will deploy our consortium's extensive network of collaborators in clinics, research institutes and public health laboratories in every other Southeast Asian country. We will conduct regular meetings with key research, public health and community leaders, including >50 leading laboratories/institutions in XX countries that are currently collaborating with core members of our consortium via other funded work (**Fig. 3**). We will use these, and our annual meetings, to share information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks across the region.

Commented [BRS7]: Show this somehow in the picture. Primary sites, collaborative linkages that extend the breadth of the EIDRC.

Current list of contacts at key human (especially) and wildlife orgs in every SE Asian country from map above – please add to this if we've missed any

Indonesia – EHA: Eijkman (Dodi Safari), IPB (Imung Joko Pamungkas);
SE China – EHA: Yunnan CDC (XX), Wuhan Inst. Virol. (Zhengli Shi);

Myanmar – Chulalongkorn (Thiravat and Supaporn) have collaborated extensively with Win Min Thit, MD (Yangoon General Hospital, Myanmar); Khin Yi Oo (National Health Laboratory, Myanmar) and also in training PREDICT Myanmar veterinarians in bat sampling under PREDICT.

Cambodia -
Laos - **CM Ltd:** Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit (Matthew Robinson)
Bangladesh – EHA: IEDCR (Meerjady Sabrina Flora, Ariful Islam), icddr,b (Mohammed Ziaur)

India – EHA: Jon's collaborators;
Vietnam – Wellcome CRU ? Liaison with Zhengli Shi for Serol assays etc.?
Philippines – EHA, **CM Ltd:** Biodiversity Management Bureau, Department of Environment and Natural Resources (Anson Tagtag),

Malaysia – **CM Ltd:** Borneo Medical and Health Research Center (Ahmed Kamruddin), Department of Wildlife and National Parks Peninsular Malaysia (Frankie Sitam, Jeffrine Japning, Rahmat Topani, Kadir Hashim, Hatta Musa), Hospital Universiti Malaysia Sabah (Helen Lasimbang), Faculty of Medicine, Universiti of Malaya (Sazaly Abu Bakar), Kota Kinabalu Public Health Laboratory (Jiloris Dony, Joel Jaimin), Ministry of Health (Chong Chee Kheong, Khebir Verasahib, Maria Suleiman), National Public Health Laboratory (Hani Mat Hussin, Noorliza Noordin, Yu Kie Chem), Queen Elizabeth Hospital (Giri Rajahram, Heng Gee Lee), Sabah State Health Department (Christina Rundi), Sabah Wildlife Department (Rosa Sipangkui, Senthilvel Nathan, Augustine Tuuga, Jumrafiah Sukor),

Thailand – **CM Ltd:** Mahidol-Oxford Tropical Medicine Research Unit, (Stuart Blacksell, Richard Maude), Ministry of Public Health (Prayuth Sudathip, Hinjoy Soawapak), Faculty of Forestry, Kasetsart University (Prateep Duengkae), Chulalongkorn University (Supaporn Wacharapluesadee).

Chulalongkorn lab is a WHO Collaborating Centre for Research and Training on Viral Zoonoses, we work closely with WHO SEARO and SEARO countries including Bangladesh, Bhutan, Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, (Thailand), Timor-Leste.
We had conducted the PREDICT lab training for Veterinary lab in the Southeast Asia region for FAO.
We conducted the bat sampling technique for scientists from Malaysia, Myanmar, the Philippines, and China.

Commented [KJO8]: From Supaporn

Aim 1: Identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife

Commented [PD9]: Remember to correct specific aims with same title

1.1 Rationale/Innovation: The vast majority of emerging zoonotic pathogens are linked directly or indirectly to wildlife host, particularly from important mammalian groups like bats, rodents and non-human primates (2). Our previous work on wildlife origin viruses demonstrates that zoonotic viral emergence tends to occur in specific regions with high diversity of wildlife that harbor viruses of zoonotic potential, and human populations that have

close contact to these wildlife (Fig 4) (*2*). In Aim 1, we will strategically conduct EID surveillance by applying risk analytics to prioritize the regions, wildlife species, and human populations that have a particularly high risk of viral spillover. We will target high-risk zoonotic host reservoirs (bats, rodents and primates) in these regions and apply novel predictive models to estimate viral host range and identify new host species that have been previously ignored by EID surveillance systems. We will strategically collect specimens from under- or not-yet-sampled species of wildlife and screen these together with some of the tens of thousands of recently collected samples we have archived in freezers in our labs for known and novel agents. We will begin screening using conserved PCR assays to identify known and novel virus in high risk RNA viral families, Coronaviridae, Paramyxoviridae, and Filoviridae. We will expand to other groups of pathogens, pending available resources, after priority setting with the EIDRC coordinating center (EIDRC-CC) and other regional EIDRCs. Using risk ranking algorithms and a series of ecological and virological risk factors, we will triage the wildlife viruses we discover for further genomic characterization and viral isolation attempts. For the subset of highly-ranked and genetically characterized viruses, we will then use *in vitro* and *in vivo* approaches (viral rescue, cell lines, and mouse models) previously developed and widely use by our team [REFS?] to predict capacity of novel viruses to] infect people and spillover. These high risk viruses will be targets for human community and clinical sampling in Aim 2 and 3, respectfully. This approach is built on substantial previous proof-of concept preliminary data:

Geographic targeting: EcoHealth Alliance has led the field in conducting analyses to indicate where the highest risk of zoonotic spillover is across the globe, identifying SE Asia as a particular high risk location for pathogen emergence (*1, 2*). For Southeast Asia, these EID hotspots are sites where high wildlife biodiversity, human population density and rapid ecologic change collide **(Fig 4a)**. Using host and ICTV viral data for all known mammalian viruses, and generalized additive models to correct for sampling bias, we are able to predict the relative number of yet-to-be-described viruses that a species harbors (*2*). Using these data to map unknown viral diversity demonstrates substantial undiscovered viral diversity in mammals across Southeast Asia, and particularly in regions of Thailand, Singapore and Malaysia **(Fig. 4b)**. In a separate paper on risk of viruses emerging from bat hosts, we found that factors driving spillover (host diversity, climate) differ from those driving spread in human populations (population density, bushmeat hunting, livestock production) (*85*). We therefore analyze capacity for viral spread as a separate risk factor, using demographic and air travel data and flight model software we have produced with Dept Homeland Security funds (*86*). In the current proposed work, we will use all the above approaches to target wildlife sampling (archive and new field collections), and to inform sites for human sampling in Aim 2, to areas of high risk for spillover and spread.

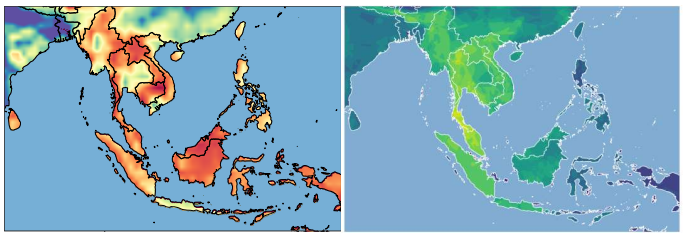


Fig. 4: Our previous work has shown proof-of-concept in geographically targeting sampling in 'hotspot' areas of highest risk for wildlife-to-human spillover and disease emergence **(Fig. 4a)**, and regions with high diversity of predicted 'missing' or as-yet undiscovered viruses, yellow = highest diversity **(Fig 4b)**. From (*1, 2*).

Commented [KJO10]: Will we? If so, make sure we mention primates more and include in vert animal sections.

Commented [KJO11]: Do you think we need to add in or mention vectors (arthropods)? We don't have big banks of these as far as I know, so maybe best not too?

Commented [PD12]: Kevin – please edit

Commented [KJO13]: Peter, consider showing this figure earlier on considering Ralph's point about highlighting the EID rich geography of SE Asia first.

Reservoir host species targeting: Our analysis of all known mammalian host-viral associations demonstrates that bats, rodents and primate represent the three most important reservoirs for zoonoses, responsible for the highest proportion of known and predicted viral diversity of zoonotic potential (*2*). The bulk of our sampling and sample testing will consist of bats, because this group is the known or hypothesized reservoir for the majority of high-impact zoonotic CoVs, henipaviruses and filoviruses (*48, 87-89*) (ADD REFS). Bats also have the highest proportion of predicted zoonotic viruses in any mammalian group (*2*). We have used a novel phylogeographic analysis, Maximum Clade Credibility (MCC) tree, to reconstruct the geographic areas of evolutionary origin for β-CoVs that we sequenced in bats (*49*). This approach allows us to identify the ancestral home of specific zoonotic viral groups in wildlife, where their diversity is likely highest. Finally, we have designed a strategy to estimate sampling gaps, using a viral 'mark-recapture' approach we previously published (*90, 91*) **(Fig. 5)**. Using prior data for bat, rodent and primate viral discovery vs. sampling rates, we can identify the likely total viral diversity for specific host groups in a region, and use this to estimate sample sizes to discovering the majority of unknown viruses within a specific family or genus. **In the current proposal,** we will use these analyses to estimate geographic and species-specific sampling targets **to more effectively identify new strains to support experimental infection studies and risk assessment.**

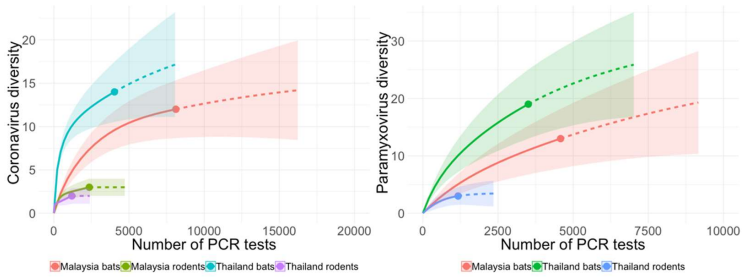


Fig. 5: Estimates of CoV (left) and PMV (right) viral species diversity in bats and rodents for Thailand and Malaysia, based on our preliminary data testing over XXXX specimens using PCR. Bats in Thailand and Malaysia are estimated to have 4X the number of novel viruses in these viral families. CoV and PMV diversity estimates are comparable, but discovery has not yet saturated in any taxonomic group or location. We used this to estimate that additional sampling of X,000 bats and X,XXX rodents will identify >80% of remaining CoV and PMV viral species from key wildlife species in these regions. We will apply this approach to our target host and viral taxa, for Thailand and Malaysia to calculate sampling targets to capture maximal viral species diversity (shown), but also for estimating targets for capturing viral strain diversity.

Sample testing to identify known and novel viruses: We have conducted extensive wildlife surveillance for novel viruses across Southeast Asia in prior NIAID funded work and as part of the USAID PREDICT project. In the first 5 years of the PREDICT program globally (2009-2014), we found more viruses (815 novel, 169 known) than the total number of viruses previously recognized in mammals by the International Committee on Taxonomy of Viruses. This includes collecting nearly 300,000 individual mammal specimens from 14 EHA-led PREDICT countries and PCR-screening more than 60,000 individual specimens(*51*). In southern China alone, this lead to the identification of 178 β-CoVs, of which 172 were novel (52 novel SARSr-CoVs). This includes members of a new β-CoV clade, "lineage E" (41), diverse HKU3-related CoVs (179 sequences) within a 'sister' clade to the SARS-CoV lineage, and the wildlife origin of a new virus (SADS-CoV) killing >20,000 pigs in Guangdong Province (*14*). Many of these bat species are found across the region. We have collected 28,760 samples from bats, rodents and primates in Thailand and 62,012 in Malaysia under PREDICT, conducting 146,503 PCR tests on a large proportion of these specimens, and archiving duplicates which are now available for use in this project(*92*). In Thailand this screening has identified XX. In Malaysia this screening has identified 77 novel viruses and 23 known viruses in wildlife reservoirs (66 novel and 19 known in Sabah and 11 novel and 9 known from Peninsular Malaysia). We used family level primers for henipaviruses, filoviruses and CoVs and have already discovered XX in Thailand and 13 novel and 5 known CoVs and 15 novel and 1 known PMV in Malaysia from wildlife. In the current proposed work, we will attempt to isolate and characterize the full genomes for those that our analyses below suggest are most likely to be able to infect humans.

Commented [PD14]: Kevin to edit

Commented [BRS15]: I would say we focus on these three species (plus vectors?). To demonstrate the capability of the team, this proposal will focus on bat surveillance (for a variety of reasons), noting that this approach will also be applied to other zoonotic vectors during the 5 year program throughout the region.

Commented [KJO16]: We can show just a single curve per country (aggregating all wildlife species) for simplicity, and could fit CoVs and PMVs on one graph.

Commented [PD17]: All to edit

Commented [KJO18]: More?

Commented [KJO19]: Latest numbers from EIDITH for P1 and P2

Commented [PD20]: Kevin – need the PREDICT numbers here please

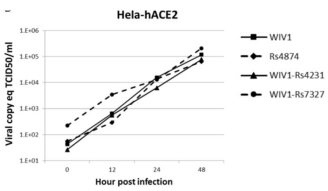
Commented [T21]: Do we want to mention other viruses we found of public health concern that are not part of the 3 viral families we are focusing on in this proposal?

Our collaborative group has worked together on these projects, as well as under DTRA funding in Singapore/Thailand (Co-Is Wacharapleusadee, Wang) to design, cross-train, and use novel serological and molecular platforms to investigate virus infection dynamics in bats and examine potential spillover events with or without human disease. For serology, our lab at Duke-NUS will be developing a phage-display method to screen antibodies for all known and related bat-borne viruses, focusing on henipaviruses, filoviruses and coronaviruses. Once various "trends" are discovered, additional platforms (including Luminex and LIPS) will be used for verification and expanded surveillance. For molecular investigation, we will combine targeted PCR approach with a discovery-driven enrichment NGS strategy to not only survey currently known viruses, but also to discover potential novel viruses. Both the phage-display serological and enrichment platforms can be rapidly updated to include new viruses identified. In Malaysia (Co-Is Hughes, Broder, Laing) the transfer of the Luminex serology platform to partner laboratories for wildlife, livestock and human samples screening to investigate the spillover of Henipaviruses and Filoviruses at high risk interfaces including farms near the forest and indigenous communities with lots of subsistence hunting in Peninsular Malaysia, preliminary screening has found serological evidence of past exposure to Nipah and Ebola antigenically-related viruses in bats, NHPs and people. In Thailand (Co-Is Broder, Laing and Wacharapluesadee) tech transfer of Luminex-based serology platform for detecting exposure to novel Asiatic filoviruses and known henipaviruses in wildlife and human samples is underway and currently being validated in our Chulalongkorn University laboratory. This is part of a planned DoD regional center of excellence for training in Thailand (details please?).

We have found 13 novel CoVs and 15 novel PMV and have begun using Illumina sequencing and virome capture sequencing to further characterize 6 of these novel CoVs and 10 of these novel PMV. To date have completed the full genome sequencing for two novel CoVs that were part of the PREDICT Deep Forest project in Sabah – PREDICT_CoV-51 found in Hipposideros cervinus and PREDICT-CoV-52 (HKU10-like) found in Hipposideros diadema. As part of our PREDICT further characterization work, we will investigate the zoonotic potential of these viruses (i.e., their ability to infect human cells). We will reverse engineer the viral RNA from the genome sequence we obtained, recover the infectious virus, and test its ability to infect a range of human cells. This involves generating a very large synthetic construct of the viral genome, which is extremely difficult and time consuming. Therefore, we use two strategies to investigate the risk of PREDICT CoVs and PMV: 1) We use surrogate experimental systems to evaluate just the important parts of the virus. In this case, we focus only on the receptor binding domain (RBD) in the spike protein, rather than the whole virus, and test whether it is able to mediate entry into human cells. The advantage of this approach is that it can be done quickly and give us a good (but preliminary) assessment of the zoonotic risk. This work is done with our partners at the NIH Rocky Mountain. 2) While this work is ongoing, we start to reverse engineer the full virus, so we can investigate cell-entry in the context of authentic virus. This work is done at the Center for Infection and Immunity, Mailman School of Public Health, Columbia University. This can take 6-12 months to generate a construct and test it which is why we use both approaches.

For Peninsular Malaysia we have 1438 serum samples from Orang Asli (1390 from current study and 48 archived samples), 560 serum samples from bats, 167 serum samples from rodents and 1022 serum samples from NHPs, 301 bat biopsy samples, 123 rodent biopsy samples and 3845 NHPs biopsy samples. For Sabah we have 10 serum samples from syndromic surveillance patients, 1179 serum samples from bats, 485 serum samples from rodents and 83 serum samples from NHPs, 294 bat biopsy samples, 114 rodent biopsy samples and 62 NHPs biopsy samples. Biopsy samples include brain, heart, lungs, liver, kidney, spleen, large and small intestine.

In vitro & in vivo *characterization viral potential for human infection for Coronaviruses:* We have used *in vitro* and *in vivo* experiments to characterize the pathogenic potential of novel SARSr-CoVs from bats. We isolated three SARSr-CoVs from bat feces: WIV1, WIV16 and Rs4874, with S protein sequences that diverged from SARS-CoV by 3% to 7% (*20,*



Commented [KJO22]: From Supaporn: I do not have this information yet, but it is possible.

Commented [PD23]: I think this is something that Linfa, Supaporn or Chris mentioned?

Commented [T24]: Waiting to hear from Simon that same technique for Cov and PMV

Commented [KJO25]: More from Tom. With this comment: **Malaysian colleagues please can you add details here of any archived samples you have that could potentially be included in this proposal using similar level of detail to the above.**

Commented [KJO26]: Think we need to shorten the CoV part of this from the previous NIH. I've added in some basic info and refs on Henipas and Filos in a separate section below (for now), but Ralph/Linfa/Dani/Chris/Eric should check and ideally we integrate into one section. WE need to specify what labs are doing what!

Commented [PD27]: Ralph, Amy – this is directly from our CoV R01 renewal – please reduce the length of this section and adapt for this proposal. We prob don't need all these figures – just one or two would do.

58, 93). We conducted full-length genome sequencing of 12 other novel SARSr-CoVs from a specific cave site in Yunnan China, some highly similar to outbreak SARS-CoV in the most variable genes: N-terminal domain and receptor binding domain (RBD) of the S gene, ORF8 and ORF3 (*58*). Using our reverse genetics system, we constructed chimeric viruses with SARSr-CoV WIV1 backbone and the S gene of different variants, including WIV1-Rs4231S and WIV1-Rs7327S. All 3 SARSr-CoV isolates and the two chimeric viruses replicated efficiently in Vero E6 cells and in HeLa cells expressing hACE2, but not in HeLa cells that don't express ACE2 (*20, 58, 93*) **(Fig. 5a)**. We used the SARS-CoV reverse genetics system (*70*) to generate a chimeric virus with a mouse-adapted SARS-CoV backbone expressing SHC014 S protein with 10% sequence divergence from SARS-CoV S. This chimera replicated in primary human airway epithelium, using the human ACE2 receptor to enter into cells (*59*) (**Fig. 5b**). Thus, **SARSr-CoVs with diverse variants of SL-CoV S protein without deletions in their RBD can use human ACE2 as receptor for cell entry.**

Fig. 5a (left): RT-PCR shows that bat SARSr-CoVs WIV1, Rs4874, and chimeras WIV1-Rs4231S, WIV1-Rs7327S grow in HeLa cells expressing human ACE2. **Fig. 5b (right):** Viral replication of SARS-CoV Urbani (black) and SARS-SHC014S (green) primary air-liquid interface human airway epithelial cell cultures at an MOI of 0.01.

We infected transgenic mice expressing hACE2 with 10^5 pfu of full-length recombinant WIV1 and three chimeric viruses (WIV1 backbone with SHC014S, WIV16S and Rs4231S). hACE2 transgenic mice challenged with rWIV1-SHC014S experienced ~20% body weight loss by 6dpi; rWIV1 and rWIV-4231S produced less body weight loss, and rWIV1-WIV16S led to no body weight loss **(Fig. 6a)**. At 2 and 4 dpi, viral loads in lung tissues of mice challenged with all three chimeras reached > 10^6 genome copies/g, significantly higher than rWIV1 infection **(Fig. 6b)**. This demonstrates that pathogenicity of SARSr-CoVs in humanized mice differs with divergent S proteins, **confirming the value of this model in assessing novel SARSr-CoV pathogenicity**.

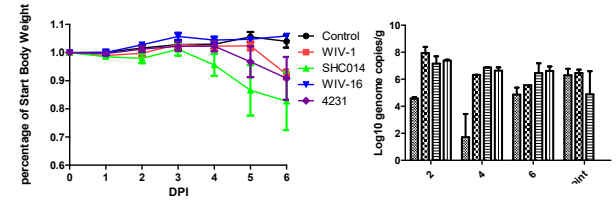


Fig. 6: *In vivo* infection of SARSr-CoVs in hACE2 transgenic mice. **6a (left)** Body weight change after infection; **6b (right)** Viral load in lung tissues.

Infection of rWIV1-SHC014S caused mild SARS-like clinical signs in the transgenic hACE2 mouse model **that weren't reduced by immune-therapeutic monoclonals that attenuate SARS-CoV pathogenecity.** Vaccination against SARS-CoV did not reduce severity of clinical signs in mice subsequently infected with rSARS-SHC014S (*59*). We found 2/4 broad human mAbs against SARS-CoV RBD cross-neutralized WIV1, but none could efficiently neutralize SHC014 which is less similar to SARS-CoV in the RBD (*94*). We repeated this virus characterization approach with chimeras using HKU3r-CoV S proteins that are ~25% divergent from SARS-CoV S, **and found that they are unable to use the ACE2 receptor**. Additionally, we were unable to culture HKU3r-CoVs in Vero E6 cells, or human cell lines. **The ability of HKU3r-CoVs to infect people, and their receptor binding target, remain unknown.**

All – what can we say about Nipah and filoviruses in this section – what is the rationale for a similar approach (e.g. we're looking for viruses somewhere between Cedar and Nipah in the Henipaviruses, and trying to assess the likelihood of some of the novel filoviruses infecting human cells).

Are we just going to use cell culture for henipas and filos, or can we look at spike protein diversity?

What about using bat cell lines and batized mice from Linfa?

In vitro & in vivo *characterization viral potential for human infection for Henipaviruses and Filoviruses*

A similar approach to the above pipeline for CoVs will be applied to novel Henipaviruses and Filovirues we discover during our research.

Henipaviruses

Commented [PD28]: Ralph, Amy, Linfa, Danielle, Chris, Eric – need details for what approach we'll use for henipas and filos

Commented [KJO29]: Think we need to shorten the CoV part above and still incorporate more for Henipas and Filos. For now some info below, but Ralph/Linfa/Dani/Chris/Eric should check and try to integrate into one section with additional info one the Cedar virus work, Mojiang, etc.. WE also need to specify what labs are doing what!

Commented [KJO30]: General question, do we want to limit to Henipas, or keep more general to Paramyxoviruses? Do we care about Rubulaviruses, etc.

Commented [KJO31]: Ralph: Am J Pathol. 2003 Dec;163(6):2371-82 Primary human lung endothelial cells are highly susceptible to EBOLA virus infection, so we can use these cells. Huh7 cells are good candidates for ebola in the liver, oftentimes used to isolate virus from clinical samples (Viruses. 2019 Feb 16;11(2). pii: E161.) Huh7 cells also ofers advantages for reverse genetic recovery of novel filoviruses (J Infect Dis. 2015 Oct 1;212 Suppl 2:S129-37.)

We have growth curves of Nipha and Hendra in Calu3 cells, a continuous lung epithelial cell, including a variety of host expression changes after infection. Likely grow in primary human airway cultures as well as they infect ciliated and secretory cells (J Gen Virol. 2016 May;97(5):1077-86.). Nipha and hendra also infect primary human microvascular endothelial cells, like mers and ebola (PMC3477106, PMC3791741). I'm less sure what kind of neural cell should be used for Nipha and Hendra.

Some evidence for nipha and Hendra replication in mouse lung, low level titers in young animals, 10 fold higher titers in aged mice (PMC4057804, PMC3393746). Thus it is likely these models could be improved in the collaborative cross mouse resource here at UNC. We have identified CC lines that are highly vulnerable to wildtype SARS and others that develop hemorhaggic disease following mouse adapted ebola infections.

Hendra virus and Nipah virus have a broad mammalian tropism with natural infection recorded in bats, horses, pigs, humans, cattle, goats and dogs. This broad species tropism is likely mediated by heniapvirus receptor usage of highly conserved ephrins ligands (e.g. ephrin-B2 and -B3) for cell entry. Ephrins are critical in evolutionary developmental process such as cell migration, axonal guidance and angiogenesis (REF), and eprin tissue distribution correlates with the multi-systematic cellular pathology, vasculitis and encephalitis disease presentation during HeV and NiV infection. The third isolated henipavirus is Cedar virus, yet unlike NiV/HeV, CedV does not cause pathogenesis in animal models. Recently, Dr. Broder and Laing have developed a reverse genetics system for Cedar henipavirus and have rescued a recombinant Cedar virus that is used as a model henipavirus tool to understand how ephrin receptor usage/tropism contributes to pathogenicity during henipavirus infections. Ephrin-B3 is distributed in the spinal cord, and usage of ephrin-B3 has been postulated to underlie the enhanced encephalitis seen during NiV infection. Further dissimilar from HeV/NiV, CedV is unable to utilize ephrin-B3 as a receptor (Marsh 2012; Laing 2018) nor does it express the interferon antagonizing virus factors: V and W proteins (Marsh 2012). However, Dr. Broder and Laing have discovered that CedV has a promiscuous/broad ephrin receptor usage and in addition to ephrin-B2 is able to utilize ephrins-B1, -A2 and -A5 for cell entry (Can include microscopy image/column graph for unpublished figure). Additionally, it was discovered that CedV can utilize mouse ephrin-A1, which differs from human ephrin-A1 by one amino acid residue in the key binding pocket, demonstrating the first evidence of henipavirus species-specific receptor use (Can include unpublished figure). Like ephrin-B3, ephrin-B1 is widely distributed throughout spinal cord tissues, however, CedV is non-pathogenic. Pathogenicity of henipaviruses is likely mediated by a contribution of both utility of ephrin receptors and expression of virulence factors V and W proteins. The full genome assembles of putative henipaviruses, Ghana virus and Mojiang virus, predict expression of V and W proteins. GhV is able to bind to ephrin-B2, but not -B2 (Lee – African emergent henipavirus B2 crystal) and the receptor for MoJV remains unknwon (Lee, idiosyncratic 2017), but is unlikely an ephrin. Thus, when we identify novel henipaviruses or paramyxoviruses, we will examine the receptor-binding proteins (e.g. envelope attachment (G) proteins) for sequence and structural homology with particular attention to conversation of amino acids that mediate ephrin receptor binding. We will prioritize molecular experimentation with novel henipaviruses that are predicted to interact with ephrin-B2 and expression V/W proteins to determine whether these viruses represent zoonotic threats. Using the recombinant Cedar virus (rCedV) platform, we will be able to rescue rCedV that express heterotypic G proteins to explore receptor utilization and tissue dissemination in animal models.

For novel Henipaviruses we will assess ability to infect human cells using a continuous lung epithelial cell line, Calu3, that our group has previously used to generate data on NiV and HeV growth curves (*95*). We will also use primary human airway cultures to assess human infection for Henipavirus and MERS- and SARS-related CoV (*96*) (*97*) . There is some evidence for Nipah and Hendra virus replication in mouse lung, low level titers in young animals, 10 fold higher titers in aged mice (*98, 99*). Thus it is likely these models could be improved in the collaborative cross mouse resource at UNC. We have identified CC lines that are highly vulnerable to wildtype SARS and others that develop hemorhaggic disease following mouse adapted ebola infections.

Am J Pathol. 2003 Dec;163(6):2371-82 Primary human lung endothelial cells are highly susceptible to EBOLA virus infection, so we can use these cells. Huh7 cells are good candidates for ebola in the liver, oftentimes used to isolate virus from clinical samples (Viruses. 2019 Feb 16;11(2). pii: E161.) Huh7 cells also ofers advantages for reverse genetic recovery of novel filoviruses (J Infect Dis. 2015 Oct 1;212 Suppl 2:S129-37.)

Attachment Glycoprotein has greater genetic diversity
Thus far, paramyxovirus attachment glycoproteins have been divided into three major groups: hemagglutinin (H), hemagglutinin-neuraminidase (HN), and attachment glycoproteins (G)

pon mapping sequence conservation between GhV-G and MojPV-G onto the GhV-G-ephrinB2 co-crystal structure, it becomes apparent that MojPV is unlikely to utilize ephrinB2 receptor (*100*)

Commented [EL32]: PMID: 16357858

Commented [EL33]: PMID: 15998730

Commented [EL34]: PMID: 16007075

Commented [EL35]: PMID: 29587789

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Commented [EL37]: PMID: 16477309
PMID: 16477309

Cedar uses Ephrin B, but doesn't cause disease. reverse genetics of Henipas - would need to be done in BSL4, pseudovirus can do in BSL 2, but no pathogenesis. Benhur Lee has done similar work to what Ralph has done, structural comparison w diff receptors for Cedar, Henipas, and ferret model. Doesn't express G protein and is therefore less pathogenic.

Mojiang diff cell binding: https://www.nature.com/articles/ncomms16060
" Furthermore, we find that MojV-G is antigenically distinct, indicating that MojV would less likely be detected in existing large-scale serological screening studies focused on well-established HNVs. "

1.2 General Approach:
We will conduct analyses to geographically- and taxonomically-target testing of samples from wild mammals that are most likely to harbor known high-profile zoonotic pathogens, or close relatives with potential to infect people. This includes selecting from tens of thousands of samples collected on recent projects and stored in freezers in our laboratories, and new collection from wildlife in high risk locales. We will use serological & PCR testing, to identify viruses, then attempt to isolate and biologically characterize viruses that phylogenetic and other data suggest have high spillover and pandemic potential. We will then conduct *in vitro* binding assays and cell culture experiments, and *in vivo* animal model infections to assess their potential to infect people and cause disease. EHA will lead the study design, targeted sampling, and data analysis; Co-Is Hughes and Wacharapluesadee will lead field sampling and in-country testing; Duke-NUS, UNC, USUS will lead the serological and PCR diagnostic development and the *in vitro* and *in vivo* analyses.

A key goal of this work will be technology transfer to the region, including cell culture, animal models, serological and PCR diagnostics, as well as knowledge transfer by embedding staff in partner labs and by regular meetings. This will leverage substantially from the other funding available to consortium partners.
A key goal of this work will be technology transfer to the region, including cell culture, animal models, serological and PCR diagnostics, as well as knowledge transfer by maintaining our current team members and embedding additional staff in partner labs, supporting one Malaysian PhD student and by regular meetings. This will leverage substantially from the other funding available to consortium partners. The PhD student Mei Ho Lee is part of the CM Ltd team and has worked with the collaborators on this project for 9 years. Mei Ho will enroll at the Faculty of Tropical Medicine at Mahidol University in Bangkok. The focus of her study will be to further investigate the infection potential of some of novel Cov and PMV viruses found in Malaysia through PREDICT.

CM/EHA helped establish and manages with SWD the Wildlife Health, Genetic and Forensic Laboratory (certified as a BSL- 2 laboratory according to the US standard for laboratory specifications) that has all the equipment necessary to store samples, run extractions, PCR and analysis on biological samples for wildlife disease surveillance. The lab is used to conduct health checks on rescued and relocated wildlife before being released into new areas or sanctuaries, to screen samples for the PREDICT and Deep Forest Project and for genetic research and forensic investigations. CM/EHA also helped establish the new molecular zoonosis laboratories (certified as a BSL- 2 laboratory according to the US standard for laboratory specifications), at DWNP's National Wildlife Forensic Laboratory. The lab is used to screen samples for the PREDICT & DTRA projects. In addition CM/EHA also has access to NPHL and KKPHL for screening human samples and the Virology lab at FVMUPM for screening Livestock samples.

Through this project we will help to establish a certified BSL-2 lab at the BMHRC. This lab can then be used for human testing to elevate pressure at KKPHL and QEH labs. It would also be an ideal location for a Luminex platform as human, wildlife and livestock samples could all be screened here. Co-Is Hughes and Kamruddin will be starting outbreak response training in August 2019 with a team from Sabah CDC, KKPHL and BMHRC. (Dr. Maria Suleiman now in PM But still collaborating with us, Dr. Muhammad Jikal current Director CDC

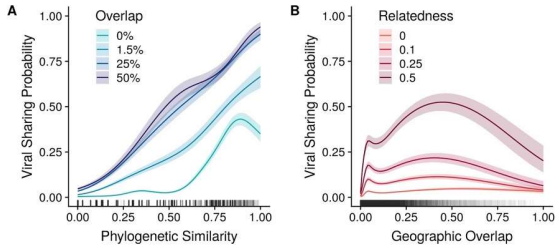
working with us and Dato' Dr Cristina Rundi Director Sabah state health Dept is supportive) this collaborative group is working to develop a Sabah outbreak response team that will help support SSHD/ CDC outbreak team and also conduct sampling for disease surveillance to be based at BMHRC and we will develop this as part of this proposal. In addition the UMS hospital will come in line in 2021 providing additional opportunities for cohort studies and capacity building.

1.3 Wildlife samples: 1.3.a Geographic and taxonomic targeting for newly collected wildlife samples

Adapt text from the rationale/innovation above and make brief statements about the work we'll do:

- Geographic targeting: we will use refined hotspot, 'missing viruses', and FLIRT analyses
- Targeting of host species/viral discovery targets: We will use phylogenetic MCC analyses and viral discovery curves
- Additional approaches: mapping of cave sites,

Using network analyses and a phylogeographic model of host range we recently developed [REF- Greg], we will prioritize additional wildlife species to be sampled and tested for the known and novel viruses we discover. Applying a generalizable model that includes just two phylogeographic traits (wildlife species range overlap and phylogenetic similarity between host species, Fig X.), we are able model the viral sharing network for 4200 mammal species and successfully predicted known hosts above 98% of other mammal species.



1.3.b **Sample size justifications for testing new and archived wildlife specimens**

To calculate sample sizes, we will use our preliminary data on average prevelance from screening bat, rodent and primate specimens for CoVs, PMVs, and FVs under PREDICT in Malaysia and Thailand, previously published data, and our viral discovery curve analyses (Fig 5) to determine the required samples sizes to maximize discovery of wildlife viruses with zoonotic potential.

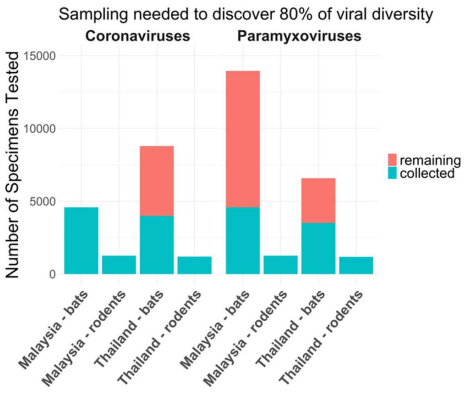


Figure X. Stacked bar plot showing number of currently-archived specimens (blue) and new specimens required (orange) to discover 80% of predicted number of CoVs and PMVs from high-risk bat and rodent taxa in Malaysia and Thailand. Estimates of predicted viral richness calculated from viral discover curve analysis (Fig 5) using data from thousands of wildlife specimens that we have previously tested and sequenced.

In Y1 we will use our bat host and viral trait modeling, phylogeographic analyses of RdRp and S Protein sequences, and geographic and host species-based viral discovery curve analyses to identify SARSr-CoV diversity hotspot regions for bat sampling. We will sample at 8 new sites in four provinces. We will use cave site data (*101*), and demographic information to identify two sites in each

Commented [KJO38]: From Tom.

Commented [KJO39]: Peter, I'll clean ups this section a little more and flesh it out while you are working on this draft.

Commented [KJO40]: From Tom: We should consider wildlife sampling at –
- Danum Valley, Sabah
- Tabin Forest Reserve, Sabah
- Around Orang Asli settlements PM (focus on Kedah, Kelantan, Pahang and Perlis),
- Around Dayak settlements in Sarawak
- Kubah National Park, Sarawak
- Batu Supu Sabah low disturbance cave and surrounding area
- Madai medium disturbance cave and surrounding area
- Gomantong high disturbance cave and surrounding area
- Caves in Peninsular Malaysia

Commented [PD41]: Please check section 1.4.b and see if parts of that should be here..

Commented [BRS42]: CoV specific, ebola and nipha/Hendra have different spike glycoprotein designations and polymerase designations.

of Yunnan, Guangxi, Guangdong, and Guizhou where humans likely have contact with bats. In Yunnan, we will identify two unsampled caves close to, but distinct from, the Jinning cave (*58*). This will provide adequate coverage of lineage 1 and 2 SARSr-CoVs, including a rich source of new HKU3r-CoVs, which have unknown potential for zoonotic spillover. Sampling will begin towards the end of Y1. We will use survey data from our previous R01 and host-specific viral accumulation curve data to target an additional 10 under-sampled *Rhinolophus* spp., 5 that were SARSr-CoV negative in our study, and a small number of related bat genera (including *Hipposideros* spp. and *Aselliscus* spp.) we previously found PCR positive for SARSr-CoVs **(Table 1)**. We will sample at least 5,000 bats from these 4 provinces (~1250 per province). Given ~5-12% prevalence of SARSr-CoVs in *Rhinolophus* spp. at our previous sites, **this sample size would give us 425 (±175) positive individual bats, and ~125 novel strains.**

1.3c Sample testing, viral isolation: Viral RNA will be extracted from bat fecal pellets/anal swabs with High Pure Viral RNA Kit (Roche). RNA will be aliquoted, and stored at -80C. One-step hemi-nested RT-PCR (Invitrogen) will be used to detect the presence of CoV sequences using primers that target a 440-nt fragment in the RNA-dependent RNA polymerase gene (RdRp) of all known α- and β-CoVs (*102*). PCR products will be gel purified and sequenced with an ABI Prism 3730 DNA analyzer.

We will attempt isolation on samples that contain viruses of interest (determined in **1.4, below**), using Vero E6 cells and primary cell culture from the wildlife taxonomic order (bat, rodent and primate cell lines). This includes the over XX cell lines maintained at Duke-NUS

1.3d Moving beyond RNA viruses: [Show how our platform is generziable to other pathogen groups and non-RNA viruses i.e. using conserved PCR assasy and vircapseq. If and when depending necessary on EID-CC research projects and outbreaks detected in the region from DNA viruses or other agents]

1.3.e Sequencing S proteins: *Our working hypothesis is that many zoonotic SARS-like, MERS-like, filoviruses and henipaviruses encode spike glycoproteins that program efficient infection of primary human cells (e.g., lung, liver, etc.).* For example, our previous R01 work identified diverse SARSr-CoVs with high propensity for human infection (*15, 58, 59*). Characterization of these suggests SARSr-CoVs that are up to 10%, but not 25% different in the spike glycoprotein use human and bat ACE2 receptors for docking and entry. Uneven sampling gaps (e.g., no strains were found with 10-25% spike variation) prevent a thorough understanding of the transition point where the most divergent strains lose human ACE2 receptor usage. Our viral discovery curves **(Fig. 4)** suggest further sampling will reveal a rich diversity of as-yet-undiscovered SARSr-CoVs. In this aim we will use phylogenetic and viral discovery analysis to specifically target bat species and regions that are undersampled to allow sufficient power to identify and characterize missing SARSr-CoV strain diversity. Our previous work also suggests that SARSr-CoVs with S proteins that are ~10% divergent from SARS-CoV resist neutralization by therapeutic mAbs and escape SARS-CoV vaccines (*20, 59, 103*), suggesting that . However, viruses with 10-25% divergence in S proteins may bind to human cell receptors, but completely evade therapeutic and vaccine effects, and could therefore be a higher risk for public health. We will sequence the S proteins of novel SARSr-CoVs to prioritize viruses for experimental work in Aim 3 to test this hypothesis. Likewise, we will test the hypothesis that phylogenetic related zoonotic spike genes of MERS-like, Ebola-like and Nipha/Hendra like viruses will also program efficient entry via human ortholog receptors.

For all novel SARSr-CoV strains, we will sequence the complete S gene by amplifying overlapping fragments using degenerate primers as shown previously (*20, 58*). Full-length genomes of selected SARSr-CoV strains (representative across subclades) will be sequenced via high throughput sequencing method followed by genome walking. The sequencing libraries will be constructed using NEBNext Ultra II DNA Library Prep Kit for Illumina and sequenced on a MiSeq sequencer. PCR will be performed to fill gaps in the genome. The full length S gene sequences, including the amount of variation in the S receptor binding residues that bind the ACE2 receptor, will be used to select strains for Aim 3 experiments.

Commented [BRS43]: Chinese locations? Do we have equivalent cool bat caves in malasia, Thailand, etc? to chase bat viruses in?

Commented [BRS44]: Don't forget about th eovel mers-like cov discovered in the region (PMC6002729) and that can use the human receptor (don't be so SARS centric).

Commented [KJO45]: I still need to rework this.

Commented [PD46]: Ralph, Amy, Linfa, Danielle, Chris, Eric - This is from our CoV R01. Please insert details that apply also to henipa and filoviruses.

Commented [KJO47]: Should we just rely on PREDICT PCR protocols for PMVs and Filos? We can add in methods from PREDICT protocols. Unless there are better screening assays to use?

Commented [PD48]: Linfa, Supaporn, Danielle – is this correct?

Commented [PD49]: Brief description of these please

Commented [KJO50]: Added this b/c noted on call w Linfa.

Commented [PD51]: Ralph et al. – I'm assuming this will still be of value for the CoVs, so a reduced version of this could be in here, but what about spike proteins of filos and henipas – is that not of any use for assessing their capacity to infect human cells. Can we do similar work with filovirus pseudotypes from sequencing the spike proteins, for example, to assess binding to human receptors? If so, please draft some text and point to some references

Commented [KJO52]: Not sure these are "Spike" genes for PMVs and Filos?

Commented [BRS53]: Way to specific. We should do spike genes of all novel CoV, including searching for elusive group 1 or group 2 CoV (group 2d, 2e, etc.) that have the potential to replicate in human cells (intestinal CaCO2 or lung HAE….noting that bat cov are enteric pathogens so human caco2 may be likely target). Same with filoviruses and nipha/Hendra like strains.

1.4. Assessing risk for spillover. 1.4.a Capacity to infect human cells: We will use a series of cell cultures to assess the capacity of target viruses to enter human cells. For CoVs, Filoviruses and *Henipaviruses*, we will use primary human ciliated airway epithelial cells (HAE) and lung endothelial cell cultures from the lungs of transplant recipients represent highly differentiated human airway epithelium containing ciliated and non-ciliated epithelial and goblet cells, grown on an air-liquid interface for several weeks prior to use (*59, 60, 104*). We will prepare HAE and lung endothelial cultures from three different patient codes in triplicate in collaboration with the tissue procurement facility at the Cystic Fibrosis Center at UNC. Cultures will be inoculated with chimeric bat SARSr-CoVs to assess efficient replication. At 72 hpi, cultures will be fixed for immunofluorescent staining using antisera to the SARS-CoV conserved nucleocapsid protein (N) or to structural genes of the other virus strains (*61, 69*). SARSr-CoVs that differ significantly in S protein sequence (11-24%) from epidemic SARS-CoV yet replicate *in vitro*, will also be evaluated for sensitivity to neutralization in Vero cells by PRNT50 assays using broadly SARS-CoV cross reactive human mAbs S227.14, S230.15, S215.17, and S109.8 (*60, 92*). As controls or if antisera is not available, the S genes of novel SARSr-CoV or structural genes of other novel viruses under investigation will be inserted into VEE 3526 replicon vectors (VRP3526-S), packaged and used to vaccinate mice (*105*). Polyclonal sera against SARS-like viruses will be harvested and tested for ability to cross neutralize SARS-CoV, GD03, WIV-1, SHC014, WIV-16, other novel SARSr-CoV and HKU3-SRBD by PRNT50 assay (*60, 106, 107*). Using PRNT50 titers with sera (n=4 each) among these viruses, antigenic cartography (*108*) will allow comparison of antigenic vs. phylogenetic distance, identifying the transition at which SARSr-CoV strains escape SARS-CoV based vaccines, informing immunotherapeutic and vaccine design strategies (*109-111*). Similar approaches will be applied to MERS-like viruses, other CoV, filoviruses or henipaviruses

Filoviruses (primary endothelial cells – ralph has primary lung endos – see if anyone has liver primary hepatocytes. Other option are monocytes) – where will we do this? BSL4? (NEIDL). Duplicate samples in culture medium from animals PCR +ve for filoviruses will be shipped to NEIDL (See letter of support) for culture and sharing with other agencies. Check on this re. wildlife filos…See if can do training opportunities so they can work with the NEIDL – visiting scholar appointments

1.4.xx Host ACE2, DPPR, Niemann-Pick C1 (NPC1) and EphrinB2/EphrinB3 receptors: We will sequence host ACE2 receptors of different bat species or different bat populations from a single species (e.g. *Rhinolophus sinicus*) to identify relative importance of different hosts of high risk SARSr-CoVs and the *intra*specific scale of host-CoV coevolution. Of particular interest is homology across 18 bat and human orthologue ACE2 contact interface residues that engage the SARS RBD as a potential indicator of SARSr-CoV cross species transmission potential and growth in human cells (*68*).

1.4.b Host-virus evolution and distribution: We will use Bayesian and Maximum Likelihood phylogenetic analyses of RdRp (or L genes), Spike glycoproteins, and full genome sequence (when available) data to reconstruct the evolutionary history of the novel bat SARSr-CoVs, bat MERSr-CoV, filoviruses and henipaviruses that we identify. We will rerun MCC analyses **(Fig. 3)** to reconstruct β-CoV evolutionary origins using our expanded dataset. Ecological niche models will be used to predict spatial distribution of PCR-positive species, identify target sites for additional bat sampling, and analyze overlap with people. We will use our viral discovery/accumulation curve approach **(Fig. 4)** to monitor progress towards discovery of >80% of predicted diversity of SARSr-CoVs (lineage 1 & 2) or other virus families within species and sites, halting sampling when this target is reached, and freeing up resources for work other work (*91, 112*).

1.4.c Viral strain prioritization: Of the expected 100-200 novel SARSr-CoV strains, we will down-select to prioritize for further characterization based on S genes that are: i) different from SHC014, WIV1, SARS-CoV with diversity ranges of 10-25%; ii) have virus S RBD that could use human/bat receptors; iii) have recombinant chimeric spikes indicative of gene flow between clade I and II strains; iv) have bat ACE2 receptors that might select for spike RBDs that can use human receptors for entry (15/18 conserved residues in human/bat ACE2 molecules that bind SARSs-CoV S RBD domains are likely more efficient receptors than 3/18 conserved sites). Using structural models based on the SARS S glycoprotein, the extent and location of

Commented [BRS54]: Can we get our hands on the live Chinese ebola like strains? We could try to use reverse genetics to recover, although I personally have no documented success with filovirus reverse genetics. Am familiar with approach and the bsl4 in boston should allow recovery.

Commented [PD55]: Ralph, Linfa, Chris – please expand and draft – I think this could be a good role for NEIDL, but don't know what the rules are…

Commented [BRS56]: Check my comments above, I covered this completely and the situation is good.

Commented [BRS57]: A lot of variation in the NPC1 receptor alters EBOV ability to infect in humans and bats, so this can be structurally mapped for prediction and downselection of strains (J Infect Dis. 2018 Nov 22;218(suppl_5):S397-S402.) PMC4709267
Second paper says: *We found signatures of positive selection in bat NPC1 concentrated at the virus-receptor interface, with the strongest signal at the same residue that controls EBOV infection in Eidolon helvum cells. Our work identifies NPC1 as a genetic determinant of filovirus susceptibility in bats, and suggests that some NPC1 variations reflect host adaptations to reduce filovirus replication and virulence.*

Nipha and Hendra use ephrin B2 or ephrin B3 as a receptor, and variation effects its ability to use human vs bat receptor molecules (PMC4418902, PMC2045465)

Commented [PD58]: Kevin/Ralph – I don't think this is relevant for our EIDRC – please let me know and delete if correct.

Commented [PD59]: KEvin/Alice – please modify this section for filos and henipas as well (and other hosts) - or does it belong in 1.3.a above?

Commented [BRS60]: Way to sars centric

antigenic variation will be annotated onto the structure, further resolving the locations of highly evolving and conserved structural motifs/epitopes that function in protective immunity (*92, 113-115*).

1.4.d Animal models: Transgenic Mice. We will use a series of mouse models to assess spillover potential of viruses. First, the Baric lab has a well-established hACE2 transgenic mouse model that we will use this to characterize the capacity of specific SARSr-CoVs to infect. This group also has transgenic mice expressing hDPP4 receptors (PMC5578707, PMC5165197). Briefly, in BSL3, n=5 10- to 20-week old hACE2 or hDPP4 transgenic mice will be intranasally inoculated with 1 x 10^4 PFU of wildtype WIV-1 or chimeric bat SARSr-CoVs with different spike proteins or MERS-CoV and MERS-like CoV, respectively, then monitored daily for weight loss, morbidity, and clinical signs of disease. Mice will be euthanized at 2, 4, and 6 dpi (endpoint of 14 dpi), organs harvested and virus quantified by NP RT-PCR. After 7 days in formalin, tissues for histopathology will be removed from the BSL3 and stained with H&E, and for immunohistochemistry using polyclonal antibodies against the N protein. We will conduct limited evaluation of existing countermeasures using therapeutic monoclonal antibodies *in vitro* and *in vivo*. Existing SARSr-CoV or MERS-CoV mAbs (PMC4769911, PMC4547275) will be diluted in DMEM starting at 1:20, and serial 2-fold dilutions mixed with an equal volume of 50 PFU of chimeric bat SARSr-CoVs with different spike proteins, then incubated on Vero E6 cells at 37°C for 1 h, then changed to a semi-solid medium (DMEM containing 1% methylcellulose and 2% FBS). Antibody neutralization titers will be evaluated by plaque quantification at 4-5dpi. hACE2 transgenic mice will be injected with SARS-CoV mAbs, and infected with chimeric bat SARSr-CoVs. Clinical signs and morbidity will be assessed and tissue pathology examined and compared with mice without treatment of mAbs to determine the therapeutic effect on SARSr-CoV infection, and protection of SARSr-CoV by wildtype SARS-S based vaccines assessed as described (*104, 116*). We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS or MERS-CoV cross neutralization, synthetically reconstruct a small subset (1-2) and evaluate the ability of nucleoside analogues to inhibit growth in HAE cultures and/or *in vivo* (*60, 104*).

Models of Outbred Populations. For novel viruses across all families, we will use the UNC collaborative cross mouse that captures 90% of the diversity within all mouse groups to assess the most suitable model for further experiments. We have used this model for CoV, filo (Ebola), Flaviviruses, and alphaviruses infections. In subpopulations it reproduces SARS weight loss, hemorrhage in EBOV, enceph in WNV, acute or chronic arthiritis in Chikungunya infection ().

Duke-NUS has developed two models for bat *in vivo* studies: a colony of *Eonycteris spelaea* (cave dwelling small fruit bat) and a bat mouse model, which contains the bat immune system in an immunodeficient mouse (*117*). Both animal systems can be used to test susceptibility, pathogenesis and immune responses of any newly discovered viruses.

1.5 Potential problems/alternative approaches: Permission to sample bats in sites or provinces we select. We have a >20-year track record of successful field work in Malaysia, Thailand and >10 years in Singapore, and have worked with local authorities and communities to ensure access. We have existing permissions in place which will be renewed at the start of this project. Due to the abundance of wildlife habitats in the region, if access to a site is withdrawn, we will rapidly identify a suitable, complementary site with similar bat species composition and abundance. Finally, we have already collected tens of thousands of wildlife samples from the region and will focus on these while waiting for permission. **We may not identify novel viruses in our sample wildlife species due to seasonality of viral shedding or other factors.** We have calculated sample sizes based on a range of PCR data and are fairly conservative in our approach. However, as a back-up, we already have identified dozens of novel viruses that are near-neighbors to known high-impact agents, and will continue characterizing these should discovery efforts yield few novel viruses. The sampling regions we have selected are subtropical, and our previous data, and even published studies in temperate regions (*118*), do not suggest a strong pattern of seasonality in CoV shedding, although there are studies that suggest this occurs in henipavirus and filovirus (MARV?) shedding (REFS?). Nonetheless to account for this we will conduct sampling evenly on quarterly basis within each province.

Commented [PD61]: Ralph – can you reduce this section to a feasible amount of work for this project, and remove a bunch of text

Commented [BRS62]: We need to do this with novel Nipha/Hendra and EBoV as well.

Commented [PD63]: Ralph – please draft a brief para explaining what we'll do with this mouse model

Commented [BRS64]: Trends Genet. 2018 Oct;34(10):777-789.

Commented [BRS65]: G3 (Bethesda). 2018 Feb 2;8(2):427-445.
G3 (Bethesda). 2017 Jun 7;7(6):1653-1663.
Genom Data. 2016 Oct 14;10:137-140.
Genom Data. 2016 Oct 14;10:114-117.
PLoS Genet. 2015 Oct 9;11(10):e1005504.
Science. 2014 Nov 21;346(6212):987-91.
PLoS Pathog. 2013 Feb;9(2):e1003196.

Commented [PD66]: Linfa/Danielle – please draft a brief para explaining how we'll use either or both…

Commented [PD67]: Kevin – please edit/check

Commented [PD68]: Kevin – please check the veracity of these comments

Aim 2: Test evidence of viral spillover in high-risk communities using novel serological assays.

2.1 Rationale/Innovation: Assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities that are the first to get infected. To enhance low statistical probability of identifying these rare events, populations will be targeted that both live in rural locations close to high wildlife biodiversity, and also engage in practices that enhance the risk of spillover (e.g. hunting and butchering wildlife). In Aim 2 we will expand from our current work in the region to identify and enroll large cross-sectional samples from human populations that have a high behavioral risk of exposure to wildlife origin viruses and live close to sites identified in Aim 1 as having high viral diversity in wildlife. We will initially identify X-X sites in each Thailand, Peninsular Malaysia, Sarawak, and Sabah for repeated biological sampling of high-risk human communities, design and deploy specific and sensitive serological assays to identify the baseline spillover of known or novel viral pathogens in these populations. In participants where symptoms are reported samples will be marked for additional PCR-based assays to identify presence of known and novel CoVs, henipaviruses and filoviruses, attempt to isolate and biologically characterize the pathogen, using the collaborative cross mouse (Aim 1) to identify an appropriate animal model to conduct preliminary pathogenesis work.

At the time of writing, a currently undiagnosed outbreak in the Batek Orang Asli (indigenious) population living in Gua Musang ("civet cat cave") district, in Kelantan, Malaysia has infected dozens of people and is currently being investigated by our colleagues at NPHL. The Orang Asli live in remote villages in the rainforest of Malaysia, with limited access to modern health care and extensive contact hunting, butchering, and eating wildlife as their primary source of protein. This outbreak, and others that will continue to emerge just like it, underscore the critical need for our EIDRC -- to improve the technological tools, training, and human capacity for rapid EID surveillance, diagnosis, and response at the frontlines of disease emergence in SE Asia.

Preliminary data human biological sampling: Our longterm collaboration in the region has included identification of key at-risk human populations using previously collected qualitative and quantitative survey data, and developing and gaining access to large archives of banked human sera and other biological samples. In the last 5 years under the USAID-PREDICT project, EHA has collected 9933 and 9269 human specimens in Malaysia and Thailand, respectively, which will be available for retrospective serological testing under our proposed work. Other studies from our research team have included the collection and testing of many thousand more specimens from community cohorts. Preliminary findings and details from these data and other research from are listed below:

Peninsular Malaysia: Data from 1390 Orang Asli samples, screened by PCR for 5 viral families found: 4 known CoVs in 16 people and one known Influenza virus in one person. Serology screening is ongoing but preliminary screening as part of training government partner to use the Luminex serology platform found serological evidence of past exposure to Nipah and Ebola antigenically-related viruses in people.

Sabah: Data from 10 Syndromic Surveillance samples, screened by PCR for 5 viral families found: 1 known virus - Human Parainfluenzavirus 2 (GenBank Accession No.AF533011) in the family Paramxyoviruses. Serology screening is yet to start.

Kamruddin (UMS Borneo Medical Health Research Center) has identified some high risk communities.

Tim, Giri, Tsin Wen has archived samples from Kudat Monkey bar project – 2,000 human sera already screened for bacteria and parasites, we've been reached out to about this. Data on macaque tracking data/human tracking data also. Encephalitis study etc Dr. Yeo Tisn Wen, DUKE-NUS, Singapore – Has worked with Giri and tim on knowlesi work with communities – 2000 selected villagers 500micros looking for

Commented [KJO69]: We can make this aim all about human serology, and 3rd aim more about molecular characterization of human pathogens and outbreak etiologies.

Commented [KJO70]: Emily: Will we be sampling participants more than once?

Commented [KJO71]: Need to decide if we do PCR on community samples, we can add in the questionnaire if you've been sick in the last 10 days and if so, then maybe test only this subset.

Commented [KJO72]: https://en.wikipedia.org/wiki/Gua_Musang_District

Commented [KJO73]: From Tom email.

Commented [PD74]: All – please read and share with collaborators to flesh out and correct details here.

Commented [T75]: Unpublished

Commented [KJO76]: Previous text from PD: We have ~25 new CoVs in Sabah. Simon is using HTS to further characterize and Tom will get the data for this proposal from Simon

Commented [T77]: **Malaysian colleagues please can you add details here of any human sampling and findings from those studies that are relevant to this proposal**

Commented [T78]: These are from wildlife so I have moved to "Identify Known and novel" section above

Commented [KJO79]: From PD: Kamruddin (UMS Borneo Medical Health Research Center) has identified some high risk communities. We can help with them and build out the BSL-2 lab, as well as work on outbreak response within DHRU (in collab with Sabah CDC?).

asymptomatic parasitemias – led by King Fonese from LSTMH – 10,000 people – archived serum samples (prob 2-3,000 – in London, KK)

Sarawak: Dr Tan (Cheng Sieng) Center for Tropical and Emerging Diseases, Faculty of Medicine at UNIMAS – high risk Dayak communities that he's identified. We will do further sampling, questionnaires etc. Has Prelim data on HPV work in Dayak communities in remote Sarawak – we can work with those communities and can reach many by car, others by 4WD or boats. Has also Enterovirus 71 paper and is interested in CoVs. Has access to caves and guano. Will look into what other archived samples he has. Will give us access to Sarawak Forestry and Health Depts.

Thailand: We have conducted longitudinal community-based surveillance for the last 5 years in two villages (Chonburi and Ratchaburi provinces) at high risk of infection from bats. In Chonburi province we sampled and tested over 300 villagers living around the largest population of flying foxes (Pteropus lylei), at a temple (Wat) roosting site where we have seasonal detections of Nipah virus from bat urine each year (with viral shedding peaking in May-July. Serological screening of monks and We also have sampled and tested over 300 villages from Ratchaburi province, including a population of bat guano miners, and workers from Department of National Parks – both of whom have extensive contact with wildlife in the area. We previously detected a MERS-like CoV in the bat guano being harvested (*48*). Molecular screening of guano miners identified human CoV, HKU1 strain, with high viral load in one healthy bat guano miner and our team was able to obtain the whole genome sequence from a clinical specimen from this individual (*119*). No evidence of novel bat CoVs have yet been detected in these populations.

US CDC (Thailand): A large cohort of children from the Kamphaeng Phet Province, Thailand were enrolled as part of a multi-year study (1998-2002) to examine the epidemiology of dengue virus (DENV) and Japanese encephalitis virus (JEV) (*120, 121*). From 2,574 paired serum samples, 784 (30%) convalescent sera were selected at random among samples drawn between 1998-2002. Among the 784 samples tested, subjects ranged in age from 6 to16 years (mean 9 years) and 51% were male. Henipavirus-specific antibodies were detected in 13 serum samples (1.7% seroprevalence) by Enzyme-linked Immunosorbent Assay (ELISA) using a soluble highly-native recombinant G glycoprotein (sG_{NiV} and sG_{HeV}) capture antigens. The ELISA endpoint titers for sG_{NiV} reactive samples ranged from 1:20 to 1:1280, with six of the thirteen samples exhibiting a titer of 1:640 or greater. Although none of the samples inhibited NiV or HeV infection *in* vitro, 3 of the 13 ELISA sero-positive patient serum samples reacted with denatured G antigen in Western blot. **The presence of henipavirus-specific antibody responses suggests prior exposure to endemic henipaviruses or a henipa-like virus in rural Thailand.** The presence of pre-existing henipavirus antibodies among these children in Thailand suggest that "background" henipavirus infections do occur and were self-limited illness without serious health impacts.

Singapore: No active human surveillance will be cnducted in Singapore, but archived human specimens (REFS, e.g. Melaka virus serosurvey) will be made available to conduct retrospective serosurveys for specific pathogens that are identified as high interest to the EIDRC, NIH, and EID-CC.

Human Contact Risk Factors: EHA is the lead global organization in the USAID-PREDICT project for characterizing the human aspects of zoonotic spillover risk. The approach began by conducting exploratory studies using standardized one-on-one semi-structured interviews and site observation data with people living in hotspot areas or engaged in high-zoonotic spillover risk activities (e.g. we interviewed 88 people involved in trading wild bats in China – REF). This allowed us to assess the local social and cultural norms and individual attitudes underlying wildlife contact. We used these study findings to develop a risk survey to accompany human biological samples to evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. We conducted cross-sectional studies in high risk populations in >15 countries during the last 5 years,

Commented [KJO80]: From Tom

Commented [PD81]: Linfa, Danielle – are we going to do human sampling in high risk communities in Singapore? If so, what would you propose and do you already have some prelim work you've done. Note these are high risk populations, i.e. high exposure to wildlife. Clinical cohorts are in Aim 3

Commented [PD82]: Hongying/Emily to edit

Commented [EH83]: For the survey and biological data collection we worked with 27 countries an managed 11

For Qualitative work we oversaw 13 countries

and study participants provided biological samples (oral/oropharyngeal swab, nasal/nasopharyngeal swab, urine sample/urogenital swab, fecal sample/rectal swab, whole blood, serum, plasma). Survey and biological samples were collected from 1,585 participants from 7 sites in China in Yunnan, Guangxi, and Guangdong provinces, 1,400 participants from 4 sites in Malaysia and 678 participants from 4 sites in Thailand. During this study serological assays were developed for implementation in China, and survey responses and demographic data suggest specific risk factors included type of occupation, keeping of pets and visiting wet markets. Seropositive individuals were mostly farmers/peasants (8/9), living in Yunnan province (7/9), 41-60 yrs old (7/9), with domestic contact with rodents (6/9), and are male (6/9). However, these characteristics were common and occurred in ~40% of survey respondents. Although these results are preliminary and don't provide detailed information on routes of exposure, they suggest that further refining of tools for serological tests coupled with survey data will identify likely routes of exposure to CoVs, novel CoVs, Henipavirus, and Filoviruses in our expanded study. The results of these surveys will be used in Aim 2 of this EIDRC proposal to better identify target populations and in Aim 2, we identify strategies to better target at-risk people, and conduct focused **human animal contact surveys and serosurveys to produce statistically significant findings for henipaviruses, CoVs and filoviruses.**

~~Behavioral risk~~: Clinical meta-data to assess human-animal contact: EHA is the global lead organization in the USAID-PREDICT project for assessing the risk of zoonotic spillover, including extensive behavioral surveys in high-risk populations under this award. Our general approach has been to conduct qualitative exploratory studies using standardized one-on-one semi-structured ethnographic interviews and observational data in among people engaged in clearly high-zoonotic risk activities (e.g. we interviewed 88 people involved in trading wild bats in China – REF). This allows us to assess local social and cultural norms and individual attitudes underlying wildlife contact. We used qualitative study findings to develop a human behavioral risk questionnaire on the type and frequency of animal contact, wildlife observed in daily life, and unusual illnesses reported over the past 12 months. Under our USAID-PREDICT project, we conducted cross-sectional studies in high risk populations in >15 countries during the last 5 years. In total, we have enrolled XX,XXX participants who have provided serum, nasopharyngeal, oral, and fecal samples and each completed behavioral risk questionnaires to assess animal contact. Questionnaires and biological samples were collected from 1390 participants from 9 sites in the Districts of Gua Musang, Kuala Lipis and Kuala Kangsar Malaysia, 10 syndromic surveillance patients at the Queen Elizabeth Hospital in Sabah, and 673 people from 3 sites in Thailand. The results of these surveys will be used in Aim 2 to better identify target populations.

Serological Evidence of Exposure: In China, we developed serological assays for HKU9 CoVs (β), SARSr-CoV Rp3 (β), HKU10 CoV (α), and MERS-CoV (β) and used ELISA and Western blot to test serum samples collected in 2016/17. **We found 7 individuals (7/733, 0.95%) living within a 6 km radius of the Jinning Cave, and 6/209 people (2.87%) at one site, with evidence of exposure to bat SARSr-CoVs.** We found evidence among human populations in Guangxi Province of people with prior exposure to the bat α-CoV HKU10 (2/412, 0.48%). This is of potential public health interest because HKU10 is known to be able to jump host species within bats, and therefore may have high propensity for emergence (*122*). However, the low seroprevalence (0.6%-2.7% at positive sites) suggests we need a larger sample size, and a focused, targeted study to maximize the likelihood of identifying seropositive cases. In Aim 2, we will use combined biological-behavioral risk surveillance in targeted populations within the community and clinical settings to 1) identify risk factors correlated with seropositivity (exposure to) and PCR positive status (infection with) henipaviruses, filoviruses and CoVs; and 2) assess possible health effects of infection in people. Obtaining this information could be a significant step in understanding the likelihood of recent 'hidden' spillover events and their public health impacts, as well as the risk of future emergence of viral disease.

Risk Factors: In China, questionnaire response and demographic data suggest specific risk factors included type of occupation, keeping of pets and visiting wet markets. Seropositive individuals were mostly farmers/peasants (8/9), living in Yunnan province (7/9), 41-60 yrs old (7/9), with domestic contact with rodents (6/9), and are male (6/9). However, these characteristics were common and occurred in ~40% of survey respondents. Although these results are preliminary and don't provide detailed information on routes of

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Commented [KJO85]: Edits from Emily.

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Commented [KJO88]: Sum up for ALL countries?

Commented [KJO89]: Same as paragraph above, but including my and Tom's edits.

Commented [PD90]: Hongying/Emily – any information from Thailand or Malaysian surveys?

exposure, they suggest that further refining use serological tests coupled with qualitative and questionnaire data will identify likely routes of exposure to novel CoVs in China. In Aim 2 of this EIDRC proposal, we identify strategies to better target at-risk people, and conduct focused **questionnaires and serosurveys to produce statistically significant findings for henipaviruses, CoVs and filoviruses.**

2.2 General Approach/Innovation: We will use a dual study design to gain in depth understanding of exposure and risk factors for viral spillover **(Fig. XX)**. In Aim 2 we will conduct community-based surveillance with a focused questionnaire to identify the prevalence and frequency of risk-factors for viral spillover in these communities in tandem with biological sampling to determine the seroprevalence of spillover viruses in at-risk human populations. We will use generalized linear models to analyze correlation between biological results and risk-factors for viral spillover. In Aim 3, we will conduct clinic-based syndromic surveillance and biological sampling at clinics/hospitals that are the catchment healthcare facilities for the community-based surveillance sites of Aim 2 (details in Aim 3). Both community-based and clinic-based syndromic surveillance programs are cross sectional case-control studies designed with the sample sizes necessary to statistically quantify (with a power of 80%) risk factors and health impacts for viral spillover, linked to serological/PCR status and symptoms.

2.3 Target population & sample sizes: We will target sites in the same geographic regions as those identified in Aim 1 that harbor wildlife with high potential zoonotic viral diversity, in regions that are EID hotspots, and are well-connected to regional travel and trade hubs and the global travel network (Fig X). We will target specific communities based on our analysis of the previously collected PREDICT behavioral questionnaire data to assess key at-risk populations and occupations with high exposure to wildlife, as well as using other publicly available data (e.g. wildlife hunting licenses sold in a region, number of guano caves accessed for fertilizer etc.). We will also expand on work currently underway in the following particularly high exposure populations:

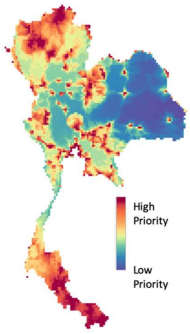

Fig X. *Preliminary analysis of geographic sites to target for human zoonotic disease surveillance in Thailand. Novel spatial analysis optimizes site selection by ranking areas with high numbers of expected viruses in wildlife and greater human access to these sites using road density data.*

Thailand: In 2018 We have conducted behavioral risk surveys and biological sampling in 117 guano miners and the communities in Ratchaburi province where MERS like CoV has been found from dry bat guano and bat rectal swab during the PREDICT study. These workers visit bat caves and dig fecal material to use as fertilizer, and are therefore highly exposed to bats and rodents in particular. At Khao Chong Pran cave in Ratchaburi we have detected and characterized novel Alpha CoVs, HKU1, and MERS like CoV (short sequence) ((*48, 119*) and Chulalongkorn, unpublished). As an initial project on our EIDRC, we will develop serology assays to the novel CoVs found in bats at this site using LIPS assays and viral genomic data, and screen archived specimens from 230 participants (2 years' collection in 2017-2018, a subset of the subjects have been sampled at multiple timepoints to allow us to assess seroconversion). Clinical syndromic surveillance at the district hospital will be conducted from the patient with respiratory symptom, the known viral and bacterial pathogens will be tested by real-time PCR commercial kit (the test result will be provided to the hospital within 48 hours for treatment), along with PCR for 3 viral family and serology for CoV found from bat in this area

The second site is at Chonburi province where Nipah virus (99% identity of whole genome sequence to NiV from Bangladesh patient) has been found from Lylei flying foxes but no infected patient been reported. Several novel Paramyxovirus and CoV have been found from bat feces roosting here. Screening using serology assays for a panel of Henipaviruses is ongoing via our existing collaborations with USUS's Luminex platform, and will be expanded under our EIDRC. The clinical syndromic surveillance at the district hospital will be conducted from the patient with encephalitis and respiratory symptom, the known viral and bacterial pathogens will be tested by real-time PCR commercial kit (the test result will be provided to the hospital within 48 hours for

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Commented [KJO93]: Think we need to be strategic and just pick a couple sites per country, so it doesn't look too ambitious given budget.

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treatment), along with PCR for 3 viral family and serology for NiV, novel CoV, novel PmV found from bat in this area.

The serology platform based on the sequence from bat need to be develop and used as an antigen for serology testing. HKU1 full genome sequence from guano miner (*119*)

Peninsular Malaysia: We have sampled Orang Asli populations during 2016 – 2018 in 3 districts, enrolling 1390 people. We will expand this to include additional communities in the Districts we have already sampled, additional Districts in the States of Kelantan Perak and Pahang and also Districts in the State of Kelantan. The Ministry of Health, Department of Wildlife and National Parks Peninsular Malaysia and Department of Veterinary Services are all supportive of this expansion and the continued surveillance and capacity building that this will entail.

Sarawak: Dr Tan (Cheng Sieng) Center for Tropical and Emerging Diseases, Faculty of Medicine at UNIMAS – high risk Dayak communities that he's identified. We will do further sampling, questionnaires etc. Has Prelim data on HPV work in Dayak communities in remote Sarawak – we can work with those communities and can reach many by car, others by 4WD or boats. Has also Enterovirus 71 paper and is interested in CoVs. Has access to caves and guano. Will look into what other archived samples he has. Will give us access to Sarawak Forestry and Health Depts.

Sabah: Queen Elizabeth hospital syndromic surveillance, UMS Hospital syndromic surveillance, BMHRC human sampling outbreak response

Singapore: No human sampling

Community-based surveillance will be conducted at XX sites in each country, a total of xx sites. From our previous work we anticipate that 10-30% of the community population will have had exposure to wildlife allowing us to capture highly exposed and non-exposure individuals at each site. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock farming, transportation, selling, or slaughtering wildlife in the surveyed areas will be targeted so that they make up ≥30% of the sampled population in each community. We will stratify sampling to ensure appropriate representation of sex, demographic, and socio-economic factors at each community site. Estimating 5% overall seroprevalence in these high-exposure populations, these sample sizes are sufficient to estimate effect sizes of risk factors of 2X or greater with 80% power. For community-based surveillance, we will enroll xxxx individuals per county/province, pooled across two sites for each, allowing us to make county/province-level comparisons of differing effects.

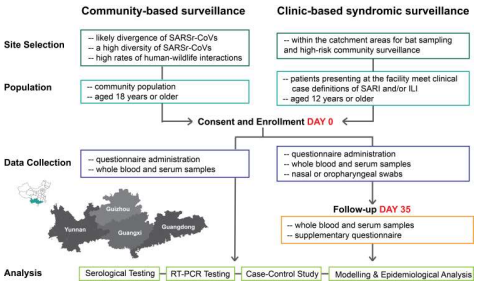

Fig. XX: Human survey and sampling study design overview (Aim 2 and 3)

2.4 Data & sample collection: Following enrollment of eligible participants and completion of the consent procedure, biological specimens (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples) will be collected and a questionnaire will be administered by trained project staff that speak appropriate local language to all participants. Through the serological findings and the survey we will investigate the details of five risk factors related to high risk wildlife exposure, to maximize the power of the analyses, based on continuing analysis of our previous work, and will focus on: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With additional consent

Commented [KJO97]: Same thing. From Supaporn in this section, but maybe move to Aim 3.

Commented [KJO98]: From Supaporn

Commented [PD99]: Tom – please start writing some plans for what we csould do.

Commented [PD100]: All – not sure if we should have one figure for community and clinical cohort surveillance (aim 2 and 3 respectively) or if we should have two separate ones?

Commented [PD101]: Hongying– this figure is from our CoV R01 renewal. Please modify to make relevant to SE Asia and henipas, filos and CoVs.

Commented [PD102]: Emily/hongying

Commented [EH103]: We will need to take more than this from people as there are not vacutainers that small. Also do we want to use a serum separator tube specifically?

For 1mL of serum we will need 2.5mL of blood plus whole blood.

considerations for participants enrolled from clinical settings we will review clinical records to collect recent data on medical history, clinical syndromes, and patient etiology.

2.5: Laboratory analysis: 2.5.a Serological testing:

One of the present challenges in emerging disease surveillance is that viremia can be short-lived, complicating viral-RNA detection (i.e. traditional PCR-based approaches), especially in zoonotic reservoir hosts, and requiring large samples sizes to understand infection patterns or host range. In contrast, antibodies are typically long-lived and can be used to assess prior virus exposure or infection with much smaller samples sizes . However, most serological assays only target a single protein, and it's not always clear which antigen is the most immunodominant during an adaptive humoral immune response – i.e. which is the best target for a serological assay. Henipaviruses, filovirus and CoVs express envelope receptor-binding proteins (e.g. CoV=spike (S) protein, filoviruses/henipaviruses=glycoprotein (GP/G)), which are the target of protective antibody responses. The Broder lab at USU, a leader in the expression and purification of native-like virus surface proteins for immunoassay application (Bossat 2008), developing monoclonal antibodies (Zhu 2008, Bossart 2009) and as subunit vaccines (Bossart 2012, Mire 2014), is presently developing a novel, multiplex microsphere immunoassay (MMIA) allowing serum samples to be simultaneously tested for antibodies reactive with henipaviruses, filoviruses and coronaviruses (Table XX).

This MMIA uses a Luminex-based multiplex platform and custom designed virus receptor-binding proteins expressed in a mammalian cell-culture system and purified as native-like oligomeric proteins (Bossart 2008, Chan YP 2009). These oligomeric antigens retain post-translational modifications and quaternary structures. This structural advantage over peptide antigens, allows the capture of conformational-dependent antibodies raised during a natural infection, which represent the majority of the protective humoral response.

Serological platforms have been historically limited by the starting antigen, and cross-reactivity between known and unknown related viruses not included in the assay. The known diversity of henipaviruses includes five presently described viruses, NiV, HeV, CedV, GhV and MojV and this assay has been expanded to include all described species (Preliminary Figure A). In the past, our collaborators have used NiV and HeV G protein antigens in a MMIA assay to identify a serological footprint of NiV-like African henipaviruses and exposure to virus(es) antigenically-related to NiV in livestock sampled in Bangladesh (Peel 2012, Chowdury 2014). To strengthen serological data interpretation, the G protein from each virus is integrated into the MMIA so that virus-specific versus henipavirus cross-reactive antibodies can be detected and address specificity of past virus exposure. Extensive cross-reactivity exists within ebolaviruses and between ebolaviruses and marburgviruses, highlighted by studies examining antibody binding between heterologous viruses (Shuh 2019, MacNeill 2011, Natesan 2016). Ongoing work is aimed at validating the MMIA pan-filovirus assay for specificity (Preliminary Figure B). The majority of ebolaviruses are endemic to Africa, however the discovery of Mengla virus in Yunnan, China increased the further demonstrated that a diversity of Asiatic filoviruses with unknown pathogenicity and zoonotic potential exists. Our past work in collaboration with Dr. Smith and Mendenhall at Duke-NUS demonstrated that three under sampled fruit bat species had serological evidence of past exposure to a filovirus(es) most antigenically-related to ebolaviruses (e.g. Ebola virus, Bundibugyo virus), but unreactive with Reston virus GP, suggesting that novel filoviruses circulate within bat hosts (Laing 2018).

The below is from our previous CoV proposal:

In our previous R01, we expressed his-tagged nucleocapsid protein (NP) of SARSr-CoV Rp3 in *E.coli* and developed a SARSr-CoV specific ELISA for serosurveillance using the purified Rp3 NP. The specificity of this assay was evaluated using polyclonal antibodies against HKU1, OC43, 229E, NL63, MERS-CoV and EBOV and no significant cross-activity was detected (*15*). **While this shows it is a specific test for Rp3, it suggests that if we can expand our serology tests to cover other bat CoVs, we may identify many more seropositive individuals.** In this renewal, we will therefore use two serological testing approaches. First, we will expand test all human sera collected from both community- and clinic- based sampling for a panel of bat CoVs that will include SARS-CoV (outbreak strain), a range of lineage 2 SARSr-CoVs (including WIV1 and SHC014), lineage 1 HKU3r-CoVs, MERS-CoV, and the α-CoVs SADS-CoV and HKU10. We previously found

Commented [PD104]: Chris, Eric, Linfa, Dani etc. please draft some language here…

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serological evidence of human exposure to HKU10 (*15*), but HKU10 is known to jump from one host bat species to another (*122*) and is therefore likely to have infected people more widely. Incoporating serological testing for SHC014 is also likely to yield higher seroprevalence because it is readily divergent from SARS-CoV wildtype and therefore unlikely to have been picked up in our earlier testing. It is possible that non-neutralizing cross reactive epitopes exist that afford an accurate measure of cross reactivity between clade1 and clade 2 strains which would allow us to target exposure to strains of 15-25% divergence from SARS-CoV. Additionally, we will test samples for antibodies to common human CoVs (HCoV NL63, OC43 – see potential pitfalls/solutions below). Secondly, we recognize that CoVs have a high propensity to recombine. To serologically target 'novel' recombinant virus exposure, we will conduct 1) ELISA screening with SARSr-CoV S or RBD; 2) confirm these results by Western blot; then 3) use NP based ELISA and LIPS assays with a diversity of SARSr-CoV NP. For NP ELISA, micro-titer plates will be coated with 100 ng/well of recombinant batCoV NP and incubated with human sera in duplicates followed by detection with HRP labeled goat anti-human IgG antibodies. For confirmation, all ELISA positive samples will be subjected to Western blot at a dilution of 1:100 by using batCoV-NP as antigen. We will use an S protein-based ELISA to distinguish the lineage of SARSr-CoV. As new SARSr-CoVs are discovered, we will rapidly design specific Luciferase immunoprecipitation system (LIPS) assays targeting the S1 genes of bat-CoV strains for follow-up serological surveillance, as per our previous work (*14*).

2.5.b RT-PCR testing. Specimens will be screened using RT-PCR for the RdRp gene (See section 1.3.b for details). Positive samples will be subjected to full genome sequencing and RT-PCR amplification of the S glycoprotein gene. Samples from the clinic-based syndromic surveillance will also be tested using RT-PCR for Influenza A & B, HCoV NL63, OC43, HKU1, SARS-CoV & 229E as rule-outs, and SADS-CoV and HKU10 as an opportunistic survey for potential spillover these CoVs as proposed above.

2.6 Epidemiological analysis: We will conduct a cross sectional case-control study to identify risk factors for Henipavirus, Filoviruses, and CoVs spillover. "Cases" are defined as participants whose samples tested positive for Henipavirus, Filoviruses, or CoVs by serological tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative for Henipavirus, Filoviruses, or CoVs. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between biological results, serological/PCR status, and risk factors related to high risk wildlife exposure including: 1) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) home or work proximity to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms lifetime and past 12mo. Additionally, we will use this procedure to determine how clinical presentation differs between Henipavirus, Filoviruses, or CoVs in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms.

2.2.2 Behavioral risks among population: Qualitative and quantitative human study have conducted among xxxx residents at xxxx community sites in Thailand and Malaysia, revealing the frequent contact with both wild (e.g., bats, rodents, non-human primates) and domestic animals (e.g., poultry, swine) among local communities (**Figure X**), which are associated with a high percentage of self-reported severe/acute respiratory illness (SARI/ARI) and Influenza-like illness (ILI) symptoms.

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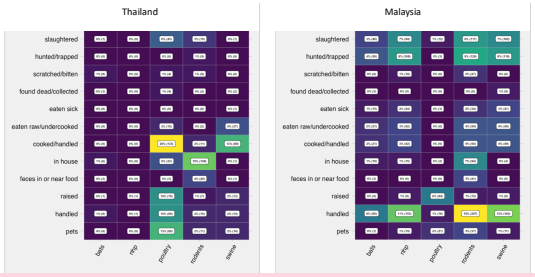

Figures of LASSO ILI/SARI analysis

Figure X High-risk human animal contact among community residents in Thailand and Malaysia

In addition, people making a living on crops production, engage in practices that enhance the risk of spillover (e.g. hunting and butchering wildlife) are at the risk of exposure and reported SARI and ILI symptoms (**Figure X – need a figure for this**). **These preliminary data suggest a target population and sites for current proposal with the correct targets for serological surveillance and risk factor surveys in Aim 2 to produce statistically significant findings**. Coupled with further refining serological tools, we will identify the likely routes of exposure to known or novel coronaviruses, henipaviruses, and filoviruses in the study region.

2.7 Biological characterization of viruses identified: XXX

2.8 Potential problems/alternative approaches: Rarity of spillover events means it may be difficult to identify sufficient seropositives to statistically analyze human risk profiles. First, we will be targeting our community-based surveillance to subpopulations with high-levels of wildlife exposure, at sites selected for diverse and prevalent viruses to increase likelihood of finding positive individuals. Second, our serology testing will include a panel of assays for a large diversity of viruses, increasing likelihood of positives. For example, in previous work our overall bat CoV PCR prevalence in the community was, 11.8%; β-CoV, 3.4%; α-CoV, 9.1%). Thus, using this broad serological panel to screen individuals prone to contact with species of higher zoonotic risk increases the potential for detecting spillover with enough power for statistical analyses, and will shed light on behaviors that predispose to spillover. Third, we will include common human CoVs, paramyxoviruses in our panel, so that even if low prevalence of wildlife viruses is found, we will be able to conduct a valuable cross-sectional study of the seroprevalence of more common human viruses. Finally, we will be able to assess relative measures of human-wildlife contact from our survey work. We will analyze intensity of contact with high-risk taxa against other risk factors to provide useful proxy information for spillover risk. **Serological testing may not match known CoVs due to recombination events.** We will use the three-tiered serological testing system outline in **2.6.a** to try to identify these 'novel' CoVs, however, we will also remain flexible on interpretation of data to ensure we account for recombination.

Aim 3: Identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts.

3.1 Rationale/Innovation: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent is not diagnosed. By working directly with individuals reporting to clinics with symptoms similar to known high-impact viral agents, we may be able to capture novel emerging diseases in at-risk communities before they spread into the general population and risk becoming pandemic. This will have clear value for public health in the region, and potentially wider. To do this, we will expand on current syndromic surveillance activities to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and present at regional clinics or hospitals with symptoms typical of high impact zoonotic viruses. We will collect detailed survey data to assess their contact type and frequency with wildlife and livestock and conduct molecular and serological diagnostic assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. We will enroll and conduct novel viral discovery assays on patients who present with unique clinical signs or relevant symptomatic cases. If novel

Commented [KJO109]: From Hongying and Emily, may go in diff section for prelim behavioral data. Showing freq and type of animal contact based on Thailand and Malayisa P2 survery data.

Commented [PD110]: All –Not sure if we should weigh too heavily on this for the community survey – or should it just be serology for Aim 2. If you want include this section, please modify and insert language from Aim 1 here.

Commented [KJO111]: I think Aim 2 should just be serology, esp. since developing new LIPS assays or applying some of the serochip stuff from Linfa and Luminex from USU is already a lot.

Commented [KJO112]: Make this more general. There's a greater challenge of interpretation of serological data, esp. if we're using many platforms (each will have its own problems that our lab colleagues are probably more familiar with). Noam may be able to generally comment on a solution here from a statistical perspective.

pathogens are identified by PCR, we will attempt to isolate, obtain full genome sequence and/or biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work.

Preliminary data clinical surveillance: Our longterm collaboration in the region has included the following activities investigating clinical syndromes:

Singapore: In collaboration with EcoHealth Alliance and colleagues from the Wuhan Virological Institute in China, we have been developing novel CoV assays for clinical surveillance. Our pipeline of novel virus detection, reservoir host identification, serological assay development, and serological testing has been optimized and streamlined to be as rapid as several months, with the development of novel serological assays from sequence data in 7 days [REF]. For example, with the discovery of SADS-CoV, we identified and full-genome sequenced a new virus, screened archived wildlife specimens to identify the natural reservoir host species (Rhinolophus spp. bats), developed LIPS serological assays, and tested human and swine samples to assess seroprevelance in less than 3 months (*14*). [Maybe a timeline figure too]?

ADD Lancet ID paper on Zika sequencing by Dani and Duke-NUS team.

Thailand: The TRC-EID laboratory is a leader in rapid detection of human clinical specimens using broadly reactive and specific assays and in-house sequencing technology. For example, in coordination with the Thailand Ministry of Health, we identified the first case of imported MERS-CoV in Thailand using sequence confirmation within 24 hours from acquiring the specimen (Fig X).

Three imported MERS cases were detected in Thailand since 2015, all cases were confirmed at Chula lab. More than 500 Zika patients were tested positive by PCR and sequence confirmation. The specimens from suspected Ebola travelers were tested negative, PREDICT filovirus protocol has been used as an additional protocol further investigation. The syndromic surveillance has been conducted and tested at Chula lab including SARI, Encephalitis, Hand Foot and Mouth disease in children and Viral diarrhea. These are research studies supported by PREDICT, US DoD and Thai government grant and the routine surveillance by the Thai Bureau of Epidemiology.

Commented [KJO113]: ?

Commented [PD114]: All – please read and share with collaborators to flesh out and correct details here.

Commented [KJO115]: Per Linfa's comments, we should highlight the work from SADS-COV from new virus detection, to assay development, to serological testing in X months.

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Commented [KJO117]: Hongying? Similar to the revised Thialand one?

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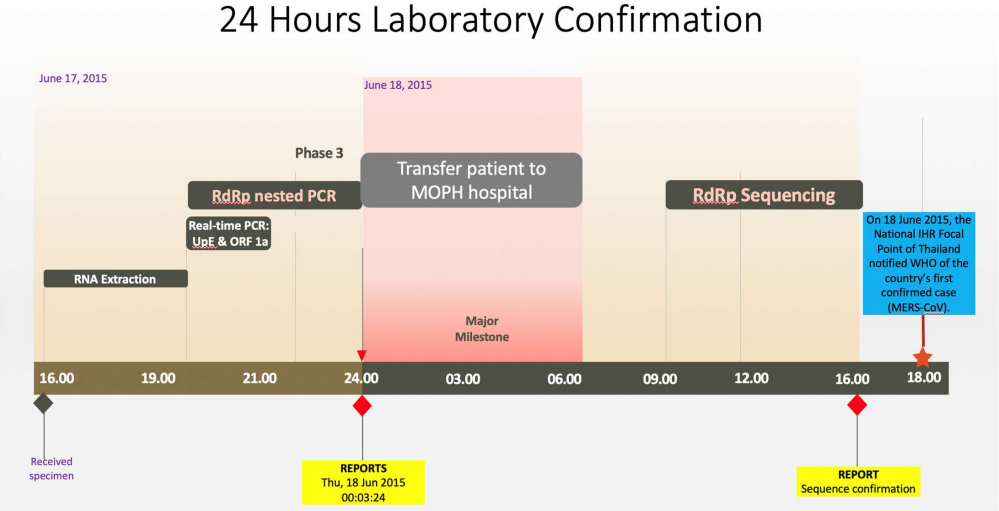


Peninsular Malaysia:

Sabah: Linfa has worked with Timothy Williams on an encephalitis cohort from Queen Elizabeth Hospital 1 & 2 (poss has worked with Linfa on these) and this is another possibility. He's doing outbreak response training in August with team from Sabah CDC (Dr. Maria now in Pen. But still collaborating with us, Jikal current director working with us and Dato Cristina dir. Sabah state health Dept and is supportive) this collaborative group is working to develop a Sabah outbreak response team – we will support that – to be based at BMHRC and we will develop this as part of this proposal. Prof. Kamruddin will summarize the serum samples he has.
POCs: Sabah State Health Dept (e.g. Dr. Giri Shan Rajahram), Queen Elizabeth Hospital (Dr. LEE Heng-Gee)

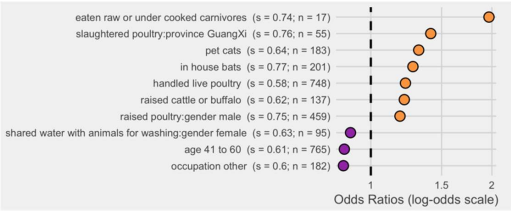
Sarawak: From Linfa – Dr. Ooi used to work with Jayne Cardosa at UNIMAS and has access to samples of patients with unusual presentation

Commented [PD119]: Tom to add – Queen Elizabeth Hospital?

Commented [KJO120]: Comment from Tom: **Sabah team please add details of any cohort work you have done**

Commented [PD121]: Tom

Commented [KJO122]: Comment from Tom: **Dr Tan have you done any clinical cohort work in Sarawak?**



Strategy for analysis of self-reported illness: In addition to biological outcomes we have developed a standardized approach in PREDICT for analyzing data on self-reported symptoms related to targeted illnesses; of fever with cough and shortness of breath or difficulty breathing (severe acute respiratory illness - SARI), fever with muscle aches (fevers of unknown origin (FUO) and, cough or sore throat (influenza-like illness - ILI) from study participants. We used a least absolute shrinkage and selection operator (LASSO) regression to identify associations between participants who reported ILI-like and/or SARI symptoms and demographics, occupation, and contact with animals in the last year. Results have clear biological relevance. In Yunnan, China, salient associated variables or combination of variables were, in descending order of odds ratio: 1) having eaten raw or undercooked carnivores; 2) having slaughtered poultry and being a resident of Guangxi province; 3) having had contact with cats or bats in the house; and 4) being a male who has raised poultry **(Fig. 11).** We will expand this approach for all syndromes in Aim 3, and use more granular targeted questions designed to assess patient's exposure to wildlife in terms that are relevant to the specific country.**Fig. 11:** Associated variables of self-reported ILI and/or SARI in prior 12 months (s = bootstrap support; n = number +ve out of 1,585 respondents). Orange circles = odds ratios > 1 (positively associated with the outcome); purple = odds ratios <1 (negatively associated with the outcome).

3.2 General Approach: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent is not identified. To capture these, we will expand on our current syndromic surveillance to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and present at regional clinics with influenza-like illness, severe respiratory illness, encephalitis, and other symptoms typical of high-impact emerging viruses. We will collect survey data tailored to the region to assess contact with wildlife, conduct molecular and serological assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. If novel pathogens are identified by PCR, we will isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work. We will then use behavioral survey data, ecological and phylogeographic analyses to assess the likely reservoir hosts to inform intervention programs.

3.3 Clinical cohorts. 3.3.a Cohort selection: We will initiate active clinic-based syndromic surveillance at XX town-level level clinics and xx provincial-level hospitals in each country, in total xx clinical/hospital sites, that all serve as the catchment healthcare facility for people in our community-based surveillance of Aim 2 and are inclusive of wildlife sampling sites in Aim 1. Patients ≥ 12 years old presenting at the health facility who meet the syndromic and clinical case definitions for SARI, ILI, encephalitis, fevers of unknown origin, or hemorrhagic fever will be recruited into the study. We will enroll a total of xxxx individuals for clinical syndromic surveillance, which accounts for up to 40% loss from follow-up. Study data will be pooled across country sites, as clinical patients are limited by the number of individuals presenting at hospitals.

3.3.b Behavioral and clinical interviews: 2.5 Clinic enrollment and follow-up: We will recruit inpatients and outpatients to participate in the study after initial screening to determine if they meet the clinical case definition of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever of unknown etiology. Once enrolled, biological samples will be collected and a questionnaire administered by trained hospital staff that speak appropriate local language. Samples will be taken concurrently when collecting samples for normative diagnostics. For inpatients, samples will be collected no more than 10 days of reported onset of illness to increase the chance of PCR detection of viruses (*123*). We will follow up 35 days after enrollment to collect an additional two 500 µL serum samples conduct a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. 35 days gives adequate time for development of IgG, which occurs <28 days after onset of symptoms for SARS patients (*124*).

3.3.c Sampling:

Commented [PD123]: Emily/Hongying

Commented [KJO124]: Emily: Will we do this in the community as well. We could also add animal contact as the outcome. We have data and figures for that as well

Commented [PD125]: Hongying/Emily

Commented [KJO126]: MeiHo: Just QEL in Sabah

Commented [PD127]: Hongying Emily

Commented [EH128]: Both inpatient and outpatient

Commented [PD129]: Need data for Nipah and filovirus patients

Following enrollment with signed consent form, biological specimens (5mL will be drawn for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples) including two nasal or oropharyngeal swabs will be collected from all eligible participants, and a questionnaire will be administered. We will investigate five risk factors, to maximize the power of the analyses, all related to high risk wildlife exposure, based on continuing analysis of our previous work, and will include: 11) occupation and occupational exposures; 2) observed or reported interactions with priority wildlife, especially bats, in/around house; 3) proximity of residence or work place to environments of increased risks, e.g. nearby bat roosts; 4) working or regular visitor to animal markets; 5) self-reported ILI/SARI clinical diagnosis or symptoms in the past 12mo and lifetime. With permission from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology.

3.4 Sample testing: PCR, Serol to link symptoms to etiologic agents

3.5 Assessing potential for pandemic spread: We will…leverage EHA's work with DHS to develop FLIRT (Flight Risk Tracker) that tracks the probable pathways for spread for viruses that are able to be transmitted among people….

Commented [PD130]: KEvin to draft

Commented [KJO131]: Pasted in a bunch of stuff, but still need to clean this up.

IBIS mines and collates data regarding on-going bioevents from biosurveillance networks and uses data on commercial air traffic to assess their risk of and likely routes of ingress into the United States. In contrast, BAT can use relatively rudimentary, high-level data, such as might be available for a newly discovered biothreat that has not yet caused a bioevent or is not subject to intense biosurveillance, in order to assess the agent's inherent pathogenicity. Thus, BAT and IBIS address distinct, but highly complementary, use cases.

Traditional approaches to pathogen assessment focus only on internal functional components of pathogens and often fail to account for the broader characteristics of host-pathogen interactions now recognized as critical for risk determination. Initial development will focus on characterization of viral pathogens, which constitute the majority of emerging diseases, especially those with pandemic potential. will use statistical models built from collated biological, ecological, and genetic data to predict the likelihood of human infection (or spillover) and pathogenicity for a diverse range of biothreats. The overarching aim of the proposed technology is to improve overall situational awareness of existing and novel infectious agents around the world, allowing DHS to more quickly identify and assess which threats pose the most significant risk to human health and thus either more rapidly deploy resources to respond to and mitigate their impact when necessary.

Lab-based virology and bacteriology studies are necessary but not sufficient because they are narrowly-focused and do not consider the broader suite of ecological, evolutionary, and host-specific factors that may affect an infectious agent's pathogenicity and transmissibility.

The initial phase of our work will build upon the existing, curated global-level pathogen and host databases currently housed at EHA. Specifically, we will employ systematic, targeted literature reviews to extend and customize these databases to include viral ecological, epidemiological, and biochemical traits to fit our statistical models and target pathogenicity predictions for the BAT. Predictor variables will span a range of data granularity, and vary according to the four information levels. For example for Level 1, variables may include location of detection (to integrate underlying EID hotspot models based on demographic and geographic factors), and non-human host range and taxonomy. For level 2, coarse-grained viral traits that are independent of sequence data may include: viral family, nucleic acid type, mode of transmission, and phylogenetic host breadth for virus (see Figure 2). For levels 3 and 4 viral sequence data will be integrated by including viral phylogenetic distance and 'nearest-known-threat' analyses (see Figure 3). For the pathogenicity model, human epidemiological data for ~300 viral species known to infect people (case fatality rate, global estimates of number of human cases per year or decade, R_0, infectious period, and primary symptoms) will be aggregated to use as predictor variables.

For ease of interpretation and for applicability across a broad range of pathogens, we propose to use two independent but related statistical models that will be used to predict pathogen spillover risk and pathogenicity, respectively. First, a model will be developed using spillover as the relevant outcome. In this case, spillover will

be defined as either a binary variable, with “0” indicating a virus that is not known to spillover into human populations and establish infection whereas “1” will indicate a virus that is capable of spillover (or is solely a human pathogen). This model will enable us to assign a spillover risk probability to a new biothreat agent that is currently not known to infect people. Given the capacity to spillover and infect humans, we would then use a second model to predict pathogenicity of the novel pathogen. We will model pathogenicity in human hosts on a discrete scale from 1 to 10, with 1 representing a pathogen having no discernible negative effect on human health (i.e., asymptomatic infection) and 10 representing the most severely pathogenic bioagents with high case fatality rates. Additional functionality for the BAT (i.e., Option Years 2-3) may include integration of models to assess pathogen spread via travel and travel networks.

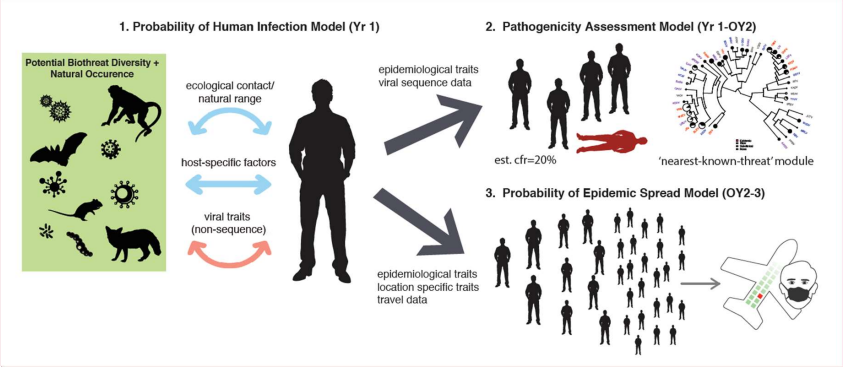


Figure 1. Conceptual framework highlighting 3 underlying models of the Biothreat Assessment Tool (BAT) to estimate: **1)** probability of human infection, or spillover, the first necessary step to understand pathogenicity when status of human infection is unknown (Year 1); **2)** pathogenicity, i.e. probability of causing clinical signs, case fatality rate estimates, and other metrics relevant to humans (or domestic animal species) (Year 1 – Option Year 2); 3) probability of pathogen spread based on ecological, epidemiological, and trade data (Option Years 2-3).

Commented [KJO132]: From our unfunded DHS proposal a couple of years ago, to modify if we want to include this.

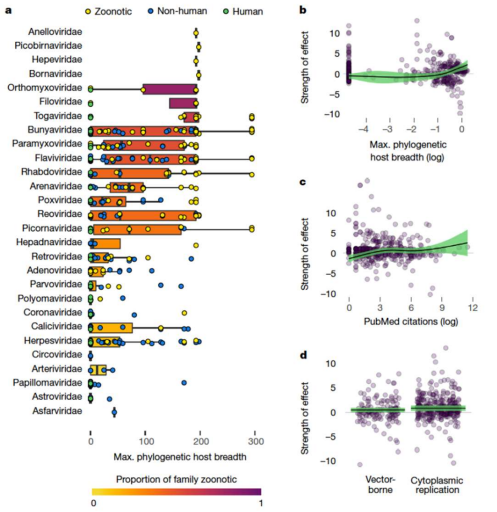


Figure 2. Example of analysis using Level 2 information (limited pathogen characterization) to assess likelihood of human infection (zoonotic potential) for a virus. Figure from Olival et al. 2017, analysis of ~500 unique mammalian viruses. Similar analyses will be developed to assess level of pathogenicity.

From CoV renewal: use data from **3.3** to identify rank SARSr-CoV strains most likely to infect people and evade therapeutic and vaccine modalities. We will use bat survey and zoological data (*101*) to build species distribution models (*125*) and predict the distribution of bat species that harbor low, medium and high risk viral strains. Stacking these modeled distributions for the ~20-30 *Rhinolophus* and related species that occur in the region will allow estimates of SARSr-CoV diversity for a given locality. We will use machine learning models (boosted regression trees) and spatial 'hotspot' mapping approaches to identify the ecological, socio-economic and other correlates of SARSr-CoV diversity and spillover (from serosurveys) (*1, 2, 85*). We will include data from our human behavioral surveys and sampling to give a direct measure of where risk of spillover to people is likely to be highest in the region.

Potential problems/alternative approaches: Patients visiting clinics may have cleared virus, but not yet developed IgG antibodies, reducing seropositive cases. Our 35 day follow-up sampling should avoid this because the maximum lag time between SARS infection and IgG development was ~28 days (*123*). We also expect that patients in rural communities will only visit clinics when symptoms have progressed, likely coinciding with late illness and onset of IgG. We will also have data from our community study, so won't be completely reliant on hospital data to identify PCR- or seropositives. Finally, the risk is outweighed by the potential public health importance of discovering active spillover of a new henipavirus, filovirus or CoV infection.

ALL – the sections below are going to be critical to demonstrate our capacity to do this work. I've not yet started drafting, but please don't hesitate to insert whole sections from other proposals you've done if they're relevant here – we'll need to be creative and get all the language we can from others…

Additional Instructions – Specific to the EIDRC FOA:

Administrative Plan

Commented [PD133]: Add data for Nipah and filo infections

Commented [PD134]: All – I've started putting a few notes in this section, but it will need substantial work. Please insert language from other proposals to fit these sections if you have them

Commented [KJO135]: Peter, I've added in some examples from other grants

In a clearly labeled section entitled **"Administrative Plan":**

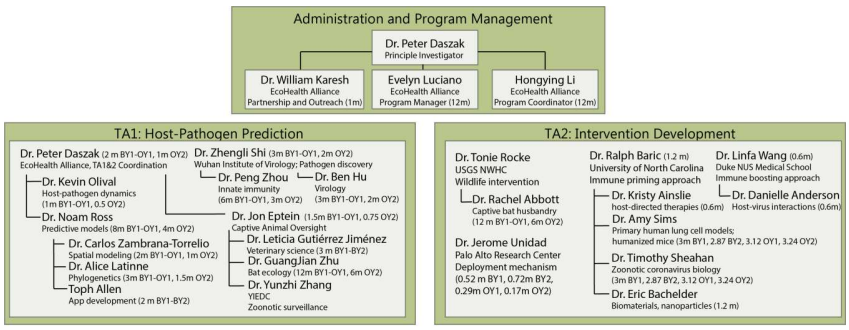
- Describe the administrative and organizational structure of the EIDCRC Administrative and Leadership Team. Include the unique features of the organizational structure that serve to facilitate accomplishment of the long range goals and objectives.
- Describe how communications will be planned, implemented, and provided to collaborators, teams, or sites. In addition, describe plans to achieve synergy and interaction within the EIDRC to ensure efficient cooperation, communication and coordination.
- Specifically address how the EIDRC Team will communicate with other EIDRCs, the EIDRC CC and NIAID and how the EIDRC will implement plans and guidance from the EIDRC CC to ensure coordination and collaboration across the Emerging Infectious Disease Program as a whole. Describe how the study site will provide information, if any, back to the local health authorities and the study subjects as needed. Note any adjustments or changes needed for rapid and flexible responses to outbreaks in emerging infectious diseases.

List out all of our involvement with outbreak investigations, ministries of health surveillance etc.

- Describe the overall management approach, including how resources will be managed, organized and prioritized, including pilot research projects.
- Highlight how the proposed staffing plan incorporates scientific and administrative expertise to fulfill the goal to develop the flexibility and capacity to respond rapidly and effectively to outbreaks in emerging infectious diseases in an international setting.

FROM DARPA grant:

Section 1.03 MANAGEMENT PLAN



Project DEFUSE lead institution is EcoHealth Alliance, New York, an international research organization focused on emerging zoonotic diseases. The PI, Dr. Daszak, has 25+ years' experience managing lab, field and modeling research projects on emerging zoonoses. Dr. Daszak will commit 2 months annually (one funded by DEFUSE, one funded by core EHA funds) to oversee and coordinate all project activities, with emphasis on modeling and fieldwork in TA1. Dr. Karesh has 40+ years' experience leading zoonotic and wildlife disease projects, and will commit 1 month annually to manage partnership activities and outreach. Dr. Epstein, with 20 years' experience working emerging bat zoonoses will coordinate animal trials across partners. Drs. Olival and Ross will manage modeling approaches for this project. Support staff includes a field surveillance team, modeling analysts, developers, data managers, and field consultants in Yunnan Province, China. The EHA team has worked extensively with other collaborators: Prof. Wang (15+ yrs); Dr. Shi (15+ yrs); Prof. Baric (5+ yrs) and Dr. Rocke (15+ yrs).

Subcontracts: #1 to Prof. Baric, UNC, to oversee reverse engineering of SARSr-CoVs, BSL-3 humanized mouse experimental infections, design and testing of targeted immune boosting treatments; **#2** to Prof. Wang, Duke-NUS, to oversee broadscale immune boosting, captive bat experiments, and analyze immunological and virological responses to immune modulators; **#3** to Dr. Shi, Wuhan Inst. Virol., to conduct PCR testing, viral discovery and isolation from bat samples collected in China, spike protein binding assays, humanized mouse work, and experimental trials on *Rhinolophus* bats; **#4** to Dr. Rocke, USGS NWHC, to refine delivery

Commented [KJO136]: Figure like this would be good, but instead of breaking down by TA, maybe by country or Specific Aim. Per FOA, Need to also show how we'll link in with the Coordinating Center and other EIDRCs.

mechanisms for immune boosting treatments. Dr. Rocke will use a captive colony of bats at NWHC for initial trials, and oversee cave experiments in the US and China. **#5** to Dr. Unidad, PARC, to develop innovative aerosal platform into a field-deployable device for large-scale inoculation of the bats. Dr. Unidad will collaborate closely with Dr. Rocke in developing a field deployable prototype for both initial trails and cave experiments in China.

Collaborator Coordination: All key personnel will join regularly-scheduled web conferencing and conference calls in addition to frequent ad hoc email and telephone interactions between individuals and groups of investigators. Regular calls will include:

- Weekly meetings between the PI and Program Manager (on project and task status)
- Weekly web/phone meetings between Program Manager and subawardee admin. staff
- Monthly web/phone conferences between EHA PIs and all subawardee PIs.
- Monthly web conferences between key personnel (research presentations/coordination)
- Four in-person partner meetings annually with key personnel at EHA and two in-person partner meetings annually between subawardees.
- Annual in-person meeting among all key personnel

Evaluation metrics will include the generation of high-quality data, successful achievement of milestones and timelines, scientific interaction and collaboration, the generation of high quality publications, and effective budget management. The PI and subawardee PIs will attend a kickoff meeting and the PI will meet regularly with DAPRA at headquarters and at site visits.

Problem identification and resolution: Regular planning, monitoring, and evaluation meetings will be the primary mechanisms for problem identification. Minor problems (e.g. delays in sample availability or test results) will be dealt with internally through appropriate action and resolution monitored by Dr. Daszak and the Project Manager. In the event of significant problems, such as prolonged poor productivity, inadequate scientific collaboration, or major disputes regarding research direction or resource allocation, EHA will assist with resolving the problem through negotiation. Should a resolution not be forthcoming, consultation with our technical advisors and DARPA Program staff may be warranted.

Risk management: Maintaining a timeline and meeting milestones will require strict and continuous oversight of all project phases, frequent and regularly scheduled communication, and the ability to make decisions and implement strategies. A project of this nature requires a different mindset from that typically associated with basic research activities that move at an incremental pace with investigators gradually optimizing experimental systems, refining data, or waiting for additional data before moving ahead with an analysis approach. To maintain our timeline, we will continually evaluate these trade-offs to make decisions about when iteration is appropriate and when necessary to move forward with current information.

Biographies:

Dr. Peter Daszak is President and Chief Scientist of EcoHealth Alliance, Chair of the NASEM Forum on Microbial Threats, member of the Executive Committee and the EHA institutional lead for the $130 million USAID-EPT-PREDICT. His >300 scientific papers include the first global map of EID hotspots(*1, 126*), estimates of unknown viral diversity(*127*), predictive models of virus-host relationships(*2*), and evidence of the bat origin of SARS-CoV(*20*) and other emerging viruses (*40, 128-130*).

Prof. Ralph Baric is a Professor in the UNC-Chapel Hill Dept. of Epidemiology and Dept. of Microbiology & Immunology. His work focuses on coronaviruses as models to study RNA virus transcription, replication, persistence, cross species transmission and pathogenesis. His group has developed a platform strategy to assess and evaluate countermeasures of the potential “pre-epidemic” risk associated with zoonotic virus cross species transmission(*60, 70, 131-134*).

Prof. Linfa Wang is the Emerging Infectious Diseases Programme Director at Duke-NUS Medical School. His research focuses on emerging bat viruses, including SARS-CoV, SADS-CoV, henipaviruses, and others(*20, 135-138*) and genetic work linking bat immunology, flight, and viral tolerance(*139-142*). A 2014 recipient of the Eureka Prize for Research in Infectious Diseases, he currently heads a Singapore Nat. Res. Foundation grant “Learning from bats” ($9.7M SGD).

Data Management Plan

In a clearly labeled section entitled **"Data Management Plan":**

- Describe internal and external data acquisition strategies to achieve harmonization of systems and procedures for data management, data quality, data analyses, and dissemination for all data and data-related materials generated by the research study with the EIDRC CC.
- Within the plan, indicate the extent to which dedicated systems or procedures will be utilized to harmonize the acquisition, curation, management, inventory and storage of data and samples. Describe how training for the data and sample collection, in terms of the use of electronic data capture systems, will be provided to all staff including those at enrollment sites.
- Describe the quality control procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens.

This includes sample labeling etc. Get a bar-coding system, everyone has a scanner (cf. PREDICT) – Ralph has this for select agents and MERS. Also need a software system

Language from NIH CoV grant:

Data Sharing Plan: Data will be available to the public and researchers without cost or registration, and be released under a CC0 license, as soon as related publications are in press. Data will be deposited for in long-term public scientific repositories – all sequence data will be made publicly available via GenBank, species location data via the Knowledge Network for Biodiversity, and other data will be deposited in appropriate topic-specific or general repositories. Computer code for modeling and statistical analysis will be made available on a code-hosting site (GitHub), and archived in the Zenodo repository under an open-source, unrestrictive MIT license. Limited human survey and clinical data will be released following anonymization and aggregation per IRB requirements. Publications will be released as open-access via deposition to PubMed commons.

Viral isolates will remain at the Wuhan Institute of Virology initially. Isolates, reagents and any other products, should they be developed, will be made available to other NIH-funded researchers via applicable Wuhan Institute of Virology and EcoHealth Alliance Material Transfer Agreements and/or licensing agreements.

Sharing Model Organisms: We do not anticipate the development of any model organisms from this study. Should any be developed, they will be made available to other NIH-funded researchers via applicable Wuhan Institute of Virology and EcoHealth Alliance Material Transfer Agreements and/or licensing agreements.

Genomic Data Sharing: We anticipate obtaining genetic sequence data for 100s of novel coronavirus genotypes, including RNA-dependent RNA polymerase (RdRp) and Spike genes for all strains/genotypes. In addition, we will generate full viral genomes for a subset of the bat SARSr-CoVs that we identify. All sequence data will be deposited in the NIH genetic sequence database, GenBank. We will ensure that all meta-data associated with these sequences, including collection locality lat/long, species-level host identification, date of collection, and sequencing protocols will also submitted. The genotype data will be made publicly available no later than the date of initial publication or six months after the receipt of final sequencing data, whichever comes first. We anticipate sequence generation will occur over the 5 year proposed project period.

Genome Wide Association Studies (GWAS): Not applicable.

Commented [KJO137]: From CoV NIH grant

Thailand TRC-EID Chulalongkorn lab:Specimen management and BioBank: The software called PACS

(Pathogen Asset Control System) supported by BTRP DTRA, is now used for managing the specimen from specimen registration (barcoding, and may move to use QR code system soon), aliquoting, tracking, short patient history record, data summary, searching, test reporting and etc. Our biobank system contains 28 freezers (-80C) and liquid N2 system. Its size is around 120 sq meters. There is a separate electric generator for our lab and biobank. There are CC TV cameras at the biobank and laboratory.

Chula TRC-EID has our own server for data management and analysis. We have the necessary software including Bioinformatic tools (Blast Standalone, SAMTOOL, GATK, AliVIEW, RaxML, FigTREE, MAFFT, Guppy, Megan, PoreChop, MEGA), Database (MySQL, MongoDB) Web, jQuery, Node.js, Express.js React, API, PHP, Ruby, Python, Java, C#, HTML5, Bootstrap, CSS, Responsive, Cloud, Linux Command, PACS (sample management system), Virtualbox virtual machines, R programming languages, Perl scripts, Bash shell scripts, Open source software, Microsoft Office running on both Apple Mac OS X, Ubuntu, Linux, and Windows Operating Systems. Chula TRC-EID has a dedicated 16-core Ram 64GB Linux server with 4TB hard drives, dual quad-core Mac Pro Server with 2TB hard drives and another dedicated iMac Pro (Retina 4K, 21.5-inch, 2017) Processor 3.6GHz quad-core Intel Core i7 (Turbo Boost up to 4.2GHz) Ra, 16GB with 512GB hard drives. TRC-EID also has HPC Linux server CPU: 4 x 26cores Intel Xeon = 104 cores 2.1GHz with 52 TB HHD and 2x3.84TB SSD with GPU 2x Tesla v100 (CUDA core = 5120 cores/GPU, Tenser core = 640 cores/GPU). TRC-EID has a dedicated 10/100/1000 of LAN (Local Area Network), 2.4G / 5G Gigabit Wi-Fi 802.11ac.

Commented [KJO138]: From Supaporn for their lab, some may go in Facilities doc?

From DARPA:

Data Management and Sharing: EcoHealth Alliance will maintain a central database of data collected and generated via all project field, laboratory, and modeling work. The database will use secure cloud hosting services and enable export to archival and platform-independent formats. It will ensure data and metadata compatibility between project components, track data versioning and annotations, and enable compliance with DARPA data requests and disclosure requirements. All archived human sample data will be de-identified. Partners will provide raw and processed data to the central database throughout the course of the project. Project partners will have access to the data they generate in the database at all times, and maintain control over local copies. Release of any data from the database to non-DARPA outside parties or for public release or publication will occur only after consultations with all project partners. EHA has extensive experience in managing data for multi-partner partner projects (PREDICT, USAID IDEEAL, the Global Ranavirus Reporting System).

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Clinical Management Plan
Clinical site selection

Our consortium partners have been conducting lab and human surveillance research, inclusive of human surveillance during outbreaks, for over the past two decades and in that time have developed strong relationships with local clinical facilities and processes. The plan for selection of clinical sites will begin in the same geographic regions as those identified in Aim 1. These are regions where we will sample wildlife due to the high potential of zoonotic viral diversity. Additionally, these will be clinical sites that serve as the catchment healthcare facilities for people in our community-based surveillance of Aim 2. In PREDICT we developed successful working relationships with the major healthcare facilities the hotspot areas in 6 different countries, including Thailand and Malaysia and we will continue to work with these sites going forward. Continuing to work with these hospitals means we will begin this project with successfully established partners and we can begin data collection quickly. This also reduces the number of additional sites needed to meet our goals within our geographic range. The capacity and capability requirements for clinical sites for this study is limited. All that is required of sites will be the ability to enroll patients that meet the clinical case definitions of interest, capacity to collect and temporarily store biological samples, and follow standards for data management and protection. We will be enrolling inpatient and outpatient participants within 10 days of the presentation of symptoms to increase the chance of PCR detection of viruses so we will not wait for advanced normative diagnostic tests,

Commented [EH140]: Is that true

that are likely unavailable in the more remote sites, to be completed. We will work with clinical sites to determine the best process for project staff, in most cases supporting the time of currently site staff. These will be persons who are locally trained and certified to collect biological specimen from participants including blood draw technique, e.g. doctor or nurse. Last, the clinical site will need the capacity to securely store all personal health information and personally identifiable research data, this will include locked offices with locked filing cabinets to store all paper records and an encrypted computer. Training on project procedures will be provided for all local project staff by local country project management staff and partners from headquarters. The study at clinical sites will begin by developing relationships with local stakeholders at the hospitals and clinics in our geographic areas of interest that serve as catchment facilities for people of within the community surveillance study. We will recruit hospital staff that will be extensively trained for project procedures including enrollment of patients, sample types to be collected, storage of samples, administration of the questionnaire, and data management plans. During the Development and onboarding of new clinical sites we will assess the physical needs of the site and what supplies will be required for collection of data (e.g. locked filing cabinet, dry shipper, secure laptop). [Did not specifically address CONDUCT]. Oversight for clinical sites will be managed by the country coordinator for each country. This will be done with regular site visits to the clinical sites and annual visits by headquarter research staff. Site visits will include monitoring and evaluating the process for patient enrollment, collecting and storage of samples, administration of the questionnaire, and secure and data management procedures for secure personal health information and research data. During these visits any additional training will also be provided.

Standardized approach oversight and implementation

Management and oversite for all study sites will be managed by the local country coordinator who is supported by staff at headquarters. Implementation of a standardized approach across all study sites, community and clinical, will begin by extensive training of staff on procedures on requirements of the project, this includes: enrollment, data collection, and data management. Our research team has over 5 years of experience building capacity on human subjects' research and has developed training resources and materials for standardized implementation of community and clinical research. This includes SOPs for screening, enrollment, and retention of participants. In addition to yearly monitory reports the country coordinator will regularly visit each clinical site to ensure quality standards and compliance of procedures are being met. Through our work with clinical sites in PREDICT we have developed culturally appropriate screening measures for clinal sites that do not disrupt the flow or quality of medical treatment received by the patient. This was done by working collectively with clinical research staff to evaluate current procedures and patient flow at the site to determine the most efficient while minimal invasive inclusion of our study into the daily working of the clinal site. Most efficiently this was done by adding minimal basic screening questions to the current clinal intake forms, which allows the clinal research officer to quickly scan charts or logs for potential patients to enroll, allowing for minimal disruption and prevents potential enrollees from being overlooked if the research staff is not on duty. At clinical sites that do scheduled outpatient days we may have a trained member of the research staff with the intake nurse to monitor the patients coming to the clinic more rapidly to no delay screening or enrollment in such a fast pace environment. Using the screening tools, we will recruit and enroll patients who present with symptoms that meet the clinical case definitions of 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever, of unknown etiology within the past 10 days. These patients will be enrolled, samples collected and survey conducted to assess the participants contact with wildlife through: 1) occupational exposures; 2) animal contact; 3) environment biosecurity. With permission from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology. The clinical cohort will be split into cases and controls. "Cases" are defined as participants whose samples tested positive for either coronaviruses, henipavirus, or filoviruses, serological tests. "Controls" will be selected from the pool of participants admitted to the studies and whose samples tested negative. We will use nearest neighbor matching to pair cases demographically (e.g. age, sex, location) with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between serological/PCR status and risk factors. In clinical study, we will use this procedure to determine how clinical presentation differs between virus-exposed

and -unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. We will model the outcomes to analyze correlation between biological results and risk factors related to high risk wildlife exposure. Additionally, we will be following up with clinical participants to determine how clinical presentation differs between coronaviruses, henipavirus, or filoviruses in exposed and unexposed participants, as well as in the time course of illness, severity of symptoms, and type of symptoms. [How do these cohorts be leveraged for new emerging pathogens] Achieving the adequate numbers will be attained through duration of the as clinical participant enrollment. While patient's enrollment is limited by the number of individuals presenting at hospitals, during the PREDICT project we had an average of 105 patients enrolled per year, ranging from 77-244. The country coordinator will be continually monitoring the project database to be sure that we are on track to reach our target.

Clinical cohort setup, recruitment, enrollment

We will recruit inpatients and outpatients from clinical sites to participate in the study after initial screening to determine if they meet the clinical case definitions for 1) severe/acute respiratory illness (SARI/ARI); 2) Influenza-like illness (ILI); 3) fever of unknown origin (FUO); 4) encephalitis; or 5) hemorrhagic fever, of unknown etiology for symptoms reported onset of illness to increase the chance of PCR detection of viruses. Once enrolled, biological samples will be collected by trained hospital staff. When possible, samples will be taken concurrently with samples being collected for normative diagnostics. We will collect 2* 5mL of blood for whole blood and serum collection, samples will be aliquoted, at least one max. 500 µL whole blood; two 500 µL serum samples and two nasal or oropharyngeal swabs will be collected. The cases are defined as participants whose samples tested positive for Henipaviruses, Filoviruses, or CoVs by PCR or serological tests. The controls will be selected from the pool of participants admitted to the studies and whose samples tested negative for coronaviruses, henipavirus, or filoviruses . We will follow up 35 days after enrollment to collect an additional 5mL blood draw for collection of two 500 µL serum samples and a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. Following up 35 days post initial collection gives adequate time for 1) development of IgG and IgM, which occurs <28 days after onset of symptoms for coronaviruses, henipavirus, or filoviruses infected patients to evaluate the immunological progression of disease and 2) further the risk assessment of the participants to monitor contact with wildlife, people, and to assess the likely reservoir hosts to collect information to inform potential intervention programs.

Utilization of collected data

Sampling of wildlife and the enrollment of community surveillance participants and a clinical cohort of participants give us the ability to assess the viruses the are circulating in each of the three populations in a similar geographic region. We will use phylogenetic analysis to compare the relationship between wildlife viruses found and viruses fond in human participants. Additionally, the questionnaire allows us to assess relative measures of human-wildlife contact from our survey work that we will analyze the intensity of contact with species known to be at a higher risk for spillover against other risk to provide useful proxy information for spillover risk.

The biological specimens to be collected (2*5mL whole blood for whole blood and serum analysis and nasal or oropharyngeal swabs) from all eligible participants and follow up specimen 35 days (3mL of whole blood for serum) after enrollment with the standardized questionnaire. Capacity for specimen is collection is not more than standard phlebotomy skills and we will collected by locally trained and certified personnel that are part of the research team in country. Staff we will trainline on ethical guidelines for conducing human subjects research and survey data collection methods. Collection of whole blood, serum, and nasal or oropharyngeal swabs allows us to test the samples for PCR to detect viruses in our priority viral families, serologically test for immunological response and historical exposure to zoonotic diseases of relevance, and the questionnaire will assess individuals evaluate the type and frequency of animal contact, wildlife exposure in daily life, occupational risk in markets and long the wildlife value chain, and unusual illnesses reported over the past 12 months. With this collective data set we aim to quantify and detect novel viruses likely to spillover regularly to people, are often unreported or misdiagnosed as their clinical manifestations are unknown to detect cryptic

Commented [EH141]: Will these cohort fulfill the goals of the EIDRC.

Commented [EH142]: PREDICT we also make the stipulation that we will collective relatvive samples if avialble from treatmean collection, eg CSF if the MD is doing a spinal tap we will request an aliqot if possible

Commented [EH143]: Please add any information that might address: types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.

Commented [EH144]: Please add any information that might address: Describe methods that might be applied to elucidate a zoonotic source

outbreaks causing previously 'hidden' clinical syndromes in people. Our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence.

Potential expansion
Expansion of the research project to other clinical sites or research areas could happen as new information is gathered, either through our research or an outbreak in the region. If expansion is required we could rapidly implement research activities in additional clinical or community sites through the same process we on-boarded the initial locations. First, by working with local stakeholders to create a working relationship and on-boarding new clinical research staff through advanced trainings of project aims, SOPs on data collection and storage, and ethical guidelines. The network of infectious disease researchers in the community and hospital settings would allow for an accelerated response to any potential infectious disease outbreaks as staff are well trained on ethical guidelines for working with human subjects, data and specimen collection, storage, and transpiration, project staff would be on the front lines ready for the call to action. Ideally, our testing for viruses in priority viral families may lead to the discovery cryptic outbreaks before it spreads in the general public. Biological results coupled with survey data on key risk factors will provided necessary information for granular understand of disease spread.

In a clearly labeled section entitled **"Clinical Management Plan":**

- Describe plans, processes, and procedures for the following activities: clinical site selection including feasibility, capacity, and capabilities, and study development, conduct, and oversight.
- Describe strategies for oversight and implementation of standardized approaches in the recruitment and clinical characterization of adequate numbers of relevant patient populations to ensure the prompt screening, enrollment, retention and completion of studies. Describe novel approaches and solutions to study enrollment. including the clinical meta-data that will be captured and describe how the staff at each enrollment/collection site will be trained and comply with the quality standards for data and sample collection and compliance with the standardized procedures. Provide a general description of the methods for establishing clinical cohorts and how cohorts may be leveraged for new emerging pathogens.
- Provide general approaches to identification of the types of clinical cohorts needed to fulfill the goals of the EIDRC. Discuss types of clinical cohorts and clinical assessments that will be utilized to study natural history and pathophysiology of disease, including characteristics of the cohort, site for recruitment, types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.
- Describe methods that might be applied to either elucidate a zoonotic source or a vector of transmission.

Our research team incredibly diverse and capable of working with a group of other viral families: Lay out all our additional vector experience incl. my WNV work, our work on Chik etc.
Baric: Flavi, Noro,
Linfa: …
EHA: …

- Describe capacity for and approaches to clinical, immunologic and biologic data and specimen collection, and how these data and specimens will address and accomplish the overall goals and objectives of the research study.

Ralph

1. Norovirus: collaboration with surveillance cohort CDC; first one to publish antigenic characterization of each new pandemic wave virus; and phase 2 and 3 trial of therapeutics

2. Tekada Sanofi Pasteur dengue
3. Nih tetravalent vaccine

- Provide plans for the potential addition of new sites and expanding scientific areas of research when needed for an accelerated response to an outbreak or newly emerging infectious disease.

Linfa – will launch VirCap platform on outbreak samples.

Statistical Analysis Plan

In a clearly labeled section within the Research Strategy entitled **"Statistical Analysis Plan":**

- Describe the overall plan for statistical analysis of preliminary research data, including observational cohort data.
- Describe the overall staffing plan for biostatistical support and systems available for following functions: 1) preliminary data analyses, 2) estimates of power and sample size, 3) research study design and protocol development.
- Describe the quality control and security procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens. Describe any unique or innovative approaches to statistical analysis that may be utilized.

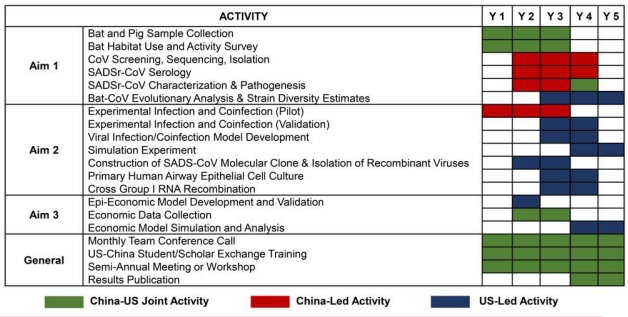
Project Milestones and Timelines

In a clearly labeled section entitled **"Project Milestones and Timelines":**

- Describe specific quantifiable milestones by annum and include annual timelines for the overall research study and for tracking progress from individual sites, collaborators, and Teams. Milestones must specify the outcome(s) for each activity. Milestones should be quantifiable and scientifically justified, and include the completion of major research study activities, including, for example, protocol development, case report forms, scheduled clinical visits, obtaining clearances study completion, and analysis of final data. Milestone criteria should not simply be a restatement of the specific aims. Using a Gantt chart or equivalent tool, describe the associated timelines and identified outcomes for the research study.

Show that for this proposal, we won't be doing the Specific Aims sequentially. We'll begin the human work (SA 2, 3) at the beginning of the project, along with SA1. We'll do the diagnostic assay development initially in Ralph's, Linfa's, Chris's lab, but then begin tech transfer in Y1….

Project Management & Timeline



PI Daszak will oversee all aspects of the project. Dr. Daszak and has two decades of experience managing international, multidisciplinary research in disease ecology, including successful multi-year projects funded by NSF, NIH and USAID. He will conduct monthly conference calls with all co-PIs, including the China team **(see Activity schedule below)**. He will be assisted by EHA Sr. Personnel Li, who is a US-based Chinese national with fluent Mandarin. Co-PI Olival will oversee Aim 1 working closely with China PI Tong, who will test samples. Co-PI Ma (China team) will coordinate and lead all Chinese experimental infections, with guidance from co-PI Saif, who will coordinate and lead all US experiments with co-PI Qiuhong Wang. Sr. Personnel Ross will conduct all modeling activities in Aim 2, working closely with co-PI Saif to parameterize the models. Co-PI Baric and Sr. Personnel Sims will lead infectious clone, recombinant viral and

culture experiments. Consultant Linfa Wang will advise on serology, PCR and other assays. China team co-PIs Shi and Zhou will oversee viral characterization in Aim 1. Sr. Personnel Feferholtz will oversee the economic modeling in close collaboration with Ross.

Commented [KJO145]: Text and figure From NSF-NIH SADS grant. Peter will adapt

Another Example of Milestones and specific tasks from a prev DHS (unfunded) proposal:

Expand existing databases to include predictor variables for pathogenicity risk model

Task 1: Collate available human clinical information from the literature for ~300 viral pathogens known to infect humans (Contract Award Date to 6 month)

Task 2: Update EHA host-viral association database to include new data for mammalian and avian species from literature 2015-present (Contract Award Date to 3 month)

Task 3: Extract and curate full and partial genomic data from Genbank for 5-6 viral families with a high proportion of human-infectious pathogens for phylogenetic analyses – e.g. 'nearest-known-threat module'. (Month 2 to 6 month)

Develop underlying pathogenicity and spillover risk assessment models

Task 4: Fit generalized linear models in Stan for multiple response variables to measure pathogen impact (Month 7 to 10 month)

Task 5: Fit generalized linear models to estimate risk of human infection (spillover) based on host, ecological, and location specific traits (Month 4 to 8 month)

Task 6: Validate model effectiveness by simulating early-outbreak, limited-information scenarios from known pandemics to confirm model behavior (Month 8 to 10 month)

Commented [KJO146]: Just example, but probably just want to use a Gantt chart format per most of our grants, depending on space.

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From:	Danielle Anderson on behalf of Danielle Anderson <danielle.anderson@duke-nus.edu.sg>
To:	Peter Daszak; Wang Linfa; Ralph Baric (rbaric@email.unc.edu); Baric, Toni C; Sims, Amy C; Christopher Broder (christopher.broder@usuhs.edu); Eric Laing; Thomas Hughes
Cc:	Aleksei Chmura; Alison Andre; Luke Hamel; Kevin Olival
Subject:	Re: CORRECTION - please ignore last email!!! re. EIDRC-SEA v.3
Date:	Sunday, June 23, 2019 6:45:45 AM
Attachments:	EIDRC Southeast Asia v3 (LW-DA).docx

Hi Peter (and all),

Here are mine and Linfa's edits. I will have to add more after some of the CoV stuff has been removed. At the moment it is too CoV specific (I know we took a lot of text from our other grant proposal), but can some of this be removed before we add in the final filo/paramyxo info- see my comments.

In the next version, can you delete the tracked comments if they have been answered or corrected in the text? I didn't delete any comments.

I should have time on Monday afternoon (SG time) to look at this again.

Cheers,
Dani

From: Peter Daszak <daszak@ecohealthalliance.org>
Sent: 21 June 2019 10:45 AM
To: Wang Linfa; Danielle Anderson; Ralph Baric (rbaric@email.unc.edu); Baric, Toni C; Sims, Amy C; Christopher Broder (christopher.broder@usuhs.edu); Eric Laing; Thomas Hughes
Cc: Aleksei Chmura; Alison Andre; Luke Hamel; Kevin Olival
Subject: CORRECTION - please ignore last email!!! re. EIDRC-SEA v.3

Many apologies – I sent the wrong attachment – here is the correct version!!!

Dear all,

Thanks to everyone for comments and text for the earlier draft at the beginning of the week. Kevin's spent the last couple of days pulling all the comments together into one draft and inserting his changes, and I've now attached a v3 of the draft. It's still rough, and now too long, but it's getting there. I wanted you to look at this rough version because I'm stuck in Liberia in meetings Friday, then flying back home Saturday. I'll continue editing this draft on the plane and will be working solely on this grant from home on Sunday, Monday, Tues, Wed, Thurs.

Please work on this rapidly if possible. If you can all push your comments through, using track changes, and get them back to me **by Sunday evening New York Time**, I will turn round a polished draft by Tuesday, giving us time for significant last go-over on Wed, Thurs. It's tight, but unfortunately, it is what it is, so please commit the time if you can!! As well as general edits (using track changes, with references in comment boxes), please do the following…

Linfa/Danielle – We especially need your input in section 1.1, 1.3, 1.4 and 2.5

Ralph/Amy - Please work your magic on this draft, inserting text, editing and reducing text, and making sure the technical details of the characterization are correct and fit the proposed work on CoVs, PMVs and Henipaviruses

Chris/Eric – Eric – congratulations on your (b) (6) and thanks for working on it at this time! Chris – please help and edit/finesse the language in the relevant sections for you

Tom – Your edits crossed over with Kevin's changes, so please insert all the bits that were new in the last version you sent to me, into this version. I think these are mainly the sections and comments from the other Malaysian colleagues, but please check and re-insert all key text. We especially need to be specific about which partner in Malaysia is doing what cohorts, and where, and about who's doing the testing

Thanks again all, and I'm looking forward to non-stop work on this as soon as I get on my flight in 24 hours…

Cheers,

Peter

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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

EcoHealth Alliance Capacity/Experience

EcoHealth Alliance (EHA) staff has over two decades' experience conducting cutting edge research on zoonotic disease emergence in international settings. This includes NIH-funded modeling, field and laboratory research on host-pathogen dynamics and spillover of Hendra, Nipah, Ebola, SARS, West Nile, avian influenza, MERS and other emerging viruses[1-12]. EHA epidemiologists, modelers and analysts have produced seminal papers analyzing the origins and hotspots of emerging zoonotic diseases[13-16], designing strategies to identify the diversity and likely reservoirs of novel viruses[17-24], and predicting patterns of EID spread[25-30]. In over 20 countries within South and Southeast Asia, Central and West Africa, and Latin America, EHA field staff has conducted human, wildlife and livestock sampling to identify known and novel pathogens, and test for serological evidence of their spillover[3,31-42]. This includes acting as a key member of the 10-yr USAID-EPT Predict consortium, managing all modeling and analytics, all human behavioral risk research, and conducting wildlife and human surveillance in 11 countries, including managing all Predict activities in Liberia, Brazil and Thailand – the sites for the current proposal. As part of Predict, EHA scientists collected >60,000 samples from humans and wildlife, organized laboratory testing for known and novel viruses (>300,000 PCR tests), and helped build, support or equip around 15 laboratories in EID hotspots, including Liberia, Brazil and Thailand.

Text for the proposal

***Introduction/Preliminary data*:** Analysis by key personnel Daszak, Olival, Ross demonstrate that the countries chosen for the NEIDL EIDRC proposal (Brazil, Liberia, Nigeria, Uganda) are hotspots of disease emergence[14]. These countries have high biodiversity of wildlife (and their viruses), high and rapidly growing human population density, and rapidly increasing levels of deforestation and agricultural intensification – globally significant drivers of EIDs[13]. One of the challenges of managing emerging zoonoses is identifying the key at-risk populations at the critical interfaces for viral spillover *within EID hotspot countries*. This is critical for building capacity where it is most likely to be needed in an outbreak, for supplying therapeutics and vaccines to populations most likely to require them, and for identifying populations with active spill-over to undertake clinical trials of novel compounds. EHA scientists have developed a series of modeling and analytical tools to better target the NEIDL's focus on in-country clinical and community sampling. This includes analysis of all known mammalian virus-host relationships from ICTV data that identifies the geographic distribution of the most wildlife reservoirs most likely to harbor novel and known zoonoses. It also includes mapping the distribution and relative number of unknown viruses likely to spillover into people in the region[24]. Prior work by EHA under USAID-EPT-PREDICT involved collection and testing of samples from >5,000 mammals (bats, rodents, primates) in Brazil, >10,000 mammals in Liberia, leading to the discovery of a reservoir host for EBOV (paper in prep.) and >50 novel paramyxoviruses, coronaviruses and viruses from other zoonotic groups[32,43]. It includes biological sample collection and behavioral risk questionnaire surveys in >500 people to assess high risk behaviors (REF). This is part of broader work that includes collection and testing of >XXXX samples from XXXX individual mammals from 14 countries, the discovery of hundreds of novel viruses from viral families with known zoonoses, and biological sampling and behavioral risk

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Commented [PD2]: Lizzie Loh's paper from Brazil, anything from Liberia?

Commented [PD3]: Include P1 L. American work for EHA

surveys from >5,000 people[43]. **These data are available to the current project to ground-truth risk mapping, and better identify populations most likely to encounter zoonotic spillover events.** Under prior and current NIAID R01 funding, key personnel Daszak, Olival, Noam have demonstrated proof-of-concept of a linked modeling-field surveillance approach to identify high risk wildlife species in China that harbor a large group of SARSr-CoVs, many capable of infecting human cells[3,8,42]. Using analyses that map wildlife reservoir diversity, behavioral risk questionnaires in rural China, and biological sampling of high-risk populations, this has resulted in the identification of a key cave system haboring bats with CoVs that have every genetic element of the SARS-CoV outbreak strain[5]. Recently our group has demonstrated serological evidence for the recent exposure of these high-risk human populations to novel bat-origin SARSr-CoVs[38]. A similar strategy will be used in this proposed work to identify high priority sites for clinical cohort and community sampling where people are at the highest risk of viral spillover.

***Workplan*: 1) Modeling to identify high priority sites for clinical trials and community sampling.** We will revise our hotspot mapping to produce fine-scale within-country maps of spillover risk for a) generalized zoonotic viral pathogens; b) specific pathogens (e.g. Lassa fever virus, bat-origin Henipaviruses, flaviviruses, filoviruses and bat-origin Coronaviruses; c) identification of the communities most likely to require interventions. Our hotspot analysis shows that land use change, biodiversity and human population density change are correlated with EID spillover risk[13,14]. We will use databases already acquired, cleaned and gridded at EHA, including annual land cover maps produced by the European Space Agency from 1992-2015 with a 300 m spatial resolution, data on biodiversity mapping from the IUCN spatial database version 2015.2, on land use change, and the CIESIN Global Rural–Urban Mapping Project human population data set, which provides gridded estimates of human population every five years for 1970–2000[44-47]. For each virus or viral group of interest, we will use ICTV data and our own PREDICT sampling data to map the distribution of each known reservoir host, and identify hotspots of reservoir density where populations are most likely to encounter spillover events. We will then use socio-economic data from >10,000 zoonotic risk questionnaires as part of USAID Predict surveillance conducted by EHA in Brazil, Liberia and other countries with similar demographic characters to pinpoint communities most likely to constitute the key wildlife-human interface for spillover.

2) Modeling to predict the identity and distribution of reservoir hosts. For many emerging viruses, poor knowledge of reservoir hosts reduces our capacity to predict which human populations are at risk of infection. Even where reservoir host identity is known or suspected, the presence of alternate hosts can lead to underestimates of risk. For example, while the hosts of henipaviruses and filoviruses are thought to be specific frugivorous bats[48-51], work by EHA and others shows that diverse bat genera, including insectivorous bats can harbor these viral families, and that viral distribution may be much wider than previously thought[40,52-54]. We will adapt our recently-published approach that uses data on all known virus-mammalian host relationships[24], as well as other modeling frameworks[55] to predict the likely hosts of known viruses of interest to the EIDRC. We will target filoviruses, henipaviruses, coronaviruses, arenaviral relatives of Lassa fever virus, known zoonotic flaviviruses, paramyxoviruses. We will

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then use ecological niche mapping and known mammalian distributions from IUCN to estimate the likely distribution of viral reservoirs, and therefore better assess the risk of spillover to people.

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3) Fieldwork/Training: Research on the potential wildlife reservoirs of lethal agents carries inherent, albeit low (given low viral prevalence in wildlife), risks of exposure. To minimize this, EHA veterinary and other staff has designed protocols, presentations, and SOPs for capacity building and cross-training in resource-poor countries, including those in the current propsal. These have been previously used for training of >300 staff in >14 countries as part of the 10-year USAID-EPT-PREDICT project, 5 NIH-funded R01s for work on select agents in resource-poor countries such as Bangladesh, Malaysia, Indonesia, India, and DTRA-funded capacity building projects in Western Asia, Malaysia, South Africa and Liberia. In the current proposed work, EHA will conduct training exercises for NEIDL and in-country field staff on the safe, humane and secure trapping, handling, and anesthesia of wildlife reservoirs, as well as high quality biological sample collection and storage and cold-chain management. This will include lectures and practicum training with live animals at the NEIDL facility, and fieldwork in Brazil, Uganda, Nigeria and Liberia to train in-country staff. Field training will be given using live individuals of species collected from locales that the modeling work suggests have a high likelihood of harboring known agents.

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From:	Peter Daszak on behalf of Peter Daszak <daszak@ecohealthalliance.org>
To:	Wang Linfa; Danielle Anderson (danielle.anderson@duke-nus.edu.sg); Ralph Baric (rbaric@email.unc.edu); Baric, Toni C; Sims, Amy C; Christopher Broder (christopher.broder@usuhs.edu); Eric Laing; Thomas Hughes
Cc:	Aleksei Chmura; Alison Andre; Luke Hamel; Kevin Olival
Subject:	EIDRC-SEA v.3
Date:	Thursday, June 20, 2019 9:40:59 PM
Attachments:	NEIDL EIDRC EHA proposed work v3.docx
Importance:	High

Dear all,

Thanks to everyone for comments and text for the earlier draft at the beginning of the week. Kevin's spent the last couple of days pulling all the comments together into one draft and inserting his changes, and I've now attached a v3 of the draft. It's still rough, and now too long, but it's getting there. I wanted you to look at this rough version because I'm stuck in Liberia in meetings Friday, then flying back home Saturday. I'll continue editing this draft on the plane and will be working solely on this grant from home on Sunday, Monday, Tues, Wed, Thurs.

Please work on this rapidly if possible. If you can all push your comments through, using track changes, and get them back to me **by Sunday evening New York Time**, I will turn round a polished draft by Tuesday, giving us time for significant last go-over on Wed, Thurs. It's tight, but unfortunately, it is what it is, so please commit the time if you can!! As well as general edits (using track changes, with references in comment boxes), please do the following…

Linfa/Danielle – We especially need your input in section 1.1, 1.3, 1.4 and 2.5

Ralph/Amy - Please work your magic on this draft, inserting text, editing and reducing text, and making sure the technical details of the characterization are correct and fit the proposed work on CoVs, PMVs and Henipaviruses

Chris/Eric – Eric – congratulations on your (b) (6) and thanks for working on it at this time! Chris – please help and edit/finesse the language in the relevant sections for you

Tom – Your edits crossed over with Kevin's changes, so please insert all the bits that were new in the last version you sent to me, into this version. I think these are mainly the sections and comments from the other Malaysian colleagues, but please check and re-insert all key text. We especially need to be specific about which partner in Malaysia is doing what cohorts, and where, and about who's doing the testing

Thanks again all, and I'm looking forward to non-stop work on this as soon as I get on my flight in 24 hours…

Cheers,

Peter

Peter Daszak
President

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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

II. Research Strategy:

1. Significance:

Coronaviruses (e.g. SARS-CoV, MERS-CoV), henipaviruses (HeV, NiV) and filoviruses (EBOV, MARV) have led to some of the most important recent emerging zoonoses (*1-12*). Their outbreaks have caused tens of thousands of deaths, and billions of dollars in economic damages (*13-15*). Like most emerging zoonoses, these pathogens originate in wildlife reservoirs, sometimes spilling over first into livestock 'amplifier' hosts, or directly into localized human populations with high levels of animal contact **(Fig. 1)**. Efforts to prevent emerging zoonoses have targeted these high-risk populations in regions prone to disease emergence (*12, 16*). However, surveillance and control is hampered by inadequate information on basic disease ecology (e.g. range of wildlife reservoirs, risk factors for spillover) and pathogen biology (viral diversity, pathogenesis), and by logistical challenges sharing novel diagnostics, animal models and reagents at sites where outbreaks begin (*17*). Countermeasure and vaccine development is challenged by the small number of isolates available, and the continuing discovery of closely-related strains, some with evidence of transmission to people but with unknown pathogenicity. For example, using NIH and other funding, we found serological evidence of exposure to African henipaviruses in 3-4% of a sample of people in Cameroon, with a significant risk factor being butchering of bats; and bat SARSr-CoVs in 6/209 people (2.87%) living close to a bat cave in Yunnan, both viruses with unknown clinical impact (*18, 19*). NiV-like virus/disease in horses and people who ate horses, Philippines. Your study (EHA) in Bangladesh, NiV serology livestock. Mojiang virus, putative henipavirus, correlatively linked with fatal human illness. Others have shown that …..Any other examples of spillover events into livestock or people, especially from SE Asia?? (REFS). Even when spillover leads to outbreaks of illness and mortality, they are often unreported, undiagnosed or misdiagnosed. Under a prior NIH R01, we initiated targeted syndromic surveillance of encephalitis patients in Bangladesh clinics and showed that NiV causes repeated annual outbreaks with an overall mortality rate of ~70%, and that it is also present in bats in India (REF). Nipah virus was originally misdiagnosed as 'aberrant measles' in West Bengal, India (REF), has now emerged repeatedly in North India, and in Kerala, South India in 2018 and 2019 (ongoing), raising the specter of future spillover at other sites across the region (*20, 21*).

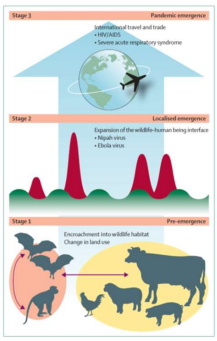

Fig. 1: Our Early Warning Approach: Most zoonotic viruses emerge from wildlife hosts, sometimes via livestock (Stage 1, lower panel), to cause small scale outbreaks (green) or larger chains (red) of human-to-human transmission (Stage 2, middle). In some cases, these spread more widely via air travel (Stage 3, upper). Our EIDRC proposal targets each stage: SA1 examines the diversity and spillover potential of viral threats in targeted SE Asian wildlife; SA2 seeks evidence of their spillover into focused high-risk human populations (stage 2); SA3 identifies their capacity to cause illness and to spread more widely (stage 3). Figure from (*16*).

Southeast Asia has significant risk of future viral emergence: it is a well-defined hotspot for emerging diseases due to its high biodiversity of wildlife and their viruses, and the presence of critical ecological and socioeconomic drivers of disease emergence (*22*). These include dense human populations living in networks of rural and urban communities, with strong cultural and behavioral connection to wildlife and livestock, and intimate connection to global travel networks (*23*). It is also a region undergoing rapid environmental and demographic change, both of which lead to increased risk of disease emergence and spread (REFS). It is therefore not surprising that a number of recent discoveries have identified near-neighbors of known agents spilling over to livestock and people through often novel pathways, leading to sometimes unusual clinical presentations **(Table 1)**.

In addition to these spillover events, relatives and near-neighbors of known viral pathogens have been reported throughout the region, including by members of our consortium. These include: henipaviruses in *Pteropus* and other? bats in Thailand, Cambodia, Philippines, Laos, Indonesia, China, other countries? (*24-30*); a novel henipavirus Mojiang virus in wild rats in Yunnan (*31*); serological evidence of filoviruses in bats in Bangladesh and Malaysia (REF); evidence of novel filoviruses in bats in Singapore (*32*) and China (*33-35*), including Mĕnglà virus that appears capable of infecting human cells (*36*); novel paramyxoviruses in bats and

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rodents consumed as bushmeat in Vietnam (*37*), a lineage C β-CoV in bats in China that uses the same cell receptor as MERS-CoV and can infect human cells *in vitro* (*38*); 52 novel SARSr-CoVs in 9 bat species in southern China (172 novel β-CoVs from >16,000 sampled bats) that are also found throughout the region (*38-41*), a new β-CoV clade ("lineage E") in bats (*41*); and XX novel wildlife-origin CoVs and paramyxoviruses in Thailand and Malaysia identified as part our work under USAID-PREDICT funding (*39, 42*). Under a current NIAID R01, we have shown that we can use analytical approaches to target surveillance of wildlife and people and identify likely sites of spillover. We used phylogenetic analysis of bat hosts and their CoVs to identify a key cave complex in SW Yunnan Province that harbors all the genetic elements of SARS-CoV (*43*), as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics (*38-41, 44-46*). We conducted behavioral risk surveys and biological sampling of human populations living close to this cave and found evidence of spillover (xx serology) in people highly exposed to wildlife. **This work provides proof-of-concept for an early warning approach that we will expand in this EIDRC to target key viral groups in one of the world's most high-risk EID hotspots.**

Viral agent	Location, date	Impact	Novelty of event	Ref.
Melaka virus & Kampar virus	Malaysia, 2006;	Caused SARI in family group, individual	1st disease due to bat-origin reoviruses (other reports Singapore, Vietnam etc.)	(*47-50*)
Reston filovirus	Philippines 2008	Seropositive people killed pigs,	No prior findings of filoviruses in pigs	(*51*)
Reston filovirus	Shanghai 2011	PCR positive pigs	Further evidence of pig RESTV infection	(*52*)
Nipah-like virus	Philippines 2015	Killed horses	No prior horse infection for NiV, known for HeV	(*53*)
SADS-CoV (HKU2)	China 2017	>25,000 pig deaths seronegative people	Novel emergence of bat-origin CoV	(*54*)
SARSr-CoV & HKU10-CoV	Yunnan, Guangxi 2015	Seropositive people	1st evidence human infection HKU10 & SARSr-CoV since 2003	(*19*)
Nipah virus	Kerala, 2018 & 2019 (REF)	XX people infected/dead	1st outbreak of NiV outside Bangladesh, W. Bengal focus	(*21*)

Table 1: Recent reports from SE Asia indicating potential for novel pathways of emergence, or unusual presentations for known or close relative viruses. OTHERS? Could include Melaka but it's in the Reoviridae and not in the RFA (should we only include pathogen groups listed in the RFA in this table?)

The overall rationale for this EIDRC proposal is that there is substantial evidence that: 1) diverse CoVs, henipaviruses and filoviruses related to known zoonotic pathogens are circulating in wildlife reservoirs in Southeast Asia; **2)** these viruses likely spillover regularly to people, are often unreported or misdiagnosed, and their clinical manifestations and potential to cause pandemics are unknown and underestimated; and **3)** our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence. The overarching goal of this proposal is to better understand the diversity of known and closely related CoVs, henipaviruses, and filoviruses in wildlife reservoirs in SE Asia, to test their capacity to infect human cells and mouse models, to develop new specific and sensitive serological and molecular diagnostic tools, and to conduct surveillance of human populations with high risk of exposure to wildlife, and clinical cohorts to identify evidence of spillover and pandemic potential, and of viruses causing previously 'hidden' clinical syndromes in people.

2. Innovation: Previous work by our consortium has developed a system to target surveillance in wildlife and people to better anticipate spillover events and pre-empt outbreaks of emerging viruses (REF). In this EIDRC we take this approach and scale it up to cover three critically high-risk EID hotspot countries in Southeast Asia, within a regional network of collaborators. The innovation in this EIDRC is in: 1) its multidisciplinary approach that combines modeling to target the geography for wildlife and human sampling, novel phylogenetic and *in vitro* and animal model approaches to identifying risk of viral spillover into people, development and transfer of novel serological and molecular diagnostics, and use of parallel large scale cohorts to identify spillover and illness due to known and novel viruses; 2) the biological characterization approach that identifies how likely viruses are to be able to spillover into people and builds on our successful work on SARSr-CoVs; and 3) the

combination of geographically targeted human populations with high risk of animal contact and syndromic clinical cohorts that present with signs of novel viral emergence, to identify risk of viral spillover in an early warning system approach. **In Aim 1, we will target viruses in new and archived wildlife samples, and characterize their risk of spillover to people**. We will use spatial and phylogeographic analyses to identify the geography and species of wildlife to test samples from, as well as further characterize viruses we have recently discovered. We will use cell culture and animal models to assess their potential for spillover into high-risk human populations. **In Aim 2, we will conduct focused, targeted surveys and sampling of human communities with high exposure to wildlife and other animals.** We will use serological tests targeting viruses identified in Aim 1 to identify the baseline spillover of known or novel CoVs, henipaviruses and filoviruses in these populations. We will use behavioral risk data, virological and ecological data to identify the likely reservoirs of viral groups identified. **In Aim 3, we will use syndromic surveillance of clinical cohorts to identify evidence of viral etiology for otherwise 'cryptic' outbreaks.** We will enroll and collect behavioral risk information and biological samples from patients in communities at high risk for disease emergence, who present with syndromes previously linked to known viral agents. We will conduct molecular and serological diagnostic assays to identify likely viral etiology of syndromes that may represent otherwise cryptic events. Where viruses are identified, we will attempt to isolate and characterize them, and use survey data, ecological and phylogeographic analysis to identify likely reservoir hosts and inform intervention programs.

3. Approach

Research team: For this EIDRC we have assembled a group of world leaders in emerging virus research with proven experience collaborating internationally on field, lab and human surveillance research **(Fig. 2)**. Over the past two decades, our consortium partners have collaborated together within the region and globally to conduct high profile research on these and other viral groups. This work includes identifying the wildlife reservoirs for Nipah and Hendra virus, MERS- and SARS-CoVs (*40, 43, 55-59*), discovering SADS-CoV (*54*), and developing an array of serological, molecular, *in vitro* and *in vivo* approaches to characterizing high-risk CoVs, henipaviruses, and filoviruses (*44-46, 60-73*). It includes substantial experience conducting human surveillance during outbreaks (e.g. - Please insert examples that you've been involved in and REFS), and as part of longitudinal efforts to pre-empt pandemics (*74, 75*).

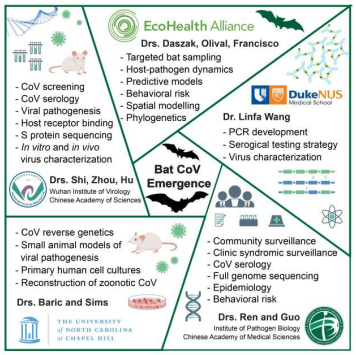

Fig. 2: Interdisciplinary team & roles in the proposed SE Asian EIDRC. PLACEHOLDER – Hongying to modify

PI Daszak has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5- 20 yrs on NIAID- and USAID-funded research. He will oversee all activities in this EIDRC. Drs. Olival, Zambrana, Ross are leaders in analytical approaches to targeting surveillance and head the modeling and analytics work for USAID-PREDICT. Drs. Wang, Baric, Broder, Anderson, Laing have developed a unique array of *in vitro* cell culture, serological and molecular reagents to identify and characterize emerging viruses in people and animals (henipaviruses and other paramyxoviruses, CoVs, filoviruses and many others). Dr. Wacharapluesedee, Hughes and collaborators have coordinated surveillance of people, wildlife, and livestock within Thailand and Malaysia for the past 10 years, in collaboration with Ministries of Health, Agriculture and Environment. Finally, this consortium has extensive external funding from DoD, USAID, NIH, DHS and other USG agencies that supports a range of laboratory, analytical and field studies

directly related to the current proposal, and will bring substantial leverage to the EIDRC **(See Section XX)**.

Fig.3 : Map of Southeast Asia indicating the three core countries for this proposed EIDRC (White: Thai, Sing, Mal) and those that Key Personnel are actively collaborating with (Green: field sites and collaborating labs indicated with asterisk). PLACEHOLDER – Hongying to modify - please use a map with part of India and Bangladesh. –This map is the perfect range

Geographical focus: The three core countries for this proposed EIDRC are Thailand, Malaysia and Singapore, three contiguous EID hotspot countries that stretch through one of the most significant foci of EID origins globally. Our proposed work includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sarawak and Sabah, and thus contains overlapping wildlife biogeography with India, Bangladesh, Myanmar, SW China, Laos, Cambodia, Vietnam and Indonesia. It encompasses diverse cultural and socioeconomic backgrounds which have correspondingly diverse and intensive interactions with wildlife reservoirs and the global travel network. We have already begun sampling of wildlife hunters, livestock workers, wildlife farmers and market workers, rural villagers living close to high risk wildlife species, people otherwise highly exposed to animals in these countries, and clinical cohorts, as part of current/prior NIH and USAID funding. To develop a larger catchment area for emerging zoonoses, we will deploy our consortium's extensive network of collaborators in clinics, research institutes and public health laboratories in every other Southeast Asian country. We will conduct regular meetings with key research, public health and community leaders, including >50 leading laboratories/institutions in XX countries that are currently collaborating with core members of our consortium via other funded work (**Fig. 3**). We will use these, and our annual meetings, to share information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks across the region.

Current list of contacts at key human (especially) and wildlife orgs in every SE Asian country from map above – please add to this and send in the full details on the form requested by Luke/Aleksei!

Indonesia – EHA: Eijkman (Dodi Safari), IPB (Imung Joko Pamungkas);
SE China – EHA: Yunnan CDC (XX), Wuhan Inst. Virol. (Zhengli Shi);
Myanmar -
Cambodia -
Laos -
Bangladesh – EHA: IEDCR (XX), XX (Arif);
India – EHA: Jon's collaborators;
Vietnam – Wellcome CRU ? Liaison with Zhengli Shi for Serol assays etc.?
Philippines –

NOTE: Everything below is a very rough draft. I've lifted heavily from our recent CoV R01 renewal submission, and indicated in comment boxes the names of the people who I believe know most about each section and can modify or write the new text needed.:

Aim 1: Identification and biological characterization of spillover risk of high zoonotic potential viruses from wildlife

1.1 Rationale/Innovation: Our previous work on wildlife origin viruses demonstrates that zoonotic viral emergence tends to occur in specific regions with high diversity of wildlife that harbor viruses of zoonotic potential, and human populations that have close contact to these wildlife (*23*). In Aim 1, we will analyze these factors to identify regions, species and human populations that have a particularly high risk of viral spillover. We will target high-risk zoonotic host reservoirs (bats, rodents and primates) in these regions and test newly collected and some of the tens of thousands of recently collected samples we have archived in freezers in our labs for known and novel agents. Using previously developed approaches to predict capacity of novel viruses to infect human cells and mouse models, we will characterize these viruses and assess their potential for spillover, using those of high risk as targets for human sampling in Aim 2 and 3. This approach is built on substantial previous proof-of concept preliminary data:

Geographic targeting: EcoHealth Alliance has led the field in conducting analyses to indicate where the highest risk of zoonotic spillover in a region is (*22, 23*). For Southeast Asia, these EID hotspots are sites with high wildlife biodiversity, human population density and undergoing rapid land use change **(Fig 4a)**. Using host and ICTV viral data for all known mammalian viruses, and generalized additive models to correct for sampling bias, we are able to predict the relative number of yet-to-be-described viruses that a species harbors (*76*). Using these data to map unknown viral diversity demonstrates substantial undiscovered viral diversity in mammals across Southeast Asia, and particularly in regions of Thailand, Singapore and Malaysia **(Fig. 4b)**. In a separate paper on risk of viruses emerging from bat hosts, we found that factors driving spillover (host diversity, climate) differ from those driving spread in human populations (population density, bushmeat hunting, livestock production) (*77*). We therefore analyze capacity for viral spread as a separate risk factor, using demographic and air travel data in an app we have produced with Dept Homeland Security funds (*78*). In the current proposed work, we will use all the above approaches to target wildlife sampling (archive and new field collections), and to inform sites for human sampling in Aim 2, to areas of high risk for spillover and spread.

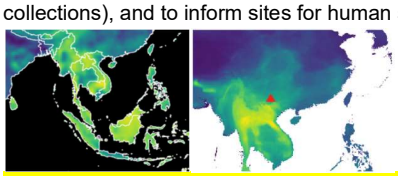

Fig. 4: Our previous work has shown proof-of-concept in geographically targeting sampling to maximize regions as high risk of wildlife-to-human spillover **(Fig. 4a)**, and regions with high diversity of predicted 'missing' or as-yet undiscovered viruses, yellow = highest diversity **(Fig 4b)**. From (*22, 23, 76*). Kevin/Alice, please insert high res hotspot map from Allen et al. (Fig 4a), expand the current map from Olival et al. to cover the same region as shown in Fig. 3, and remove the red triangle of course.

Reservoir host species targeting: Our analysis of all known mammalian host-viral associations demonstrates that bats, rodents and primate represent the three most important reservoirs for zoonoses, responsible for the highest proportion of known and predicted viral diversity of zoonotic potential (*76*). The bulk of our sampling and sample testing will consist of bats, because this group is the known or hypothesized reservoir for the majority of high-impact zoonotic CoVs, henipaviruses and filoviruses (*79-82*) (ADD REFS). Bats also have the highest proportion of predicted zoonotic viruses in any mammalian group (*76*). We have used a novel phylogeographic analysis, Maximum Clade Credibility (MCC) tree, to reconstruct the geographic areas of evolutionary origin for β-CoVs that we sequenced in bats (*39*). This approach allows us to identify the ancestral home of specific zoonotic viral groups in wildlife, where their diversity is likely highest. Finally, we have designed a strategy to estimate sampling gaps, using a viral 'mark-recapture' approach we previously published (*83, 84*) **(Fig. 5)**. Using prior data for bat, rodent and primate viral discovery vs. sampling rates, we can identify the likely total viral diversity for specific host groups in a region, and use this to estimate sample sizes to discovering the majority of unknown viruses within a specific family or genus. **In the current proposal,** we will use these analyses to estimate geographic and species-specific sampling targets **to more effectively identify new strains to support experimental infection studies and risk assessment.**

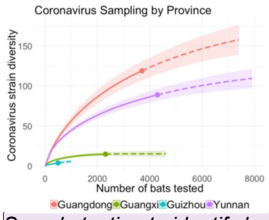

Fig. 5: Estimates of SARSr-CoV total strain diversity in bats we sampled in China under a NIAID R01 (strain defined as >10% sequence divergence in RdRp gene). GD and YN harbor highest CoV diversity, but discovery has not yet saturated. We used this to estimate that additional sampling of 5,000 bats will identify >80% of remaining β-CoV strains in bat hosts from these regions. We will apply this approach to our target host and viral taxa, for Thailand, Singapore and Malaysia to calculate sampling targets and success rates.

Sample testing to identify known and novel viruses: We have conducted extensive wildlife surveillance for novel viruses across Southeast Asia in prior NIAID funded work and as part of the USAID PREDICT project. This includes sampling and PCR-screening >16,000 individual bats from 6 families (16 genera) in southern China for coronaviruses, identifying 178 β-CoVs, of which 172 were novel (52 novel SARSr-CoVs). This includes members of a new β-CoV clade, "lineage E" (41), diverse HKU3-related CoVs (179 sequences) within a 'sister' clade to the SARS-CoV lineage, and the wildlife origin of a new virus (SADS-CoV) killing >20,000 pigs in Guangdong Province (*54*). Many of these bat species are found across the region. We have collected XX samples from bats, rodents and primates in Thailand and XX in Malaysia under PREDICT, screening XX and

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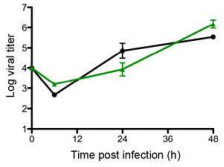
archiving duplicates of all of these which are now available for use in this project. We used family level primers for henipaviruses, filoviruses and CoVs and have already discovered XX, YY and ZZ in these two countries. In the current proposed work, we will attempt to isolate and characterize those that our analyses below suggest are most likely to be able to infect humans. Our collaborative group has worked together on these projects, as well as under DTRA funding in Singapore/Thailand (Co-Is Wacharapleusadee, Wang) to design, cross-train, and use novel serological and PCR tests to investigate xxx, and in Thailand (Co-Is Broder, Laing and Wacharapluesadee) on tech transfer of Luminex-based serology platform for detecting exposure to novel Asiatic filoviruses and known henipaviruses in wildlife and human samples. This is part of a planned DoD regional center of excellence for training in Thailand (details please?).

In vitro & in vivo *characterization viral potential for human infection:* We have used *in vitro* and *in vivo* experiments to characterize the pathogenic potential of novel SARSr-CoVs from bats. We isolated three SARSr-CoVs from bat feces: WIV1, WIV16 and Rs4874, with S protein sequences that diverged from SARS-CoV by 3% to 7% (*40, 43, 85*). We conducted full-length genome sequencing of 12 other novel SARSr-CoVs from a specific cave site in Yunnan China, some highly similar to outbreak SARS-CoV in the most variable genes: N-terminal domain and receptor binding domain (RBD) of the S gene, ORF8 and ORF3 (*43*). Using our reverse genetics system, we constructed chimeric viruses with SARSr-CoV WIV1 backbone and the S gene of different variants, including WIV1-Rs4231S and WIV1-Rs7327S. All 3 SARSr-CoV isolates and the two chimeric viruses replicated efficiently in Vero E6 cells and in HeLa cells expressing hACE2, but not in HeLa cells that don't express ACE2 (*40, 43, 85*) **(Fig. 5a)**. We used the SARS-CoV reverse genetics system (*64*) to generate a chimeric virus with a mouse-adapted SARS-CoV backbone expressing SHC014 S protein with 10% sequence divergence from SARS-CoV S. This chimera replicated in primary human airway epithelium, using the human ACE2 receptor to enter into cells (*44*) (**Fig. 5b**). Thus, **SARSr-CoVs with diverse variants of SL-CoV S protein without deletions in their RBD can use human ACE2 as receptor for cell entry.**



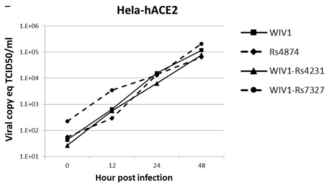


Fig. 5a (left): RT-PCR chimeras WIV1-expressing human Urbani (black) and human airway epithelial shows that bat SARSr-CoVs WIV1, Rs4874, and Rs4231S, WIV1-Rs7327S grow in HeLa cells ACE2. **Fig. 5b (right):** Viral replication of SARS-CoV SARS-SHC014S (green) primary air-liquid interface cell cultures at an MOI of 0.01.

We infected transgenic mice expressing hACE2 with 10^5 pfu of full-length recombinant WIV1 and three chimeric viruses (WIV1 backbone with SHC014S, WIV16S and Rs4231S). hACE2 transgenic mice challenged with rWIV1-SHC014S experienced ~20% body weight loss by 6dpi; rWIV1 and rWIV-4231S produced less body weight loss, and rWIV1-WIV16S led to no body weight loss **(Fig. 6a)**. At 2 and 4 dpi, viral loads in lung tissues of mice challenged with all three chimeras reached > 10^6 genome copies/g, significantly higher than rWIV1 infection **(Fig. 6b)**. This demonstrates that pathogenicity of SARSr-CoVs in humanized mice differs with divergent S proteins, **confirming the value of this model in assessing novel SARSr-CoV pathogenicity**.

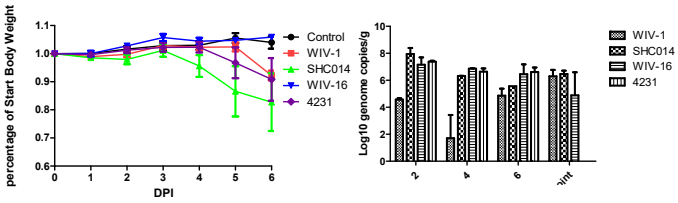


Fig. 6: *In vivo* infection of SARSr-CoVs in hACE2 transgenic mice. **6a (left)** Body weight change after infection; **6b (right)** Viral load in lung tissues.

Infection of rWIV1-SHC014S caused mild SARS-like clinical signs in the transgenic hACE2 mouse model **that weren't reduced by immune-therapeutic monoclonals that attenuate SARS-CoV pathogenecity.** Vaccination against SARS-CoV did not reduce severity of clinical signs in mice subsequently infected with rSARS-SHC014S (*44*). We found 2/4 broad human mAbs against SARS-CoV RBD cross-neutralized WIV1, but none could efficiently neutralize SHC014 which is less similar to SARS-CoV in the RBD (*86*). We repeated

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Commented [PD12]: I think this is something that Linfa, Supaporn or Chris mentioned?

Commented [PD13]: Ralph, Amy – this is directly from our CoV R01 renewal – please reduce the length of this section and adapt for this proposal. We prob don't need all these figures – just one or two would do.

this virus characterization approach with chimeras using HKU3r-CoV S proteins that are ~25% divergent from SARS-CoV S, **and found that they are unable to use the ACE2 receptor**. Additionally, we were unable to culture HKU3r-CoVs in Vero E6 cells, or human cell lines. **The ability of HKU3r-CoVs to infect people, and their receptor binding target, remain unknown.**

All – what can we say about Nipah and filoviruses in this section – what is the rationale for a similar approach (e.g. we're looking for viruses somewhere between Cedar and Nipah in the Henipaviruses, and trying to assess the likelihood of some of the novel filoviruses infecting human cells).

Are we just going to use cell culture for henipas and filos, or can we look at spike protein diversity?

Henipaviruses

Hendra virus and Nipah virus have a broad mammalian tropism with natural infection recorded in bats, horses, pigs, humans, cattle, goats and dogs. This broad species tropism is likely mediated by heniapvirus receptor usage of highly conserved ephrins ligands (e.g. ephrin-B2 and -B3) for cell entry. Ephrins are critical in evolutionary developmental process such as cell migration, axonal guidance and angiogenesis (REF), and eprin tissue distribution correlates with the multi-systematic cellular pathology, vasculitis and encephalitis disease presentation during HeV and NiV infection. The third isolated henipavirus is Cedar virus, yet unlike NiV/HeV, CedV does not cause pathogenesis in animal models. Recently, Dr. Broder and Laing have developed a reverse genetics system for Cedar henipavirus and have rescued a recombinant Cedar virus that is used as a model henipavirus tool to understand how ephrin receptor usage/tropism contributes to pathogenicity during henipavirus infections. Ephrin-B3 is distributed in the spinal cord, and usage of ephrin-B3 has been postulated to underlie the enhanced encephalitis seen during NiV infection. Further dissimilar from HeV/NiV, CedV is unable to utilize ephrin-B3 as a receptor (Marsh 2012; Laing 2018) nor does it express the interferon antagonizing virus factors: V and W proteins (Marsh 2012). However, Dr. Broder and Laing have discovered that CedV has a promiscuous/broad ephrin receptor usage and in addition to ephrin-B2 is able to utilize ephrins-B1, -A2 and -A5 for cell entry (Can include microscopy image/column graph for unpublished figure). Additionally, it was discovered that CedV can utilize mouse ephrin-A1, which differs from human ephrin-A1 by one amino acid residue in the key binding pocket, demonstrating the first evidence of henipavirus species-specific receptor use (Can include unpublished figure). Like ephrin-B3, ephrin-B1 is widely distributed throughout spinal cord tissues, however, CedV is non-pathogenic. Pathogenicity of henipaviruses is likely mediated by a contribution of both utility of ephrin receptors and expression of virulence factors V and W proteins. The full genome assembles of putative henipaviruses, Ghana virus and Mojiang virus, predict expression of V and W proteins. GhV is able to bind to ephrin-B2, but not -B2 (Lee – African emergent henipavirus B2 crystal) and the receptor for MoJV remains unknwon (Lee, idiosyncratic 2017), but is unlikely an ephrin. Thus, when we identify novel henipaviruses or paramyxoviruses, we will examine the receptor-binding proteins (e.g. envelope attachment (G) proteins) for sequence and structural homology with particular attention to conversation of amino acids that mediate ephrin receptor binding. We will prioritize molecular experimentation with novel henipaviruses that are predicted to interact with ephrin-B2 and expression V/W proteins to determine whether these viruses represent zoonotic threats. Using the recombinant Cedar virus (rCedV) platform, we will be able to rescue rCedV that express heterotypic G proteins to explore receptor utilization and tissue dissemination in animal models.

What about using bat cell lines and batized mice from Linfa?

1.2 General Approach:

We will conduct analyses to geographically- and taxonomically-target testing of samples from wild mammals that are most likely to harbor known high-profile zoonotic pathogens, or close relatives with potential to infect people. This includes selecting from tens of thousands of samples collected on recent projects and stored in freezers in our laboratories, and new collection from wildlife in high risk locales. We will use serological & PCR testing, to identify viruses, then attempt to isolate and biologically characterize viruses that phylogenetic and

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other data suggest have high spillover and pandemic potential. We will then conduct *in vitro* binding assays and cell culture experiments, and *in vivo* animal model infections to assess their potential to infect people and cause disease. EHA will lead the study design, targeted sampling, and data analysis; Co-Is Hughes and Wacharapluesadee will lead field sampling and in-country testing; Duke-NUS, UNC, USUS will lead the serological and PCR diagnostic development and the *in vitro* and *in vivo* analyses. A key goal of this work will be technology transfer to the region, including cell culture, animal models, serological and PCR diagnostics, as well as knowledge transfer by embedding staff in partner labs and by regular meetings. This will leverage substantially from the other funding available to consortium partners.

1.3 Wildlife samples: 1.3.a Site selection & sample sizes for newly collected and archived samples:

Adapt text from the rationale/innovation above and make brief statements about the work we'll do:

- Geographic targeting: we will use refined hotspot, 'missing viruses', and FLIRT analyses
- Targeting of host species/viral discovery targets: We will use phylogenetic MCC analyses and viral discovery curves
- Additional approaches: mapping of cave sites,

To calculate sample sizes, we will use previous work on SARSr-CoVs in bats in China, the results of bat, rodent and primate sampling under PREDICT in Malaysia and Thailand, and published data. For SARSr-CoVs in China, we found 6.7% mean PCR prevalence of SARSr-CoVs across bat hosts, with a small number of *Rhinolophus* spp. horseshoe bats having significantly higher PCR prevalence than other species sampled (REF).

Kevin – below is the language from our R01 CoV renewal proposal as a model:

In Y1 we will use our bat host and viral trait modeling, phylogeographic analyses of RdRp and S Protein sequences, and geographic and host species-based viral discovery curve analyses to identify SARSr-CoV diversity hotspot regions for bat sampling. We will sample at 8 new sites in four provinces. We will use cave site data (*87*), and demographic information to identify two sites in each of Yunnan, Guangxi, Guangdong, and Guizhou where humans likely have contact with bats. In Yunnan, we will identify two unsampled caves close to, but distinct from, the Jinning cave (*43*). This will provide adequate coverage of lineage 1 and 2 SARSr-CoVs, including a rich source of new HKU3r-CoVs, which have unknown potential for zoonotic spillover. Sampling will begin towards the end of Y1. We will use survey data from our previous R01 and host-specific viral accumulation curve data to target an additional 10 under-sampled *Rhinolophus* spp., 5 that were SARSr-CoV negative in our study, and a small number of related bat genera (including *Hipposideros* spp. and *Aselliscus* spp.) we previously found PCR positive for SARSr-CoVs **(Table 1)**. We will sample at least 5,000 bats from these 4 provinces (~1250 per province). Given ~5-12% prevalence of SARSr-CoVs in *Rhinolophus* spp. at our previous sites, **this sample size would give us 425 (±175) positive individual bats, and ~125 novel strains.**

1.3b Sample testing, viral isolation: Viral RNA will be extracted from bat fecal pellets/anal swabs with High Pure Viral RNA Kit (Roche). RNA will be aliquoted, and stored at -80C. One-step hemi-nested RT-PCR (Invitrogen) will be used to detect the presence of CoV sequences using primers that target a 440-nt fragment in the RNA-dependent RNA polymerase gene (RdRp) of all known α- and β-CoVs (*88*). PCR products will be gel purified and sequenced with an ABI Prism 3730 DNA analyzer.

We will attempt isolation on samples that contain viruses of interest (determined in **1.4, below**), using Vero E6 cells and primary cell culture from the wildlife taxonomic order (bat, rodent and primate cell lines). This includes the over XX cell lines maintained at Duke-NUS

1.3.c Sequencing S proteins: Our previous R01 work identified diverse SARSr-CoVs with high propensity for human infection (*19, 43, 44*). Characterization of these suggests SARSr-CoVs that are up to 10%, but not 25% different in the spike glycoprotein use human and bat ACE2 receptors for docking and entry. Uneven sampling gaps (e.g., no strains were found with 10-25% spike variation) prevent a thorough understanding of the transition point where the most divergent strains lose human ACE2 receptor usage. Our viral discovery curves **(Fig. 4)** suggest further sampling will reveal a rich diversity of as-yet-undiscovered SARSr-CoVs. In this aim we will use phylogenetic and viral discovery analysis to specifically target bat species and regions that are

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Commented [PD24]: Linfa, Supaporn, Danielle – is this correct?

Commented [PD25]: Brief description of these please

Commented [PD26]: Ralph et al. – I'm assuming this will still be of value for the CoVs, so a reduced version of this could be in here, but what about spike proteins of filos and henipas – is that not of any use for assessing their capacity to infect human cells. Can we do similar work with filovirus pseudotypes from sequencing the spike proteins, for example, to assess binding to human receptors? If so, please draft some text and point to some references

undersampled to allow sufficient power to identify and characterize missing SARSr-CoV strain diversity. Our previous work also suggests that SARSr-CoVs with S proteins that are ~10% divergent from SARS-CoV resist neutralization by therapeutic mAbs and escape SARS-CoV vaccines (*40, 44, 89*), suggesting that . However, viruses with 10-25% divergence in S proteins may bind to human cell receptors, but completely evade therapeutic and vaccine effects, and could therefore be a higher risk for public health. We will sequence the S proteins of novel SARSr-CoVs to prioritize viruses for experimental work in Aim 3 to test this hypothesis.

For all novel SARSr-CoV strains, we will sequence the complete S gene by amplifying overlapping fragments using degenerate primers as shown previously (*40, 43*). Full-length genomes of selected SARSr-CoV strains (representative across subclades) will be sequenced via high throughput sequencing method followed by genome walking. The sequencing libraries will be constructed using NEBNext Ultra II DNA Library Prep Kit for Illumina and sequenced on a MiSeq sequencer. PCR will be performed to fill gaps in the genome. The full length S gene sequences, including the amount of variation in the S receptor binding residues that bind the ACE2 receptor, will be used to select strains for Aim 3 experiments.

1.4. Assessing risk for spillover. 1.4.a Capacity to infect human cells: We will use a series of cell cultures to assess the capacity of target viruses to enter human cells. For CoVs, we will use primary human ciliated airway epithelial cells (HAE) cultures from the lungs of transplant recipients represent highly differentiated human airway epithelium containing ciliated and non-ciliated epithelial and goblet cells, grown on an air-liquid interface for several weeks prior to use (*44, 45, 90*). We will prepare HAE cultures from three different patient codes in triplicate in collaboration with the tissue procurement facility at the Cystic Fibrosis Center at UNC. Cultures will be inoculated with chimeric bat SARSr-CoVs to assess efficient replication. At 72 hpi, cultures will be fixed for immunofluorescent staining using antisera to the SARS-CoV conserved nucleocapsid protein (N) (*46, 63*). SARSr-CoVs that differ significantly in S protein sequence (11-24%) from epidemic SARS-CoV yet replicate *in vitro*, will also be evaluated for sensitivity to neutralization in Vero cells by PRNT50 assays using broadly SARS-CoV cross reactive human mAbs S227.14, S230.15, S215.17, and S109.8 (*45, 91*). As controls, the S genes of novel SARSr-CoV will be inserted into VEE 3526 replicon vectors (VRP3526-S), packaged and used to vaccinate mice (*92*). Polyclonal sera will be harvested and tested for ability to cross neutralize SARS-CoV, GD03, WIV-1, SHC014, WIV-16, other novel SARSr-CoV and HKU3-SRBD by PRNT50 assay (*45, 93, 94*). Using PRNT50 titers with sera (n=4 each) among these viruses, antigenic cartography (*95*) will allow comparison of antigenic vs. phylogenetic distance, identifying the transition at which SARSr-CoV strains escape SARS-CoV based vaccines, informing immunotherapeutic and vaccine design strategies (*96-98*).

Filoviruses (primary endothelial cells – ralph has primary lung endos – see if anyone has liver primary hepatocytes. Other option are monocytes) – where will we do this? BSL4? (NEIDL). Duplicate samples in culture medium from animals PCR +ve for filoviruses will be shipped to NEIDL (See letter of support) for culture and sharing with other agencies. Check on this re. wildlife filos…See if can do training opportunities so they can work with the NEIDL – visiting scholar appointments

Commented [PD27]: Ralph, Linfa, Chris – please expand and draft – I think this could be a good role for NEIDL, but don't know what the rules are…

1.4.xx Host ACE2 receptors: We will sequence host ACE2 receptors of different bat species or different bat populations from a single species (e.g. *Rhinolophus sinicus*) to identify relative importance of different hosts of high risk SARSr-CoVs and the *intra*specific scale of host-CoV coevolution. Of particular interest is homology across 18 bat and human orthologue ACE2 contact interface residues that engage the SARS RBD as a potential indicator of SARSr-CoV cross species transmission potential and growth in human cells (*62*).

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1.4.b Host-virus evolution and distribution: We will use Bayesian and Maximum Likelihood phylogenetic analyses of RdRp, Spike, and full genome sequence (when available) data to reconstruct the evolutionary history of the novel bat SARSr-CoVs we identify. We will rerun MCC analyses **(Fig. 3)** to reconstruct β-CoV evolutionary origins using our expanded dataset. Ecological niche models will be used to predict spatial distribution of PCR-positive species, identify target sites for additional bat sampling, and analyze overlap with people. We will use our viral discovery/accumulation curve approach **(Fig. 4)** to monitor progress towards discovery of >80% of predicted diversity of SARSr-CoVs (lineage 1 & 2) within species and sites, halting sampling when this target is reached, and freeing up resources for work other work (*84, 99*).

Commented [PD29]: KEvin/Alice – please modify this section for filos and henipas as well (and other hosts) - or does it belong in 1.3.a above?

1.4.c Viral strain prioritization: Of the expected 100-200 novel SARSr-CoV strains, we will down-select to prioritize for further characterization based on S genes that are: i) different from SHC014, WIV1, SARS-CoV with diversity ranges of 10-25%; ii) have virus S RBD that could use human/bat receptors; iii) have recombinant chimeric spikes indicative of gene flow between clade I and II strains; iv) have bat ACE2 receptors that might select for spike RBDs that can use human receptors for entry (15/18 conserved residues in human/bat ACE2 molecules that bind SARSs-CoV S RBD domains are likely more efficient receptors than 3/18 conserved sites). Using structural models based on the SARS S glycoprotein, the extent and location of antigenic variation will be annotated onto the structure, further resolving the locations of highly evolving and conserved structural motifs/epitopes that function in protective immunity (*91, 100-102*).

1.4.d Animal models: We will use a series of mouse models to assess spillover potential of viruses. First, the Baric lab has a well-established hACE2 transgenic mouse model that we will use this to characterize the capacity of specific SARSr-CoVs to infect. Briefly, in BSL3, n=5 10- to 20-week old hACE2 transgenic mice will be intranasally inoculated with 1 x 10^4 PFU of wildtype WIV-1 or chimeric bat SARSr-CoVs with different spike proteins, then monitored daily for weight loss, morbidity, and clinical signs of disease. Mice will be euthanized at 2, 4, and 6 dpi (endpoint of 14 dpi), organs harvested and virus quantified by SARS-CoV NP RT-PCR. After 7 days in formalin, tissues for histopathology will be removed from the BSL3 and stained with H&E, and for immunohistochemistry using polyclonal antibodies against the N protein. We will conduct limited evaluation of existing countermeasures using therapeutic monoclonal antibodies *in vitro* and *in vivo*. Existing SARSr-CoV mAbs will be diluted in DMEM starting at 1:20, and serial 2-fold dilutions mixed with an equal volume of 50 PFU of chimeric bat SARSr-CoVs with different spike proteins, then incubated on Vero E6 cells at 37°C for 1 h, then changed to a semi-solid medium (DMEM containing 1% methylcellulose and 2% FBS). Antibody neutralization titers will be evaluated by plaque quantification at 4-5dpi. hACE2 transgenic mice will be injected with SARS-CoV mAbs, and infected with chimeric bat SARSr-CoVs. Clinical signs and morbidity will be assessed and tissue pathology examined and compared with mice without treatment of mAbs to determine the therapeutic effect on SARSr-CoV infection, and protection of SARSr-CoV by wildtype SARS-S based vaccines assessed as described (*90, 103*). We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS cross neutralization, synthetically reconstruct a small subset (1-2) and evaluate the ability of nucleoside analogues to inhibit growth in HAE cultures and/or *in vivo* (*45, 90*).

For novel viruses across all families, we will use the UNC collaborative cross mouse that captures 90% of the diversity within all mouse groups to assess the most suitable model for further experiments. We have used this model for CoV, filo (Ebola), Flaviviruses, alphaviruses infections. In subpopulations it reproduces SARS weight loss, hemorrhage in EBOV, enceph in WNV, acute or chronic arthiritis in Chikungunya infection.

Duke-NUS has develop two models for bat *in vivo* culture: the 'batized' mouse model (Desciption) and a colony of *Eonycteris spelaea* (cave dwelling small fruit bat).

1.5 Potential problems/alternative approaches: Permission to sample bats in sites or provinces we select. We have a >20-year track record of successful field work in Malaysia, Thailand and >10 years in Singapore, and have worked with local authorities and communities to ensure access. We have existing permissions in place which will be renewed at the start of this project. Due to the abundance of wildlife habitats in the region, if access to a site is withdrawn, we will rapidly identify a suitable, complementary site with similar bat species composition and abundance. Finally, we have already collected tens of thousands of wildlife samples from the region and will focus on these while waiting for permission. **We may not identify novel viruses in our sample wildlife species due to seasonality of viral shedding or other factors**. We have calculated sample sizes based on a range of PCR data and are fairly conservative in our approach. However, as a back-up, we already have identified dozens of novel viruses that are near-neighbors to known high-impact agents, and will continue characterizing these should discovery efforts yield few novel viruses. The sampling regions we have selected are subtropical, and our previous data, and even published studies in temperate

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Commented [PD31]: Ralph – please draft a brief para explaining what we'll do with this mouse model

Commented [PD32]: Linfa/Danielle – please draft a brief para explaining how we'll use either or both…

Commented [PD33]: Kevin – please edit/check

Commented [PD34]: Kevin – please check the veracity of these comments

regions (*104*), do not suggest a strong pattern of seasonality in CoV shedding, although there are studies that suggest this occurs in henipavirus and filovirus (MARV?) shedding (REFS?). Nonetheless to account for this we will conduct sampling evenly on quarterly basis within each province.

Aim 2: New serological and PCR tools to test evidence of viral spillover in high-risk communities..

2.1 Rationale/Innovation: Assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities that are the first to get infected. To enhance low statistical probability of identifying these rare events, populations could be targeted that both live in rural locations close to high wildlife biodiversity, and also engage in behaviors that enhance the risk of spillover (e.g. hunting and butchering wildlife). In Aim 2 we will expand from our current work in the region to identify and enroll large cross-sectional samples from human populations that have high behavioral risk of exposure to wildlife origin viruses and live close to sites identified in Aim 1 as having high viral diversity in wildlife. We will use behavioral surveys and repeated biological sampling, design and deploy specific and sensitive serological assays to identify the baseline spillover of known or novel viral pathogens in these populations. Where symptoms are found, we will use PCR-based assays to identify presence of known and novel CoVs, henipaviruses and filoviruses, attempt to isolate and biologically characterize the pathogen, using the collaborative cross mouse (Aim 1) to identify an appropriate animal model to conduct preliminary pathogenesis work.

Preliminary data human biological sampling: Our longterm collaboration in the region has included identification of key at-risk human populations from which we have already collected qualitative and quantitative survey data, and biological samples. These include:

Peninsular Malaysia: Data from 1,300 Orang Asli samples, PCR for 5 viral families: 5 known CoVs in 16 people, one Influenza virus. Serology on its way incl. filovirus reactors…

Sabah: We have ~25 new CoVs in Sabah. Simon is using HTS to further characterize and Tom will get the data for this proposal from Simon.
Tom will work out all serum samples we have, incl. samples from dead bats.
Kamruddin (UMS Borneo Medical Health Research Center) has identified some high risk communities. We can help with them and build out the BSL-2 lab, as well as work on outbreak response within DHRU (in collab with Sabah CDC?).
Tom will speak with Tim Williams …He has archived samples from Kudat Monkey bar project – 2,000 human sera already screened for bacteria and parasites, we've been reached out to about this. Data on macaque tracking data/human tracking data also.

Sarawak: Dr Tan (Cheng Sieng) Center for Tropical and Emerging Diseases, Faculty of Medicine at UNIMAS – high risk Dayak communities that he's identified. We will do further sampling, questionnaires etc. Has Prelim data on HPV work in Dayak communities in remote Sarawak – we can work with those communities and can reach many by car, others by 4WD or boats. Has also Enterovirus 71 paper and is interested in CoVs. Has access to caves and guano. Will look into what other archived samples he has. Will give us access to Sarawak Forestry and Health Depts.

Thailand: Imported MERS cases..Zika patients. 10 yrs ago AFRIMS in Thailand found serological NiV +ve in Children far from the bat colony, prob with publication of data because didn't do neg controls – could do SNT at NEIDL or Wuhan – couldn't get permission Have emailed Andrew Hickey about this.
High-risk community cohorts – Loei Province (Guano miners) – whole community

Singapore:

Behavioral risk: EHA is the global lead organization in the USAID-PREDICT project for assessing human behavioral risk of zoonotic spillover. The general approach has been to conduct qualitative exploratory studies using standardized one-on-one semi-structured ethnographic interviews and observational data in among people engaged in clearly high-zoonotic risk activities (e.g. we interviewed 88 people involved in trading wild bats in China – REF). This allows us to assess local social and cultural norms and individual attitudes underlying wildlife contact. We used qualitative study findings to develop a human behavioral risk questionnaire on the type and frequency of animal contact, wildlife observed in daily life, and unusual illnesses

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Commented [PD36]: Linfa, Danielle – are we going to do human sampling in high risk communities in Singapore? If so, what would you propose and do you already have some prelim work you've done. Note these are high risk populations, i.e. high exposure to wildlife. Clinical cohorts are in Aim 3

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reported over the past 12 months. We conducted cross-sectional studies in high risk populations in >15 countries during the last 5 years, and study participants also provided biological samples (oropharynx swab, serum, plasma). Questionnaires and biological samples were collected from 1,585 participants from 7 sites in Yunnan, Guangxi, and Guangdong provinces, XX from XX sites in Malaysia and XX from XX sites in Thailand. The results of these surveys will be used in Aim 2 to better identify target populations.

Serological Evidence of Exposure: In China, we developed serological assays for HKU9 CoVs (β), SARSr-CoV Rp3 (β), HKU10 CoV (α), and MERS-CoV (β) and used ELISA and Western blot to test serum samples collected in 2016/17. **We found 7 individuals (7/733, 0.95%) living within a 6 km radius of the Jinning Cave, and 6/209 people (2.87%) at one site, with evidence of exposure to bat SARSr-CoVs.** We found evidence among human populations in Guangxi Province of people with prior exposure to the bat α-CoV HKU10 (2/412, 0.48%). This is of potential public health interest because HKU10 is known to be able to jump host species within bats, and therefore may have high propensity for emergence (*105*). However, the low seroprevalence (0.6%-2.7% at positive sites) suggests we need a larger sample size, and a focused, targeted study to maximize the likelihood of identifying seropositive cases. In Aim 2, we will use combined biological-behavioral risk surveillance in targeted populations within the community and clinical settings to 1) identify risk factors correlated with seropositivity (exposure to) and PCR positive status (infection with) henipaviruses, filoviruses and CoVs; and 2) assess possible health effects of infection in people. Obtaining this information could be a significant step in understanding the likelihood of recent 'hidden' spillover events and their public health impacts, as well as the risk of future emergence of viral disease.

Risk Factors: In China, questionnaire response and demographic data suggest specific risk factors included type of occupation, keeping of pets and visiting wet markets. Seropositive individuals were mostly farmers/peasants (8/9), living in Yunnan province (7/9), 41-60 yrs old (7/9), with domestic contact with rodents (6/9), and are male (6/9). However, these characteristics were common and occurred in ~40% of survey respondents. Although these results are preliminary and don't provide detailed information on routes of exposure, they suggest that further refining use serological tests coupled with qualitative and questionnaire data will identify likely routes of exposure to novel CoVs in China. In Aim 2 of this EIDRC proposal, we identify strategies to better target at-risk people, and conduct focused **questionnaires and serosurveys to produce statistically significant findings for henipaviruses, CoVs and filoviruses.**

Commented [PD38]: Hongying/Emily – any information from Thailand or Malaysian surveys?

2.2 General Approach/Innovation: We will use a dual study design to gain in depth understanding of exposure and risk factors for viral spillover **(Fig. XX)**. In Aim 2 we will conduct community-based surveillance with more focused questionnaires and biological sampling to determine the seroprevalence of spillover viruses in at-risk human populations, and to identify risk-factors for viral spillover in these communities. In Aim 3, we will conduct clinic-based syndromic surveillance and biological sampling at sites that include the community-based surveillance sites within their catchment (details in Aim 3). Both community-based and clinic-based syndromic surveillance programs are case-control studies designed with the sample sizes necessary to statistically quantify (with a power of 80%) risk factors and health impacts for viral spillover, linked to serological status and symptoms.

2.3 Target population & sample sizes: We will target sites in the same geographic regions as those identified in Aim 1 that harbor wildlife with high potentially zoonotic viral diversity, in regions that are EID hotspots, and are well-connected to regional travel and trade hubs and the global travel network. We will target specific communities based on our PREDICT behavioral analysis questionnaire data to assess key at-risk populations with high exposure to wildlife, as well as other publicly available data (e.g. wildlife hunting licenses sold in a region, number of guano caves accessed for fertilizer etc.). We will also expand on work currently underway in the following particularly high exposure populations:

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Thailand: We have conducted behavioral risk surveys and biological sampling in XX guano miners in Loei. These workers visit bat caves and dig fecal material to use as fertilizer, and are therefore highly exposed to bats and rodents in particular.

Commented [PD40]: Supaporn – please draft plans for what we should do to scale up this work We could also include other high-risk communities – any ideas?

Peninsular Malaysia: We have sampled Orang Asli populations for the past 2 years, enrolling XX people. We will expand this to includeXXX

Sarawak: Dr Tan (Cheng Sieng) Center for Tropical and Emerging Diseases, Faculty of Medicine at UNIMAS – high risk Dayak communities that he's identified. We will do further sampling, questionnaires etc. Has Prelim data on HPV work in Dayak communities in remote Sarawak – we can work with those communities and can reach many by car, others by 4WD or boats. Has also Enterovirus 71 paper and is interested in CoVs. Has access to caves and guano. Will look into what other archived samples he has. Will give us access to Sarawak Forestry and Health Depts.

Sabah:

Singapore:?

Community-based surveillance will be conducted at XX sites in each country, a total of xx sites. From our previous work we anticipate that 10-30% of the community population will have had exposure to wildlife allowing us to capture highly exposed and non-exposure individuals at each site. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock farming, transportation, selling, or slaughtering wildlife in the surveyed areas will be targeted so that they make up ≥30% of the sampled population in each community. We will stratify sampling to ensure appropriate representation of sex, demographic, and socio-economic factors at each community site. Estimating 5% overall seroprevalence in these high-exposure populations, these sample sizes are sufficient to estimate effect sizes of behavioral risk factors of 2X or greater with 80% power. For community-based surveillance, we will enroll xxxx individuals per county/province, pooled across two sites for each, allowing us to make county/province-level comparisons of differing effects.

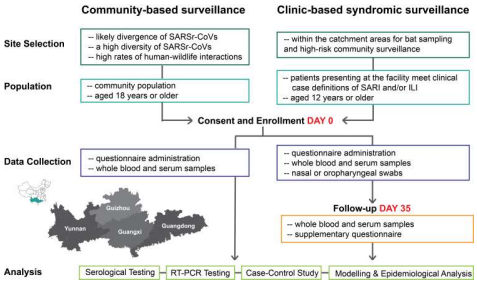

Fig. XX: Human survey and sampling study design overview (Aim 2 and 3)

2.4 Data & sample collection: Following enrollment with signed consent form, biological specimens (two whole blood samples, one max. 500 µL; two 500 µL serum samples) will be collected from all eligible participants, and a questionnaire will be administered. We will investigate five risk factors, so as to maximize the power of the analyses, all related to high risk wildlife exposure, based on continuing analysis of our previous work, and will include: 1) occupation; 2) observed or reported interactions with bats in/around house; 3) proximity to nearby bat roosts; 4) working or regular visit to animal markets; 5) self-reported ILI/SARI. With consent from participants, we will review clinical records to collect recent data on medical history, clinical syndromes, and patient etiology.

2.5: Laboratory analysis: 2.5.a Serological testing:

One of the present challenges in emerging disease surveillance is that viremia can be short-lived, complicating viral-RNA detection (i.e. traditional PCR-based approaches), especially in zoonotic reservoir hosts, and requiring large samples sizes to understand infection patterns or host range. In contrast, antibodies are typically long-lived and can be used to assess prior virus exposure or infection with much smaller samples sizes . However, most serological assays only target a single protein, and it's not always clear which antigen is the most immunodominant during an adaptive humoral immune response – i.e. which is the best target for a serological assay. Henipaviruses, filovirus and CoVs express envelope receptor-binding proteins (e.g. CoV=spike (S) protein, filoviruses/henipaviruses=glycoprotein (GP/G)), which are the target of protective antibody responses. The Broder lab at USU, a leader in the expression and purification of native-like virus surface proteins for immunoassay application (Bossat 2008), developing monoclonal antibodies (Zhu 2008, Bossart 2009) and as subunit vaccines (Bossart 2012, Mire 2014), is presently developing a novel, multiplex

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Commented [EL46]: Nations, F. a. A. O. o. t. U. in *FAO Animal Production and Health Manual* Vol. No. 12 (ed H.E. Field S.H. Newman, C.E. de Jong and J.H. Epstein.) (Rome, 2011).

microsphere immunoassay (MMIA) allowing serum samples to be simultaneously tested for antibodies reactive with henipaviruses, filoviruses and coronaviruses (Table XX).

This MMIA uses a Luminex-based multiplex platform and custom designed virus receptor-binding proteins expressed in a mammalian cell-culture system and purified as native-like oligomeric proteins (Bossart 2008, Chan YP 2009). These oligomeric antigens retain post-translational modifications and quaternary structures. This structural advantage over peptide antigens, allows the capture of conformational-dependent antibodies raised during a natural infection, which represent the majority of the protective humoral response.

Serological platforms have been historically limited by the starting antigen, and cross-reactivity between known and unknown related viruses not included in the assay.

The known diversity of henipaviruses includes five presently described viruses, NiV, HeV, CedV, GhV and MojV and this assay has been expanded to include all described species (Preliminary Figure A). In the past, our collaborators have used NiV and HeV G protein antigens in a MMIA assay to identify a serological footprint of NiV-like African henipaviruses and exposure to virus(es) antigenically-related to NiV in livestock sampled in Bangladesh (Peel 2012, Chowdury 2014). To strengthen serological data interpretation, the G protein from each virus is integrated into the MMIA so that virus-specific versus henipavirus cross-reactive antibodies can be detected and address specificity of past virus exposure. Extensive cross-reactivity exists within ebolaviruses and between ebolaviruses and marburgviruses, highlighted by studies examining antibody binding between heterologous viruses (Shuh 2019, MacNeill 2011, Natesan 2016). Ongoing work is aimed at validating the MMIA pan-filovirus assay for specificity (Preliminary Figure B). The majority of ebolaviruses are endemic to Africa, however the discovery of Mengla virus in Yunnan, China increased the further demonstrated that a diversity of Asiatic filoviruses with unknown pathogenicity and zoonotic potential exists. Our past work in collaboration with Dr. Smith and Mendenhall at Duke-NUS demonstrated that three under sampled fruit bat species had serological evidence of past exposure to a filovirus(es) most antigenically-related to ebolaviruses (e.g. Ebola virus, Bundibugyo virus), but unreactive with Reston virus GP, suggesting that novel filoviruses circulate within bat hosts (Laing 2018).

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The below is from our previous CoV proposal:

In our previous R01, we expressed his-tagged nucleocapsid protein (NP) of SARSr-CoV Rp3 in *E.coli* and developed a SARSr-CoV specific ELISA for serosurveillance using the purified Rp3 NP. The specificity of this assay was evaluated using polyclonal antibodies against HKU1, OC43, 229E, NL63, MERS-CoV and EBOV and no significant cross-activity was detected (*19*). **While this shows it is a specific test for Rp3, it suggests that if we can expand our serology tests to cover other bat CoVs, we may identify many more seropositive individuals.** In this renewal, we will therefore use two serological testing approaches. First, we will expand test all human sera collected from both community- and clinic- based sampling for a panel of bat CoVs that will include SARS-CoV (outbreak strain), a range of lineage 2 SARSr-CoVs (including WIV1 and SHC014), lineage 1 HKU3r-CoVs, MERS-CoV, and the α-CoVs SADS-CoV and HKU10. We previously found serological evidence of human exposure to HKU10 (*19*), but HKU10 is known to jump from one host bat species to another (*105*) and is therefore likely to have infected people more widely. Incoporating serological testing for SHC014 is also likely to yield higher seroprevalence because it is readily divergent from SARS-CoV wildtype and therefore unlikely to have been picked up in our earlier testing. It is possible that non-neutralizing cross reactive epitopes exist that afford an accurate measure of cross reactivity between clade1 and clade 2 strains which would allow us to target exposure to strains of 15-25% divergence from SARS-CoV. Additionally, we will test samples for antibodies to common human CoVs (HCoV NL63, OC43 – see potential pitfalls/solutions below). Secondly, we recognize that CoVs have a high propensity to recombine. To serologically target 'novel' recombinant virus exposure, we will conduct 1) ELISA screening with SARSr-CoV S or RBD; 2) confirm these results by Western blot; then 3) use NP based ELISA and LIPS assays with a diversity of SARSr-CoV NP. For NP ELISA, micro-titer plates will be coated with 100 ng/well of recombinant batCoV NP and incubated with human sera in duplicates followed by detection with HRP labeled goat anti-

human IgG antibodies. For confirmation, all ELISA positive samples will be subjected to Western blot at a dilution of 1:100 by using batCoV-NP as antigen. We will use an S protein-based ELISA to distinguish the lineage of SARSr-CoV. As new SARSr-CoVs are discovered, we will rapidly design specific Luciferase immunoprecipitation system (LIPS) assays targeting the S1 genes of bat-CoV strains for follow-up serological surveillance, as per our previous work (*54*).

2.5.b RT-PCR testing. Specimens will be screened using RT-PCR for the RdRp gene (See section 1.3.b for details). Positive samples will be subjected to full genome sequencing and RT-PCR amplification of the S glycoprotein gene. Samples from the clinic-based syndromic surveillance will also be tested using RT-PCR for Influenza A & B, HCoV NL63, OC43, HKU1, SARS-CoV & 229E as rule-outs, and SADS-CoV and HKU10 as an opportunistic survey for potential spillover these CoVs as proposed above.

2.6 Epidemiological analysis: We will conduct a case-control study to identify risk factors for SARSr-CoVs spillover. "Cases" are defined as participants whose samples tested positive for SARSr-CoVs by serological tests. "Controls" will be selected from the pool of participants admitted to the studies but testing negative. We will use nearest neighbor matching to pair cases demographically with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between serological/PCR status and risk factors including: Activities with likely exposure to 1) bats; 2) livestock; and 3) locations of residence and work. We will use the same procedure to determine how clinical presentation differs between SARSr-COVs-exposed and unexposed enrollees, in the time course of illness, severity of symptoms, and type of symptoms.

2.7 Biological characterization of viruses identified: XXX

2.8 Potential problems/alternative approaches: Rarity of spillover events means it may be difficult to identify sufficient seropositives to statistically analyze risk behavior. First, we will be targeting our community-based surveillance to subpopulations with high-levels of wildlife exposure, at sites selected for diverse and prevalent viruses to increase likelihood of finding positive individuals. Second, our serology testing will include a panel of assays for a large diversity of viruses, increasing likelihood of positives. For example, in previous work our overall bat CoV PCR prevalence in the community was, 11.8%; β-CoV, 3.4%; α-CoV, 9.1%). Thus, using this broad serological panel to screen individuals in likely contact with these species increases the potential for detecting spillover with enough power for statistical analyses, and will shed light on behaviors that predispose to spillover. Third, we will include common human CoVs, paramyxoviruses in our panel, so that even if low prevalence of wildlife viruses is found, we will be able to conduct a valuable cross-sectional study of the seroprevalence of more common human viruses. Finally, we will be able to assess relative measures of human-wildlife contact from our survey work. We will analyze intensity of contact against other risk factors and clinical outcomes to provide useful proxy information for spillover risk. **Serological testing may not match known CoVs due to recombination events.** We will use the three-tiered serological testing system outline in **2.6.a** to try to identify these 'novel' CoVs, however, we will also remain flexible on interpretation of data to ensure we account for recombination.

Aim 3: Clinical cohorts to identify evidence of viral etiology for 'cryptic' outbreaks.

3.1 Rationale/Innovation: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent is not diagnosed. By working with syndromic cohorts reporting to clinics with symptoms similar to known high-impact viral agents, we may be able to capture novel emerging diseases in at-risk communities before they spread into the general population and risk becoming pandemic. This will have clear value for public health in the region, and potentially wider. To do this, we will expand on current syndromic surveillance activities to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and present at regional clinics with symptoms typical of high impact zoonotic viruses. We will collect detailed interview data to assess their likely contact with wildlife and livestock and conduct molecular and serological diagnostic assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. We'll conduct novel viral discovery on unique clinical + symptomatic cases (maybe w high animal contact too) where testing has already ruled out all normative diseases? If novel pathogens are identified by PCR, we will isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work.

Commented [PD48]: Please modify for Henipas, filos and CoVs

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Preliminary data clinical surveillance: Our longterm collaboration in the region has included the following activities investigating clinical syndromes:

Thailand: Sick people cohorts – Chulalongkorn Hospital; EID Center for Thailand – Outbreak investigation Lab surveillance Min public Health Dept of Disease Control, 3 projects testing samples from people (encephalitis, Hand foot mouth disease, viral diarrhea, Influenza) = outbreak identification Dr. Rone. Any cluster of unusual disease samples will be sent to Supaporn. Have been using PREDICT protocol to identify pathogens. Dr. Parvenee doing lab investigation

Peninsular Malaysia:

Sabah: Linfa has worked with Timothy Williams on an encephalitis cohort from Queen Elizabeth Hospital 1 & 2 (poss has worked with Linfa on these) and this is another possibility. He's doing outbreak response training in August with team from Sabah CDC (Dr. Maria now in Pen. But still collaborating with us, Jikal current director working with us and Dato Cristina dir. Sabah state health Dept and is supportive) this collaborative group is working to develop a Sabah outbreak response team – we will support that – to be based at BMHRC and we will develop this as part of this proposal. Prof. Kamruddin will summarize the serum samples he has.
POCs: Sabah State Health Dept (e.g. Dr. Giri Shan Rajahram), Queen Elizabeth Hospital (Dr. LEE Heng-Gee)

Sarawak: From Linfa – Dr. Ooi used to work with Jayne Cardosa at UNIMAS and has access to samples of patients with unusual presentation

Strategy for analysis of self-reported illness: We have developed a standardized approach in PREDICT to analyzing data on self-reported symptoms of fever with cough and shortness of breath or difficulty breathing (severe acute respiratory illness - SARI), and fever with muscle aches, cough, or sore throat (influenza-like illness - ILI) from study participants. We used a least absolute shrinkage and selection operator (LASSO) regression to identify associations between ILI and/or SARI symptoms and contact with animals in the last year. Results have clear biological relevance. In Yunann, China, salient predictors or combination of predictors were, in descending order of odds ratio: 1) having eaten raw or undercooked carnivores; 2) having slaughtered poultry and being a resident of Guangxi province; 3) having had contact with cats or bats in the house; and 4) being a male who has raised poultry **(Fig. 11).** We will expand this approach for all syndromes in Aim 3, and with questions designed to assess patient's exposure to wildlife in terms that are relevant to the specific country.

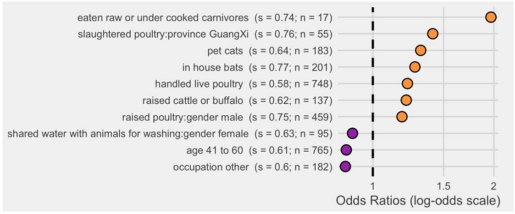

Fig. 11: Predictors of self-reported ILI and/or SARI in prior 12 months (s = bootstrap support; n = number +ve out of 1,585 respondents). Orange circles = odds ratios > 1 (positively associated with the outcome); purple = odds ratios <1 (negatively associated with the outcome).

3.2 General Approach: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent is not identified. To capture these, we will expand on our current syndromic surveillance to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and present at regional clinics with influenza-like illness, severe respiratory illness, encephalitis, and other symptoms typical of high-impact emerging viruses. We will collect interview data tailored to the region to assess contact with wildlife, conduct molecular and serological assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. If novel pathogens are identified by PCR, we will isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work. We will then use behavioral survey data, ecological and phylogeographic analyses to assess the likely reservoir hosts to inform intervention programs.

3.3 Clinical cohorts. 3.3.a Cohort selection: We will initiate active clinic-based syndromic surveillance at XX town-level level clinics and xx provincial-level hospital in each country, in total xx hospital sites, all within the

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catchment areas for wildlife sampling, and which are used by people in our community-based surveillance. Patients ≥ 12 years old presenting at the health facility who meet the syndromic and clinical case definitions for SARI, ILI, encephalitis, hemorrhagic fever will be recruited into the study. We will enroll a total of at least xxxx individuals for clinical studies, which accounts for up to 40% loss from follow-up. Study data will be pooled across sites, as clinical patients are limited by the number of individuals presenting at hospitals.

3.3.b Behavioral and clinical interviews: 2.5 Clinic enrollment and follow-up: We will recruit inpatients and outpatients after initial screening to meet the clinical case definition of 1) severe/acute respiratory illness (SARI/ARI) of unknown etiology; or 2) Influenza-like illness (ILI) of unknown etiology. Once enrolled, biological samples will be collected and a questionnaire administered by trained hospital staff that speak appropriate local language. Samples will be taken concurrently when collecting samples for normative diagnostics. For inpatients, samples will be collected within 10 days of reported onset of illness to increase the chance of PCR detection of viruses (*106*). We will follow up 35 days after enrollment to collect an additional two 500 µL serum samples conduct a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. 35 days gives adequate time for development of IgG, which occurs <28 days after onset of symptoms for SARS patients (*107*).

3.3.c Sampling: Following enrollment with signed consent form, biological specimens (two whole blood samples, one max. 500 µL; two 500 µL serum samples) will be collected from all eligible participants, and a questionnaire will be administered. We will investigate five risk factors, so as to maximize the power of the analyses, all related to high risk wildlife exposure, based on continuing analysis of our previous work, and will include: 1) occupation; 2) observed or reported interactions with bats in/around house; 3) proximity to nearby bat roosts; 4) working or regular visit to animal markets; 5) self-reported ILI/SARI. An additional two nasal or oropharyngeal swabs will be collected from syndromic patients. With permission from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology.

3.4 Sample testing: PCR, Serol to link symptoms to etiologic agents

3.5 Assessing potential for pandemic spread: We will…leverage EHA's work with DHS to develop FLIRT (Flight Risk Tracker) that tracks the probable pathways for spread for viruses that are able to be transmitted among people….

From CoV renewal: use data from **3.3** to identify rank SARSr-CoV strains most likely to infect people and evade therapeutic and vaccine modalities. We will use bat survey and zoological data (*87*) to build species distribution models (*108*) and predict the distribution of bat species that harbor low, medium and high risk viral strains. Stacking these modeled distributions for the ~20-30 *Rhinolophus* and related species that occur in the region will allow estimates of SARSr-CoV diversity for a given locality. We will use machine learning models (boosted regression trees) and spatial 'hotspot' mapping approaches to identify the ecological, socio-economic and other correlates of SARSr-CoV diversity and spillover (from serosurveys) (*22, 23, 77*). We will include data from our human behavioral surveys and sampling to give a direct measure of where risk of spillover to people is likely to be highest in the region.

Potential problems/alternative approaches: Patients visiting clinics may have cleared virus, but not yet developed IgG antibodies, reducing seropositive cases. Our 35 day follow-up sampling should avoid this because the maximum lag time between SARS infection and IgG development was ~28 days (*106*). We also expect that patients in rural communities will only visit clinics when symptoms have progressed, likely coinciding with late illness and onset of IgG. We will also have data from our community study, so won't be completely reliant on hospital data to identify PCR- or seropositives. Finally, the risk is outweighed by the potential public health importance of discovering active spillover of a new henipavirus, filovirus or CoV infection.

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Commented [PD57]: Need data for Nipah and filovirus patients

Commented [PD58]: KEvin to draft

Commented [PD59]: Add data for Nipah and filo infections

ALL – the sections below are going to be critical to demonstrate our capacity to do this work. I've not yet started drafting, but please don't hesitate to insert whole sections from other proposals you've done if they're relevant here – we'll need to be creative and get all the language we can from others…

Additional Instructions – Specific to the EIDRC FOA:

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Administrative Plan

In a clearly labeled section entitled **"Administrative Plan":**

- Describe the administrative and organizational structure of the EIDCRC Administrative and Leadership Team. Include the unique features of the organizational structure that serve to facilitate accomplishment of the long range goals and objectives.
- Describe how communications will be planned, implemented, and provided to collaborators, teams, or sites. In addition, describe plans to achieve synergy and interaction within the EIDRC to ensure efficient cooperation, communication and coordination.
- Specifically address how the EIDRC Team will communicate with other EIDRCs, the EIDRC CC and NIAID and how the EIDRC will implement plans and guidance from the EIDRC CC to ensure coordination and collaboration across the Emerging Infectious Disease Program as a whole. Describe how the study site will provide information, if any, back to the local health authorities and the study subjects as needed. Note any adjustments or changes needed for rapid and flexible responses to outbreaks in emerging infectious diseases.

List out all of our involvement with outbreak investigations, ministries of health surveillance etc.

- Describe the overall management approach, including how resources will be managed, organized and prioritized, including pilot research projects.
- Highlight how the proposed staffing plan incorporates scientific and administrative expertise to fulfill the goal to develop the flexibility and capacity to respond rapidly and effectively to outbreaks in emerging infectious diseases in an international setting.

Data Management Plan

In a clearly labeled section entitled **"Data Management Plan":**

- Describe internal and external data acquisition strategies to achieve harmonization of systems and procedures for data management, data quality, data analyses, and dissemination for all data and data-related materials generated by the research study with the EIDRC CC.
- Within the plan, indicate the extent to which dedicated systems or procedures will be utilized to harmonize the acquisition, curation, management, inventory and storage of data and samples. Describe how training for the data and sample collection, in terms of the use of electronic data capture systems, will be provided to all staff including those at enrollment sites.
- Describe the quality control procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens.

This includes sample labeling etc. Get a bar-coding system, everyone has a scanner (cf. PREDICT) – Ralph has this for select agents and MERS. Also need a software system

Clinical Management Plan

In a clearly labeled section entitled **"Clinical Management Plan":**

- Describe plans, processes, and procedures for the following activities: clinical site selection including feasibility, capacity, and capabilities, and study development, conduct, and oversight.
- Describe strategies for oversight and implementation of standardized approaches in the recruitment and clinical characterization of adequate numbers of relevant patient populations to ensure the prompt screening, enrollment, retention and completion of studies. Describe novel approaches and solutions to study enrollment. including the clinical meta-data that will be captured and describe how the staff at each enrollment/collection site will be trained and comply with the quality standards for data and

sample collection and compliance with the standardized procedures. Provide a general description of the methods for establishing clinical cohorts and how cohorts may be leveraged for new emerging pathogens.

- Provide general approaches to identification of the types of clinical cohorts needed to fulfill the goals of the EIDRC. Discuss types of clinical cohorts and clinical assessments that will be utilized to study natural history and pathophysiology of disease, including characteristics of the cohort, site for recruitment, types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.
- Describe methods that might be applied to either elucidate a zoonotic source or a vector of transmission.

Our research team incredibly diverse and capable of working with a group of other viral families: Lay out all our additional vector experience incl. my WNV work, our work on Chik etc.
Baric: Flavi, Noro,
Linfa: …
EHA: …

- Describe capacity for and approaches to clinical, immunologic and biologic data and specimen collection, and how these data and specimens will address and accomplish the overall goals and objectives of the research study.

Ralph

1. Norovirus: collaboration with surveillance cohort CDC; first one to publish antigenic characterization of each new pandemic wave virus; and phase 2 and 3 trial of therapeutics
2. Tekada Sanofi Pasteur dengue
3. Nih tetravalent vaccine

- Provide plans for the potential addition of new sites and expanding scientific areas of research when needed for an accelerated response to an outbreak or newly emerging infectious disease.

Linfa – will launch VirCap platform on outbreak samples.

Statistical Analysis Plan

In a clearly labeled section within the Research Strategy entitled **"Statistical Analysis Plan":**

- Describe the overall plan for statistical analysis of preliminary research data, including observational cohort data.
- Describe the overall staffing plan for biostatistical support and systems available for following functions: 1) preliminary data analyses, 2) estimates of power and sample size, 3) research study design and protocol development.
- Describe the quality control and security procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens. Describe any unique or innovative approaches to statistical analysis that may be utilized.

Project Milestones and Timelines

In a clearly labeled section entitled **"Project Milestones and Timelines":**

- Describe specific quantifiable milestones by annum and include annual timelines for the overall research study and for tracking progress from individual sites, collaborators, and Teams. Milestones must specify the outcome(s) for each activity. Milestones should be quantifiable and scientifically justified, and include the completion of major research study activities, including, for example, protocol development, case report forms, scheduled clinical visits, obtaining clearances study completion, and analysis of final data. Milestone criteria should not simply be a restatement of the specific aims. Using a Gantt chart or equivalent tool, describe the associated timelines and identified outcomes for the

research study.

Show that for this proposal, we won't be doing the Specific Aims sequentially. We'll begin the human work (SA 2, 3) at the beginning of the project, along with SA1. We'll do the diagnostic assay development initially in Ralph's, Linfa's, Chris's lab, but then begin tech transfer in Y1….

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From: Laing, Eric on behalf of Laing, Eric <eric.laing@usuhs.edu>
To: Peter Daszak
Cc: Broder, Christopher
Subject: Re: Gavin and Ian collab at DukeNUS
Date: Tuesday, June 18, 2019 8:44:21 PM
Attachments: EIDRC Southeast Asia v2-edl.docx

Hey Peter,

Have a look at some of the proposal suggestions. My (b) (6) [redacted]. I'm running on fumes right now and having difficulty finding time to incorporate suggestions, apologies if the word-dump is disconnected. If these are on point I'll send over some accompanying data figures you can embed.

- Eric

Eric D. Laing, Ph.D.
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On Sun, Jun 16, 2019 at 2:59 PM Peter Daszak <daszak@ecohealthalliance.org> wrote:

> Thanks Eric,
>
> As you go through the text of the grant making revisions, can you add in a bit on preliminary data at the appropriate point re. prior collab with DukeNUS with Gavin and Ian. For example, if this work is on developing the Luminex strategy, put it in that section in Aim 1 under preliminary data (section 1.1) and say something like:
>
> "In a collaboration between USUHS and Duke-NUS, Co-Is Laing and Broder have refined and tested the Luminex serology platform on 500 samples of bats and humans with Duke-NUS (Smith and Mendenhall). The results suggest that we're all doomed….etc…"
>
> I'll be talking with Linfa and Danielle tonight and will check in with them on how/if to formally include Gavin and Ian in the proposal.

Cheers,

Peter

Peter Daszak

President

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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

From: Laing, Eric [mailto:eric.laing@usuhs.edu]
Sent: Monday, June 10, 2019 12:00 PM
To: Peter Daszak
Cc: Broder, Christopher
Subject: Re: EIDRC SE Asia V. Rough first draft for edits

Hi Peter,

I wanted to pass along the names our two collaborators at Duke-NUS: Drs. Gavin Smith and Ian Mendenhall. Gavin is a Professor in the EID Programme that Linfa is chair of and Ian is a Principal Research Scientist in Gavin's lab. Chris and I have worked with Ian and Gavin on 2 wildlife/human interface surveillance projects in SE/South Asia and Gavin/Ian have a large footprint in Cambodia (bats/rats/collaborators). Ian and I also have a small GEIS project thru NAMRU-2 Phnom Penh, Their surveillance projects are independent of Linfa's research at Duke-NUS. I'll leave the decision to fold them into this proposal yours to make. Here are there contact addresses: gavin.smith@duke-nus.edu.sg ian.mendenhall@duke-nus.edu.sg

Best regards,

Eric

Eric D. Laing, Ph.D.

Research Assistant Professor

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On Mon, Jun 10, 2019 at 3:59 AM Peter Daszak <daszak@ecohealthalliance.org> wrote:

> Dear all – here's the first rough draft.
>
> Please start editing as fast and furious as you all can. I've based this on our previous conversations, so there are lots of bits for you to expand on, insert references etc. Also, you'll see that lots of the text in the Aims is from our recent CoV proposal and will need extending for Henipas, CoVs and Filos, and then editing back on the SARSr-CoV text. Please get stuck into that.
>
> Don't worry about all editing separately – I can collate this, although it is great if some of you go rapidly and others build on theirs.
>
> Main thing – please draft as much as you can, please use TRACK CHANGES, and please don't mess up the ENDNOTE! Prob best for references that you just insert them into comment boxes. It's a bit of a pain, but it's less work than if the whole library decomposes…
>
> Anyway – thanks for being great collaborators, and I look forward to what you send back
>
> NB Supaporn and Tom – please share with your collaborators, and cc me, luke, aleksei and evelyn so I have their email addresses.
>
> Cheers,

Peter

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The overarching goal of this proposal is to better understand the diversity of known and closely related CoVs, henipaviruses, and filoviruses in wildlife reservoirs in SE Asia, to test their capacity to infect human cells and mouse models, to develop new specific and sensitive serological and molecular diagnostic tools, and to conduct surveillance of human populations with high risk of exposure to wildlife, and clinical cohorts to identify evidence of spillover and pandemic potential, and of viruses causing ~~previously 'hidden'~~ 'cryptic' clinical syndromes in people. Our work will follow three specific aims:

Specific Aim 1: Identification and biological characterization of spillover risk of high zoonotic potential viruses from wildlife. Building on our extensive previous work, we will: 1) analyze some of the tens of thousands of archived wildlife samples in our laboratories in the region, and conduct geographically- and taxonomically-targeted field surveillance in wild mammals (bats, rodents, primates), and use serological & PCR testing~~, and isolation~~ to identify viruses that are known high-profile zoonotic pathogens, or close relatives with potential to infect people.~~ This includes accessing tens of thousands of samples collected on recent projects and stored in freezers in our laboratories~~; 2) attempt to isolate and biologically characterize viruses that we have recently identified of that we discover, where~~that~~ our analyses suggest they have high spillover and pandemic potential; and 3) conduct *in vitro* binding assays and cell culture experiments, and *in vivo* animal model infections using humanized mice and the collaborative cross mouse to assess their potential to infect people and cause disease.

Specific Aim 2: New serological and PCR tools to test evidence of viral spillover in high-risk communities. Assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities and approaches that can deal with the low statistical probability of identifying rare events. To achieve this, we will expand current work in the region to 1) identify and enroll large cross-sectional samples from human populations that have high risk of exposure to wildlife origin viruses in the region, conducting behavioral surveys and taking repeated ~~clinical~~ biological samples; 2) design and deploy specific and sensitive serological assays to identify the baseline spillover of known or novel viral pathogens in these populations; and 3) use novel PCR-based assays to identify presence of known and novel CoVs, henipaviruses and filoviruses in particularly highly-exposed people (OR DITCH PCR for Aim 2). When spillover of novel pathogens is identified, we will isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work.

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Specific Aim 3: Clinical cohorts to identify evidence of viral etiology for '~~hidden~~' cryptic' outbreaks. Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent is not identified. To capture these, we will expand on our current syndromic surveillance to: 1) enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and present at regional clinics with influenza-like illness, severe respiratory illness, encephalitis, and other specific symptoms; 2) collect detailed interview data to assess their likely contact with wildlife and livestock; 3) conduct molecular and serological diagnostic assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. we'll do novel viral discovery on unique clinical + symptomatic cases (maybe w high animal contact too) where testing has already ruled out all normative diseases?If novel pathogens are identified by PCR, we will isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work. We will then use behavioral survey data, ecological and phylogeographic analyses to assess the likely reservoir hosts to inform intervention programs.~~.~~

In addition, we will conduct regular meetings with key research, public health and community leaders in our collaborative consortium's network across other clinics, research institutes and public health laboratories within Thailand, Singapore and Malaysia, and across SE Asia. This includes >50 leading laboratories/institutions in XX countries that are currently collaborating on other funded work with core members of our consortium. We will use these, and our annual meetings, to share information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and any information from the greater network on likely outbreaks of novel disease.

From:	Peter Daszak on behalf of Peter Daszak <daszak@ecohealthalliance.org>
To:	Hickey, Andrew (CDC/DDID/NCHHSTP/DHPSE); Broder, Christopher; Eric Laing; MacArthur, John R. (CDC/DDPHSIS/CGH/DGHP)
Cc:	Aleksei Chmura; Luke Hamel; Kevin Olival
Subject:	RE: Introduction. Andrew. Regards to Ecohealth NIAID submission.
Date:	Friday, June 14, 2019 10:41:38 AM
Attachments:	EIDRC SE Asia Specific aims v3.docx

Great – here are the high level aims (still a draft, of course!).

Much appreciate you moving ahead on this.

Cheers,

Peter

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From: Hickey, Andrew (CDC/DDID/NCHHSTP/DHPSE) [mailto:ANH9@cdc.gov]
Sent: Wednesday, June 12, 2019 11:41 PM
To: Peter Daszak; Broder, Christopher; Eric Laing; MacArthur, John R. (CDC/DDPHSIS/CGH/DGHP)
Cc: Aleksei Chmura; Luke Hamel; Kevin Olival
Subject: RE: Introduction. Andrew. Regards to Ecohealth NIAID submission.

Sounds great. I'm working on another grant with a Friday deadline - so I will put together some language for you this weekend after I get that out. John is currently traveling, though he is aware of our conversation. He will be back in Thailand next week and I will discuss the potential LOS with him ASAP. Do you have a high level summary and Aims that I can share with John for our discussion? I would also use this to draft the LOS.

Andrew

From: Peter Daszak <daszak@ecohealthalliance.org>
Sent: Thursday, June 13, 2019 8:44 AM
To: Hickey, Andrew (CDC/DDID/NCHHSTP/DHPSE) <ANH9@cdc.gov>; Broder, Christopher <christopher.broder@usuhs.edu>; Eric Laing <eric.laing@usuhs.edu>; MacArthur, John R. (CDC/DDPHSIS/CGH/DGHP) <zae5@cdc.gov>
Cc: Aleksei Chmura <chmura@ecohealthalliance.org>; Luke Hamel <hamel@ecohealthalliance.org>; Kevin Olival <olival@ecohealthalliance.org>
Subject: RE: Introduction. Andrew. Regards to Ecohealth NIAID submission.
Importance: High

Thanks very much for the rapid response Andrew, and a pleasure to virtually meet you John.

I really appreciate your willingness to provide a letter of support and I will absolutely respect your caveats below, of course. I think this is a great opportunity because NIAID are looking for leverage and partnerships, and although there's always that issue about one federal agency not being allowed to fund another, there are plenty of ways we can use this to help build up your capacity through cross training and joint research projects.

I've answered some of your questions in the body of your email below, and again thank you for your openness for collaboration.

Cheers,

Peter

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From: Hickey, Andrew (CDC/DDID/NCHHSTP/DHPSE) [mailto:ANH9@cdc.gov]
Sent: Wednesday, June 12, 2019 12:26 AM
To: Peter Daszak; Broder, Christopher; Eric Laing; MacArthur, John R. (CDC/DDPHSIS/CGH/DGHP)
Cc: Aleksei Chmura; Luke Hamel; Kevin Olival
Subject: RE: Introduction. Andrew. Regards to Ecohealth NIAID submission.

Hi Peter. Thanks for the email. I cannot provide a letter of support individually, but we can consider this as the CDC country office in Thailand.
[Peter Daszak] That's great, because NIAID is looking for us to leverage partnerships
I'm adding Dr. John MacArthur who is filling multiple roles currently, including Director- CDC Thailand/SE Asia regional office, HHS Country Rep for Thailand, and Director for the CDC-Thailand Div. of Global Health Protection.

I am happy to share information to help support your grant, but request this be used for the grant only. The data potentially could be published eventually and I don't want to jeopardize that possibility. What kind of information/figures do you need? What is your deadline for this LOS and this information?
[Peter Daszak] We'll definitely keep this solely for this grant and will maintain confidentiality. Given the space constraints, ideally if you could send a few sentences with some explanation of the site, subjects, number of individuals/other data with a reference, and a figure and I'll insert that or reduce a bit. The deadline is June 28th, but we're trying to get a final draft by the 19th, so if you can get me something by then that would be ideal. Luke will send a sample of the sort of info for the Letter of Support, but it's always best if it's written in your own words…

Chris may have mentioned, our CDC Director recently visited the laboratory and expressed his interest in our office starting some research on bat-borne viruses with the MOPH.
[Peter Daszak] That's very interesting and great news also.
My goal is to make sure the CDC office is positioned to work well with the groups already performing this research in Thailand and not duplicating efforts. I reached out to John already and we plan to talk in July when he comes to Thailand for the bat meeting. Are you coming for the meeting as well? If so, it would be great to connect with you while you are here.
[Peter Daszak] That's perfect re. CDC office collaborating on bat research. We've been working with Supaporn for many years, but there is so much to do there's little chance of duplication and tons of opportunity for collaboration. We are definitely interested in supporting your efforts any way we can. I won't be able to be there in July – but I'll check in with others in the office here to see if any of our staff will be there and can meet with you. I'll be in Malaysia in September and maybe that's a chance to meet also.

Again – thanks for your help and I look forward to future collaboration!

Andrew

From: Peter Daszak <daszak@ecohealthalliance.org>
Sent: Saturday, June 8, 2019 3:26 AM
To: Broder, Christopher <christopher.broder@usuhs.edu>; Hickey, Andrew (CDC/DDID/NCHHSTP/DHPSE) <ANH9@cdc.gov>; Eric Laing <eric.laing@usuhs.edu>
Cc: Aleksei Chmura <chmura@ecohealthalliance.org>; Luke Hamel <hamel@ecohealthalliance.org>; Kevin Olival <olival@ecohealthalliance.org>
Subject: RE: Introduction. Andrew. Regards to Ecohealth NIAID submission.
Importance: High

Hi Andrew and thanks for the introduction Chris.

We're pulling together an EIDRC proposal with the goal of funding work to identify evidence of hidden, misdiagnosed, or under-reported spillover events in SE Asia. We're working with Supaporn in Thailand, Tom Hughes in Malaysia and Linfa Wang in Singapore. In the USA, we'll be collaborating with Ralph Baric, Chris and Eric and the NEIDL lab (just for sample submission).

As Chris mentioned, I heard about the interesting findings from Supaporn, but wasn't sure what the details are – Chris filled me in. It's particularly interesting to me because our whole premis for this proposal is that viruses like henipaviruses, CoVs and filoviruses are found in wildlife hosts in SE Asia, and likely spillover regularly but the cases are either clinically inconsequential, don't get reported, or get misdiagnosed (e.g. like the original Nipah virus outbreaks in India which were called 'aberrant measles', or like the outbreaks in Bangladesh which we know how occur annually, but prior to 1999 must have been undiagnosed).

We don't have spare funds right now, given that we're pretty close to the deadline and have a full set of partners with their budgets already fixed. But, if you are interested in drafting a letter of support, maybe we could mention the preliminary findings, and make the case that these samples would benefit from further work with this collaborative group. I'm not sure of all the details, but Chris has a good idea of how we could move forwards, and given that we'll be collaborating with him, NEIDL, Linfa, Ralph, I'm sure that there would be availability of resources to run the next step of the diagnostic path to move this to a validated finding. Chris – please let me know if that's correct, or if this is a case of running SNTs, which we could do through NEIDL.

If you were able to sign on, we would certainly appreciate your involvement, and make sure that there would be opportunity for co-authorship of some of the work we're planning, as well as trying to mobilize the findings that you've already made..

Cheers,

Peter

Peter Daszak

President

EcoHealth Alliance
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New York, NY 10001

Tel. +1 212-380-4474
Website: www.ecohealthalliance.org
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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

From: Broder, Christopher [mailto:christopher.broder@usuhs.edu]
Sent: Thursday, June 6, 2019 3:35 PM
To: Hickey, Andrew (CDC/OPHPR/DEO) (CTR); Peter Daszak; Eric Laing
Subject: Introduction. Andrew. Regards to Ecohealth NIAID submission.

Hey Andrew,

I know you are only just getting back to Bangkok.
Eric and I just had another TelCon with Peter (cc'd here)

Peter is going to submit this RFA:

Letter of Intent: RFA-AI-19-028 Emerging Infectious Diseases Research Centers
This letter of intent indicates that we plan to submit a proposal in response to the FOA RFA-AI-19-028 "Emerging Infectious Diseases Research Centers". Below are the details requested in the RFA:

Descriptive Title of Proposed Activity
Understanding risk of zoonotic virus emergence in EID hotspots of Southeast Asia

Supaporn is a part of this network. Eric and I will be as well but with only a small budget.
In his chat with Supaporn she mentioned preliminary data of NiV spillover evidence in children in Thailand.
This is your data, with Julie. I briefly explained that and how it was left unpublished and told him
you and I chatted again about that just yesterday when discussing the CDC NiV ELISA vs Bioplex in
her bat samples (perhaps we should by some of those ELISAs)

I suggested to Peter that he include you and your folks in this proposal. and also mention that DTRA wants to fund you and get you started as well.

Peter can share with you the LOI and other documents and perhaps have a chat with you.

cheers

Chris

Andrew Hickey, PhD, MPH, LCDR, USPHS.
Chief, HIV/STD Laboratory Research Section,
HIV/STD Research Program, Division of HIV/AIDS Prevention,
CDC, Thailand MOPH-US CDC.
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Christopher C. Broder, Ph.D.
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[redacted]
fax - 301-295-3773

II. Research Strategy:

1. Significance:

Coronaviruses (e.g. SARS-CoV, MERS-CoV), henipaviruses (HeV, NiV) and filoviruses (EBOV, MARV) have led to some of the most important recent emerging zoonoses (*1-12*). Their outbreaks have caused tens of thousands of deaths, and billions of dollars in economic damages (*13-15*). Like most emerging zoonoses, these pathogens originate in wildlife reservoirs, sometimes spilling over first into livestock 'amplifier' hosts, or directly into localized human populations with high levels of animal contact **(Fig. 1)**. Efforts to prevent emerging zoonoses have targeted these high-risk populations in regions prone to disease emergence (*12, 16*). However, surveillance and control is hampered by inadequate information on basic disease ecology (e.g. range of wildlife reservoirs, risk factors for spillover) and pathogen biology (viral diversity, pathogenesis), and by logistical challenges sharing novel diagnostics, animal models and reagents at sites where outbreaks begin (*17*). Countermeasure and vaccine development is challenged by the small number of isolates available, and the continuing discovery of closely-related strains, some with evidence of transmission to people but with unknown pathogenicity. For example, using NIH and other funding, we found serological evidence of exposure to African henipaviruses in 3-4% of a sample of people in Cameroon, with a significant risk factor being butchering of bats; and bat SARSr-CoVs in 6/209 people (2.87%) living close to a bat cave in Yunnan, both viruses with unknown clinical impact (*18, 19*). Others have shown that …..Any other examples of spillover events into livestock or people, especially from SE Asia?? (REFS). Even when spillover leads to outbreaks of illness and mortality, they are often unreported, undiagnosed or misdiagnosed. Under a prior NIH R01, we initiated targeted syndromic surveillance of encephalitis patients in Bangladesh clinics and showed that NiV causes repeated annual outbreaks with an overall mortality rate of ~70%, and that it is also present in bats in India (REF). Nipah virus was originally misdiagnosed as 'aberrant measles' in West Bengal, India (REF), has now emerged repeatedly in North India, and in Kerala, South India in 2018 and 2019 (ongoing), raising the specter of future spillover at other sites across the region (*20, 21*).

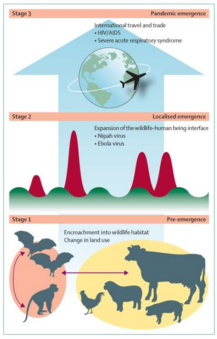

Fig. 1: Our Early Warning Approach: Most zoonotic viruses emerge from wildlife hosts, sometimes via livestock (Stage 1, lower panel), to cause small scale outbreaks (green) or larger chains (red) of human-to-human transmission (Stage 2, middle). In some cases, these spread more widely via air travel (Stage 3, upper). Our EIDRC proposal targets each stage: SA1 examines the diversity and spillover potential of viral threats in targeted SE Asian wildlife; SA2 seeks evidence of their spillover into focused high-risk human populations (stage 2); SA3 identifies their capacity to cause illness and to spread more widely (stage 3). Figure from (*16*).

Southeast Asia has significant risk of future viral emergence: it is a well-defined hotspot for emerging diseases due to its high biodiversity of wildlife and their viruses, and the presence of critical ecological and socioeconomic drivers of disease emergence (*22*). These include dense human populations living in networks of rural and urban communities, with strong cultural and behavioral connection to wildlife and livestock, and intimate connection to global travel networks (*23*). It is also a region undergoing rapid environmental and demographic change, both of which lead to increased risk of disease emergence and spread (REFS). It is therefore not surprising that a number of recent discoveries have identified near-neighbors of known agents spilling over to livestock and people through often novel pathways, leading to sometimes unusual clinical presentations **(Table 1)**.

In addition to these spillover events, relatives and near-neighbors of known viral pathogens have been reported throughout the region, including by members of our consortium. These include: henipaviruses in *Pteropus* and other? bats in Thailand, Cambodia, Philippines, Laos, Indonesia, China, other countries? (*24-30*); a novel henipavirus Mojiang virus in wild rats in Yunnan (*31*); serological evidence of filoviruses in bats in Bangladesh and Malaysia (REF); evidence of novel filoviruses in bats in Singapore (*32*) and China (*33-35*), including Měnglà virus that appears capable of infecting human cells (*36*); novel paramyxoviruses in bats and rodents consumed as bushmeat in Vietnam (*37*), a lineage C β-CoV in bats in China that uses the same cell receptor as MERS-CoV and can infect human cells *in vitro* (*38*); 52 novel SARSr-CoVs in 9 bat species in

southern China (172 novel β-CoVs from >16,000 sampled bats) that are also found throughout the region (*38-41*), a new β-CoV clade ("lineage E") in bats (*41*); and XX novel wildlife-origin CoVs and paramyxoviruses in Thailand and Malaysia identified as part our work under USAID-PREDICT funding (*39, 42*). Under a current NIAID R01, we have shown that we can use analytical approaches to target surveillance of wildlife and people and identify likely sites of spillover. We used phylogenetic analysis of bat hosts and their CoVs to identify a key cave complex in SW Yunnan Province that harbors all the genetic elements of SARS-CoV (*43*), as well as a series of SARSr-CoVs that can infect human cells *in vitro*, cause SARS-like clinical signs in humanized mice, and evade vaccine treatment and therapeutics (*38-41, 44-46*). We conducted behavioral risk surveys and biological sampling of human populations living close to this cave and found evidence of spillover (xx serology) in people highly exposed to wildlife. **This work provides proof-of-concept for an early warning approach that we will expand in this EIDRC to target key viral groups in one of the world's most high-risk EID hotspots.**

Viral agent	Location, date	Impact	Novelty of event	Ref.
Melaka virus & Kampar virus	Malaysia, 2006;	Caused SARI in family group, individual	1st disease due to bat-origin reoviruses (other reports Singapore, Vietnam etc.)	(*47-50*)
Reston filovirus	Philippines 2008	Seropositive people killed pigs,	No prior findings of filoviruses in pigs	(*51*)
Reston filovirus	Shanghai 2011	PCR positive pigs	Further evidence of pig RESTV infection	(*52*)
Nipah-like virus	Philippines 2015	Killed horses	No prior horse infection for NiV, known for HeV	(*53*)
SADS-CoV (HKU2)	China 2017	>25,000 pig deaths seronegative people	Novel emergence of bat-origin CoV	(*54*)
SARSr-CoV & HKU10-CoV	Yunnan, Guangxi 2015	Seropositive people	1st evidence human infection HKU10 & SARSr-CoV since 2003	(*19*)
Nipah virus	Kerala, 2018 & 2019 (REF)	XX people infected/dead	1st outbreak of NiV outside Bangladesh, W. Bengal focus	(*21*)

Table 1: Recent reports from SE Asia indicating potential for novel pathways of emergence, or unusual presentations for known or close relative viruses. OTHERS? Could include Melaka but it's in the Reoviridae and not in the RFA (should we only include pathogen groups listed in the RFA in this table?)

The overall rationale for this EIDRC proposal is that there is substantial evidence that: 1) diverse CoVs, henipaviruses and filoviruses related to known zoonotic pathogens are circulating in wildlife reservoirs in Southeast Asia; **2)** these viruses likely spillover regularly to people, are often unreported or misdiagnosed, and their clinical manifestations and potential to cause pandemics are unknown and underestimated; and **3)** our strategy for targeted surveillance and detection of spillover and illness in at-risk human populations can be used as an 'early warning system' to conduct public health interventions and disrupt disease emergence. The overarching goal of this proposal is to better understand the diversity of known and closely related CoVs, henipaviruses, and filoviruses in wildlife reservoirs in SE Asia, to test their capacity to infect human cells and mouse models, to develop new specific and sensitive serological and molecular diagnostic tools, and to conduct surveillance of human populations with high risk of exposure to wildlife, and clinical cohorts to identify evidence of spillover and pandemic potential, and of viruses causing previously 'hidden' clinical syndromes in people.

2. Innovation: Previous work by our consortium has developed a system to target surveillance in wildlife and people to better anticipate spillover events and pre-empt outbreaks of emerging viruses (REF). In this EIDRC we take this approach and scale it up to cover three critically high-risk EID hotspot countries in Southeast Asia, within a regional network of collaborators. The innovation in this EIDRC is in: 1) its multidisciplinary approach that combines modeling to target the geography for wildlife and human sampling, novel phylogenetic and *in vitro* and animal model approaches to identifying risk of viral spillover into people, development and transfer of novel serological and molecular diagnostics, and use of parallel large scale cohorts to identify spillover and illness due to known and novel viruses; 2) the biological characterization approach that identifies how likely viruses are to be able to spillover into people and builds on our successful work on SARSr-CoVs; and 3) the combination of geographically targeted human populations with high risk of animal contact and syndromic clinical cohorts that present with signs of novel viral emergence, to identify risk of viral spillover in an early

warning system approach. **In Aim 1, we will target viruses in new and archived wildlife samples, and characterize their risk of spillover to people**. We will use spatial and phylogeographic analyses to identify the geography and species of wildlife to test samples from, as well as further characterize viruses we have recently discovered. We will use cell culture and animal models to assess their potential for spillover into high-risk human populations. **In Aim 2, we will conduct focused, targeted surveys and sampling of human communities with high exposure to wildlife and other animals.** We will use serological tests targeting viruses identified in Aim 1 to identify the baseline spillover of known or novel CoVs, henipaviruses and filoviruses in these populations. We will use behavioral risk data, virological and ecological data to identify the likely reservoirs of viral groups identified. **In Aim 3, we will use syndromic surveillance of clinical cohorts to identify evidence of viral etiology for otherwise 'cryptic' outbreaks.** We will enroll and collect behavioral risk information and biological samples from patients in communities at high risk for disease emergence, who present with syndromes previously linked to known viral agents. We will conduct molecular and serological diagnostic assays to identify likely viral etiology of syndromes that may represent otherwise cryptic events. Where viruses are identified, we will attempt to isolate and characterize them, and use survey data, ecological and phylogeographic analysis to identify likely reservoir hosts and inform intervention programs.

3. Approach

Research team: For this EIDRC we have assembled a group of world leaders in emerging virus research with proven experience collaborating internationally on field, lab and human surveillance research **(Fig. 2)**. Over the past two decades, our consortium partners have collaborated together within the region and globally to conduct high profile research on these and other viral groups. This work includes identifying the wildlife reservoirs for Nipah and Hendra virus, MERS- and SARS-CoVs (*40, 43, 55-59*), discovering SADS-CoV (*54*), and developing an array of serological, molecular, *in vitro* and *in vivo* approaches to characterizing high-risk CoVs, henipaviruses, and filoviruses (*44-46, 60-73*). It includes substantial experience conducting human surveillance during outbreaks (e.g. - Please insert examples that you've been involved in and REFS), and as part of longitudinal efforts to pre-empt pandemics (*74, 75*).

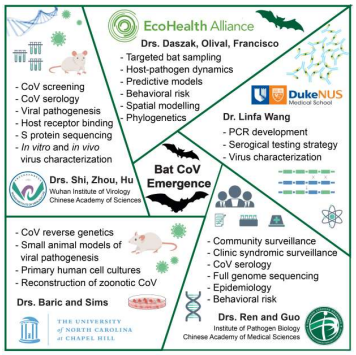

Fig. 2: Interdisciplinary team & roles in the proposed SE Asian EIDRC. PLACEHOLDER – Hongying to modify

PI Daszak has >20 years' experience managing international research collaborations on emerging zoonoses, and has collaborated with all partners in the EIDRC consortium for 5- 20 yrs on NIAID- and USAID-funded research. He will oversee all activities in this EIDRC. Drs. Olival, Zambrana, Ross are leaders in analytical approaches to targeting surveillance and head the modeling and analytics work for USAID-PREDICT. Drs. Wang, Baric, Broder, Anderson, Laing have developed a unique array of *in vitro* cell culture, serological and molecular reagents to identify and characterize emerging viruses in people and animals (henipaviruses and other paramyxoviruses, CoVs, filoviruses and many others). Dr. Wacharapluesedee, Hughes and collaborators have coordinated surveillance of people, wildlife, and livestock within Thailand and Malaysia for the past 10 years, in collaboration with Ministries of Health, Agriculture and Environment. Finally, this consortium has extensive external funding from DoD, USAID, NIH, DHS and other USG agencies that supports a range of laboratory, analytical and field studies directly related to the current proposal, and will bring substantial leverage to the EIDRC **(See Section XX)**.

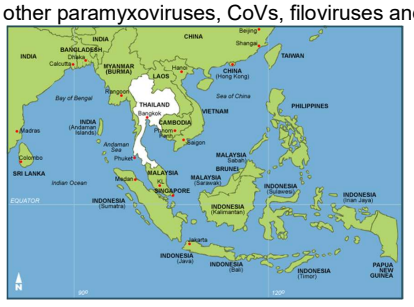

Fig.3 : Map of Southeast Asia indicating the three core countries for this proposed EIDRC (White: Thai, Sing, Mal) and those that Key Personnel are actively collaborating with (Green: field sites and collaborating labs

indicated with asterisk). PLACEHOLDER – Hongying to modify - please use a map with part of India and Bangladesh. – This map is the perfect range

Geographical focus: The three core countries for this proposed EIDRC are Thailand, Malaysia and Singapore, three contiguous EID hotspot countries that stretch through one of the most significant foci of EID origins globally. Our proposed work includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sarawak and Sabah, and thus contains overlapping wildlife biogeography with India, Bangladesh, Myanmar, SW China, Laos, Cambodia, Vietnam and Indonesia. It encompasses diverse cultural and socioeconomic backgrounds which have correspondingly diverse and intensive interactions with wildlife reservoirs and the global travel network. We have already begun sampling of wildlife hunters, livestock workers, wildlife farmers and market workers, rural villagers living close to high risk wildlife species, people otherwise highly exposed to animals in these countries, and clinical cohorts, as part of current/prior NIH and USAID funding. To develop a larger catchment area for emerging zoonoses, we will deploy our consortium's extensive network of collaborators in clinics, research institutes and public health laboratories in every other Southeast Asian country. We will conduct regular meetings with key research, public health and community leaders, including >50 leading laboratories/institutions in XX countries that are currently collaborating with core members of our consortium via other funded work (**Fig. 3**). We will use these, and our annual meetings, to share information on novel research and diagnostic approaches, pathogens that are of key pandemic potential, regions or populations at high risk of spillover, and information from the greater network on likely outbreaks of novel disease. This platform will coordinate sample sharing and diagnostic platforms and help build a rapid response to outbreaks across the region.

Current list of contacts at key human (especially) and wildlife orgs in every SE Asian country from map above – please add to this and send in the full details on the form requested by Luke/Aleksei!

Indonesia – EHA: Eijkman (Dodi Safari), IPB (Imung Joko Pamungkas);
SE China – EHA: Yunnan CDC (XX), Wuhan Inst. Virol. (Zhengli Shi);
Myanmar -
Cambodia -
Laos -
Bangladesh – EHA: IEDCR (XX), XX (Arif);
India – EHA: Jon's collaborators;
Vietnam – Wellcome CRU ? Liaison with Zhengli Shi for Serol assays etc.?
Philippines –

NOTE: Everything below is a very rough draft. I've lifted heavily from our recent CoV R01 renewal submission, and indicated in comment boxes the names of the people who I believe know most about each section and can modify or write the new text needed.:

Aim 1: Identification and biological characterization of spillover risk of high zoonotic potential viruses from wildlife

1.1 Rationale/Innovation: Our previous work on wildlife origin viruses demonstrates that zoonotic viral emergence tends to occur in specific regions with high diversity of wildlife that harbor viruses of zoonotic potential, and human populations that have close contact to these wildlife (*23*). In Aim 1, we will analyze these factors to identify regions, species and human populations that have a particularly high risk of viral spillover. We will target high-risk zoonotic host reservoirs (bats, rodents and primates) in these regions and test newly collected and some of the tens of thousands of recently collected samples we have archived in freezers in our labs for known and novel agents. Using previously developed approaches to predict capacity of novel viruses to infect human cells and mouse models, we will characterize these viruses and assess their potential for spillover, using those of high risk as targets for human sampling in Aim 2 and 3. This approach is built on substantial previous proof-of concept preliminary data:

Geographic targeting: EcoHealth Alliance has led the field in conducting analyses to indicate where the highest risk of zoonotic spillover in a region is (*22, 23*). For Southeast Asia, these EID hotspots are sites with high wildlife biodiversity, human population density and undergoing rapid land use change **(Fig 4a)**. Using host and

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ICTV viral data for all known mammalian viruses, and generalized additive models to correct for sampling bias, we are able to predict the relative number of yet-to-be-described viruses that a species harbors (*76*). Using these data to map unknown viral diversity demonstrates substantial undiscovered viral diversity in mammals across Southeast Asia, and particularly in regions of Thailand, Singapore and Malaysia **(Fig. 4b)**. In a separate paper on risk of viruses emerging from bat hosts, we found that factors driving spillover (host diversity, climate) differ from those driving spread in human populations (population density, bushmeat hunting, livestock production) (*77*). We therefore analyze capacity for viral spread as a separate risk factor, using demographic and air travel data in an app we have produced with Dept Homeland Security funds (*78*). In the current proposed work, we will use all the above approaches to target wildlife sampling (archive and new field collections), and to inform sites for human sampling in Aim 2, to areas of high risk for spillover and spread.

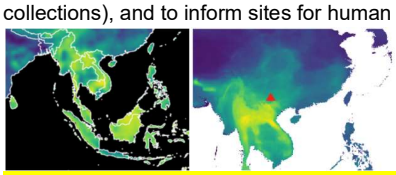

Fig. 4: Our previous work has shown proof-of-concept in geographically targeting sampling to maximize regions as high risk of wildlife-to-human spillover **(Fig. 4a)**, and regions with high diversity of predicted 'missing' or as-yet undiscovered viruses, yellow = highest diversity **(Fig 4b)**. From (*22, 23, 76*). Kevin/Alice, please insert high res hotspot map from Allen et al. (Fig 4a), expand the current map from Olival et al. to cover the same region as shown in Fig. 3, and remove the red triangle of course.

Reservoir host species targeting: Our analysis of all known mammalian host-viral associations demonstrates that bats, rodents and primate represent the three most important reservoirs for zoonoses, responsible for the highest proportion of known and predicted viral diversity of zoonotic potential (*76*). The bulk of our sampling and sample testing will consist of bats, because this group is the known or hypothesized reservoir for the majority of high-impact zoonotic CoVs, henipaviruses and filoviruses (*79-82*) (ADD REFS). Bats also have the highest proportion of predicted zoonotic viruses in any mammalian group (*76*). We have used a novel phylogeographic analysis, Maximum Clade Credibility (MCC) tree, to reconstruct the geographic areas of evolutionary origin for β-CoVs that we sequenced in bats (*39*). This approach allows us to identify the ancestral home of specific zoonotic viral groups in wildlife, where their diversity is likely highest. Finally, we have designed a strategy to estimate sampling gaps, using a viral 'mark-recapture' approach we previously published (*83, 84*) **(Fig. 5)**. Using prior data for bat, rodent and primate viral discovery vs. sampling rates, we can identify the likely total viral diversity for specific host groups in a region, and use this to estimate sample sizes to discovering the majority of unknown viruses within a specific family or genus. **In the current proposal,** we will use these analyses to estimate geographic and species-specific sampling targets **to more effectively identify new strains to support experimental infection studies and risk assessment.**

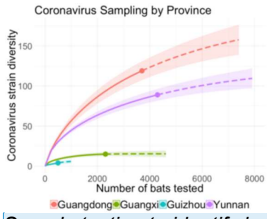

Fig. 5: Estimates of SARSr-CoV total strain diversity in bats we sampled in China under a NIAID R01 (strain defined as >10% sequence divergence in RdRp gene). GD and YN harbor highest CoV diversity, but discovery has not yet saturated. We used this to estimate that additional sampling of 5,000 bats will identify >80% of remaining β-CoV strains in bat hosts from these regions. We will apply this approach to our target host and viral taxa, for Thailand, Singapore and Malaysia to calculate sampling targets and success rates.

Sample testing to identify known and novel viruses: We have conducted extensive wildlife surveillance for novel viruses across Southeast Asia in prior NIAID funded work and as part of the USAID PREDICT project. This includes sampling and PCR-screening >16,000 individual bats from 6 families (16 genera) in southern China for coronaviruses, identifying 178 β-CoVs, of which 172 were novel (52 novel SARSr-CoVs). This includes members of a new β-CoV clade, "lineage E" (41), diverse HKU3-related CoVs (179 sequences) within a 'sister' clade to the SARS-CoV lineage, and the wildlife origin of a new virus (SADS-CoV) killing >20,000 pigs in Guangdong Province (*54*). Many of these bat species are found across the region. We have collected XX samples from bats, rodents and primates in Thailand and XX in Malaysia under PREDICT, screening XX and archiving duplicates of all of these which are now available for use in this project. We used family level primers for henipaviruses, filoviruses and CoVs and have already discovered XX, YY and ZZ in these two countries. In the current proposed work, we will attempt to isolate and characterize those that our analyses below suggest

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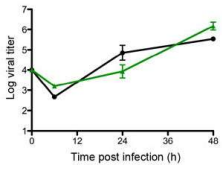
are most likely to be able to infect humans. Our collaborative group has worked together on these projects, as well as under DTRA funding in Singapore/Thailand (Co-Is Wacharapleusadee, Wang) to design, cross-train, and use novel serological and PCR tests to investigate xxx, and in Thailand (Co-Is Broder, Laing and Wacharapluesadee)on tech transfer of Luminex serology platform for wildlife and human samples. This is part of a planned DoD regional center of excellence for training in Thailand (details please?).

In vitro & in vivo *characterization viral potential for human infection:* We have used *in vitro* and *in vivo* experiments to characterize the pathogenic potential of novel SARSr-CoVs from bats. We isolated three SARSr-CoVs from bat feces: WIV1, WIV16 and Rs4874, with S protein sequences that diverged from SARS-CoV by 3% to 7% (*40, 43, 85*). We conducted full-length genome sequencing of 12 other novel SARSr-CoVs from a specific cave site in Yunnan China, some highly similar to outbreak SARS-CoV in the most variable genes: N-terminal domain and receptor binding domain (RBD) of the S gene, ORF8 and ORF3 (*43*). Using our reverse genetics system, we constructed chimeric viruses with SARSr-CoV WIV1 backbone and the S gene of different variants, including WIV1-Rs4231S and WIV1-Rs7327S. All 3 SARSr-CoV isolates and the two chimeric viruses replicated efficiently in Vero E6 cells and in HeLa cells expressing hACE2, but not in HeLa cells that don't express ACE2 (*40, 43, 85*) **(Fig. 5a)**. We used the SARS-CoV reverse genetics system (*64*) to generate a chimeric virus with a mouse-adapted SARS-CoV backbone expressing SHC014 S protein with 10% sequence divergence from SARS-CoV S. This chimera replicated in primary human airway epithelium, using the human ACE2 receptor to enter into cells (*44*) (**Fig. 5b**). Thus, **SARSr-CoVs with diverse variants of SL-CoV S protein without deletions in their RBD can use human ACE2 as receptor for cell entry.**



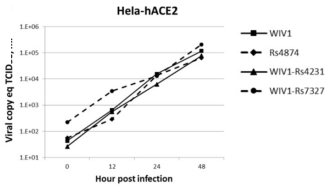


Fig. 5a (left): RT-PCR shows that bat SARSr-CoVs WIV1, Rs4874, and chimeras WIV1-Rs4231S, WIV1-Rs7327S grow in HeLa cells expressing human ACE2. **Fig. 5b (right):** Viral replication of SARS-CoV Urbani (black) and SARS-SHC014S (green) primary air-liquid interface human airway epithelial cell cultures at an MOI of 0.01.

We infected transgenic mice expressing hACE2 with 10^5 pfu of full-length recombinant WIV1 and three chimeric viruses (WIV1 backbone with SHC014S, WIV16S and Rs4231S). hACE2 transgenic mice challenged with rWIV1-SHC014S experienced ~20% body weight loss by 6dpi; rWIV1 and rWIV-4231S produced less body weight loss, and rWIV1-WIV16S led to no body weight loss **(Fig. 6a)**. At 2 and 4 dpi, viral loads in lung tissues of mice challenged with all three chimeras reached > 10^6 genome copies/g, significantly higher than rWIV1 infection **(Fig. 6b)**. This demonstrates that pathogenicity of SARSr-CoVs in humanized mice differs with divergent S proteins, **confirming the value of this model in assessing novel SARSr-CoV pathogenicity**.

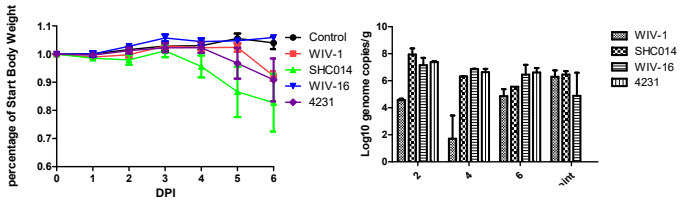


Fig. 6: *In vivo* infection of SARSr-CoVs in hACE2 transgenic mice. **6a (left)** Body weight change after infection; **6b (right)** Viral load in lung tissues.

Infection of rWIV1-SHC014S caused mild SARS-like clinical signs in the transgenic hACE2 mouse model **that weren't reduced by immune-therapeutic monoclonals that attenuate SARS-CoV pathogenecity.** Vaccination against SARS-CoV did not reduce severity of clinical signs in mice subsequently infected with rSARS-SHC014S (*44*). We found 2/4 broad human mAbs against SARS-CoV RBD cross-neutralized WIV1, but none could efficiently neutralize SHC014 which is less similar to SARS-CoV in the RBD (*86*). We repeated this virus characterization approach with chimeras using HKU3r-CoV S proteins that are ~25% divergent from SARS-CoV S, **and found that they are unable to use the ACE2 receptor**. Additionally, we were unable to culture HKU3r-CoVs in Vero E6 cells, or human cell lines. **The ability of HKU3r-CoVs to infect people, and their receptor binding target, remain unknown.**

Commented [PD6]: Linfa, Danielle, Supaporn – please expand

Commented [PD7]: Chris, Eric, Supaporn please expand

Commented [PD8]: I think this is something that Linfa, Supaporn or Chris mentioned?

Commented [PD9]: Ralph, Amy – this is directly from our CoV R01 renewal – please reduce the length of this section and adapt for this proposal. We prob don't need all these figures – just one or two would do.

All – what can we say about Nipah and filoviruses in this section – what is the rationale for a similar approach (e.g. we're looking for viruses somewhere between Cedar and Nipah in the Henipaviruses, and trying to assess the likelihood of some of the novel filoviruses infecting human cells).

Are we just going to use cell culture for henipas and filos, or can we look at spike protein diversity?

What about using bat cell lines and batized mice from Linfa?

1.2 General Approach:
We will conduct analyses to geographically- and taxonomically-target testing of samples from wild mammals that are most likely to harbor known high-profile zoonotic pathogens, or close relatives with potential to infect people. This includes selecting from tens of thousands of samples collected on recent projects and stored in freezers in our laboratories, and new collection from wildlife in high risk locales. We will use serological & PCR testing, to identify viruses, then attempt to isolate and biologically characterize viruses that phylogenetic and other data suggest have high spillover and pandemic potential. We will then conduct *in vitro* binding assays and cell culture experiments, and *in vivo* animal model infections to assess their potential to infect people and cause disease. EHA will lead the study design, targeted sampling, and data analysis; Co-Is Hughes and Wacharapluesadee will lead field sampling and in-country testing; Duke-NUS, UNC, USUS will lead the serological and PCR diagnostic development and the *in vitro* and *in vivo* analyses. A key goal of this work will be technology transfer to the region, including cell culture, animal models, serological and PCR diagnostics, as well as knowledge transfer by embedding staff in partner labs and by regular meetings. This will leverage substantially from the other funding available to consortium partners.

1.3 Wildlife samples: 1.3.a Site selection & sample sizes for newly collected and archived samples:

Adapt text from the rationale/innovation above and make brief statements about the work we'll do:

- Geographic targeting: we will use refined hotspot, 'missing viruses', and FLIRT analyses
- Targeting of host species/viral discovery targets: We will use phylogenetic MCC analyses and viral discovery curves
- Additional approaches: mapping of cave sites,

To calculate sample sizes, we will use previous work on SARSr-CoVs in bats in China, the results of bat, rodent and primate sampling under PREDICT in Malaysia and Thailand, and published data. For SARSr-CoVs in China, we found 6.7% mean PCR prevalence of SARSr-CoVs across bat hosts, with a small number of *Rhinolophus* spp. horseshoe bats having significantly higher PCR prevalence than other species sampled (REF).

Kevin – below is the language from our R01 CoV renewal proposal as a model:

In Y1 we will use our bat host and viral trait modeling, phylogeographic analyses of RdRp and S Protein sequences, and geographic and host species-based viral discovery curve analyses to identify SARSr-CoV diversity hotspot regions for bat sampling. We will sample at 8 new sites in four provinces. We will use cave site data (*87*), and demographic information to identify two sites in each of Yunnan, Guangxi, Guangdong, and Guizhou where humans likely have contact with bats. In Yunnan, we will identify two unsampled caves close to, but distinct from, the Jinning cave (*43*). This will provide adequate coverage of lineage 1 and 2 SARSr-CoVs, including a rich source of new HKU3r-CoVs, which have unknown potential for zoonotic spillover. Sampling will begin towards the end of Y1. We will use survey data from our previous R01 and host-specific viral accumulation curve data to target an additional 10 under-sampled *Rhinolophus* spp., 5 that were SARSr-CoV negative in our study, and a small number of related bat genera (including *Hipposideros* spp. and *Aselliscus* spp.) we previously found PCR positive for SARSr-CoVs **(Table 1)**. We will sample at least 5,000 bats from these 4 provinces (~1250 per province). Given ~5-12% prevalence of SARSr-CoVs in *Rhinolophus* spp. at our previous sites, **this sample size would give us 425 (±175) positive individual bats, and ~125 novel strains.**

1.3b Sample testing, viral isolation: Viral RNA will be extracted from bat fecal pellets/anal swabs with High Pure Viral RNA Kit (Roche). RNA will be aliquoted, and stored at -80C. One-step hemi-nested RT-PCR (Invitrogen) will be used to detect the presence of CoV sequences using primers that target a 440-nt fragment

Commented [PD10]: Ralph, Amy, Linfa, Danielle, Chris, Eric – need details for what approach we'll use for henipas and filos

Commented [PD11]: Kevin – please lead the drafting of this section. I've left in the section from our CoV R01 for you to adapt.

Commented [PD12]: Please check section 1.4.b and see if parts of that should be here..

Commented [PD13]: Ralph, Amy, Linfa, Danielle, Chris, Eric - This is from our CoV R01. Please insert details that apply also to henipa and filoviruses.

in the RNA-dependent RNA polymerase gene (RdRp) of all known α- and β-CoVs (*88*). PCR products will be gel purified and sequenced with an ABI Prism 3730 DNA analyzer.

We will attempt isolation on samples that contain viruses of interest (determined in **1.4, below**), using Vero E6 cells and primary cell culture from the wildlife taxonomic order (bat, rodent and primate cell lines). This includes the over XX cell lines maintained at Duke-NUS

1.3.c Sequencing S proteins: Our previous R01 work identified diverse SARSr-CoVs with high propensity for human infection (*19, 43, 44*). Characterization of these suggests SARSr-CoVs that are up to 10%, but not 25% different in the spike glycoprotein use human and bat ACE2 receptors for docking and entry. Uneven sampling gaps (e.g., no strains were found with 10-25% spike variation) prevent a thorough understanding of the transition point where the most divergent strains lose human ACE2 receptor usage. Our viral discovery curves **(Fig. 4)** suggest further sampling will reveal a rich diversity of as-yet-undiscovered SARSr-CoVs. In this aim we will use phylogenetic and viral discovery analysis to specifically target bat species and regions that are undersampled to allow sufficient power to identify and characterize missing SARSr-CoV strain diversity. Our previous work also suggests that SARSr-CoVs with S proteins that are ~10% divergent from SARS-CoV resist neutralization by therapeutic mAbs and escape SARS-CoV vaccines (*40, 44, 89*), suggesting that . However, viruses with 10-25% divergence in S proteins may bind to human cell receptors, but completely evade therapeutic and vaccine effects, and could therefore be a higher risk for public health. We will sequence the S proteins of novel SARSr-CoVs to prioritize viruses for experimental work in Aim 3 to test this hypothesis.

For all novel SARSr-CoV strains, we will sequence the complete S gene by amplifying overlapping fragments using degenerate primers as shown previously (*40, 43*). Full-length genomes of selected SARSr-CoV strains (representative across subclades) will be sequenced via high throughput sequencing method followed by genome walking. The sequencing libraries will be constructed using NEBNext Ultra II DNA Library Prep Kit for Illumina and sequenced on a MiSeq sequencer. PCR will be performed to fill gaps in the genome. The full length S gene sequences, including the amount of variation in the S receptor binding residues that bind the ACE2 receptor, will be used to select strains for Aim 3 experiments.

1.4. Assessing risk for spillover. 1.4.a Capacity to infect human cells: We will use a series of cell cultures to assess the capacity of target viruses to enter human cells. For CoVs, we will use primary human ciliated airway epithelial cells (HAE) cultures from the lungs of transplant recipients represent highly differentiated human airway epithelium containing ciliated and non-ciliated epithelial and goblet cells, grown on an air-liquid interface for several weeks prior to use (*44, 45, 90*). We will prepare HAE cultures from three different patient codes in triplicate in collaboration with the tissue procurement facility at the Cystic Fibrosis Center at UNC. Cultures will be inoculated with chimeric bat SARSr-CoVs to assess efficient replication. At 72 hpi, cultures will be fixed for immunofluorescent staining using antisera to the SARS-CoV conserved nucleocapsid protein (N) (*46, 63*). SARSr-CoVs that differ significantly in S protein sequence (11-24%) from epidemic SARS-CoV yet replicate *in vitro*, will also be evaluated for sensitivity to neutralization in Vero cells by PRNT50 assays using broadly SARS-CoV cross reactive human mAbs S227.14, S230.15, S215.17, and S109.8 (*45, 91*). As controls, the S genes of novel SARSr-CoV will be inserted into VEE 3526 replicon vectors (VRP3526-S), packaged and used to vaccinate mice (*92*). Polyclonal sera will be harvested and tested for ability to cross neutralize SARS-CoV, GD03, WIV-1, SHC014, WIV-16, other novel SARSr-CoV and HKU3-SRBD by PRNT50 assay (*45, 93, 94*). Using PRNT50 titers with sera (n=4 each) among these viruses, antigenic cartography (*95*) will allow comparison of antigenic vs. phylogenetic distance, identifying the transition at which SARSr-CoV strains escape SARS-CoV based vaccines, informing immunotherapeutic and vaccine design strategies (*96-98*).

Filoviruses (primary endothelial cells – ralph has primary lung endos – see if anyone has liver primary hepatocytes. Other option are monocytes) – where will we do this? BSL4? (NEIDL). Duplicate samples in culture medium from animals PCR +ve for filoviruses will be shipped to NEIDL (See letter of support) for culture and sharing with other agencies. Check on this re. wildlife filos…See if can do training opportunities so they can work with the NEIDL – visiting scholar appointments

Commented [PD14]: Linfa, Supaporn, Danielle – is this correct?

Commented [PD15]: Brief description of these please

Commented [PD16]: Ralph et al. – I'm assuming this will still be of value for the CoVs, so a reduced version of this could be in here, but what about spike proteins of filos and henipas – is that not of any use for assessing their capacity to infect human cells. Can we do similar work with filovirus pseudotypes from sequencing the spike proteins, for example, to assess binding to human receptors? If so, please draft some text and point to some references

Commented [PD17]: Ralph, Linfa, Chris – please expand and draft – I think this could be a good role for NEIDL, but don't know what the rules are…

1.4.xx Host ACE2 receptors: We will sequence host ACE2 receptors of different bat species or different bat populations from a single species (e.g. *Rhinolophus sinicus*) to identify relative importance of different hosts of high risk SARSr-CoVs and the *intra*specific scale of host-CoV coevolution. Of particular interest is homology across 18 bat and human orthologue ACE2 contact interface residues that engage the SARS RBD as a potential indicator of SARSr-CoV cross species transmission potential and growth in human cells (*62*).

1.4.b Host-virus evolution and distribution: We will use Bayesian and Maximum Likelihood phylogenetic analyses of RdRp, Spike, and full genome sequence (when available) data to reconstruct the evolutionary history of the novel bat SARSr-CoVs we identify. We will rerun MCC analyses **(Fig. 3)** to reconstruct β-CoV evolutionary origins using our expanded dataset. Ecological niche models will be used to predict spatial distribution of PCR-positive species, identify target sites for additional bat sampling, and analyze overlap with people. We will use our viral discovery/accumulation curve approach **(Fig. 4)** to monitor progress towards discovery of >80% of predicted diversity of SARSr-CoVs (lineage 1 & 2) within species and sites, halting sampling when this target is reached, and freeing up resources for work other work (*84, 99*).

1.4.c Viral strain prioritization: Of the expected 100-200 novel SARSr-CoV strains, we will down-select to prioritize for further characterization based on S genes that are: i) different from SHC014, WIV1, SARS-CoV with diversity ranges of 10-25%; ii) have virus S RBD that could use human/bat receptors; iii) have recombinant chimeric spikes indicative of gene flow between clade I and II strains; iv) have bat ACE2 receptors that might select for spike RBDs that can use human receptors for entry (15/18 conserved residues in human/bat ACE2 molecules that bind SARSs-CoV S RBD domains are likely more efficient receptors than 3/18 conserved sites). Using structural models based on the SARS S glycoprotein, the extent and location of antigenic variation will be annotated onto the structure, further resolving the locations of highly evolving and conserved structural motifs/epitopes that function in protective immunity (*91, 100-102*).

1.4.d Animal models: We will use a series of mouse models to assess spillover potential of viruses. First, the Baric lab has a well-established hACE2 transgenic mouse model that we will use this to characterize the capacity of specific SARSr-CoVs to infect. Briefly, in BSL3, n=5 10- to 20-week old hACE2 transgenic mice will be intranasally inoculated with 1 x 10^4 PFU of wildtype WIV-1 or chimeric bat SARSr-CoVs with different spike proteins, then monitored daily for weight loss, morbidity, and clinical signs of disease. Mice will be euthanized at 2, 4, and 6 dpi (endpoint of 14 dpi), organs harvested and virus quantified by SARS-CoV NP RT-PCR. After 7 days in formalin, tissues for histopathology will be removed from the BSL3 and stained with H&E, and for immunohistochemistry using polyclonal antibodies against the N protein. We will conduct limited evaluation of existing countermeasures using therapeutic monoclonal antibodies *in vitro* and *in vivo*. Existing SARSr-CoV mAbs will be diluted in DMEM starting at 1:20, and serial 2-fold dilutions mixed with an equal volume of 50 PFU of chimeric bat SARSr-CoVs with different spike proteins, then incubated on Vero E6 cells at 37°C for 1 h, then changed to a semi-solid medium (DMEM containing 1% methylcellulose and 2% FBS). Antibody neutralization titers will be evaluated by plaque quantification at 4-5dpi. hACE2 transgenic mice will be injected with SARS-CoV mAbs, and infected with chimeric bat SARSr-CoVs. Clinical signs and morbidity will be assessed and tissue pathology examined and compared with mice without treatment of mAbs to determine the therapeutic effect on SARSr-CoV infection, and protection of SARSr-CoV by wildtype SARS-S based vaccines assessed as described (*90, 103*). We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS cross neutralization, synthetically reconstruct a small subset (1-2) and evaluate the ability of nucleoside analogues to inhibit growth in HAE cultures and/or *in vivo* (*45, 90*).

For novel viruses across all families, we will use the UNC collaborative cross mouse that captures 90% of the diversity within all mouse groups to assess the most suitable model for further experiments. We have used this model for CoV, filo (Ebola), Flaviviruses, alphaviruses infections. In subpopulations it reproduces SARS weight loss, hemorrhage in EBOV, enceph in WNV, acute or chronic arthiritis in Chikungunya infection.

Duke-NUS has develop two models for bat *in vivo* culture: the 'batized' mouse model (Desciption) and a colony of *Eonycteris spelaea* (cave dwelling small fruit bat).

Commented [PD18]: Kevin/Ralph – I don't think this is relevant for our EIDRC – please let me know and delete if correct.

Commented [PD19]: KEvin/Alice – please modify this section for filos and henipas as well (and other hosts) - or does it belong in 1.3.a above?

Commented [PD20]: Ralph – can you reduce this section to a feasible amount of work for this project, and remove a bunch of text

Commented [PD21]: Ralph – please draft a brief para explaining what we'll do with this mouse model

Commented [PD22]: Linfa/Danielle – please draft a brief para explaining how we'll use either or both…

1.5 Potential problems/alternative approaches: Permission to sample bats in sites or provinces we select. We have a >20-year track record of successful field work in Malaysia, Thailand and >10 years in Singapore, and have worked with local authorities and communities to ensure access. We have existing permissions in place which will be renewed at the start of this project. Due to the abundance of wildlife habitats in the region, if access to a site is withdrawn, we will rapidly identify a suitable, complementary site with similar bat species composition and abundance. Finally, we have already collected tens of thousands of wildlife samples from the region and will focus on these while waiting for permission. **We may not identify novel viruses in our sample wildlife species due to seasonality of viral shedding or other factors.** We have calculated sample sizes based on a range of PCR data and are fairly conservative in our approach. However, as a back-up, we already have identified dozens of novel viruses that are near-neighbors to known high-impact agents, and will continue characterizing these should discovery efforts yield few novel viruses. The sampling regions we have selected are subtropical, and our previous data, and even published studies in temperate regions (*104*), do not suggest a strong pattern of seasonality in CoV shedding, although there are studies that suggest this occurs in henipavirus and filovirus (MARV?) shedding (REFS?). Nonetheless to account for this we will conduct sampling evenly on quarterly basis within each province.

Commented [PD23]: Kevin – please edit/check

Commented [PD24]: Kevin – please check the veracity of these comments

Aim 2: New serological and PCR tools to test evidence of viral spillover in high-risk communities..

2.1 Rationale/Innovation: Assessing the spillover of rare or novel zoonotic agents will require targeted surveillance of high-risk communities that are the first to get infected. To enhance low statistical probability of identifying these rare events, populations could be targeted that both live in rural locations close to high wildlife biodiversity, and also engage in behaviors that enhance the risk of spillover (e.g. hunting and butchering wildlife). In Aim 2 we will expand from our current work in the region to identify and enroll large cross-sectional samples from human populations that have high behavioral risk of exposure to wildlife origin viruses and live close to sites identified in Aim 1 as having high viral diversity in wildlife. We will use behavioral surveys and repeated biological sampling, design and deploy specific and sensitive serological assays to identify the baseline spillover of known or novel viral pathogens in these populations. Where symptoms are found, we will use PCR-based assays to identify presence of known and novel CoVs, henipaviruses and filoviruses, attempt to isolate and biologically characterize the pathogen, using the collaborative cross mouse (Aim 1) to identify an appropriate animal model to conduct preliminary pathogenesis work.

Preliminary data human biological sampling: Our longterm collaboration in the region has included identification of key at-risk human populations from which we have already collected qualitative and quantitative survey data, and biological samples. These include:

Commented [PD25]: All – please read and share with collaborators to flesh out and correct details here.

Peninsular Malaysia: Data from 1,300 Orang Asli samples, PCR for 5 viral families: 5 known CoVs in 16 people, one Influenza virus. Serology on its way incl. filovirus reactors…

Sabah: We have ~25 new CoVs in Sabah. Simon is using HTS to further characterize and Tom will get the data for this proposal from Simon.
Tom will work out all serum samples we have, incl. samples from dead bats.
Kamruddin (UMS Borneo Medical Health Research Center) has identified some high risk communities. We can help with them and build out the BSL-2 lab, as well as work on outbreak response within DHRU (in collab with Sabah CDC?).
Tom will speak with Tim Williams …He has archived samples from Kudat Monkey bar project – 2,000 human sera already screened for bacteria and parasites, we've been reached out to about this. Data on macaque tracking data/human tracking data also.

Sarawak: Dr Tan (Cheng Sieng) Center for Tropical and Emerging Diseases, Faculty of Medicine at UNIMAS – high risk Dayak communities that he's identified. We will do further sampling, questionnaires etc. Has Prelim data on HPV work in Dayak communities in remote Sarawak – we can work with those communities and can reach many by car, others by 4WD or boats. Has also Enterovirus 71 paper and is interested in CoVs. Has access to caves and guano. Will look into what other archived samples he has. Will give us access to Sarawak Forestry and Health Depts.

Thailand: Imported MERS cases..Zika patients. 10 yrs ago AFRIMS in Thailand found serological NiV +ve in Children far from the bat colony, prob with publication of data because didn't do neg controls – could do SNT at NEIDL or Wuhan – couldn't get permission Have emailed Andrew Hickey about this.
High-risk community cohorts – Loei Province (Guano miners) – whole community

Singapore:

Behavioral risk: EHA is the global lead organization in the USAID-PREDICT project for assessing human behavioral risk of zoonotic spillover. The general approach has been to conduct qualitative exploratory studies using standardized one-on-one semi-structured ethnographic interviews and observational data in among people engaged in clearly high-zoonotic risk activities (e.g. we interviewed 88 people involved in trading wild bats in China – REF). This allows us to assess local social and cultural norms and individual attitudes underlying wildlife contact. We used qualitative study findings to develop a human behavioral risk questionnaire on the type and frequency of animal contact, wildlife observed in daily life, and unusual illnesses reported over the past 12 months. We conducted cross-sectional studies in high risk populations in >15 countries during the last 5 years, and study participants also provided biological samples (oropharynx swab, serum, plasma). Questionnaires and biological samples were collected from 1,585 participants from 7 sites in Yunnan, Guangxi, and Guangdong provinces, XX from XX sites in Malaysia and XX from XX sites in Thailand. The results of these surveys will be used in Aim 2 to better identify target populations.

Serological Evidence of Exposure: In China, we developed serological assays for HKU9 CoVs (β), SARSr-CoV Rp3 (β), HKU10 CoV (α), and MERS-CoV (β) and used ELISA and Western blot to test serum samples collected in 2016/17. **We found 7 individuals (7/733, 0.95%) living within a 6 km radius of the Jinning Cave, and 6/209 people (2.87%) at one site, with evidence of exposure to bat SARSr-CoVs.** We found evidence among human populations in Guangxi Province of people with prior exposure to the bat α-CoV HKU10 (2/412, 0.48%). This is of potential public health interest because HKU10 is known to be able to jump host species within bats, and therefore may have high propensity for emergence (*105*). However, the low seroprevalence (0.6%-2.7% at positive sites) suggests we need a larger sample size, and a focused, targeted study to maximize the likelihood of identifying seropositive cases. In Aim 2, we will use combined biological-behavioral risk surveillance in targeted populations within the community and clinical settings to 1) identify risk factors correlated with seropositivity (exposure to) and PCR positive status (infection with) henipaviruses, filoviruses and CoVs; and 2) assess possible health effects of infection in people. Obtaining this information could be a significant step in understanding the likelihood of recent 'hidden' spillover events and their public health impacts, as well as the risk of future emergence of viral disease.

Risk Factors: In China, questionnaire response and demographic data suggest specific risk factors included type of occupation, keeping of pets and visiting wet markets. Seropositive individuals were mostly farmers/peasants (8/9), living in Yunnan province (7/9), 41-60 yrs old (7/9), with domestic contact with rodents (6/9), and are male (6/9). However, these characteristics were common and occurred in ~40% of survey respondents. Although these results are preliminary and don't provide detailed information on routes of exposure, they suggest that further refining use serological tests coupled with qualitative and questionnaire data will identify likely routes of exposure to novel CoVs in China. In Aim 2 of this EIDRC proposal, we identify strategies to better target at-risk people, and conduct focused **questionnaires and serosurveys to produce statistically significant findings for henipaviruses, CoVs and filoviruses.**

2.2 General Approach/Innovation: We will use a dual study design to gain in depth understanding of exposure and risk factors for viral spillover **(Fig. XX)**. In Aim 2 we will conduct community-based surveillance with more focused questionnaires and biological sampling to determine the seroprevalence of spillover viruses in at-risk human populations, and to identify risk-factors for viral spillover in these communities. In Aim 3, we will conduct clinic-based syndromic surveillance and biological sampling at sites that include the community-based surveillance sites within their catchment (details in Aim 3). Both community-based and clinic-based syndromic surveillance programs are case-control studies designed with the sample sizes necessary to statistically quantify (with a power of 80%) risk factors and health impacts for viral spillover, linked to serological status and symptoms.

Commented [PD26]: Linfa, Danielle – are we going to do human sampling in high risk communities in Singapore? If so, what would you propose and do you already have some prelim work you've done. Note these are high risk populations, i.e. high exposure to wildlife. Clinical cohorts are in Aim 3

Commented [PD27]: Hongying/Emily to edit

Commented [PD28]: Hongying/Emily – any information from Thailand or Malaysian surveys?

2.3 Target population & sample sizes: We will target sites in the same geographic regions as those identified in Aim 1 that harbor wildlife with high potentially zoonotic viral diversity, in regions that are EID hotspots, and are well-connected to regional travel and trade hubs and the global travel network. We will target specific communities based on our PREDICT behavioral analysis questionnaire data to assess key at-risk populations with high exposure to wildlife, as well as other publicly available data (e.g. wildlife hunting licenses sold in a region, number of guano caves accessed for fertilizer etc.). We will also expand on work currently underway in the following particularly high exposure populations:

Thailand: We have conducted behavioral risk surveys and biological sampling in XX guano miners in Loei. These workers visit bat caves and dig fecal material to use as fertilizer, and are therefore highly exposed to bats and rodents in particular.

Peninsular Malaysia: We have sampled Orang Asli populations for the past 2 years, enrolling XX people. We will expand this to includeXXX

Sarawak: Dr Tan (Cheng Sieng) Center for Tropical and Emerging Diseases, Faculty of Medicine at UNIMAS – high risk Dayak communities that he's identified. We will do further sampling, questionnaires etc. Has Prelim data on HPV work in Dayak communities in remote Sarawak – we can work with those communities and can reach many by car, others by 4WD or boats. Has also Enterovirus 71 paper and is interested in CoVs. Has access to caves and guano. Will look into what other archived samples he has. Will give us access to Sarawak Forestry and Health Depts.

Sabah:

Singapore:?

Community-based surveillance will be conducted at XX sites in each country, a total of xx sites. From our previous work we anticipate that 10-30% of the community population will have had exposure to wildlife allowing us to capture highly exposed and non-exposure individuals at each site. Individuals living or working around wildlife habitats (e.g. bat caves/roosts), who hunt wildlife, work with wildlife or livestock farming, transportation, selling, or slaughtering wildlife in the surveyed areas will be targeted so that they make up ≥30% of the sampled population in each community. We will stratify sampling to ensure appropriate representation of sex, demographic, and socio-economic factors at each community site. Estimating 5% overall seroprevalence in these high-exposure populations, these sample sizes are sufficient to estimate effect sizes of behavioral risk factors of 2X or greater with 80% power. For community-based surveillance, we will enroll xxxx individuals per county/province, pooled across two sites for each, allowing us to make county/province-level comparisons of differing effects.

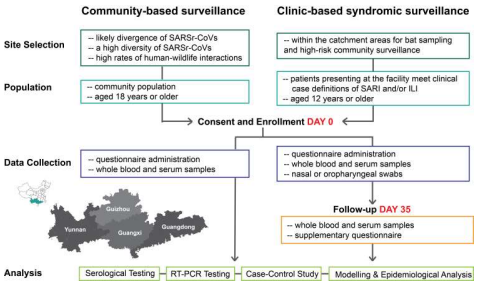


Fig. XX: Human survey and sampling study design overview (Aim 2 and 3)

2.4 Data & sample collection: Following enrollment with signed consent form, biological specimens (two whole blood samples, one max. 500 µL; two 500 µL serum samples) will be collected from all eligible participants, and a questionnaire will be administered. We will investigate five risk factors, so as to maximize the power of the analyses, all related to high risk wildlife exposure, based on continuing analysis of our previous work, and will include: 1) occupation; 2) observed or reported interactions with bats in/around house; 3) proximity to nearby bat roosts; 4) working or regular visit to animal markets; 5) self-reported ILI/SARI. With consent from participants, we will review clinical records to collect recent data on medical history, clinical syndromes, and patient etiology.

2.5: Laboratory analysis: 2.5.a Serological testing:

Commented [PD29]: Hongying/Emily to edit

Commented [PD30]: Supaporn – please draft plans for what we should do to scale up this work We could also include other high-risk communities – any ideas?

Commented [PD31]: Tom – please start writing some plans for what we csould do.

Commented [PD32]: All – not sure if we should have one figure for community and clinical cohort surveillance (aim 2 and 3 respectively) or if we should have two separate ones?

Commented [PD33]: Hongying– this figure is from our CoV R01 renewal. Please modify to make relevant to SE Asia and henipas, filos and CoVs.

Commented [PD34]: Emily/hongying

Commented [PD35]: Chris, Eric, Linfa, Dani etc. please draft some language here…

The below is from our previous CoV proposal:

In our previous R01, we expressed his-tagged nucleocapsid protein (NP) of SARSr-CoV Rp3 in *E.coli* and developed a SARSr-CoV specific ELISA for serosurveillance using the purified Rp3 NP. The specificity of this assay was evaluated using polyclonal antibodies against HKU1, OC43, 229E, NL63, MERS-CoV and EBOV and no significant cross-activity was detected (*19*). **While this shows it is a specific test for Rp3, it suggests that if we can expand our serology tests to cover other bat CoVs, we may identify many more seropositive individuals.** In this renewal, we will therefore use two serological testing approaches. First, we will expand test all human sera collected from both community- and clinic- based sampling for a panel of bat CoVs that will include SARS-CoV (outbreak strain), a range of lineage 2 SARSr-CoVs (including WIV1 and SHC014), lineage 1 HKU3r-CoVs, MERS-CoV, and the α-CoVs SADS-CoV and HKU10. We previously found serological evidence of human exposure to HKU10 (*19*), but HKU10 is known to jump from one host bat species to another (*105*) and is therefore likely to have infected people more widely. Incoporating serological testing for SHC014 is also likely to yield higher seroprevalence because it is readily divergent from SARS-CoV wildtype and therefore unlikely to have been picked up in our earlier testing. It is possible that non-neutralizing cross reactive epitopes exist that afford an accurate measure of cross reactivity between clade1 and clade 2 strains which would allow us to target exposure to strains of 15-25% divergence from SARS-CoV. Additionally, we will test samples for antibodies to common human CoVs (HCoV NL63, OC43 – see potential pitfalls/solutions below). Secondly, we recognize that CoVs have a high propensity to recombine. To serologically target 'novel' recombinant virus exposure, we will conduct 1) ELISA screening with SARSr-CoV S or RBD; 2) confirm these results by Western blot; then 3) use NP based ELISA and LIPS assays with a diversity of SARSr-CoV NP. For NP ELISA, micro-titer plates will be coated with 100 ng/well of recombinant batCoV NP and incubated with human sera in duplicates followed by detection with HRP labeled goat anti-human IgG antibodies. For confirmation, all ELISA positive samples will be subjected to Western blot at a dilution of 1:100 by using batCoV-NP as antigen. We will use an S protein-based ELISA to distinguish the lineage of SARSr-CoV. As new SARSr-CoVs are discovered, we will rapidly design specific Luciferase immunoprecipitation system (LIPS) assays targeting the S1 genes of bat-CoV strains for follow-up serological surveillance, as per our previous work (*54*).

2.5.b RT-PCR testing. Specimens will be screened using RT-PCR for the RdRp gene (See section 1.3.b for details). Positive samples will be subjected to full genome sequencing and RT-PCR amplification of the S glycoprotein gene. Samples from the clinic-based syndromic surveillance will also be tested using RT-PCR for Influenza A & B, HCoV NL63, OC43, HKU1, SARS-CoV & 229E as rule-outs, and SADS-CoV and HKU10 as an opportunistic survey for potential spillover these CoVs as proposed above.

2.6 Epidemiological analysis: We will conduct a case-control study to identify risk factors for SARSr-CoVs spillover. "Cases" are defined as participants whose samples tested positive for SARSr-CoVs by serological tests. "Controls" will be selected from the pool of participants admitted to the studies but testing negative. We will use nearest neighbor matching to pair cases demographically with controls at a 1-to-3 ratio or greater. We will use generalized linear models to analyze correlation between serological/PCR status and risk factors including: Activities with likely exposure to 1) bats; 2) livestock; and 3) locations of residence and work. We will use the same procedure to determine how clinical presentation differs between SARSr-COVs-exposed and unexposed enrollees, in the time course of illness, severity of symptoms, and type of symptoms.

2.7 Biological characterization of viruses identified: XXX

2.8 Potential problems/alternative approaches: Rarity of spillover events means it may be difficult to identify sufficient seropositives to statistically analyze risk behavior. First, we will be targeting our community-based surveillance to subpopulations with high-levels of wildlife exposure, at sites selected for diverse and prevalent viruses to increase likelihood of finding positive individuals. Second, our serology testing will include a panel of assays for a large diversity of viruses, increasing likelihood of positives. For example, in previous work our overall bat CoV PCR prevalence in the community was, 11.8%; β-CoV, 3.4%; α-CoV, 9.1%). Thus, using this broad serological panel to screen individuals in likely contact with these species increases the potential for detecting spillover with enough power for statistical analyses, and will shed light on behaviors that

Commented [PD36]: Please modify for Henipas, filos and CoVs

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predispose to spillover. Third, we will include common human CoVs, paramyxoviruses in our panel, so that even if low prevalence of wildlife viruses is found, we will be able to conduct a valuable cross-sectional study of the seroprevalence of more common human viruses. Finally, we will be able to assess relative measures of human-wildlife contact from our survey work. We will analyze intensity of contact against other risk factors and clinical outcomes to provide useful proxy information for spillover risk. **Serological testing may not match known CoVs due to recombination events.** We will use the three-tiered serological testing system outline in **2.6.a** to try to identify these 'novel' CoVs, however, we will also remain flexible on interpretation of data to ensure we account for recombination.

Aim 3: Clinical cohorts to identify evidence of viral etiology for 'cryptic' outbreaks.

3.1 Rationale/Innovation: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent is not diagnosed. By working with syndromic cohorts reporting to clinics with symptoms similar to known high-impact viral agents, we may be able to capture novel emerging diseases in at-risk communities before they spread into the general population and risk becoming pandemic. This will have clear value for public health in the region, and potentially wider. To do this, we will expand on current syndromic surveillance activities to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and present at regional clinics with symptoms typical of high impact zoonotic viruses. We will collect detailed interview data to assess their likely contact with wildlife and livestock and conduct molecular and serological diagnostic assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. We'll conduct novel viral discovery on unique clinical + symptomatic cases (maybe w high animal contact too) where testing has already ruled out all normative diseases? If novel pathogens are identified by PCR, we will isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work.

Preliminary data clinical surveillance: Our longterm collaboration in the region has included the following activities investigating clinical syndromes:

Thailand: Sick people cohorts – Chulalongkorn Hospital; EID Center for Thailand – Outbreak investigation Lab surveillance Min public Health Dept of Disease Control, 3 projects testing samples from people (encephalitis, Hand foot mouth disease, viral diarrhea, Influenza) = outbreak identification Dr. Rone. Any cluster of unusual disease samples will be sent to Supaporn. Have been using PREDICT protocol to identify pathogens. Dr. Parvenee doing lab investigation

Peninsular Malaysia:

Sabah: Linfa has worked with Timothy Williams on an encephalitis cohort from Queen Elizabeth Hospital 1 & 2 (poss has worked with Linfa on these) and this is another possibility. He's doing outbreak response training in August with team from Sabah CDC (Dr. Maria now in Pen. But still collaborating with us, Jikal current director working with us and Dato Cristina dir. Sabah state health Dept and is supportive) this collaborative group is working to develop a Sabah outbreak response team – we will support that – to be based at BMHRC and we will develop this as part of this proposal. Prof. Kamruddin will summarize the serum samples he has.
POCs: Sabah State Health Dept (e.g. Dr. Giri Shan Rajahram), Queen Elizabeth Hospital (Dr. LEE Heng-Gee)

Sarawak: From Linfa – Dr. Ooi used to work with Jayne Cardosa at UNIMAS and has access to samples of patients with unusual presentation

Strategy for analysis of self-reported illness: We have developed a standardized approach in PREDICT to analyzing data on self-reported symptoms of fever with cough and shortness of breath or difficulty breathing (severe acute respiratory illness - SARI), and fever with muscle aches, cough, or sore throat (influenza-like illness - ILI) from study participants. We used a least absolute shrinkage and selection operator (LASSO) regression to identify associations between ILI and/or SARI symptoms and contact with animals in the last year. Results have clear biological relevance. In Yunann, China, salient predictors or combination of predictors were, in descending order of odds ratio: 1) having eaten raw or undercooked carnivores; 2) having slaughtered poultry and being a resident of Guangxi province; 3) having had contact with cats or bats in the house; and 4) being a male who has raised poultry **(Fig. 11).** We will expand this approach for all syndromes in Aim 3, and

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with questions designed to assess patient's exposure to wildlife in terms that are relevant to the specific country.

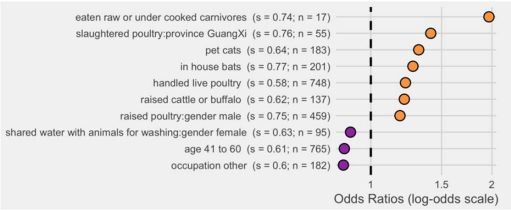

Fig. 11: Predictors of self-reported ILI and/or SARI in prior 12 months (s = bootstrap support; n = number +ve out of 1,585 respondents). Orange circles = odds ratios > 1 (positively associated with the outcome); purple = odds ratios <1 (negatively associated with the outcome).

3.2 General Approach: Our previous research suggests that there are substantial numbers of outbreak cases for which the etiologic agent is not identified. To capture these, we will expand on our current syndromic surveillance to enroll, and collect high quality biological samples from patients who live in rural communities linked to Aim 2 and present at regional clinics with influenza-like illness, severe respiratory illness, encephalitis, and other symptoms typical of high-impact emerging viruses. We will collect interview data tailored to the region to assess contact with wildlife, conduct molecular and serological assays to test causal links between their syndromes and the known and novel viral agents identified in Aim 1. If novel pathogens are identified by PCR, we will isolate and biologically characterize the pathogen, using the collaborative cross to identify an appropriate animal model to conduct preliminary pathogenesis work. We will then use behavioral survey data, ecological and phylogeographic analyses to assess the likely reservoir hosts to inform intervention programs.

3.3 Clinical cohorts. 3.3.a Cohort selection: We will initiate active clinic-based syndromic surveillance at XX town-level level clinics and xx provincial-level hospital in each country, in total xx hospital sites, all within the catchment areas for wildlife sampling, and which are used by people in our community-based surveillance. Patients ≥ 12 years old presenting at the health facility who meet the syndromic and clinical case definitions for SARI, ILI, encephalitis, hemorrhagic fever will be recruited into the study. We will enroll a total of at least xxxx individuals for clinical studies, which accounts for up to 40% loss from follow-up. Study data will be pooled across sites, as clinical patients are limited by the number of individuals presenting at hospitals.

3.3.b Behavioral and clinical interviews: 2.5 Clinic enrollment and follow-up: We will recruit inpatients and outpatients after initial screening to meet the clinical case definition of 1) severe/acute respiratory illness (SARI/ARI) of unknown etiology; or 2) Influenza-like illness (ILI) of unknown etiology. Once enrolled, biological samples will be collected and a questionnaire administered by trained hospital staff that speak appropriate local language. Samples will be taken concurrently when collecting samples for normative diagnostics. For inpatients, samples will be collected within 10 days of reported onset of illness to increase the chance of PCR detection of viruses (*106*). We will follow up 35 days after enrollment to collect an additional two 500 µL serum samples conduct a standardized questionnaire supplement to collect additional data on the course of symptoms in the interim period. 35 days gives adequate time for development of IgG, which occurs <28 days after onset of symptoms for SARS patients (*107*).

3.3.c Sampling: Following enrollment with signed consent form, biological specimens (two whole blood samples, one max. 500 µL; two 500 µL serum samples) will be collected from all eligible participants, and a questionnaire will be administered. We will investigate five risk factors, so as to maximize the power of the analyses, all related to high risk wildlife exposure, based on continuing analysis of our previous work, and will include: 1) occupation; 2) observed or reported interactions with bats in/around house; 3) proximity to nearby bat roosts; 4) working or regular visit to animal markets; 5) self-reported ILI/SARI. An additional two nasal or oropharyngeal swabs will be collected from syndromic patients. With permission from each clinic, and consent from participants, we will review clinical records to collect data on medical history, clinical syndromes, and patient etiology.

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Commented [PD45]: Need data for Nipah and filovirus patients

3.4 Sample testing: PCR, Serol to link symptoms to etiologic agents

3.5 Assessing potential for pandemic spread: We will…leverage EHA's work with DHS to develop FLIRT (Flight Risk Tracker) that tracks the probable pathways for spread for viruses that are able to be transmitted among people….

From CoV renewal: use data from **3.3** to identify rank SARSr-CoV strains most likely to infect people and evade therapeutic and vaccine modalities. We will use bat survey and zoological data (*87*) to build species distribution models (*108*) and predict the distribution of bat species that harbor low, medium and high risk viral strains. Stacking these modeled distributions for the ~20-30 *Rhinolophus* and related species that occur in the region will allow estimates of SARSr-CoV diversity for a given locality. We will use machine learning models (boosted regression trees) and spatial 'hotspot' mapping approaches to identify the ecological, socio-economic and other correlates of SARSr-CoV diversity and spillover (from serosurveys) (*22, 23, 77*). We will include data from our human behavioral surveys and sampling to give a direct measure of where risk of spillover to people is likely to be highest in the region.

Potential problems/alternative approaches: Patients visiting clinics may have cleared virus, but not yet developed IgG antibodies, reducing seropositive cases. Our 35 day follow-up sampling should avoid this because the maximum lag time between SARS infection and IgG development was ~28 days (*106*). We also expect that patients in rural communities will only visit clinics when symptoms have progressed, likely coinciding with late illness and onset of IgG. We will also have data from our community study, so won't be completely reliant on hospital data to identify PCR- or seropositives. Finally, the risk is outweighed by the potential public health importance of discovering active spillover of a new henipavirus, filovirus or CoV infection.

ALL – the sections below are going to be critical to demonstrate our capacity to do this work. I've not yet started drafting, but please don't hesitate to insert whole sections from other proposals you've done if they're relevant here – we'll need to be creative and get all the language we can from others…

Additional Instructions – Specific to the EIDRC FOA:

Administrative Plan

In a clearly labeled section entitled **"Administrative Plan":**

- Describe the administrative and organizational structure of the EIDCRC Administrative and Leadership Team. Include the unique features of the organizational structure that serve to facilitate accomplishment of the long range goals and objectives.
- Describe how communications will be planned, implemented, and provided to collaborators, teams, or sites. In addition, describe plans to achieve synergy and interaction within the EIDRC to ensure efficient cooperation, communication and coordination.
- Specifically address how the EIDRC Team will communicate with other EIDRCs, the EIDRC CC and NIAID and how the EIDRC will implement plans and guidance from the EIDRC CC to ensure coordination and collaboration across the Emerging Infectious Disease Program as a whole. Describe how the study site will provide information, if any, back to the local health authorities and the study subjects as needed. Note any adjustments or changes needed for rapid and flexible responses to outbreaks in emerging infectious diseases.

List out all of our involvement with outbreak investigations, ministries of health surveillance etc.

- Describe the overall management approach, including how resources will be managed, organized and prioritized, including pilot research projects.
- Highlight how the proposed staffing plan incorporates scientific and administrative expertise to fulfill the goal to develop the flexibility and capacity to respond rapidly and effectively to outbreaks in emerging infectious diseases in an international setting.

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Commented [PD47]: Add data for Nipah and filo infections

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Data Management Plan

In a clearly labeled section entitled **"Data Management Plan":**

- Describe internal and external data acquisition strategies to achieve harmonization of systems and procedures for data management, data quality, data analyses, and dissemination for all data and data-related materials generated by the research study with the EIDRC CC.
- Within the plan, indicate the extent to which dedicated systems or procedures will be utilized to harmonize the acquisition, curation, management, inventory and storage of data and samples. Describe how training for the data and sample collection, in terms of the use of electronic data capture systems, will be provided to all staff including those at enrollment sites.
- Describe the quality control procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens.

This includes sample labeling etc. Get a bar-coding system, everyone has a scanner (cf. PREDICT) – Ralph has this for select agents and MERS. Also need a software system

Clinical Management Plan

In a clearly labeled section entitled **"Clinical Management Plan":**

- Describe plans, processes, and procedures for the following activities: clinical site selection including feasibility, capacity, and capabilities, and study development, conduct, and oversight.
- Describe strategies for oversight and implementation of standardized approaches in the recruitment and clinical characterization of adequate numbers of relevant patient populations to ensure the prompt screening, enrollment, retention and completion of studies. Describe novel approaches and solutions to study enrollment. including the clinical meta-data that will be captured and describe how the staff at each enrollment/collection site will be trained and comply with the quality standards for data and sample collection and compliance with the standardized procedures. Provide a general description of the methods for establishing clinical cohorts and how cohorts may be leveraged for new emerging pathogens.
- Provide general approaches to identification of the types of clinical cohorts needed to fulfill the goals of the EIDRC. Discuss types of clinical cohorts and clinical assessments that will be utilized to study natural history and pathophysiology of disease, including characteristics of the cohort, site for recruitment, types of samples collected and how they can be used for disease surveillance or provide the basis for discovery of novel pathogens, along with a detailed description on how samples can be utilized for immunologic studies, pathogenesis and reagent and diagnostic assay development.
- Describe methods that might be applied to either elucidate a zoonotic source or a vector of transmission.

Our research team incredibly diverse and capable of working with a group of other viral families: Lay out all our additional vector experience incl. my WNV work, our work on Chik etc.
Baric: Flavi, Noro,
Linfa: …
EHA: …

- Describe capacity for and approaches to clinical, immunologic and biologic data and specimen collection, and how these data and specimens will address and accomplish the overall goals and objectives of the research study.

Ralph

1. Norovirus: collaboration with surveillance cohort CDC; first one to publish antigenic characterization of each new pandemic wave virus; and phase 2 and 3 trial of therapeutics
2. Tekada Sanofi Pasteur dengue
3. Nih tetravalent vaccine

- Provide plans for the potential addition of new sites and expanding scientific areas of research when

needed for an accelerated response to an outbreak or newly emerging infectious disease.
Linfa – will launch VirCap platform on outbreak samples.

Statistical Analysis Plan

In a clearly labeled section within the Research Strategy entitled **"Statistical Analysis Plan":**

- Describe the overall plan for statistical analysis of preliminary research data, including observational cohort data.
- Describe the overall staffing plan for biostatistical support and systems available for following functions: 1) preliminary data analyses, 2) estimates of power and sample size, 3) research study design and protocol development.
- Describe the quality control and security procedures for the data and biological specimens, and how to identify and resolve issues with quality control that maintains integrity of data and specimens. Describe any unique or innovative approaches to statistical analysis that may be utilized.

Project Milestones and Timelines

In a clearly labeled section entitled **"Project Milestones and Timelines":**

- Describe specific quantifiable milestones by annum and include annual timelines for the overall research study and for tracking progress from individual sites, collaborators, and Teams. Milestones must specify the outcome(s) for each activity. Milestones should be quantifiable and scientifically justified, and include the completion of major research study activities, including, for example, protocol development, case report forms, scheduled clinical visits, obtaining clearances study completion, and analysis of final data. Milestone criteria should not simply be a restatement of the specific aims. Using a Gantt chart or equivalent tool, describe the associated timelines and identified outcomes for the research study.

Show that for this proposal, we won't be doing the Specific Aims sequentially. We'll begin the human work (SA 2, 3) at the beginning of the project, along with SA1. We'll do the diagnostic assay development initially in Ralph's, Linfa's, Chris's lab, but then begin tech transfer in Y1….

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Dear all – here's the first rough draft.

Please start editing as fast and furious as you all can. I've based this on our previous conversations, so there are lots of bits for you to expand on, insert references etc. Also, you'll see that lots of the text in the Aims is from our recent CoV proposal and will need extending for Henipas, CoVs and Filos, and then editing back on the SARSr-CoV text. Please get stuck into that.

Don't worry about all editing separately – I can collate this, although it is great if some of you go rapidly and others build on theirs.

Main thing – please draft as much as you can, please use TRACK CHANGES, and please don't mess up the ENDNOTE! Prob best for references that you just insert them into comment boxes. It's a bit of a pain, but it's less work than if the whole library decomposes…

Anyway – thanks for being great collaborators, and I look forward to what you send back

NB Supaporn and Tom – please share with your collaborators, and cc me, luke, aleksei and evelyn so I have their email addresses.

Cheers,

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EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that prevent pandemics and promote conservation.

Nipah virus dynamics in bats and implications for zoonotic spillover to humans

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Abstract

Nipah virus (NiV) is an emerging bat-borne zoonotic virus with pandemic potential that causes near-annual outbreaks of fatal encephalitis in South Asia – one of the most populous regions on Earth. In Bangladesh, infection commonly occurs when people drink date palm sap contaminated with bat excreta. However, outbreaks are sporadic and the factors driving their temporal and spatial distribution poorly understood. We analyzed data on host ecology, molecular epidemiology, serological dynamics, and viral genetics to characterize spatio-temporal patterns of NiV dynamics in its wildlife reservoir, *Pteropus medius* bats. We found that NiV transmission occurred throughout the country and throughout the year, but local transmission dynamics were driven by density-dependent transmission, acquired immunity, and recrudescence. Increased transmission followed multi-year periods of declining seroprevalence due to bat population turnover and individual loss of humoral immunity. Individual bats had smaller host ranges than other *Pteropus* spp., which likely contributed to the relatively high diversity of NiV strains we observed, including a Malaysia-clade strain in eastern Bangladesh. We conclude that discrete multi-annual local epidemics in bat populations contribute to the sporadic nature of Nipah virus outbreaks in South Asia. At the same time, the broad spatial and temporal extent of NiV transmission highlights the continued risk of spillover to humans and the importance of limiting human-wildlife contact to reduce zoonotic viral spillover risk.

Keywords: bats, emerging infectious diseases, henipavirus, Nipah virus, *Pteropus medius, Pteropus giganteus,* satellite telemetry, zoonoses, phylogeny, disease dynamics, modeling

Introduction.

Outbreaks of zoonotic diseases are often sporadic, rare events that are inherently difficult to predict, but can have devastating consequences (*1*). Several emerging viral zoonoses with wildlife reservoirs have become pandemics, including HIV/AIDS, SARS coronavirus, and 1918 Pandemic Influenza H1N1 (*2-4*). Bats are important hosts for many zoonotic viruses (*5*) including Ebola virus, SARS-CoV, and Nipah virus, but the ecological drivers and transmission dynamics of these viruses in their reservoir hosts are poorly understood (*6-10*). A better understanding of the transmission dynamics of zoonotic pathogens in their natural reservoirs may help anticipate and prevent outbreaks (*9, 11*).

Nipah virus (NiV) is an emerging zoonotic paramyxovirus (genus *Henipavirus*) that has repeatedly spilled over from bats to cause outbreaks in people and livestock with high case fatality rates across a broad geographic range, making it a significant threat to global health. Nipah virus was first discovered in Malaysia in 1998 where it caused a significant outbreak in pigs and people (*12*). Subsequently it has caused repeated outbreaks in Bangladesh and India, with a mean case fatality rate greater than 70% (*13-15*). A single genus of frugivorous bats (*Pteropus*) appears to be the main reservoir for henipaviruses throughout Asia and Australia (*16-20*), including *Pteropus medius* (formerly *Pteropus giganteus* (*21*)) in Bangladesh and India (*22-25*). Nipah virus has several characteristics that make it a significant threat to human and animal health (*26-28*): 1) Its bat reservoir hosts are widely distributed throughout Asia, overlapping dense human and livestock populations, providing broad opportunity to cause outbreaks; 2) it can be transmitted directly to humans by bats, or via domestic animals; 3) it can be transmitted from person to person; 4) spillover has repeatedly occurred in highly populous and internationally connected regions, giving it pandemic potential; 5) it is associated with high mortality rates in people; and 6) there are currently no commercially available vaccines or therapeutics. As a result, the World Health Organization has listed Nipah virus among the ten most significant threats to global health (*29*). To date, human Nipah virus infections have been identified in India, Bangladesh, Malaysia, Singapore, and the Philippines (*12, 13, 23, 30, 31*). In May 2018, an outbreak of Nipah virus encephalitis associated with a 91% mortality rate, occurred in a new location - Kerala, India - more than 1,100 km southwest of previous Indian and Bangladeshi outbreaks (*32*).

Although the primary pathway of spillover among bats, humans and livestock has been identified, the underlying spatio-temporal dynamics in bats and the extent and importance of genetic variation in the virus has not. Consumption of cultivated food resources contaminated with bat excreta such as mangoes in Malaysia and date palm sap in Bangladesh and India have been identified as the

predominant cause of spillover to pigs and people respectively (*33*). Human outbreaks occur almost annually in Bangladesh and the seasonal timing (November-April) and spatial distribution of outbreaks coincide with patterns of raw date palm sap consumption in a region termed the "Nipah belt" (*34*). However, there is variability in the number of spillover events that occur each year (*35*), and spillover has occasionally occurred outside the predominant season and region of date-palm sap consumption (*36*). The drivers of temporal patterns of viral transmission in bats, including seasonal and year-to-year variation, and the geographic extent of viral circulation within Bangladesh, and indeed within the range of this species (e.g. southern India) are poorly understood, which limits our understanding of how these processes influence spillover into humans.

Previous research on the transmission dynamics of Nipah and Hendra viruses (both in the genus *Henipavirus*) in *Pteropus* spp. bats have produced mixed and sometimes contradictory findings. Nipah virus, like Ebola, Marburg, Hendra and some bat coronaviruses, might be hypothesized to have seasonal spikes in infection that coincide with annual or semi-annual synchronous birth pulses (*16, 37-42*). Seasonal periods of Nipah virus shedding were observed in *P. lylei* in Thailand (*43*), but not in *P. vampyrus* or *P. hypomelanus* in Peninsular Malaysia (*20*). Hendra virus prevalence in Australian pteropid bats has shown multi-year inter-epidemic periods where very little virus can be detected, followed by periods of increased viral shedding, suggesting that viral dynamics are not annual (*44-46*). It has been hypothesized that multi-year periodicity in infection dynamics could arise from a build-up and waning of herd immunity in the reservoir host, with re-introduction of virus via immigration or recrudescence or viral persistence due to large population sizes (*10, 47, 48*). Some pteropid bat species are migratory and interconnected colonies form a metapopulation which could allow for viral re-introductions (*9, 20, 49, 50*). In addition, NiV recrudescence has been observed in *P. vampyrus*, and either of these phenomena could allow it to persist regionally during periods of high local immunity (*51*). However, no study has yet shown evidence in free-ranging bat populations that favors one or the other hypothesis in driving NiV transmission dynamics.

Two distinct clades of NiV have been described: A Bangladesh clade, which includes sequences identified in India and Bangladesh; and a Malaysian clade, which comprises sequences from Malaysia, Cambodia, and Thailand (*52*). Strains of NiV from these two clades are associated with differences in pathogenesis, epidemiological and clinical profiles in humans and animal models and observed shedding patterns in bats (*43, 53-57*). Phenotypic variation in Nipah virus could influence observed human outbreak patterns by altering transmission to, or pathogenesis in, humans, and the likelihood of smaller

outbreaks being identified or reported (*58*). In Bangladesh, human-to-human transmission via contact with respiratory and other secretions has been regularly observed (*59*), whereas transmission among people was not a common feature of the Malaysia outbreak, despite close contact between cases and health care providers (*60, 61*). Primate models and human clinical data indicate that the Malaysia strain is less pathogenic than the Bangladesh strain and has less upper respiratory tract involvement, which may influence transmission (*57*). However, Nipah virus cases in Bangladesh have shown more strain diversity than in the Malaysia outbreak (*62*), and the diversity of NiV strains in *P. medius* has not been well characterized. Characterizing the diversity of NiV in bats may help determine whether more virulent or transmissible genotypes of NiV exist in bats that may eventually emerge in humans (*27, 34*).

The goal of the current study was to understand the distribution and drivers of NiV infection dynamics in *Pteropus medius* in Bangladesh. We examined spatial, temporal and demographic variation in serological dynamics in bats over a six-year period to determine the spatio-temporal drivers and dynamics of virus transmission. We also studied local and long-range movements of individual bats and analyzed NiV phlyogenetics to understand patterns of spatial mixing, and strain diversity.

Results

Cross sectional Nipah virus study of bats inside and outside the Nipah Belt and concurrent longitudinal bat study inside the Nipah Belt (2006-2012)

We caught and tested 883 *P. medius* (approximately 100 per district) from eight different districts across Bangladesh. We detected anti-Nipah IgG antibodies in all colonies (**Figure 1**). Viral detection was rare. Overall, we detected NiV RNA in 11 individuals, 3 pooled oropharyngeal samples, and 21 pooled roost urine samples. We detected viral RNA in bats from four study sites: from individual bats in Faridpur and Rajbari and from pooled samples from Thakurgaon and Comilla. We also detected virus in pooled urine collected from bats associated with human outbreaks in Bhanga and Joypurhat (Table 1). The viral prevalence in Rajbari in January 2006 was 3.8% (95% CI: 0% -11%; n=78). In Faridpur, where we conducted an intensive longitudinal study (see below), viral prevalence estimates ranged from 0% to 3% (95% CI: 0%-10%; n=100 at each of 18 sampling times) (**Table 1** and **Figure 2**). Nipah virus RNA was detected in individual bats from inside (Rajbari, Thakurgaon, and Faridpur) and outside (Comilla) the Nipah Belt (**Figure 1 and 2**). Urine samples provided the highest detection rate. Detection rates in individual bats by sample type were: urine/urogenital swab = 4% (n=2,126); oropharyngeal swab 3%

(n=2,088); and rectal swab = 1.3% (n=79). The estimated detection rate from pooled urine samples across the entire study was 2.7% (+/- 1.6%; n=829).

Factors associated with NiV IgG *serostatus in* P. medius

Among adult and juvenile bats across the cross-sectional study (n=844), including the eight colonies previously described, seropositivity was 2.4 times more likely among adults than juveniles, and 1.6 times more likely among males than females. Seropositivity was higher in pup-carrying (4 times) and pregnant (1.5 times) females (**Figure 2**). Weight or forearm length did not consistently correlate with seropositivity. Seroprevalence varied among sites within regions, but there was no effect on seroprevalence based on being inside or outside of the Nipah belt (**Figure 2).** Finally, serostatus was strongly correlated in mother-pup pairs, with 71/80 pairs (89%) having matching status.

NiV serodynamics over time in a population of P. medius*, Faridpur district (2006-2012)*

We conducted an intensive longitudinal study of NiV serology in a population of bats in the Faridpur district and used flexible generalized additive models (GAMs) to characterize changes over time. There were significant fluctuations in adult and juvenile seroprevalence over the six-year study period (**Figure 3A**). Juvenile seroprevalence ranged from 0% to 44% (95%CI 37%-51%), and decreased over the first year of life for bats born in each year, consistent with loss of maternal antibodies in juveniles. A more pronounced decrease occurred from mid-October to mid-December. However, the GAM indicating this had only marginal better fit (ΔAIC < 1), than one with a linear decrease over the whole year (**Figure 3B**). While the effect of birth cohort was significant on overall seroprevalence.

Adult seroprevalence ranged from 31% (95% CI: 20%-46%) to 82% (95% CI: 77%-87%) and went through three periods of significant increase over the course of the study (**Figure 3A**). We found no evidence of regular seasonal fluctuations; a GAM with annual cyclic terms fit worse than one without (ΔAIC > 10). Each of these periods was preceded by a period of significantly decreasing seroprevalence. Viral RNA detections occurred in periods of both increasing, decreasing, and stable seroprevalence.

We fitted a series of age-stratified mechanistic models to examine different biological processes influencing serodynamics, including density- vs. frequency-dependent transmission, recrudescence, immigration of infected individuals, and seroreversion (loss of antibodies) in both juveniles and adults (**Figure 4**). Density-dependent models were a far better fit to the data than frequency-dependent models (difference in log-likelihood 10.0; ΔAIC = 20.0), suggesting that movements of bats and fluctuations in colony size alter spatio-temporal variation in the risk of NiV epidemic spillover to humans.

In this colony during the period of sampling, the roost population declined from approximately 300 bats to 185, which decreased transmission **(Figure S1)**. In the six-year study period, the number of infected bats increased when the seroprevalence of adults fell below 72% (when bat counts were highest - in 2006) and 52% (when bat counts were lowest). R_0 in adult bats was estimated to decrease from 3.5 to 2.1 as the number of bats in the colony decreased. Serodynamics in juveniles were strongly driven by inheritance and loss of maternal antibodies. The rate of loss of maternal antibodies was 17.6 weeks (95% CI: 13.7-25.0), which was much quicker than the loss of antibodies in adults (290.8 weeks, 95% CI: 245.0-476.4) (**Table S1**). Finally, models with recrudescence fit the data better than models without recrudescence (**Table S1;** difference in log-likelihood 32.6; ΔAIC = 30.6), and models with recrudescence fit the data better than models with immigration (ΔAIC = 3.76).

Mark-recapture and seroconversion/seroreversion

A total of 2,345 bats from the Faridpur/Rajbari region were sampled and microchipped. Between 2007 and 2012 there were 56 recapture events (**Table S2**). Thirty-one bats were recaptured at a roost other than the original capture location. This network of roosts or "roost complex" formed a polygon covering approximately 80 Km^2 and included 15 roosts sampled during the longitudinal study (**Figure S2**). Ten instances of seroconversion (change from IgG negative to IgG positive) and 9 instances of seroreversion (positive to negative) were observed (**Table S2**). The mean time between positive and negative tests in *adults* (excluding juveniles with maternal antibodies) was 588 days (n=6) (range: 124-1,082 days).

Homerange and inter-colony connectivity analysis

Home range analysis of satellite telemetry data from 14 bats showed that the majority of bats roosted within 10 km of where the bats were collared for the Faridpur (Nipah belt) colony, within 7 km of the bats were originally collared for the Cox's Bazaar colony (315km east of Faridpur). The average foraging radius was 18.7 km (s.d. 21.5 km) for the Faridpur bats and 10.8 km (s.d. 11.9 km) for the Cox's Bazaar bats (**Figure S2**). Homerange analysis suggests that bats in Faridpur and Cox's Bazar would have a <5% probability of intermingling (**Figure 5**). Homerange size was significantly larger during the wet season than the dry season (2,746 km^2 vs. 618 km^2) (**Figures S3 & S4**).

Phylogenetic analysis.

Phylogenetic analysis of NiV sequences from a 224nt section of the N gene (nt position 1290-1509 [position ref gb|FJ513078.1| India]) suggests that two clusters are present in Bangladesh bats – one that aligns with previously reported sequences from Bangladesh and India, including the 2018 Kerala outbreak virus (group I), and one that aligns with those previously reported from Malaysia and Thailand (group II) (**Figure 6**). This finding is supported by an analysis of near-whole N gene sequences (~1720 nt) from bats, pigs, and humans, including those from a subset of *P. medius* from this and a more recent study by our group (**Figure S5**) (*63*). Eleven 224nt N gene sequences obtained from bats between 2006 and 2012 (all from the Faridpur population) were identical. Overall, the N gene sequences identified from the Faridpur, Rajbari and Bhanga colonies between 2006 and 2011 had 98.21%-100% shared nucleotide identity. Sequences from Rajbari district obtained in January 2006 and January 2011 had only a single nucleotide difference resulting in a synonymous substitution (G to A) at position 1304, which was found in four other bat NiV sequences from this study, as well as in the NiV isolate from *P. vampyrus* in Malaysia. The five Human NiV N gene sequences collected from various locations within the Nipah belt over the same time period as our bat study show more nucleotide diversity than those from the Faridpur *P. medius* population, but across the country and including in Kerala, India, human NiV sequences were nested within the diversity found in *P. medius* (**Figure 6**). By contrast, the sequences found in Comilla, a location 150Km to the east of Faridpur, showed 80.8%-82.59% shared nucleotide identity with sequences from *P. medius* in Faridpur and clustered within the Malaysia group of NiV sequences. The two Comilla sequences were identical to each other, and had up to 87.95% shared nucleotide identity to sequences from *P. lylei* in Thailand. *Pteropus lylei* bats in Thailand were also found to carry NiV strains from both Malaysia and Bangladesh groups.

Discussion

Our study provided new insights into Nipah virus (NiV) transmission dynamics within bat hosts in Bangladesh, and demonstrates how understanding viral dynamics in wildlife hosts can help explain patterns of outbreaks in people. Firstly, we undertook the most geographically extensive survey of bat roosts in Bangladesh to date, and demonstrated that NiV transmission occurs throughout the country. This has important implications for NiV spillover because previous studies have shown that human NiV outbreaks occur only within a defined region in western Bangladesh, termed the "Nipah belt" (*14, 34, 64*). Our sample testing data show no significant difference in bat NiV seroprevalence between colonies inside and outside the Nipah belt. This suggests infection patterns in bats within the region of human NiV outbreaks are not unique, and that other factors such as differences in intensity or timing of date

palm sap collection are more likely to underpin the heightened spillover risk within the Nipah belt (*34, 36, 58*). We did find variation in seroprevalence among roosts, but these are likely to be a product of the timing of sample collection which was only conducted once in sites other than Faridpur and Comilla.

Secondly, analysis of serological data from our longitudinal study suggests that the underlying mechanism driving the timing of NiV transmission in bats and its spillover to people is the waning of herd immunity in discrete bat populations allowing heightened viral transmission. Our modeling indicates that NiV is primarily driven by density-dependent transmission dynamics among bats, with cycles of higher seroprevalence that would dampen intra-colony transmission followed by waning of antibody titers within individuals. Our large sample size provided the first data from recapture of sampled and released bats leading to the first reported evidence for the loss of detectable NiV IgG antibodies in recaptured individual free-ranging bats. The model outputs suggest that spikes in viral transmission occur after virus is reintroduced into the colony via recrudescence of viral transmission within bats that have previously been infected. This would likely occur in individuals with antibody titers that have waned below a neutralizing titer, and would lead to outbreaks only when overall population-level (herd) immunity drops below a level that would dampen transmission. Previous authors have proposed a number of mechanisms that could lead to maintenance of acute viral infections in bat metapopulations, including synchronous birthing and subsequent loss of maternal antibodies (*10, 38, 40*), lowered immunity within pregnant females due to stress, nutritional stress and other factors (*42*) immigration of infected individuals from other colonies (*49, 65, 66*), and recrudescence within previously-infected individuals (*10, 51, 67*). Our findings and analyses provide strong multi-year data to support the hypothesis that recrudescence and natural loss of immunity due to waning antibodies are the primary cause of NiV transmission spikes within bats, with immigration as another likely factor in maintenance. The consistently lower and decreasing seroprevalence in juveniles suggests that they lose maternal antibodies over their first year, and likely in the first 6-7 months, consistent with other studies of maternal antibodies against henipaviruses in pteropodid bats (*42, 68, 69*). However, our analysis refutes the hypothesis that seasonal pulses of these new seronegative individuals are the primary driver of new outbreaks in adults (*40*). Recrudescence of henipavirus infection has been observed for NiV in captive *P. vampyrus* (*51*), for henipavirus in captive *E. helvum* (*70*), and has also been observed in humans infected by NiV (*71*) and Hendra virus (*72*).

Studying local and migratory bat movements can provide important ecological information related to viral transmission, including how bats move between different colonies (*49*). Our satellite telemetry data suggest that *P. medius* exists as a metapopulation, but we found that *Pteropus medius* appear to travel shorter distances and remain within a smaller home range (321.46km^2 and 2,865.27km^2 for two groups) compared to *P. vampyrus* in Malaysia (64,000 km^2 and 128,000 km^2) and *Acerodon jubatus* in the Philippines which are similarly sized fruit bats (*49, 73*). Pteropodid bat migration is primarily driven by seasonal food resource availability (*50, 74-76*). In Bangladesh, *P. medius* prefer to roost in human-dominated environments in highly fragmented forests (*77*). The anthropogenic colonization and conversion of land over recent human history has likely led to increased food availability for *P. medius* and reduced necessity for long-distance migration (*33*). This may reflect a similar adaptation to anthropogenic food resources as observed over the last few decades in Australian *Pteropus* species (*66*). It may also create a more patchy landscape of viral dynamics and diversity across bat populations in Bangladesh. Less connectivity among flying fox populations on NiV transmission may be longer inter-epidemic periods and larger amplitude fluctuations in population level immunity compared to more migratory species (*66*).

There are limitations to these field studies. Firstly, our study size limits comparison of male and female bat migratory trends, whereas previous studies report that males disperse more often from roost sites to search for new territory, exposing them to different populations (*78*). This phenomenon could affect NiV transmission dynamics as we found that male bats have higher seroprevalence than females, in contrast to other henipaviruses that appear to have no sex-based difference in seroprevalence (*42, 45, 79*). Males may be more likely to be exposed to NiV through territorial bouts with other males at multiple roost sites and mating interactions with females (*78, 80*). Secondly, the observation of bats at multiple roost sites suggests that bats sampled from nearby roosts could be considered part of the same population. It also suggests that changes in roost size, which our models suggest impacts transmission dynamics, could reflect local shifting rather than true fluctuations in population due to wider dispersal – a limitation in our model parameterization where the latter interpretation was used.

Analyzing viral strain diversity in wildlife hosts may have relevance to identifying viral genetic markers associated with specific clinical outcomes in humans. We found that there is more N-gene diversity in bat and human NiV sequences from disparate locations compared to the nearly identical sequences in bats at our longitudinal site over the same six-year period. This suggests that there may be locally prevalent NiV genotypes that persist over time within bat colonies. Human genotype diversity is

297 likely a reflection of the relative diversity of the NiV strains in the local bats where outbreaks begin (*9*).
298 Our report of a NiV strain in *P. medius* from Comilla that clusters phylogenetically within the Malaysia
299 NiV clade corroborates a similar finding of two NiV strains in *P. lylei* in Thailand (*81*). It suggests that
300 there has been historical interaction among bats in eastern Bangladesh and those in Myanmar and
301 Thailand. Genetic studies of *P. medius* show interbreeding between these two species (Olival *et al*., in
302 review.). Connectivity among bat populations in eastern Bangladesh, Myanmar and Thailand may
303 explain the genetic diversity observed between NiV sequences from Faridpur and Comilla. Malaysia and
304 Bangladesh NiV strains have been associated with differences in pathogenesis, epidemiological and
305 clinical profiles in humans and animal models. Animal models as well as human clinical data indicate
306 strain differences among Bangladesh and Malaysia NiV, with the latter being less pathogenic, resulting in
307 less oral secretion and upper respiratory tract involvement – factors that may influence transmission
308 (*53-57, 82*). The N gene of the Comilla NiV strain differs from those reported from bats in the Nipah belt
309 by 20%, whereas NiV Malaysia and NiV Bangladesh differ by 6-9% despite different clinical
310 outcomes. Therefore it is plausible that the clinical profile of a 20% divergent NiV strain differs
311 significantly from known strains. Mild or asymptomatic cases would be unlikely to be detected by
312 national surveillance systems and it's possible that cryptic spillover has occurred in Bangladesh (*62*).
313 Future studies comparing NiV genotypes with clinical and epidemiological outcomes may provide
314 important insights about the ability of specific strains found in bats to cause significant outbreaks.
315 Finally, our study may explain the sporadic nature of NiV outbreaks with multi-year inter-
316 epidemic periods in South Asia. Firstly, PCR tests show that overall NiV incidence in *P. medius* is low,
317 similar to previous studies of Hendra virus (*42, 45, 79*). The data and our modeling suggests that PCR-
318 positive samples are only likely to be identified during viral transmission spikes after periodic
319 reintroduction into populations that have become susceptible through waning population-level
320 immunity (*10*). This is likely a rare or at least sporadic event. In the current study, observed
321 seroprevalence patterns and the fitted model suggest that three periods of transmission occurred over
322 the 6 years of sampling, each of which followed periods of low adult seroprevalence, though not all
323 measurements of low seroprevalence were followed by outbreaks. We detected NiV RNA during
324 periods of both increasing and decreasing seroprevalence, supporting the fitted model which suggested
325 that shedding can occur at low levels in bats even in periods without sustained transmission. Our
326 observation that not every instance of rising seroprevalence resulted in detectable viral shedding
327 suggests that not all episodes of viral circulation in bats are of equal magnitude, and that other factors
328 (e.g. variation in human-bat contact and exposure) may affect likelihood of spillover. Together, this

evidence suggests that outbreaks can occur in bats when the population falls below a protective threshold of immunity in any season, but variability in how many bats become infected may impact the likelihood of spillover to humans, assuming a route of transmission is available. This could explain variation in the number of human outbreaks from year-to year in Bangladesh. Thus, the timing of multiple factors involved in driving transmission dynamics needs to align for intra-colony NiV transmission events and further align with human behavior and availability of a route of spillover for human outbreaks to occur, as previously hypothesized (*83*). This, and the seasonality and specific geography of date palm sap consumption in Bangladesh likely explains the somewhat sporadic nature of human outbreaks in the region, albeit that when spillover occurs, it is within the well-defined date palm sap collection season and geographic zone (*34*).

These findings suggest that Nipah virus outbreaks in other regions where *Pteropus* spp. bats occur, are also likely to be sporadic and rare, leading to under-reporting or a lack or reporting, particularly given that human neurologic symptoms are similar to other common infections, e.g. Japanese encephalitis, malaria, measles (*84*). Our work and other reports suggest that Nipah virus transmission is possible wherever *Pteropus* spp. bats and humans live in close association and at any time of year, provided there is an available route of transmission, as illustrated by the May 2018 outbreak in Kerala, India – more than 1100km from Bangladesh and prior Indian outbreaks (*15*). In countries where NiV surveillance occurs, the absence of a sufficient bat-human interface may explain the lack of cases. However, improving capacity for NiV surveillance where it is enzootic is crucial for determining whether cryptic human infections are indeed occurring – especially if it is a relatively rare event (*85*). Interventions should focus on raising awareness of the potential for contamination of fruit or date palm sap in regions where pteropid bats are present, and in protecting food resources to limit the opportunity for human or livestock exposure to Nipah virus. These interventions are critical to reducing the risk of a more transmissible strain of Nipah virus, or a yet undiscovered virus, from emerging and causing an epidemic with significant human and animal mortality.

Methods

The study period was between January 2006 and November 2012. The study was conducted under Tufts University IACUC protocol #G929-07. Locations were selected based on whether the district had any previously recorded human NiV encephalitis clusters at the time of this study (Rajbari, Tangail, Thakurgaon, and Kushtia) or whether they had not (Comilla, Khulna, Sylhet, and Chittagong) so that infection rates could be compared between regions inside and outside of the "Nipah Belt." The Thakurgaon study was conducted as part of an NiV outbreak investigation, and coincided with ongoing human transmission (*86*). Between 2006-2012, three different studies of *Pteropus medius*, with similar bat sampling protocols were performed: 1) a cross-sectional spatial study with a single sampling event in each of the eight locations listed above; 2) a longitudinal study of a Faripur bat colony with repeated sampling approximately every 3 months from July 2007 to November 2012; and 3) a longitudinal study of the Rajbari colony with repeated sampling at a monthly interval between 12 month period between April 2010 and May 2011. Opportunistic sampling of *Pteropus medius* was also performed during this time period during NiV outbreak investigations [Bangha, Faridpur (Feb 2010), Joypurhat (Jan 2012), Rajbari (Dec 2009), West Algi, Faridpur (Jan 2010)]. These data are included here because NiV testing of bat samples during these investigations supported the aims of the study. Bats were captured using mist nets at locations within eight different districts across Bangladesh between January 2006 and December 2012 (**Figure 1**).

Capture and sample collection

For the country-wide cross-sectional and Faridpur longitudinal study, on average, 100 bats were sampled at each sampling event, which lasted 7-10 days. This sample size allowed us to detect at least one exposed bat (IgG antibody-positive) given a seroprevalence of 10% with 95% confidence. Bats were captured using a custom made mist net of approximately 10 m x 15 m suspended between bamboo poles which were mounted atop trees close to the target bat roost. Catching occurred between 11 pm and 5 am as bats returned from foraging. To minimize bat stress and chance of injury, nets were continuously monitored and each bat was extracted from the net immediately after entanglement. Bats were placed into cotton pillow cases and held for a maximum of 6 hours before being released at the site of capture. Bats were sampled at the site of capture using a field lab setup. Bats were anesthetized using isoflurane gas (*87*) and blood, urine, oropharyngeal swabs were collected. For some sampling periods, rectal swabs were collected but due to resource constraints, these samples were deemed to likely be lower yield than saliva and urine for NiV, and therefore were not collected consistently. For

each bat sampled we recorded age, weight, sex, physiologic and reproductive status, and morphometric measurements as described previously (*22*). Bats were classified as either juveniles (approximately four to six months - the age by which most pups are weaned) to two years old (the age when most *Pteropus* species reach sexual maturity) or adults (sexually mature) based on body size and the presence of secondary sexual characteristics, pregnancy, or lactation - indicating reproductive maturity (*22, 88*). Up to 3.0 mL of blood were collected from the brachial vein and placed into serum tubes with serum clot activator (Vaccutainer, USA). Blood tubes were stored vertically on ice packs in a cold box and serum was allowed to separate overnight. Serum was drawn from the tube after 24 hours and placed in a screw-top cryovial (Corning, USA) and stored in a liquid nitrogen dewar (Princeton Cryogenics, NJ, USA). Sterile pediatric swabs with polyester tips and aluminum shafts were used to collect urogenital and rectal samples, and larger polyester swabs with plastic shafts (Fisher, USA) were used to collect oropharyngeal samples. All swabs were collected in duplicate, with one set being placed individually in cryotubes containing lysis buffer (either tri-reagent or NucliSENS Lysis buffer, BIOMERIEUX, France) and the second set in viral transport medium. All tubes were stored in liquid nitrogen in the field then transferred to a -80C freezer.

During each sampling event, pooled urine samples were collected beneath bat roosts using polyethylene sheets (2' x 3') distributed evenly under the colony between 3 am and 6 am. Urine was collected from each sheet either using a sterile swab to soak up droplets, or a sterile disposable pipette. Swabs or syringed urine from a single sheet were combined to represent a pooled sample. Each urine sample was divided in half and aliquoted into lysis buffer or VTM at an approximate ratio of one part sample to two parts preservative.

Serological and molecular assays

Sera from the cross-sectional survey were heat inactivated at 56^{o}C for 30 minutes as described (*89*) prior to shipment to the Center for Infection and Immunity at Columbia University (New York, USA) for analysis. Samples were screened for anti-NiV IgG antibodies using an enzyme linked immunosorbent assay (ELISA) as described in (*22*). Sera from the longitudinal studies were sent to the Australian Animal Health Laboratory and were gamma irradiated upon receipt. Because of the large sample size and development of a high throughput multiplex assay of comparable specificity and sensitivity, for these samples we used a Luminex-based assay to detect anti-Nipah G IgG antibodies reactive to a purified NiV soluble G protein reagent (*90, 91*).

Total nucleic acids from swabs and urine samples were extracted and cDNA synthesized using Superscript III (Invitrogen) according to manufacturer's instructions. A nested RT-PCR and a real-time assay targeting the N gene were used to detect NiV RNA in samples (*92*). A RT-qPCR designed to detect the nucleocapsid gene of all known NiV isolates was also utilized (*93*). Oligonucleotide primers and probe were as described (*93*). Assays were performed using AgPath-ID One-StepRT-PCR Reagents (Thermofisher) with 250 nM probe, 50 nM forward and 900 nM reverse primers. Thermal cycling was 45◦C for 10 min, followed by 45 cycles of 95◦C for 15 s and 60◦C for 45 s. Cut-off values were cycle threshold (C_T) ≤40 for positive and CT ≥45 for negative. Results with C_T values between 40 and 45 were deemed indeterminate, i.e. not conclusively positive or negative. Samples with NiV RNA detected by real time PCR were confirmed by gel electrophoresis and product sequencing.

A subset of NiV-positive samples was processed by high-throughput sequencing (HTS) on the Ion Torrent PGM platform in order to obtain additional NiV genomic sequence. Libraries were prepared according to the manufacturer's instructions, and 1 million reads allocated per sample. HTS reads were aligned against host reference databases to remove host background using bowtie2 mapper, and host-subtracted reads primer trimmed and filtered based on quality, GC content and sequence complexity. The remaining reads were de novo assembled using Newbler (v2.6) and mapped to the full length NiV genome. Contigs and unique singletons were also subjected to homology search using MegaBlast against the GenBank nucleotide database, in case variance in parts of the genome precluded efficient mapping. From these data, N, P, and G gene consensus sequences were constructed using Geneious v 7.1, and used for phylogenetic analyses.

Phylogenetic analysis

All *P. medius* NiV sequences will be submitted to Genbank and included in supplemental material upon acceptance for publication. Sequence alignments were constructed using ClustalW in Geneious v7.1 software (*94*). Phylogenetic trees of NiV N-gene, P-gene, and G-gene sequences were constructed using Neighbor-Joining, Maximum-Likelihood algorithms in MEGA v. 5 (*95*) and figures constructed in FigTree 1.4.2.

Satellite telemetry and homerange analysis

We developed a collar system to attach 12g solar-powered Platform Terminal Transmitters (PTT) (Microwave Telemetry, Colombia, MD, USA) to adult bats using commercial nylon feline collars with the buckle removed and 0-gauge nylon suture to attach the PTT to the collar and to fasten the collar around

the bat's neck. Collars were fitted to the bat such that there was enough space to allow for normal neck movement and swallowing, but so that the collar would not slip over the head of the animal (**Figure S6**). PTTs were programmed with a duty cycle of 10 hours on and 48 hours off. Data was accessed via the Argos online data service (argos-system.org; France). A total of 14 collars were deployed as follows: Feb 2009: 3 males, 3 females from a colony in Shuvarampur, Faridpur district; Feb 2011: 3 males, 2 females from the same colony; and April 2011 Cox's Bazaar, 3 bats from a colony in Cox's Bazaar, Chittagong district. Adult bats were selected based on size such that the total weight of the collar (~21g) was less than 3% of the bat's body mass.

The individual telemetry dataset was combined for each region and its aggregate utilization distributions (UD) computed in R using package 'adehabitatHR' (*96*). Population-specific home range is represented by the *95% area enclosure of its UD's volume. The volume of intersection between the colonies quantifies the extent of home range overlap. To evaluate the potential for contact with the Sylhet colony, we calculated the most likely distance moved ('mldm') for each sampled bat at Faridpur where the population was more intensively monitored. Movement distance was measured in kilometers with respect to a centroid location (ω) shared by the whole colony; assuming random spatial distribution in tracking rate, we estimated its kernel density function per individual and defined mldm as the mode.

Statistical approach – cross-sectional study

We fit a full Bayesian generalized linear model with a logit link and a Bernoulli distribution to identify potential predictors which influenced a bat's serostatus. We included age, sex, age- and sex-normalized mass and forearm length, mass:forearm ratio, body condition, and whether the bat was pregnant, lactating, or carrying a pup. We included location of sampling a random effect nested within Nipah Belt or non-Nipah Belt regions. We fit the models and performed posterior predictive checks in R 3.4.3 , using the **brms** and **rstan** packages.

Statistical approach – longitudinal study

We fit binomial general additive models (GAMs) (*97*) to the time series of adult and juvenile seroprevalence in the longitudinal study. For juveniles, we modeled the birth cohort of bats as separate random effects in a pooled model of juveniles' dynamics starting from June of their birth year, June being the earliest month we sampled free-flying juveniles in any cohort. We determined the cohort year of juveniles by using cluster analysis to group individuals by weight, assuming those in the smallest group were born in the year prior to sampling and those in the larger group were born the previous

year. 92% of juveniles captured were yearlings. For adults, we modeled dynamics of adults as a single pool over the entire course of the study. We tested models with and without annual cyclic effects.

Where time series had significant temporal autocorrelation (adults only), we aggregated data by week. We determined periods of significant increase in decrease as those where the 95% confidence interval of the GAM prediction's derivative did not overlap zero. We fit the models and performed checks in R 3.4.3, using the **mgcv** package.

We fit an age-structured (adult and juvenile) maternally immune (M)-susceptible (S)-infected (I)-recovered (R) model with recrudescence (R to I) and loss of immunity (R to S) to the seroprevalence data on a weekly timescale:

$$\frac{dS_J}{dt} = -S_J(\beta_{JJ}I_J + \beta_{AJ}I_A) - \mu_J S_J + bN_A(t-5)\frac{S_A}{N_A} - bN_A(t-52)\frac{S_J}{N_J}(1-\mu_J)^{52} + \lambda M_J$$

$$\frac{dI_J}{dt} = S_J(\beta_{JJ}I_J + \beta_{AJ}I_A) - \gamma I_J - bN_A(t-52)\frac{I_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dR_J}{dt} = \gamma I_J - \mu_J S_J - \mu_J R_J - bN_A(t-52)\frac{R_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dM_J}{dt} = bA(t-5)\frac{R_A}{N_A} - \lambda M_J - \mu_J M_J - bN_A(t-52)\frac{R_A}{N_A}(1-\mu_J)^{52}\lambda^{52}$$

$$\frac{dS_A}{dt} = -S_A(\beta_{AA}I_A + \beta_{JA}I_J) - \mu_A S_A + \tau R_A + bN_A(t-52)\frac{S_J}{N_J}(1-\mu_J)^{52}$$

$$\frac{dI_A}{dt} = S_A(\beta_{AA}I_A + \beta_{JA}I_J) - \gamma I_J - \mu_A I_A + bN_A(t-52)\frac{I_J}{N_J}(1-\mu_J)^{52} + \Delta R_A$$

$$\frac{dR_A}{dt} = \gamma I_A - \mu_A R_A + bN_A(t-52)\frac{R_J}{N_J}(1-\mu_J)^{52} - \tau R_A - \Delta R_A$$

We included a class M for the density of juvenile bats with maternal antibodies to allow for the biological possibility that maternal antibodies are lost at a much higher rate than antibodies acquired following infection. The subscripts J refer to juveniles and A to adults, β is the transmission rate, γ is the recovery rate, μ is the mortality rate, τ is the rate of loss of adult immunity, λ is the rate of loss of maternal antibodies(*68*), Δ is the adult recrudescence rate (R to I), b is the birth rate (pups join the juvenile population after 5 weeks). Juveniles transition to adults after 52 weeks. We included terms for loss of antibody in adults (τ, S to R), and viral recrudescence (Δ, R to I) based on previous studies on captive bats that demonstrated the existence of these processes without providing enough data to characterize them precisely (*51, 69*). We fit this deterministic model to the seroprevalence data by maximum likelihood, which assumes that deviations from the model are due to observation error. We estimated the

confidence intervals around maximum likelihood parameter estimates using likelihood profiles using the *profile* function in package *bbmle* in R v3.2.2.

We used model fitting and model comparison to examine the need for several of the biological processes in the model above that could influence NiV dynamics. First, we examined both density and frequency-dependent transmission by comparing the fit of the model above to one with transmission terms that have population size (N_A or N_J) in the denominator. Second, we examined the confidence intervals of the parameters describing viral recrudescence, loss of antibodies in adult bats, and loss of antibodies in juvenile bats. If the confidence bounds for these parameters included zero, then these biological processes are not needed to explain the serological dynamics. Finally, we examined the confidence bounds for parameters describing the loss of maternal and non-maternal antibodies (τ and λ), to determine if the rate of loss of these two types of immunity were different. We note that this model structure has similarities to a susceptible-infected-latently infected(L)-infected (SILI) model (if latently infected individuals are seropositive), but the model above differs in allowing for the possibility of individuals to transition from the R class back to the S class.

Code availability

SIR model code written in R is available upon request.

Data availability

All molecular sequences will be available via Genbank. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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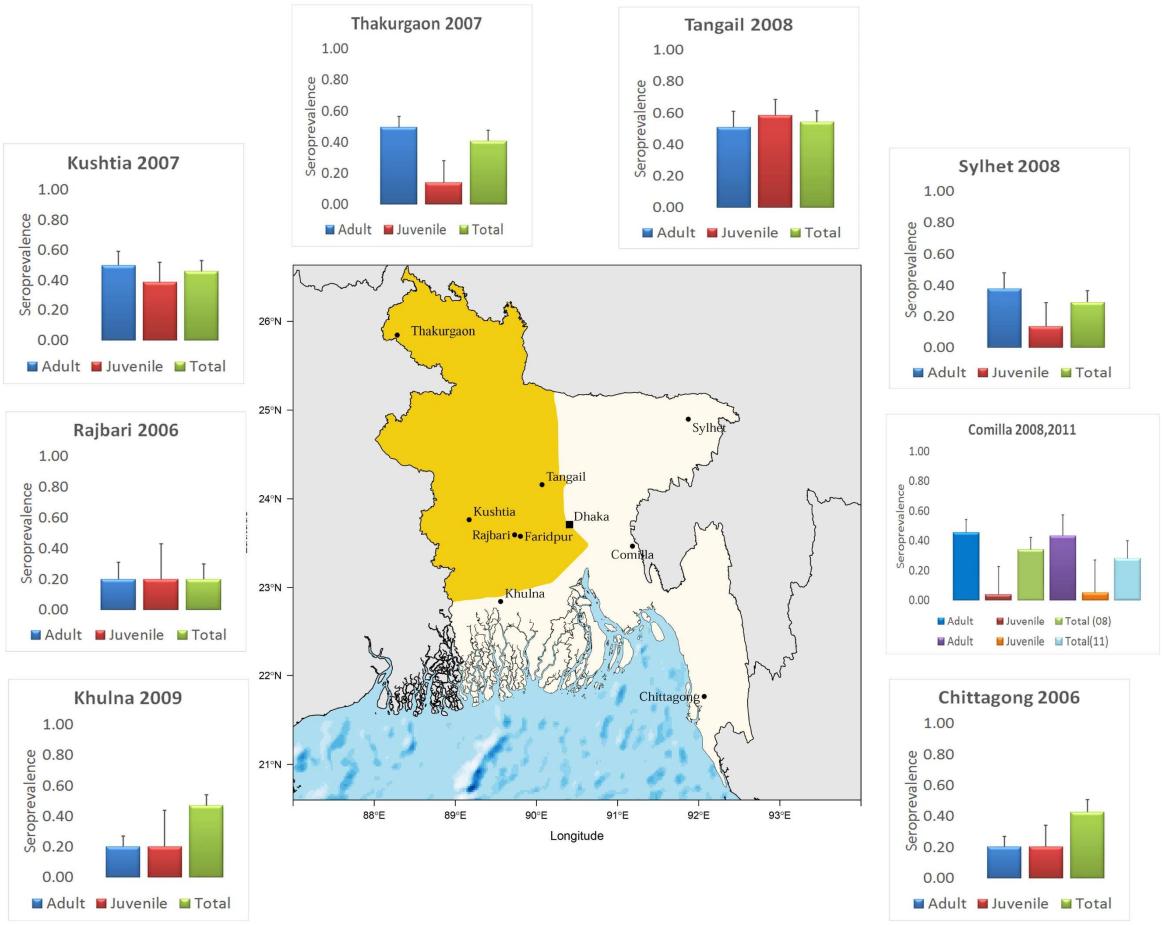
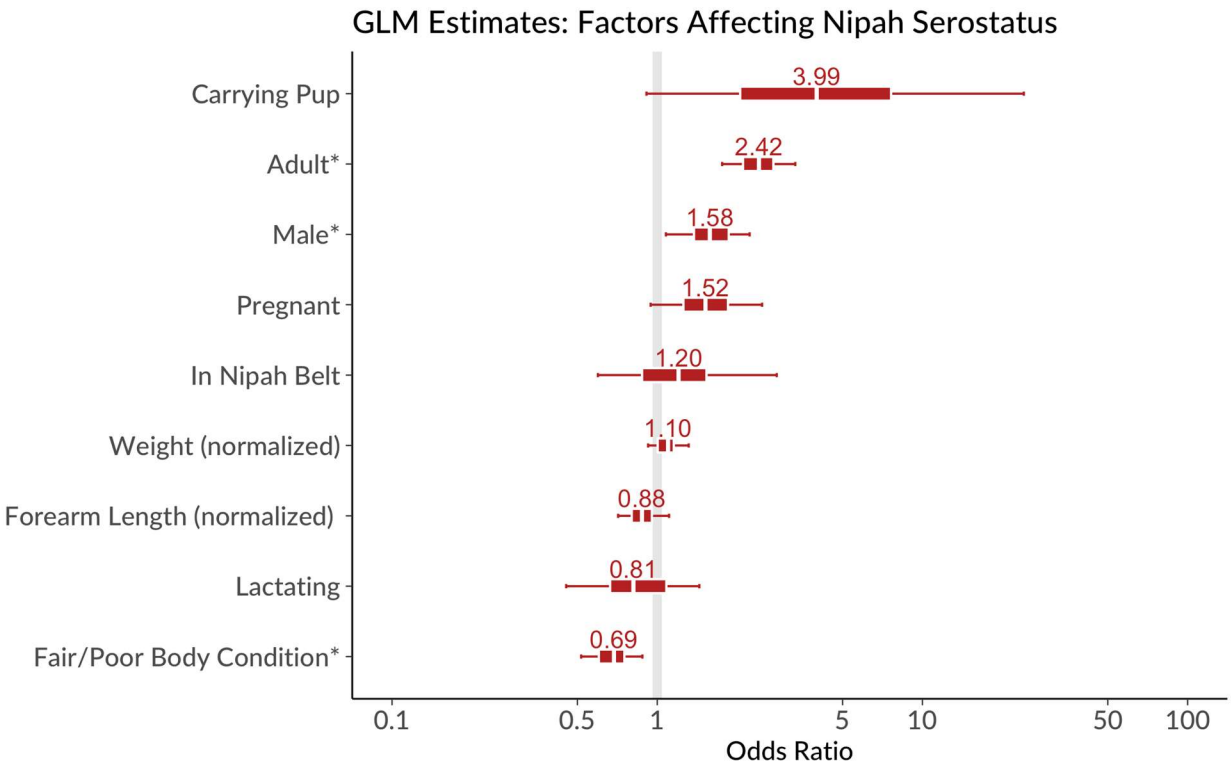


Figure 1. Map showing age-stratified seroprevalence in *Pteropus medius* colonies, Bangladesh. Bats from eight colonies were sampled and tested for anti-NiV IgG antibodies: four within the "Nipah Belt" (orange shaded) and four outside. Seroprevalence of adults (blue, purple), juveniles (red, orange) and total seroprevalence (green, light blue) are shown. Number (n) of Adult, Juvenile, and Total bats sampled (clockwise): Tangail [53,41,94], Sylhet [63, 37, 100], Comilla 2008 [73,27,100], Comilla 2011 [30,20,50], Chittagong [72,24,96], Khulna[85,15,100], Rajbari [65,15, 80], Kushtia [64,36,100], Thakurgaon [89,29,118]. The shaded region represents the "Nipah Belt" where previous human NiV outbreaks have been reported. Seroprevalence varied by location ($\chi^2 = 55.61$, $p<.001$), but there was no statistical difference between seroprevalence in bas inside the Nipah Belt and outside. Adult bats had equal or greater seroprevalence than juveniles in each location.



781

782 **Figure 2.** Results of Bayesian GLM of factors affecting Nipah serostatus in bats in cross-sectional study. Bars
783 indicate odds ratios and 50% (inner) and 95% (outer) credible intervals for model parameters. Factors with
784 asterisks (*) have 95% CIs that do not overlap 1. Model intercept (predicted probability of seropositivity for a
785 juvenile, female, bat outside the Nipah belt of mean size and good body condition) was 0.26, (95% CI 0.12-0.56).
786 Random effect of location (not shown) had an estimated SD of 0.63 (95% CI 0.29-1.27)

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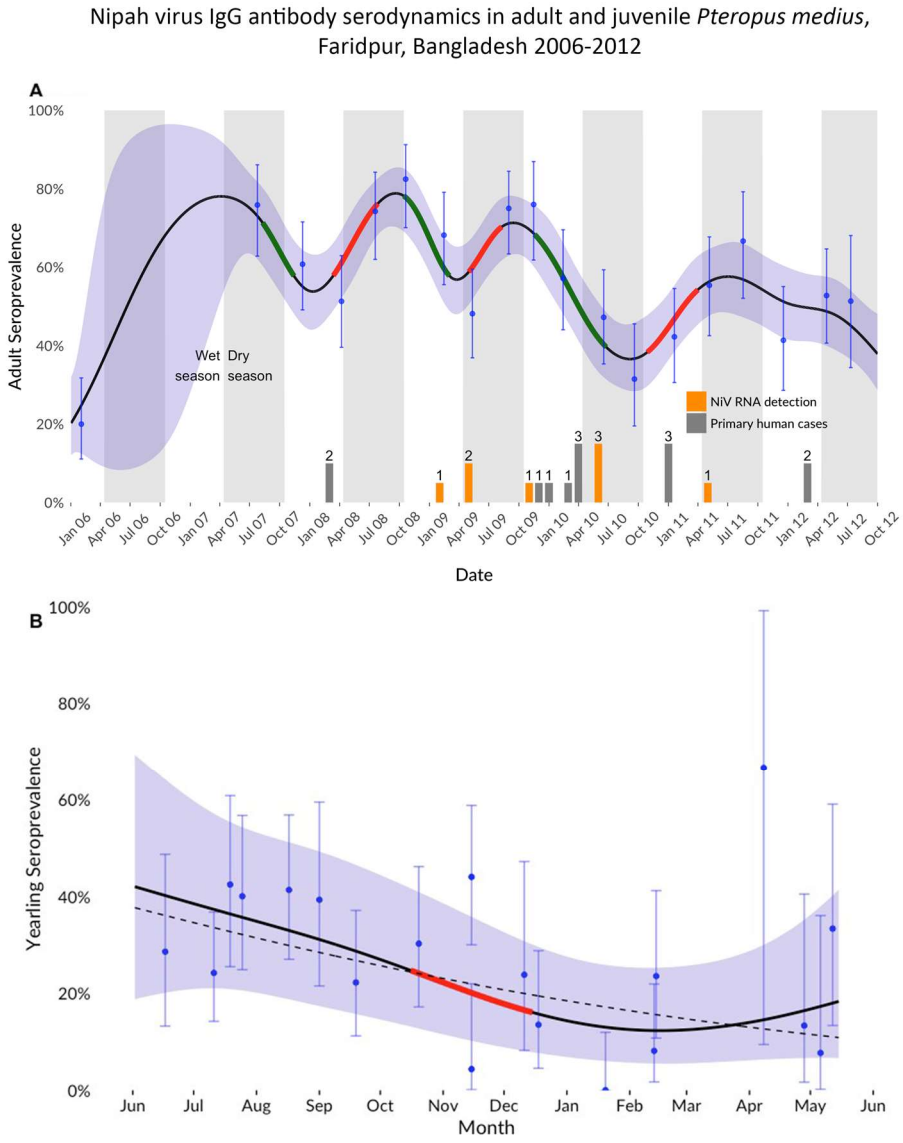

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789 **Figure 3 A & B.** Serodynamics of the Faridpur bat population. (A) Adult serodynamics, with measured values and
790 95% CI in blue, and mean GAM prediction and 95% shown with line and surrounding shaded areas. Periods of
791 significant increase (red) and decrease (green) shown where the GAM derivative's 95% CI does not overlap zero.
792 Counts of primary human cases from local district (orange, and bat viral detections (dark grey, see Table 1), shown
793 on bottom. (B) Juvenile serodynamics during the first year of life, with all years' measurements overlain to show
794 cohort-level dynamics across all years. Measured values and 95% CI in blue, and mean and 95% CI for the GAM
795 model pooled across cohorts shown with line and surrounded shaded areas. The period of significant decline in
796 the GAM is shown in red. Also shown is the mean prediction of a model with only a linear mean term, with similar
797 fit (ΔAIC < 1) as the GAM (dotted line).

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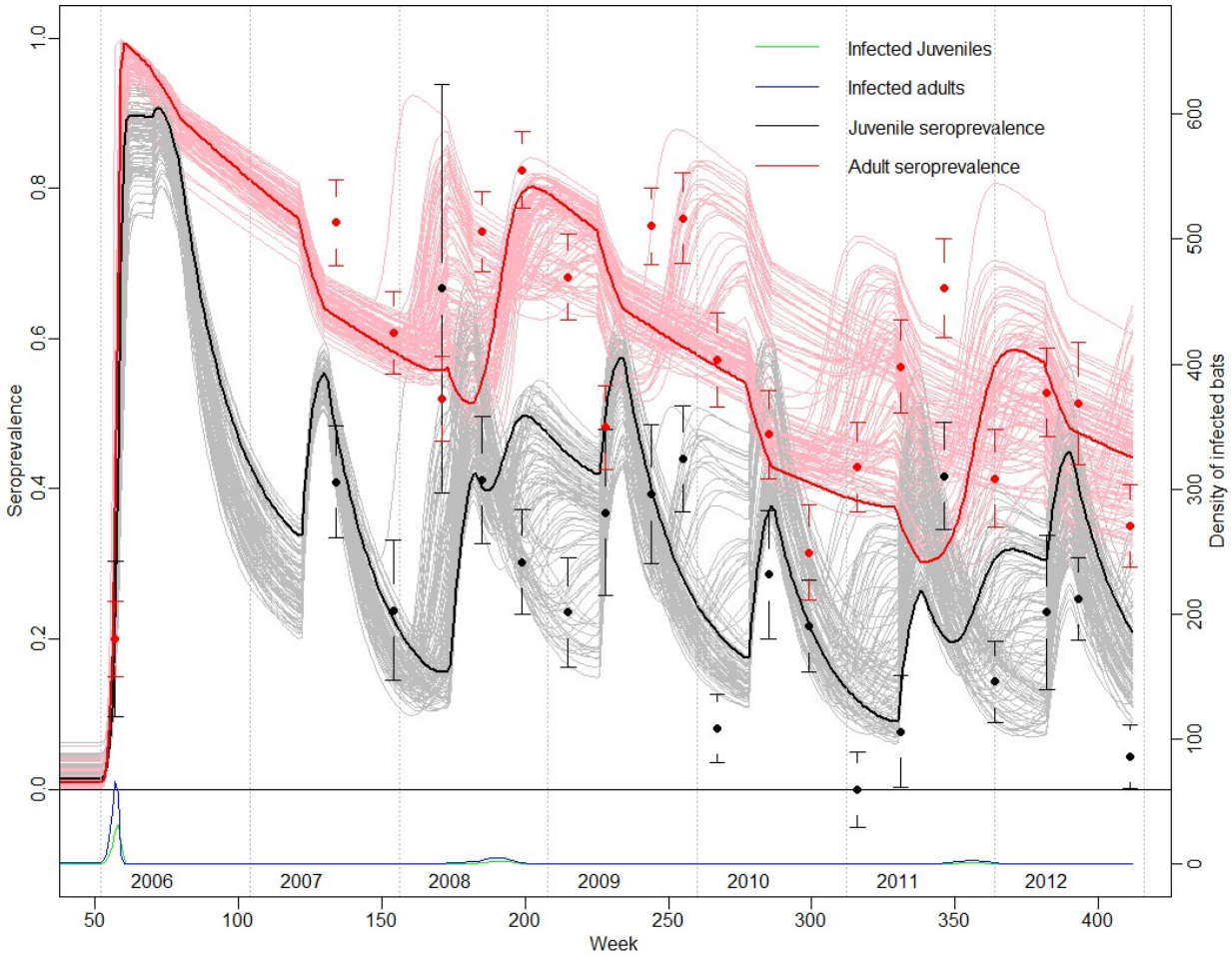


Figure 4. **NiV serological dynamics in adult and juvenile bats.** The observed data (red and black points ±1 SE) and model fit (solid lines) for the fraction of adults and juveniles seropositive for NiV (left axis), and the model estimated density of infected adult and juvenile bats (bottom panel and right axis). See Methods for details of model structure.

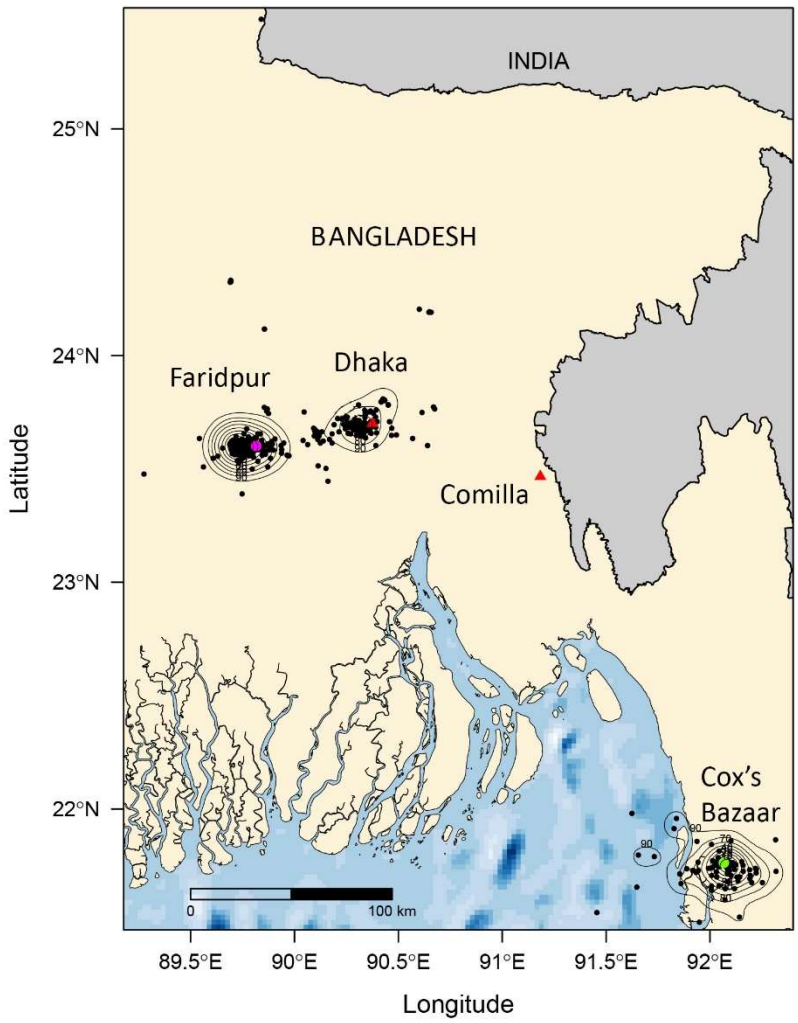


Figure 5. Satellite telemetry and homerange analysis. Location data from satellite collars (n=14) placed on 11 bats from Faridpur and 3 bats from Cox's Bazaar, Chittagong collected between 2009 and 2011, were used to calculate local and long-range movement patterns and home range for these two groups.

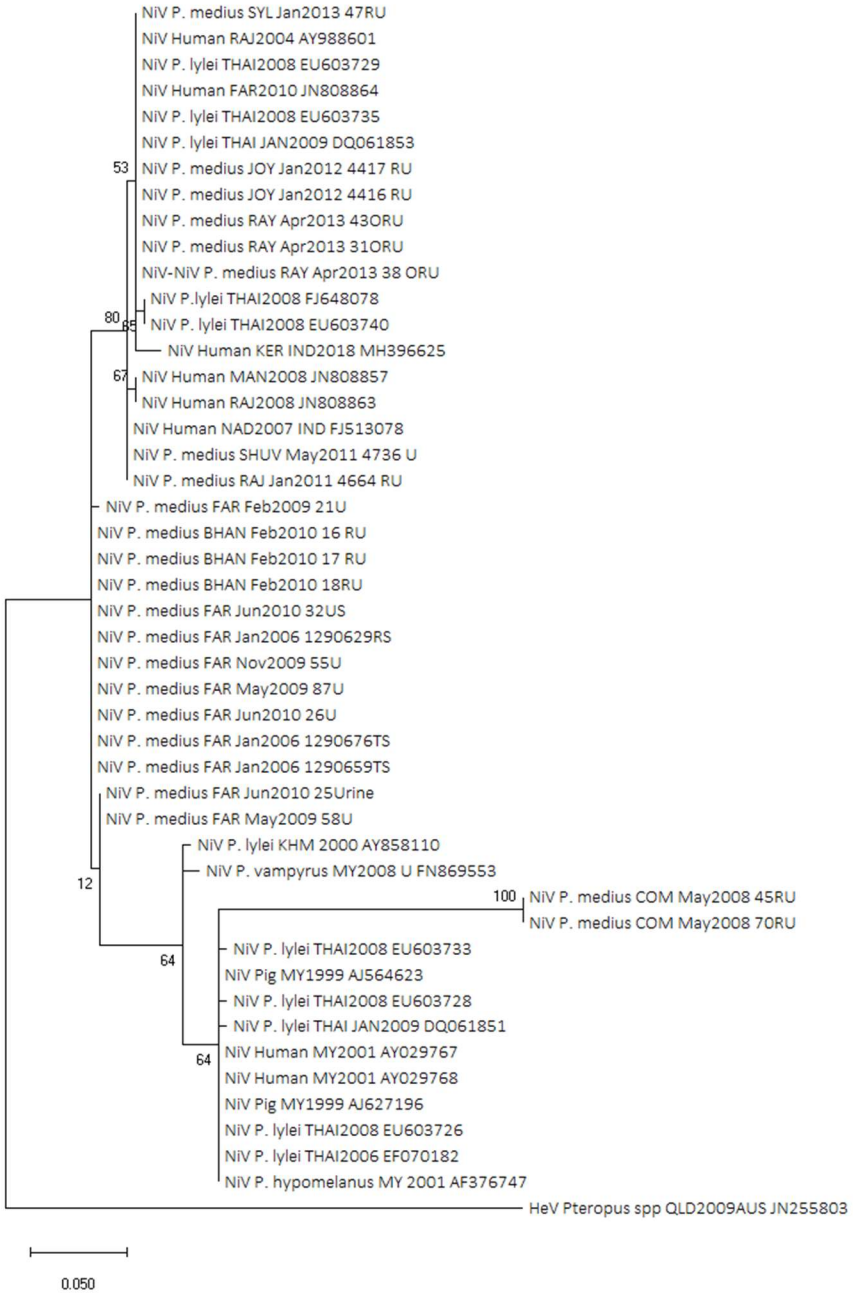


Figure 6. **Nipah Virus N gene phylogenetic tree (224nt):** Tree created in MEGA X using the Maximum Likelihood method and Tamura-Nei model (*98*). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Hendra virus was used as an outgroup. Evolutionary analyses were conducted in MEGA X (*99*). Genbank accession numbers are included in the label for each sequence.

863 **Table 1**. PCR detection of NiV RNA in *Pteropus medius* 2006-2012.

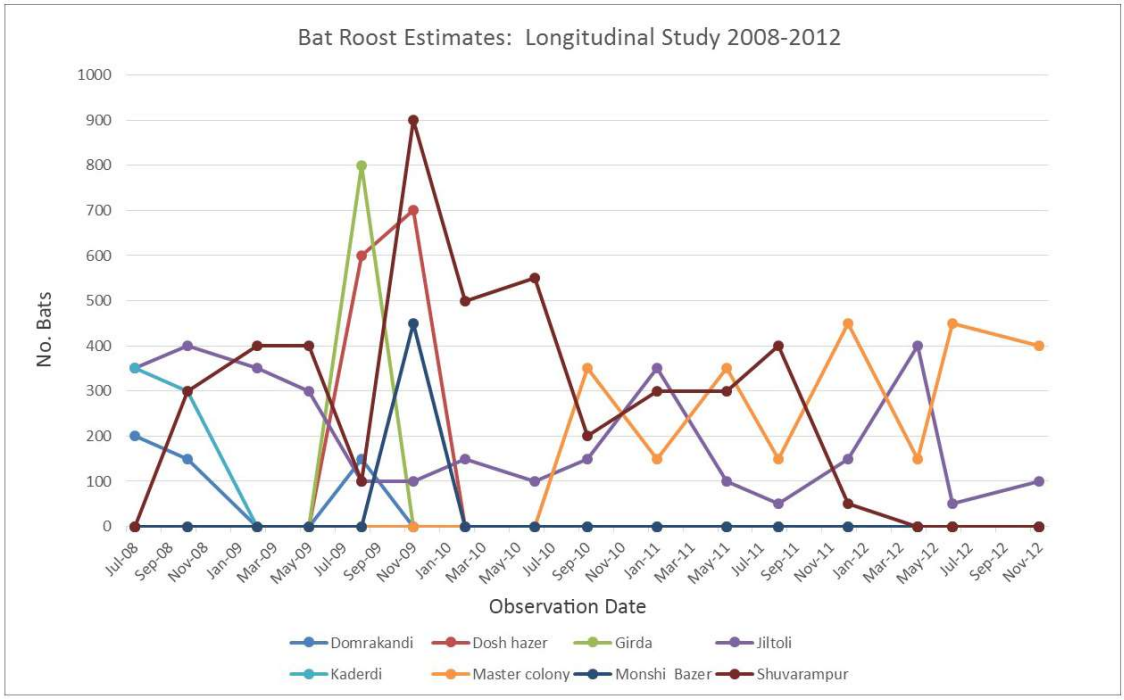
Location	date	Bats Sampled	Throat Tested	Throat Pos	Urine Tested	Urine Pos	Rectal Tested	Rectal Pos	Paired samples	Pos. Bats	Bats w multi pos samples	prev.	95% CI	Roost Urine	Roost Urine pos.
Spatial Study															
Rajbari	Jan-06	99	79	**3**	78	0	79	**1**	78	3	**1**	**0.04**	**0.11**	-	-
Thakurgaon	Mar-07	118	115	**3***	72	0	-	-	70	unk.	0	0.00	-	-	-
Kushtia	Aug-07	101	100	0	99	0	-	-	98	0	0	0.00	-	-	-
Tangail	Jun-08	100	61	0	77	0	-	-	60	0	0	0.00	-	81	0
Chittagong	Aug-06	115	19	0	-	-	-	-	-	0	-	-	-	-	0
Comilla	May-08	100	0	0	50	0	-	-	0	0	-	-	-	100	**2**
Sylhet	Sep-08	100	100	0	49	0	-	-	48	0	0	0.00	-	100	0
Khulna	Jan-09	100	50	0	80	0	-	-	32	0	0	0.00	-	50	0
Comilla	Mar-11	50	50	0	50	0	-	-	0	0	0	0.00	-	-	-
Outbreak Investigation															
Bangha	Feb-10													19	3
Joypurhat	Jan-12													19	**16**[α]
Rajbari	Dec-09													35	0
West Algi	Jan-10													31	0
Longitudinal Study															
Faridpur	Jul-07	102	64	0	50	0	-	-	22	0	0	0.00			
Faridpur	Dec-07	101	N/A	N/A	N/A	-	-	-		0					
Faridpur	Apr-08	100	64	0	88	0	-	-	54	0	0	0.00		51	0
Faridpur	Jul-08	100	58	0	74	0	-	-	54	0	0	0.00			
Faridpur	Oct-08	100	98	0	99	0	-	-	98	0	0	0.00			
Faridpur	Feb-09	100	50	0	100	**1**	-	-	49	**1**	0	**0.01**	**0.10**	50	0
Faripdur	May-09	101	100	0	99	**2**	-	-	99	**2**	**0**	**0.02**	**0.10**	9	0
Faridpur	Aug-09	100	100	0	99	0	-	-	95	0	0	0.00		3	0
Faridpur	Nov-09	100	100	0	82	**1**	-	-	82	**1**	**0**	**0.01**	**0.11**	50	0
Faridpur	Feb-10	100	100	0	100	0	-	-	100	0	0	0.00		45	0
Faridpur	Jun-10	100	100	0	100	**3**	-	-	100	**3**	**0**	**0.03**	**0.10**	25	0
Faridpur	Sep-10	100	100	0	100	0	-	-	-	0	-	-		20	0
Faridpur	Jan-11	100	100	0	100	0	-	-	0	0	0	0.00		15	0
Faridpur	May-11	102	102	0	102	**1**	-	-	0	**1**	0	**0.01**	**0.10**	20	0
Faridpur	Aug-11	100	100	0	100	0	-	-	-	0	-	-		10	0
Faridpur	Dec-11	100	100	0	100	0	-	-	-	0	-	-		16	0
Faridpur	Apr-12	100	78	0	78	0	-	-	-	0	-	-		50	0
Faridpur	Jul-12	100	100	0	100	0	-	-	-	0	-	-		30	0
Faridpur	Nov-12	100	100	0	100	0	-	-	-	0	0			34	0
Total		**2789**	**2088**	**6**	**2126**	**8**	**79**	**1**		**11**	**1**	**0.005**	**0.02**	**829**	

864 *NiV RNA was detected in three pooled oropharyngeal samples, confirmed by sequencing, although confirmation from individual samples could

865 not be made. These data re not used in prevalence estimates. [α] Detection by qPCR, Ct ranges 20-38.

866 Supplemental Data

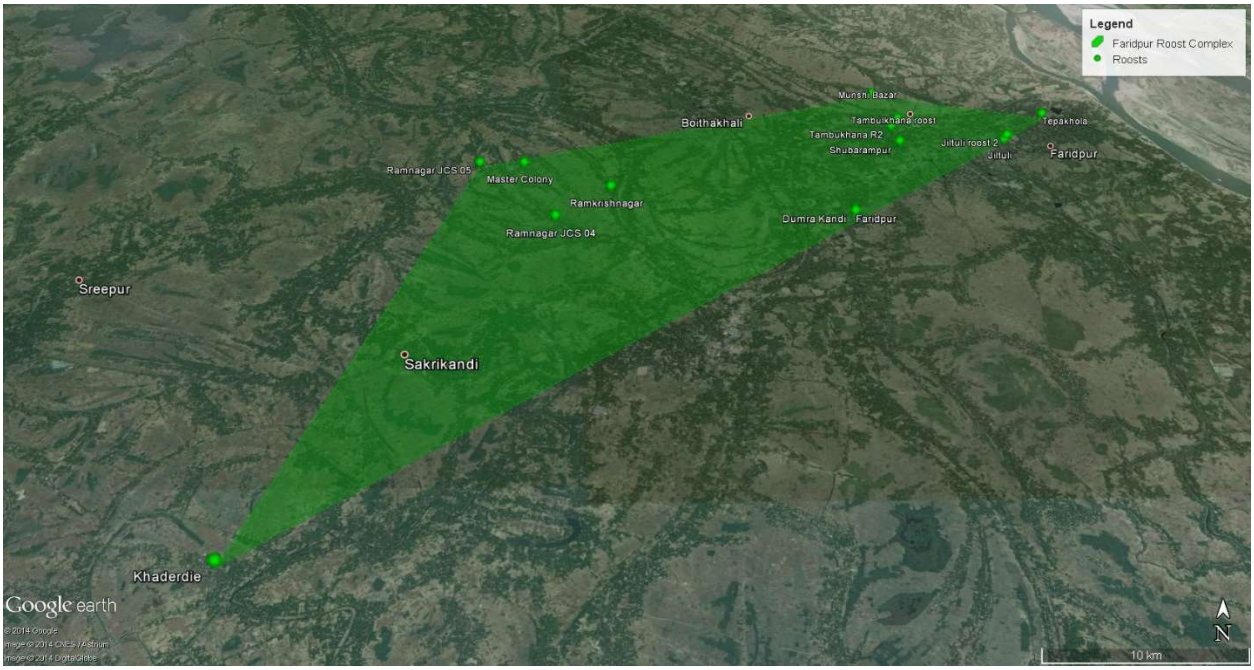
867



868 **Figure S1**. *Pteropus medius* counts from roosting sites within the Faridpur roost complex: 2008-2012.
869 Domrakandi and Kaderdi were the two primary roost sites sampled for the longitudinal study, although recapture
870 data showed intermixing among all of these roost sites in the Faridpur Roost Complex.

871

872



873

874 **Figure S2.** Faridpur Roost Complex. 51 Individual bats were recaptured during the longitudinal study at various
875 locations. 33 bats were recaptured at a different site from where they were originally sampled. 15 unique roosts
876 within an 80km^2 area were identified.
877

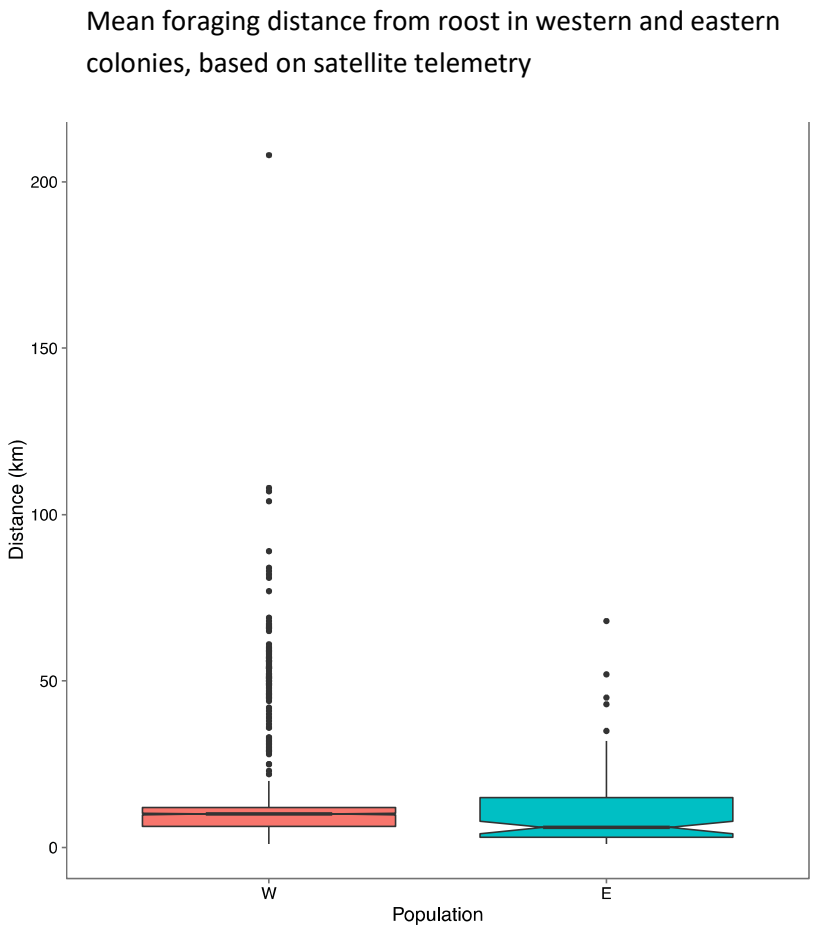


Figure S3. Mean foraging distance of western (W) and eastern (E) bat populations, based on satellite telemetry locations obtained between 1800h and 0600h, when *P. medius* typically forages.

Home range of *Pteropus medius* in wet and dry seasons.

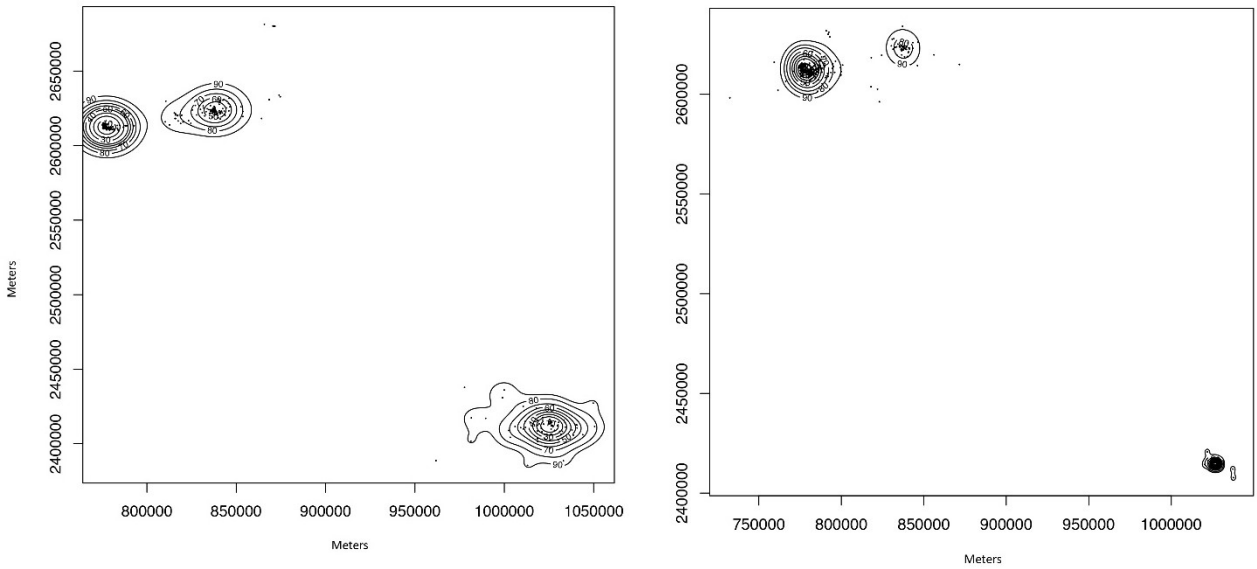


Figure S4. a) Homerange of *Pteropus medius* during the wet season (left) and dry season (right). Maps are projected in UTM (Universal Transverse Mercator) Zone 45 where units are represented in meters. The mean wet season homerange size was 2,746 km^2. Homerange size in the dry season is contracted and represents less than a quarter (618 km^2) of the homerange in the wet season.

Nipah virus phylogenetic tree, based on near complete N gene sequences

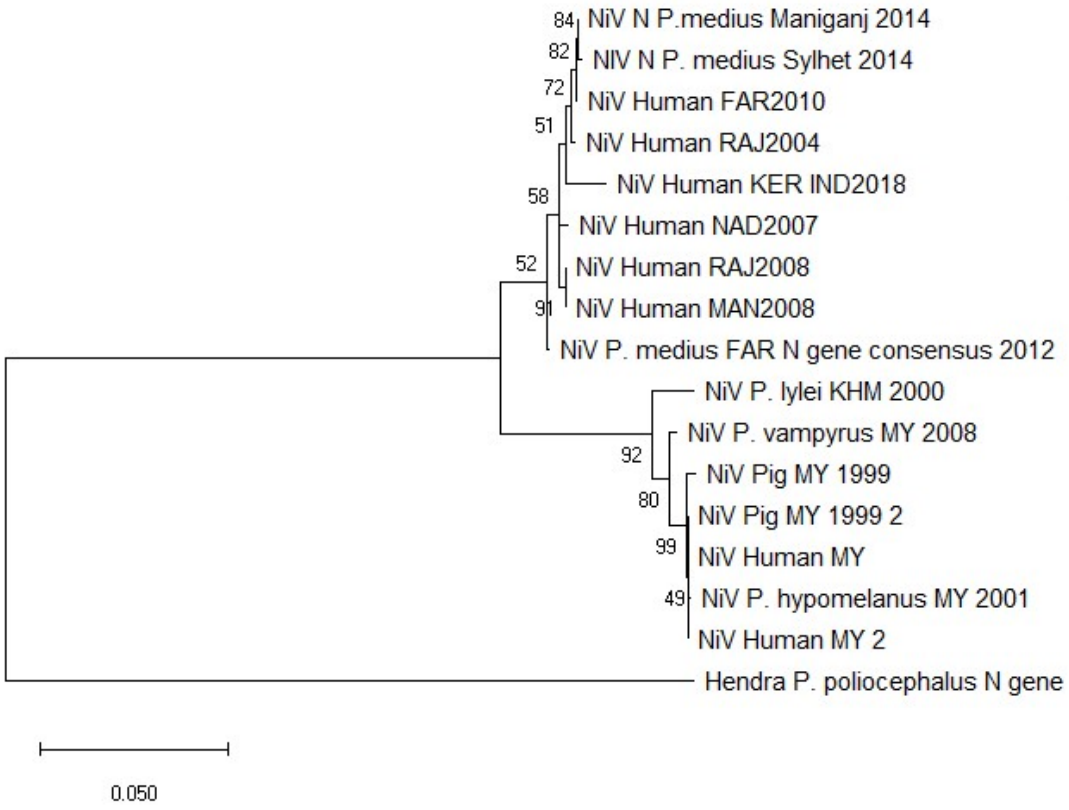


Figure S5. Nipah Virus phylogenetic tree, N gene: Clustal W alignment using nearly whole N gene consensus sequence from *P. medius* (1,592 nt) using Geneious Prime 2019 (*100*). The evolutionary history was inferred by using the Maximum Likelihood method and Hasegawa-Kishino-Yano model (*101*). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X (*99*). Genbank accession numbers for sequences (from top to bottom): *P. medius* Maniganj & Sylhet pending (63); JN808864, AY988601, MH396625, FJ513078, JN808863, JN808857, AY858110, FN869553, AJ627196, AJ564623, AY029767, AF376747, AY029768, JN255803.

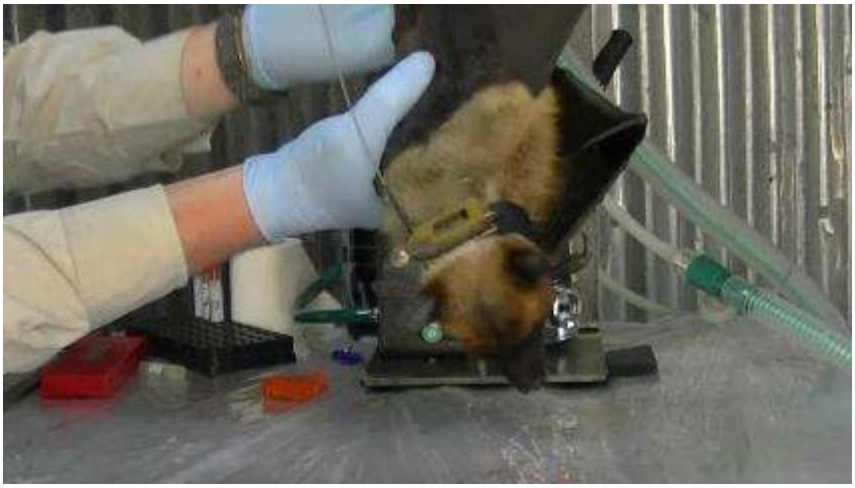


Figure S6. Platform terminal transmitter (PTT) and collar attachment on an anesthetized adult *Pteropus medius*, Bangladesh.

Table S1. Maximum likelihood estimates of fitted parameter values and (95% CI). All rates are on a weekly timestep unless otherwise indicated.

Parameter	Name	Maximum likelihood estimate	Lower 95% CI	Upper 95% CI
B_{jj}	Transmision rate, juveniles→juveniles	0.012584	0.00958	0.013021
B_{ja}	Transmision rate, juveniles→adults	0.030008	0.023707	0.03444
B_{aj}	Transmision rate, adults→juveniles	0.002417	0.00195	0.002937
B_{aa}	Transmision rate, adults→adults	0.000465	0	0.004092
R_A/N_A (t=0)	Initial adult seroprevalence	0.018752	0	0.067691
Δ	Recrudescence	2.3E-07	1.41E-08	7.1E-07
$(1-\mu)^{52}$	Adult annual survival	0.754971	0.718554	0.798346
λ	Rate of maternal antibody loss	0.05688	0.040029	0.072817
τ	Rate of adult antibody loss	0.003438	0.002099	0.004082

Param	MLE	2.50%	97.50%
Bjj	0.012584	0.00958	0.013021
Bja	0.030008	0.023707	0.03444
Baj	0.002417	0.00195	0.002937
Baa	0.000465	0	0.004092
Ias	0.018752	0	0.067691
r	2.3E-07	1.41E-08	7.1E-07
SA_A	0.754971	0.718554	0.798346
MAlr	0.05688	0.040029	0.072817
LAA	0.003438	0.002099	0.004082

Table S2. Recaptured bats and NiV IgG sero-status from the Faridpur population

Bat Chip ID	Age1	Sex	Capture Date	Location 1	Serostatus1	Age2	Capture Date 2	Serostatus2	Location 2	Status Change	Age3	Capture Date 3	Location 3	Serostatus 3	Status Change 2
17044540	A	M	24/07/08	JH	0	A	19/09/10	1	SH	C					
26774096	A	M	05/10/09	JH	1	A	13/11/09	1	SH	N					
26783883	J	F	15/05/09	SH	1	A	14/02/10	0	SH	R					
26789012	A	M	15/05/09	SH	0	A	02/10/10	1	SH	C					
26791784	A	F	14/05/09	SH	1	A	30/04/12	0	JH	R					
26816627	A	F	11/05/09	SH	0	A	26/08/09	0	DM	N					
26824582	J	M	09/05/09	JH	0	A	20/09/10	0	TP	N	A	05/05/11	TP	0	N
27099360	A	M	24/09/10	RM_MC	0	A	18/08/11	1	TB	C	A	18/11/12	JH	0	R
27102063	A	M	16/11/09	SH	1	A	16/02/10	1	SH	N	A	19/12/11	TP	1	N
27103623	J	M	21/09/10	TP	1	A	17/12/11	0	TP	R					
27105342	J	M	21/09/10	TP	0	A	19/12/11	1	TP	C					
27105562	P	M	24/04/10	RM_JCS	0	J	20/10/10	0	RM_JCS 05	N					
27110270	A	M	24/07/10	RM_JCS	0	A	04/04/11	0	RM_JCS	N					
27111334	A	M	23/07/10	RM_JCS	0	A	19/10/10	0	RM_JCS	N					
27123779	J	F	21/06/10	RM_JCS2	0	J	28/02/11	0	RK	N					
27123803	J	F	21/08/10	RM_JCS1	0	J	28/02/11	0	RK	N					
27123868	J	M	18/02/10	SH	0	A	26/04/12	1	JH	C					
27126256	A	F	10/02/10	SH	0	A	18/12/11	0	TP	N					
27259351	A	M	20/09/10	TP	0	A	30/04/12	0	JH	N					
27259370	A	M	22/07/10	RM_JCS1	0	A	04/04/11	0	RM_JCS1	N					
27261073	J	F	22/06/10	RM_JCS2	1	J	22/07/10	1	RM_JCS1	N					
27261577	A	M	21/09/10	TP	0	A	17/12/11	1	TP	C					
27266775	A	M	21/08/10	RM_JCS1	1	A	18/11/12	0	JH	R					
27291793	A	M	24/09/10	RM_JCS1	0	A	22/01/11	1	RM_JCS1	C					
27296568	A	M	12/11/09	SH	1	A	07/05/11	1	SH	N					
27296851	A	M	22/04/10	RM_JCS1	0	A	25/05/10	0	RM_JCS2	N					
27301580	A	M	17/06/10	SH	1	A	05/05/11	1	TP	N					
27301857	A	M	11/02/10	SH	0	A	22/04/10	1	RM_JCS1	C	A	03/05/11	SH	0	R

Location Codes: Location codes: Jhiltuli (JH); Jiltuli Roost 2 (JH2); Shuvarampur (SH); RamnagarMC (RM); Tepakhola (TP); Ramnagar Roost JCS (RM_JCS); Ramnagar JCS 1 (RM_JCS1); Ramnagar JCS 02 (RM_JCS2); Tambukhana R2 (TB2); Domrakhandi (DM); Khaderdi (KD); Ramkrishnagar (RK); Tepakhola Master Colony (TPMC). Status Change: C = seroconversion, N = No Change, R = sero-reversion.

Table S2 (cont…). Recaptured bats and NiV IgG sero-status from the Faridpur population

Bat Chip ID	Age1	Sex	Capture Date	Location 1	Serostatus1	Age2	Capture Date 2	Serostatus2	Location 2	Status Change	Age3	Capture Date 3	Location 3	Serostatus 3	Status Change 2
27305044	J	M	22/06/10	RM_JCS2	1	A	15/11/12	0	JH	R					
27306794	A	M	15/06/10	SH	1	A	11/07/12	1	TPMC	N					
27306824	A	M	23/07/10	RM_JCS1	1	A	24/09/10	1	RM_JCS1	N	A	18/11/12	JH	1	N
54867532	A	M	23/01/11	JH2	1	A	18/11/12	0	JH	R					
54872600	A	M	19/10/10	RM_JCS2	0	A	30/04/11	0	RM_MC	N					
54877598	J	F	18/01/11	SH	0	A	01/05/12	0	JH	N					
65770323	J	M	04/04/11	RM_JCS1	0	J	01/05/11	0	RM_MC	N					
65780555	A	M	05/05/11	TP	1	A	13/11/12	1	JH	N					
68608827	J	M	14/08/11	TB2	1	J	18/12/11	1	TP	N					
68612032	J	M	15/07/12	TPMC	0		18/11/12	0	JH	N					
80825550	A	M	11/12/07	DM	0	A	13/04/08	1	DM	R					
80855347	A	M	06/12/07	DM	1	A	22/07/08	1	KD	N					
80867630	A	M	11/12/07	DM	0	A	14/05/09	0	SH	N					
80876042	A	M	06/12/07	DM	0	A	14/08/11	0	TB2	N					
80877779	A	M	07/12/07	DM	1	A	21/07/08	1	DM	N					
81030044	A	M	06/02/06	RM_JCS1		A	24/07/10	0	RM_JCS1	NA		04/04/11	RM_JCS1	0	N
81055270	A	F	12/12/07	DM	0	A	20/12/11	1	TP	C					
81095300	A	M	09/12/07	DM	1	A	18/07/08	1	DM	N					
99605347	A	M	15/12/07	DM	0	A	07/04/08	0	DM	N					
99618528	A	M	20/07/08	DM	1	A	16/02/10	1	SH	N					
103821120	A	F	12/04/08	DM	0	A	16/05/09	1	SH	C					
104083112	A	M	03/04/08	DM	1	A	20/07/08	1	DM	N					
65777367	P	M	30/04/11	RM_MC	0	J	14/11/12	0	JH	N					

Location Codes: Location codes: Jhiltuli (JH); Jiltuli Roost 2 (JH2); Shuvarampur (SH); RamnagarMC (RM); Tepakhola (TP); Ramnagar Roost JCS (RM_JCS); Ramnagar JCS 1 (RM_JCS1); Ramnagar JCS 02 (RM_JCS2); Tambukhana R2 (TB2); Domrakhandi (DM); Khaderdi (KD); Ramkrishnagar (RK); Tepakhola Master Colony (TPMC). Status Change: C = seroconversion, N = No Change, R = sero-reversion.

From:	Jon Epstein on behalf of Jon Epstein <epstein@ecohealthalliance.org>
To:	Anthony, Simon J.; Ariful Islam; A. Marm Kilpatrick; Shahneaz Ali Khan; Maria Sanchez Leon; Noam Ross; ina.smith@csiro.au; Carlos M. Zambrana-Torrelio MSc; Yun Tao; Ausraful Islam; Phenix-Lan Quan; Kevin Olival, PhD; Salah Uddin Khan; Emily Gurley; Dr. Jahangir Hossain; Hume Field; Fielder, Mark; Thomas Briese; Mahmud Rahman; Christopher Broder; Gary Crameri; Linfa Wang; Stephen Luby; Ian Lipkin; Peter Daszak
Subject:	Nipah dynamics manuscript re-submitted to Science Advances
Date:	Wednesday, February 13, 2019 2:22:55 AM
Attachments:	Nipah virus dynamics in bats_manuscript + suppl_02132019.docx

All,
I wanted to update you that our manuscript on Nipah virus dynamics in bats is being re-submitted to *Science Advances.* Although it was originally rejected, the reviews were positive enough that the Deputy Editor has agreed to receive the revised manuscript as a new submission and send it out again for review. Thank you all for your input into this manuscript in its various stages of development. I'll follow up once we receive reviewers' comments.

Attached is the submitted version.

Cheers,
Jon

--

Jonathan H. Epstein DVM, MPH, PhD

Vice President for Science and Outreach

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New York, NY 10001

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Twitter: @epsteinjon

Budget Justification

PERSONNEL

Key Personnel

Dr. Christopher Broder will serve as Co-PI at 2.5% effort. Dr. Broder will be responsible for supervising the activity of all USU personnel engaged in the project and providing project oversight. Dr. Broder will not require salary support since he is an employee of the federal government.

Dr. Eric Laing will serve as a scientist on this project with 10% of effort. He will be responsible for the day to day USU project oversight related to production of recombinant protein materials, and actively direct and participate in the development of the immunoassay, validation and data analysis. Also, he will be responsible for long-term milestone planning, and supervising the research activities of the technical staff. Dr. Laing will also lead material and technology transfer and in-country training at Jordan University of Science and Technology (JUST), Jordan and R. Lugar Center, Georgia in Years 1 and 2. Additionally, Dr. Laing will participate in data analysis of serological data generated throughout the performance period and data analysis training on-site at JUST and Lugar in Year 3 with EcoHealth Alliance scientists and PI. Salary support for Dr. Laing will not be requested.

Other Personnel

Laingyang Yan will serve as the senior research technician on this project with 20% effort.

Spencer Sterling will serve as the research assistant II on this project with 20% effort.

A TBD research assistant will provide 100% effort.

The research assistants will be responsible for the recombinant materials at the USU laboratory, and will perform quality-control experiments, and data analysis prior to deliverance of reagents to Jordan University of Science and Technology (JUST), Jordan and R. Lugar Center, Georgia. This will enhance early virus detection through serological biosurveillance at Jordan University of Science and Technology (JUST), Jordan and R. Lugar Center, Georgia, and improve situational awareness for Middle Eastern Respiratory Syndrome (MERS)-coronavirus in USCENTCOM and USEUCOM theaters. The personnel listed above will provide custom reagent support and on-site staff training with Luminex-based technologies and a multiplex microsphere immunoassay that we have developed.

The salary support requested does budget for a small merit increase per year for each research assistant position.

SUPPLIES

The proposed budget includes costs for the production of coronavirus surface proteins and nucleoprotein, MagPIX magnetic beads, reagents necessary for the purification of polyclonal antibodies that complement the custom virus proteins as controls and laboratory disposables required in the production process and assay validation. The current proposal includes the production costs of 10 virus protein antigens, including production of custom MERS, SARS, MERS-related and SARS-related coronavirus spike proteins. The Principal Investigator (PI), Kevin Olival, EcoHealth Alliance, has indicated that the projected sample size of this biosurveillance study is ~2400 individual sera samples and the proposed budget has been designed to meet this sample size. Additionally, included is the cost of ordering 10 uniquely colored compatible MagPIX magnetic beads to correspond with each virus protein. Size exclusion and affinity purification are used to purify each virus protein.

	Year 1	Year 2	Year 3
Protein Production (Cov) ~1500/each	$19,000.00	$15,000.00	$7,500.00
Magnetic beads (725/bottle - 10 plex)	$7,250.00		
Cell culture materials - disposables	$10,000.00	$10,000.00	$10,000.00
Polyclonal rabbit antisera	$7,200.00		
Affinity mattrices and control IgG materials	$4,000.00	$4,000.00	$4,000.00
Shipments	$3,000.00	$3,000.00	$3,000.00
TOTAL	$50,450.00	$32,000.00	$24,500.00

TRAVEL

USU/HJF staff will travel to JUST, Jordan and the R. Lugar Center, Georgia to complete on-site training of research staff and laboratory personnel. We anticipate travel and training during each fiscal year of funding. In Year 1 we will require separate trips to perform trainings at both JUST and R. Lugar Center; in Year 2 we will host a second training in Jordan and anticipate attendance at a conference where data generated from this project will be presented; and in Year 3 we will conduct a data analysis training at the R. Lugar Center. In this budget we are estimating sums for per diem, airfare, and lodging costs to cover 7 days of staff travel including 5 days of on-site training for two USU/HJF scientists per year. The costs associated with travel has been generated using the published GSA rates.

	Year 1	Year 2	Year 3
Foreign / International Travel	$ 14,400.00	$15,000.00	$8,200.00

OTHER DIRECT COSTS

An AKTA chromatography machine is used for purification and a service contract is required for proper machine maintenance. Instrument (Bio-Rad Bio-Plex 200) usage fees at the USU Biomedical Instrumentation Center (BIC) have been estimated on a yearly usage basis. The BIC instrumentation is necessary to validate and perform control test with the virus antigens produced in the USU laboratory. The estimated cost will be **$4,500 per year**, for all 3 years.

Indirect and Fringe Benefit

The Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc. (HJF) indirect cost (IDC) is calculated based on the value-added cost base overhead rates. The IDC applied is 29.48% (USU On-site Overhead) or 4.19% (USU Off-site Overhead) for all allowable direct costs, less sub-awards. Additionally, a 14.30% (Companywide G&A) rate is applied on the total direct costs less subawards plus the USU On-site Overhead rate. For proposals including subawards, 1.60% is applied to the total subaward costs.

The fringe rate is 31.70% for Tier 1 HJF employees, and 5.45% for Tier 2 HJF employees. All HJF employees on this proposal are Tier 1.

The above provisional indirect cost and fringe benefit rates for FY 2019 were approved by the U.S. Army Medical Research Acquisition Activity on December 12, 2017.

RESEARCH & RELATED BUDGET - Budget Period 1

OMB Number: 4040-0001
Expiration Date: 6/30/2016

ORGANIZATIONAL DUNS: 1446765660000 **Enter name of Organization:** Henry M. Jackson Foundation

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 1** **Start Date:** 04/01/2019 **End Date:** 03/31/2020

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Christopher		Broder			0.00			0.00	0.00	0.00
Project Role:	Co-Investigator										
	Eric		Laing						0.00	0.00	0.00
Project Role:	Scientist										

Additional Senior Key Persons: Add Attachment | Delete Attachment | View Attachment

Total Funds requested for all Senior Key Persons in the attached file

Total Senior/Key Person 0.00

B. Other Personnel

Number of Personnel	Project Role	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Post Doctoral Associates						
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						
1	Senior Research Assistant	2.40			12,000.00	3,804.00	15,804.00
1	Research Assistant II	2.40			9,491.00	3,009.00	12,500.00
1	Research Assistant	12.00			39,562.00	12,541.00	52,103.00

3 **Total Number Other Personnel**

Total Other Personnel 80,407.00

Total Salary, Wages and Fringe Benefits (A+B) 80,407.00

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment item	Funds Requested ($)

Additional Equipment: Add Attachment | Delete Attachment | View Attachment

Total funds requested for all equipment listed in the attached file	
Total Equipment	

D. Travel

	Funds Requested ($)
1. Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	
2. Foreign Travel Costs	14,400.00
Total Travel Cost	14,400.00

E. Participant/Trainee Support Costs

	Funds Requested ($)
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other	
Number of Participants/Trainees / **Total Participant/Trainee Support Costs**	

F. Other Direct Costs

		Funds Requested ($)
1.	Materials and Supplies	50,450.00
2.	Publication Costs	
3.	Consultant Services	
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.	Equipment Maintenance	4,500.00
9.		
10.		
	Total Other Direct Costs	54,950.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	149,757.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
On Site	29.48	149,757.00	44,148.00
HJF G&A Rate	14.30	193,905.00	27,728.00

Total Indirect Costs 71,876.00

Cognizant Federal Agency (Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	221,633.00

J. Fee

Funds Requested ($)

K. Budget Justification

(Only attach one file.) VLP Duke Sempowski Justification.pdf [Add Attachment] [Delete Attachment] [View Attachment]

RESEARCH & RELATED BUDGET - Budget Period 2

OMB Number: 4040-0001
Expiration Date: 6/30/2016

ORGANIZATIONAL DUNS: 1446765660000 **Enter name of Organization:** Henry M. Jackson Foundation

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 2** **Start Date:** 04/01/2020 **End Date:** 03/31/2021

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Christopher		Broder			0.00			0.00	0.00	0.00
Project Role:	Co-Investigator										
	Eric		Laing						0.00	0.00	0.00
Project Role:	Scientist										

Additional Senior Key Persons: Add Attachment | Delete Attachment | View Attachment

Total Funds requested for all Senior Key Persons in the attached file

Total Senior/Key Person 0.00

B. Other Personnel

Number of Personnel	Project Role	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Post Doctoral Associates						
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						
1	Senior Research Assistant	2.40			12,360.00	3,918.00	16,278.00
1	Research Assistant II	2.40			9,776.00	3,099.00	12,875.00
1	Research Assistant	12.00			40,749.00	12,917.00	53,666.00

3 **Total Number Other Personnel**

Total Other Personnel 82,819.00

Total Salary, Wages and Fringe Benefits (A+B) 82,819.00

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment item	Funds Requested ($)

Additional Equipment: Add Attachment | Delete Attachment | View Attachment

Total funds requested for all equipment listed in the attached file	
Total Equipment	

D. Travel

	Funds Requested ($)
1. Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	
2. Foreign Travel Costs	15,000.00
Total Travel Cost	15,000.00

E. Participant/Trainee Support Costs

	Funds Requested ($)
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other	
Number of Participants/Trainees — **Total Participant/Trainee Support Costs**	

F. Other Direct Costs

		Funds Requested ($)
1.	Materials and Supplies	32,000.00
2.	Publication Costs	
3.	Consultant Services	
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.	Equipment Maintenance	4,500.00
9.		
10.		
	Total Other Direct Costs	36,500.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	134,319.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
On Site	29.48	134,319.00	39,597.00
HJF G&A Rate	14.30	173,916.00	24,870.00

Total Indirect Costs 64,467.00

Cognizant Federal Agency (Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	198,786.00

J. Fee

Funds Requested ($)

K. Budget Justification

(Only attach one file.) VLP Duke Sempowski Justification.pdf [Add Attachment] [Delete Attachment] [View Attachment]

RESEARCH & RELATED BUDGET - Budget Period 3

OMB Number: 4040-0001
Expiration Date: 6/30/2016

ORGANIZATIONAL DUNS: 1446765660000 **Enter name of Organization:** Henry M. Jackson Foundation

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 3** **Start Date:** 04/01/2021 **End Date:** 03/31/2022

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Christopher		Broder			0.00			0.00	0.00	0.00
Project Role:	Co-Investigator										
	Eric		Laing						0.00	0.00	0.00
Project Role:	Scientist										

Additional Senior Key Persons: Add Attachment | Delete Attachment | View Attachment

Total Funds requested for all Senior Key Persons in the attached file:

Total Senior/Key Person 0.00

B. Other Personnel

Number of Personnel	Project Role	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Post Doctoral Associates						
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						
1	Senior Research Assistant	2.40			12,731.00	4,036.00	16,767.00
1	Research Assistant II	2.40			10,069.00	3,192.00	13,261.00
1	Research Assistant	12.00			41,971.00	13,305.00	55,276.00

3 **Total Number Other Personnel**

Total Other Personnel 85,304.00

Total Salary, Wages and Fringe Benefits (A+B) 85,304.00

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment item	Funds Requested ($)

Additional Equipment: Add Attachment | Delete Attachment | View Attachment

Total funds requested for all equipment listed in the attached file	
Total Equipment	

D. Travel

	Funds Requested ($)
1. Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	
2. Foreign Travel Costs	8,200.00
Total Travel Cost	8,200.00

E. Participant/Trainee Support Costs

	Funds Requested ($)
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other	
Number of Participants/Trainees / **Total Participant/Trainee Support Costs**	

F. Other Direct Costs

		Funds Requested ($)
1.	Materials and Supplies	21,500.00
2.	Publication Costs	
3.	Consultant Services	
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.	Equipment Maintenance	4,500.00
9.		
10.		
	Total Other Direct Costs	26,000.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	119,504.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
On Site	29.48	119,504.00	35,230.00
HJF G&A Rate	14.30	154,734.00	22,127.00

Total Indirect Costs	57,357.00

Cognizant Federal Agency (Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	176,861.00

J. Fee

Funds Requested ($)

K. Budget Justification

(Only attach one file.) VLP Duke Sempowski Justification.pdf [Add Attachment] [Delete Attachment] [View Attachment]

RESEARCH & RELATED BUDGET - Cumulative Budget

		Totals ($)
Section A, Senior/Key Person		0.00
Section B, Other Personnel		248,530.00
Total Number Other Personnel	9	
Total Salary, Wages and Fringe Benefits (A+B)		248,530.00
Section C, Equipment		
Section D, Travel		37,600.00
1. Domestic		
2. Foreign	37,600.00	
Section E, Participant/Trainee Support Costs		
1. Tuition/Fees/Health Insurance		
2. Stipends		
3. Travel		
4. Subsistence		
5. Other		
6. Number of Participants/Trainees		
Section F, Other Direct Costs		117,450.00
1. Materials and Supplies	103,950.00	
2. Publication Costs		
3. Consultant Services		
4. ADP/Computer Services		
5. Subawards/Consortium/Contractual Costs		
6. Equipment or Facility Rental/User Fees		
7. Alterations and Renovations		
8. Other 1	13,500.00	
9. Other 2		
10. Other 3		
Section G, Direct Costs (A thru F)		403,580.00
Section H, Indirect Costs		193,700.00
Section I, Total Direct and Indirect Costs (G + H)		597,280.00
Section J, Fee		

ORG: Uniformed Services University of the Health Sciences
DUNS: 931258755
ADDRESS: 4301 Jones Bridge Road, Bethesda, MD 20814-4719
CONGRESSIONAL DISTRICT: MD-008

Please note the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc. will manage this award.

OMB Number: 4040-0010

Project/Performance Site Location(s)

Project/Performance Site Primary Location

☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name:

DUNS Number:

* Street1:

Street2:

* City: County:

* State:

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: * Project/ Performance Site Congressional District:

BIOGRAPHICAL SKETCH

NAME: Broder, Christopher C.

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6)

POSITION TITLE: Professor of Microbiology, Immunology and Emerging Infectious Diseases

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Florida Institute of Technology, Melbourne, FL	B.S.	06/1983	Biological Science
Florida Institute of Technology, Melbourne, FL	M.S.	12/1985	Molecular Biology
University of Florida, Gainesville, FL	Ph.D.	05/1989	Immunology and Med-Micro

A. Personal Statement

The main objective of this proposal is to determine the seroprevalence of MERS-CoV across the human-camel value chain in Jordan and be able to inform locations, seasons, and epidemiological interfaces of greatest risk. We will make use of a Luminex-based microsphere multiplex assay using immunologically relevant soluble and native-like viral envelope glycoproteins. We are well experienced in making soluble viral glycoprotein spikes from henipaviruses, filoviruses and other viruses, and we already have related soluble SARS coronavirus S proteins multiplexed. Once developed we can transfer the technology and multiplex serological assay to partner nation laboratories (Jordan and Georgia) to accelerate early detection capabilities for emerging zoonotic diseases. The proposed project will also leverage an ongoing DoD-funded bat-borne disease surveillance project in CENTCOM and EUCOM countries to screen specimens for coronaviruses using multiplex serological assays. We have been collaborating with EcoHealth Alliance along similar serological surveillance projects for more than 6 years with a focus on the detection of henipaviruses and filoviruses in wildlife, livestock and indigenous human populations. I have been an active researcher in enveloped virus-host cell interactions for the past 29 years and, together with my collaborators, have made significant contributions to this field. I developed the first oligomeric HIV-1 gp140 glycoprotein subunit vaccine, the vaccinia virus-based reporter gene assay for measuring viral glycoprotein-mediated membrane fusion, defined the fusion tropism of HIV-1 followed by the discovery of the HIV-1 coreceptors (CXCR4 and CCR5). My lab has studied the entry and inhibition of Australian bat lyssavirus (ABLV) a rabies-like virus, including the first reverse genetics system to rescue ABLV and also the henipavirus, Cedar virus. In 1999, I established a collaborative international group of experts in Hendra virus and Nipah virus research, in areas from structural biochemistry, animal models and *in vivo* pathogenesis, to the development and testing of vaccines and therapeutics. My work includes the discovery of the Hendra and Nipah virus entry receptors (ephrin-B2/B3), and the development of the feline, ferret and African green monkey models of Hendra and Nipah virus pathogenesis with my collaborators. My lab's henipavirus glycoprotein work, with collaborators, have made the structural solutions and characterization of the F and the G-ephrin receptor glycoprotein interactions, and the discovery and development of antiviral human monoclonal antibodies to ABLV and Hendra and Nipah viruses; one (m102.4) having a Phase I clinical trial completed in May 2016, and has been used by emergency protocol in 13 people in Australia and one in the U.S. because of significant risk of infection. I also developed the Hendra/Nipah subunit vaccine based on soluble Hendra G glycoprotein (HeV-sG); called Equivac® HeV (Zoetis, Inc.) the first commercialized vaccine to a BSL-4 agent, and now being developed as a human use Nipha/Hendra vaccine supported by CEPI. More recently, and relevant to the present proposal, my lab has developed a panel of nearly 20 different soluble viral envelope glycoproteins for serological surveillance studies, and have the tools required to conduct the studies in the present proposal.

1. Bonaparte, M. I., A. S. Dimitrov, K. N. Bossart, G. Crameri, B. A. Mungall, K. A. Bishop, V. Choudhry, D. S. Dimitrov, L.-F. Wang, B. T. Eaton, and **C. C. Broder*.** Ephrin-B2 Ligand is a Functional Receptor for Hendra Virus and Nipah Virus. Proc Natl Acad Sci U S A. 102(30):10652-7. 2005. (***from the cover***)

2. Middleton D, Pallister J, Klein R, Feng YR, Haining J, Arkinstall R, Frazer L, Huang JA, Edwards N, Wareing M, Elhay M, Hashmi Z, Bingham J, Yamada M, Johnson D, White J, Foord A, Heine HG, Marsh GA, **Broder CC**, Wang LF. Hendra virus vaccine, a one health approach to protecting horse, human, and environmental health. Emerg Infect Dis. 2014 Mar;20(3). PMCID: PMC3944873
3. Xu K, Chan YP, Bradel-Tretheway B, Akyol-Ataman Z, Zhu Y, Dutta S, Yan L, Feng Y, Wang LF, Skiniotis G, Lee B, Zhou ZH, **Broder CC**, Aguilar HC, Nikolov DB. Crystal Structure of the Pre-fusion Nipah Virus Fusion Glycoprotein Reveals a Novel Hexamer-of-Trimers Assembly. PLoS Pathog. 2015 Dec 8;11(12):e1005322. doi: 10.1371/journal.ppat.1005322. PMID: 26646856
4. Mire CE, Satterfield BA, Geisbert JB, Agans KN, Borisevich V, Yan L, Chan YP, Cross RW, Fenton KA, **Broder CC**, Geisbert TW. Pathogenic Differences between Nipah Virus Bangladesh and Malaysia Strains in Primates: Implications for Antibody Therapy. Sci Rep. 2016 Aug 3;6:30916. doi: 10.1038/srep30916.

B. Positions and Honors

Positions and Employment

1990-1992 National Research Council, Research Associate, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland.
1993-1996 IRTA Fellow, LVD, NIAID, NIH, Bethesda, Maryland.
1996-2000 Assistant Professor, Department of Microbiology and Immunology, Joint appointment, Molecular and Cell Biology Graduate Program, Uniformed Services University, Bethesda, Maryland.
2000-2005 Associate Professor, Department of Microbiology and Immunology, Joint appointment, Emerging Infectious Diseases Graduate Program, USUHS, Bethesda, Maryland.
2005-pres. Professor, Department of Microbiology and Immunology, Joint appointment, Emerging Infectious Diseases Graduate Program, USUHS, Bethesda, Maryland.
2006-pres. Director, Emerging Infectious Diseases Graduate Program, USUHS, Bethesda, Maryland.

Other Experience and Professional Affiliations

2009 Member, National Veterinary Stockpile Nipah virus Countermeasures Workshop; United States Department of Agriculture. Geelong, Australia.
2011 Member, Discontools Nipah Virus Infection Panel Expert Group. Gap analysis. International Federation for Animal Health Europe, Brussels, Belgium.
2011 Invited expert, National Academies, Washington, DC. Evaluation of site-specific risk assessment for the National Bio- and Agro-Defense Facility (NBAF) in Manhattan, Kansas.
2007- Editorial board, *Journal of Virology*.
2010- Editorial board, *Virology*.
2011- Editorial board, *Viruses*.
2011- Editorial board, *Pathogens*.
2012- Editorial board, *Virologica Sinica*

Honor and Awards

1987-1988 National Institutes of Health Training Grant Award
1989 Medical Guild Graduate Research Award, University of Florida College of Medicine
1990-1992 National Research Council Research Associateship Award
1993-1996 NIH Intramural Research Training Award (IRTA) Fellowship
1996 The Fellows Award for Research Excellence, Office of Science Education, NIH
1995-1996 American Association for the Advancement of Science: Breakthrough of the Year, Science Magazine; Newcomb Cleveland Prize
2001 Outstanding Instructor in Virology, USUHS, School of Medicine.
2008 The Henry Wu Award for Excellence in Basic Science Research.
2013 The 3rd Sidney Pestka Lecture; 22nd Annual Philadelphia Infection & Immunity Forum.
2013 The 2013 Federal Laboratory Consortium Award for Excellence in Technology Transfer.
2013 Second Finalist for the Australian Infectious Diseases Research Centre Eureka Prize.
2013 The CSIRO Chairman's Medal. The Commonwealth Scientific and Industrial Research Organisation (CSIRO); Australia's national science agency.
2014 The Cinda Helke Award for Excellence in Graduate Student Advocacy.
2016 The James J. Leonard Award for Excellence in Translational/Clinical Research.

Patents issued and pending

- Bacterial plasmin receptors as fibrinolytic agents: U.S. Pat. No. 5,237,050.

- Oligomeric HIV-1 envelope glycoproteins (gp140): U.S. Pat. Nos. 6,039,957 and 6,171,596.
- Modified Oligomeric HIV-1 gp140 Envelope Proteins. U.S. Serial No. 60/608,144; pending.
- CCR5 DNA, new animal models and therapeutic agents for HIV infection: U.S. Pat. No. 7,151,087.
- Cells expressing both human CD4 and CXCR4 associated with HIV infection: U.S. Pat. No. 6,197,578.
- Compositions and methods for the inhibition of membrane fusion by paramyxoviruses: U.S. Pat. Nos. 7,666,431 and 8,114,410.
- Soluble forms of Hendra and Nipah virus G glycoproteins. U.S. Pat. Nos. 8,865,171; 9,045,532; 9,533,038; Australian Patent No. 2005327194.
- Human monoclonal antibodies against Hendra and Nipah viruses. U.S. Pat. Nos. 8,313,746, 8,858,938.
- Soluble Forms of Hendra and Nipah Virus F Glycoprotein and Uses Thereof: Australian Patent No. 2013276968. U.S. Patent No. 10,040,825.
- Antibodies against F glycoprotein of Hendra and Nipah viruses. U.S. Patent No. 9,982,038.
- Novel Paramyxovirus and Methods of Use (Cedar virus). U.S. Patent app. No. 61/667,194.

C. Contributions to Science

1. My Ph.D. thesis centered on the characterization of a specific receptor for human plasmin on Group A Streptococci that I discovered during a rotation project as a first year student. My studies revealed that certain group A streptococci elaborated receptors that could bind selectively a key fibrinolytic enzyme, plasmin, while having no binding ability for the zymogen precursor plasminogen or other serine proteases. The bacterium-bound plasmin remained enzymatically active including its ability to hydrolyze a fibrin clot. Bound plasmin could not be inhibited by its physiological regulator, alpha 2-plasmin inhibitor. Thus, since these organisms produced streptokinase, a protein that complexes with plasminogen producing an active enzyme that can convert plasminogen to plasmin, they could accelerate the destruction of the extracellular matrix environment: findings that formed a molecular-pathogenic model for the "flesh-eating streptococci".

   a. Lottenberg, R., **C.C. Broder**, and M.D.P. Boyle. Identification of a Specific Receptor for Plasmin on a Group A Streptococcus. Infection and Immunity. 55(8):1914-1918, 1987.
   b. **Broder, C.C.**, R. Lottenberg, and M.D.P. Boyle. Mapping of the Domain of Human Plasmin Recognized by its Unique Group A Streptococcal Receptor. Infection and Immunity. 57(9): 2597-2605, 1989.
   c. **Broder, CC**, R Lottenberg, GO vonMering, K. Johnston and MDP Boyle. Isolation of a prokaryotic plasmin receptor: relationship to a plasminogen activator produced by the same microorganism. J. Biol. Chem. 266:4922-28, 1991.
   d. Lottenberg, R., **C.C. Broder**, M.D.P. Boyle, S.J. Kain, B.L. Schroeder, and R. Curtiss III. Cloning, Sequence Analysis, and Expression in *Escherichia coli* of a Streptococcal Plasmin Receptor. J. Bacteriology. 174:5204-5210, 1992.

2. My independent work as a postdoctoral fellow focused on the early stages of HIV-1 envelope glycoprotein mediated membrane fusion as a surrogate model of HIV-1 entry. I established a vaccinia virus-based reporter gene assay for measuring viral (HIV-1) glycoprotein-mediated membrane fusion and generated the first panel of T-cell tropic and Macrophage-tropic HIV-1 envelope glycoprotein (Env) encoding recombinant vaccinia virus vectors and I used these tools to be the first to hypothesize that the cellular tropism of HIV-1 could be explained by specific membrane fusion factors required for the different classes of HIV-1 Envs. I also developed the first soluble and secreted full-length oligomeric HIV-1 gp140 glycoprotein and explored the importance of its native oligomeric structure in terms of its presentation of conformational and virus-neutralizing epitopes through the development and characterization of more than 100 murine mAbs.

   a. **Broder, C.C.,** D.S. Dimitrov, R. Blumenthal, and E.A. Berger. The block to HIV-1 envelope glycoprotein-mediated membrane fusion in animal cells expressing human CD4 can be overcome by a human cell component(s). Virology. 193:483-491, 1993.
   b. Nussbaum, O., **C.C. Broder,** and E.A. Berger. HIV-1 Envelope Glycoprotein/CD4 Mediated Cell Fusion: A Novel Recombinant Vaccinia Virus-Based Assay Measuring Activation of a Reporter Gene by Bacterio-phage T7 RNA Polymerase Selectively In Fused Cells. J.Virol. 68:5411-5422, 1994.
   c. **Broder, C.C.,** P.L. Earl, D. Long, B. Moss, and R.W. Doms. Antigenic implications of HIV-1 envelope glycoprotein quaternary structure: oligomer-specific and -sensitive mAbs. PNAS. 91:11699-11703, 1994.
   d. **Broder, C.C.** and E.A. Berger. Fusogenic Selectivity of the Envelope Glycoprotein is a Major Determinant of HIV-1 Tropism for CD4+ T-Cell Lines vs. Macrophages. PNAS. USA. 92:9004-08, 1995.

3. My early studies on the cellular and viral membrane fusion tropism of HIV-1 and the development of a sensitive and specific reporter gene assay of cell-cell membrane fusion facilitated the discovery of the first membrane fusion accessory factor (fusin, now known as CXCR4) that we earlier hypothesized existed, and this rapidly led to the discovery by us and others of the second factor for macrophage-tropic Envs (CCR5); the HIV-1 coreceptors. These findings were a significant breakthrough in HIV research leading to numerous new directions in understanding HIV-1 pathogenesis as well as new therapeutic strategies.

   a. Feng, Y., **C.C. Broder**, P.E. Kennedy, and E.A. Berger. HIV-1 Entry Cofactor: Functional cDNA Cloning of a Seven-Transmembrane, G Protein-Coupled Receptor. Science. 272:872-877, 1996.
   b. Alkhatib*, G., C. Combadiere*, **C.C. Broder***, Y. Feng*, P.E. Kennedy*, P.M. Murphy, and E.A. Berger. CC CKR5: a RANTES, MIP-1α , MIP-1β Receptor as a Fusion Cofactor for Macrophage-Tropic HIV-1. Science. 272:1955-1958, 1996. (*equal contribution).
   c. Rucker, J., M. Samson, B. J. Doranz, F. Libert, J. F. Berson, Y. Yi, R. G. Collman, **C. C. Broder**, G. Vassart,, R. W. Doms, and M. Parmentier. Regions in β-chemokine Receptors CCR-5 and CCR-2b that Determine HIV-1 Cofactor Specificity. Cell. 87:1-10, 1996.
   d. Edinger, A.L., A. Amedee, K. Miller, B.J. Doranz, M. Endres, M. Sharron, M. Samson, Z-h. Lu, J.E. Clements, M. Murphey-Corb, S.C. Peiper, M. Parmentier, **C.C. Broder**, and R.W. Doms. Differential utilization of CCR5 by macrophage and T cell tropic simian immunodeficiency virus strains. PNAS. USA. 94:4005-4010, 1997.

4. My initial work on HIV-1 entry led to further independent studies which focused on follow-up investigations characterizing the roles of the HIV-1 coreceptors in the virus entry process. These studies revealed the interplay between the HIV-1 entry receptors, mapped important domains of the coreceptors involved in HIV-1 Env interaction, and also revealed possible avenues of how an HIV-1 Env might engage and differently utilize the CXCR4 and CCR5 coreceptors for infection. In addition, I also engaged in collaborative follow-up studies exploring the utility of soluble oligomeric HIV-1 envelope glycoproteins as subunit vaccine immunogens (gp140) which I initiated at NIH while a postdoctoral fellow. This work on the unusual R2 HIV-1 Env isolate provided important data on the renewed potential of a subunit vaccine approach against HIV-1 and our work with the laboratory of Dr. Gerald Quinnan led to the first NIAID program project grant at USU.

   a. Chabot, D.J., P-F. Zhang, G.V. Quinnan, and **C.C. Broder**. Mutagenesis of CXCR4 Identifies Important Domains for HIV-1 X4 Isolate Envelope-Mediated Membrane Fusion and Virus Entry and Reveals Cryptic Coreceptor Activity for R5 Isolates. J. Virol. 73:6598-6609, 1999.
   b. Xiao, X., L. Wu, T.S. Stantchev, Y-R. Feng, S. Ugolini, H. Chen, Z. Shen, **C.C. Broder**, Q.J. Sattentau, and D.S. Dimitrov. Constitutive cell surface association between CD4 and CCR5. PNAS. 96:7496-7501, 1999.
   c. Chabot, D.J., H. Chen, D.S. Dimitrov, and **C.C. Broder**. N-linked Glycosylation in CXCR4 Masks Coreceptor Function for CCR5-Dependent HIV-1 Isolates. J. Virol. 74:4404-4413, 2000.
   d. Zhang, P.F., Cham, F., Dong, M., Choudhary, A., Bouma, P., Zhang, Z., Shao, Y., Feng, Y.R., Wang, L., Mathy, N., Voss, G., **Broder, C.C**., Quinnan, G.V., Jr. Extensively cross-reactive anti-HIV-1 neutralizing antibodies induced by gp140 immunization. PNAS. USA. 104(24):10193-8. 2007.

5. More recently, my research efforts have centered on emerging viruses that impact human and domestic livestock populations; including Australian bat lyssavirus (rabies-like virus), filoviruses (Ebola and Marburg) and the henipaviruses (Hendra and Nipah). In 1999 I established a collaborative international group of experts in Hendra virus and Nipah virus biology. My lab was the first to publish on Hendra virus outside of Australia. Initial work focused on the structural and functional analysis of the Hendra glycoproteins F and G. I obtained the first NIAID funded project providing monetary support on select agent research to an overseas laboratory (2003). Henipavirus research has been the major focus of my laboratory over the past 15 years, covering areas from structural biochemistry, *in vivo* pathogenesis and animal model development to the development and testing of vaccines and therapeutics. My group developed the first peptide henipavirus fusion inhibitors, subunit vaccine and neutralizing human monoclonal antibodies (mAb), and supported the development of the feline and ferret models of Hendra and Nipah infection and pathogenesis and helped support the development of the first nonhuman primate model for Hendra and Nipah. We and our collaborators tested the *in vivo* efficacy of the Hendra soluble G glycoprotein vaccine (HeV-sG) and an anti-henipavirus G-specific neutralizing human mAb. One human mAb (m102.4) has been used by compassionate emergency protocol in 13 people in Australia and one individual in the United States; a Phase I clinical trial was completed in May, 2016. The henipavirus subunit vaccine, HeV-sG, has was launched; called Equivac® HeV (Pfizer Animal Health (Zoetis, Inc.)) and is the first commercialized and deployed vaccine to a BSL-4 agent. My group's additional findings include the discovery of the henipavirus

cellular receptors (ephrin-B2/B3) and produced soluble versions of the G and F glycoproteins facilitating their structural solutions with our collaborators; developed the first reverse genetics system for an authentic Cedar virus based BSL-2 virus system; and these native like soluble viral membrane glycoproteins have now been used to develop a panel of more than 16 different envelope glycoproteins from all the filoviruses and henipaviruses for serological surveillance studies. Altogether I have contributed to more than 60 publications and book chapters on Hendra and Nipah virus since 2002.

a. Bossart KN, Rockx B, Feldmann F, Brining D, Scott D, Lacasse R, Geisbert JB, Feng YR, Chan YP, Hickey AC, **Broder CC*,** Feldmann H, Geisbert TW. A Hendra virus G glycoprotein subunit vaccine protects African green monkeys from Nipah virus challenge. Sci Transl Med. 4(146):146ra107. 2012 *corresponding. (***from the cover**) PMCID: PMC3516289.
b. Geisbert TW*, Mire CE, Geisbert JB, Chan YP, Agans KN, Feldmann F, Fenton KA, Zhu Z, Dimitrov DS, Scott DP, Bossart KN, Feldmann H, **Broder CC***. Therapeutic treatment of Nipah virus infection in nonhuman primates with a neutralizing human monoclonal antibody. Sci Transl Med. 2014, *corresponding author (***from the cover**) 6(242):242ra82. PMCID: PMC4467163.
c. Laing ED, Mendenhall IH, Linster M, Low DHW, Chen Y, Yan L, Sterling SL, Borthwick S, Neves ES, Lim JSL, Skiles M, Lee BPY, Wang LF, **Broder CC**, Smith GJD. Serologic Evidence of Fruit Bat Exposure to Filoviruses, Singapore, 2011-2016. Emerg Infect Dis. 2018 Jan; 24(1):114-117. doi: 10.3201/eid2401.170401. PMCID: PMC5749470
d. Laing ED, Amaya M, Navaratnarajah CK, Feng YR, Cattaneo R, Wang LF, **Broder CC***. Rescue and characterization of recombinant cedar virus, a non-pathogenic Henipavirus species. Virol J. 2018 Mar 27;15(1):56. doi: 10.1186/s12985-018-0964-0. PMCID: PMC5869790

A more complete list of published work in MyBibliography: 164 publications; total citations: >19,200
http://www.ncbi.nlm.nih.gov/sites/myncbi/christopher.broder.1/bibliography/41141103/public/?sort=date&direction=ascending

D. Research Support

Ongoing

"Serological Biosurveillance for Spillover of Henipaviruses and Filoviruses at Agricultural and Hunting Human-Animal Interfaces in Peninsular Malaysia"
Co-Principal Investigator: Christopher C. Broder, Ph.D.
Agency: DTRA: HDTRA1-17-10037: With: Ecohealth Alliance, New York, New York. Period:05/01/17-04/30/20
Major goals: The overarching goal is to characterize the distribution and detect the spillover of henipaviruses and filoviruses among indigenous farming and hunting communities in Peninsular Malaysia. As part of this process, we will build capacity at key government labs in human and animal health sectors to enhance serological surveillance in animals and human populations for these high consequence pathogens.

“Collaborative development and evaluation of an equine vaccine against Hendra virus”
Principal Investigator: Christopher C. Broder, Ph.D.
Agency: Pfizer (Zoetis)/CRADA Period: July 1, 2012 to September 30, 2040
A cooperative research and development agreement for of an equine vaccine against Hendra virus.

“A Recombinant Cedar Virus-based Henipavirus Replication Platform for High-throughput Inhibitor Screening”
Agency: NIH/NIAID: R21 AI137813-01 Period: 04/01/2018 to 03/31/20
Major goals: Develop, characterize and adapt a rCedPV luciferase reporter virus for use in HTS; Optimize the HTS parameters of recombinant virus infection and reporter activities; Pilot a HTS assay using a small molecule library.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799
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Christopher C. Broder, Ph.D.
Professor and Chair

Tele: 301-295-3401 / Fax: 301-295-3773
E-mail: Christopher.broder@usuhs.edu

December 6, 2018

Kevin J. Olival, PhD
Vice President for Research
EcoHealth Alliance
460 West 34th Street – 17th floor
New York, NY 10001

Dear Kevin,

We are very excited to expand my group's collaboration with you and EcoHealth Alliance on our emerging virus surveillance activities. Your new proposal entitled "*Strengthening biosurveillance and early detection capabilities for MERS-CoV and other coronaviruses in USCENTCOM and USEUCOM*" to the FOA Program Announcement identification information (*Global Health Engagement Research Initiative (GHERI) FOA, VP-18-011*) will be an ideal opportunity for us to expand on our combined efforts with a focus on Middle East Respiratory Syndrome Coronavirus (MERS-CoV), a critically important pathogenic emerging virus threat. Indeed, MERS-CoV has recently been recognized by the WHO as an emerging threat with pandemic potential and one needing immediate research focus. Further, the Coalition for Epidemic Preparedness Innovations (CEPI) has also made MERS-CoV vaccine research a priority this year.

We will develop recombinant MERS-CoV envelope spike protein for a multiplex microsphere-based serological assay. Using this new method for MERS-CoV we will be able determine the seroprevalence of MERS-CoV across the human-camel value chain in Jordan and be able to inform locations, seasons, and epidemiological interfaces of greatest risk. As you know we are well experienced in making native-like viral glycoprotein spikes from henipaviruses, filoviruses and others, and we already have related soluble SARS coronavirus S proteins multiplexed. Once developed we can transfer the technology and multiplex serological assay to partner nation laboratories (Jordan and Georgia) to accelerate early detection capabilities for emerging zoonotic diseases.

As always, I and my group will continue to provide our expertise and available resources to you and this exciting project, and I look forward to this continued collaboration.

Sincerely,

(b) (6)

Christopher C. Broder, Ph.D.
Professor and Chair
Department of Microbiology and Immunology

Statement of Work (USU-Broder)

Major Milestones, Tasks and Timeline.

We have laid-out a series of aims and hypotheses that will contribute significant insight into the prevalence of Middle Eastern Respiratory Syndrome (MERS)-coronavirus (MERS-CoV) and related Severe Acute Respiratory Syndrome (SARS)-CoV at human-animal interfaces in Jordan and Georgia. A Luminex-based, multiplex immunoassay will be the primary serological approach for the detection of antiviral antibodies to quantify the presence of MERS-CoV in camels, bats and human populations. The findings generated from the proposed studies will facilitate the development of a sustainable approach to serological biosurveillance and early detection of MERS-CoV at Department of Defense partner laboratories in Jordan (Jordan University of Science and Technology, JUST) and Georgia (R. Lugar Center). Epidemiological data generated through this serological approach will inform a risk analysis of MERS-CoV hotspots in these regions that will ultimately be used to improve situational awareness and force health protection. The major Milestones and associated tasks to be conducted by Uniformed Services University, along with a timeline of this proposal's goals are shown below.

Major Milestones:

•*Milestone 1*: Complete the production of 6 coronavirus spike protein constructs, develop polyclonal antiserums, and test all new antigens for utility in Luminex bead-based platforms, ELISA and Western blot assays: FY19-20.

Five recombinant coronavirus surface spike (S) proteins will be designed and constructed for the purposes of producing two high quality, native like, oligomeric virus S proteins and two monomeric S subunit proteins. An additional MERS-CoV nucleoprotein (N) will be designed and constructed as an additional virus antigen. These recombinant viral S and N proteins will be used as the capture antigens in the proposed serological, multiplex microsphere immunoassays as well as secondary or validation assays including, ELISA and Western blotting. The details of the nature and unique properties of these glycoproteins are provided in the Technical Proposal. The individual subtasks linked to this specific *Milestone 1* for USU are:

- *Subtask 1.1*: Construct and produce the full-length, native-like, trimeric MERS-CoV S protein.
- *Subtask 1.2*: Construct and produce the full-length, native-like, trimeric SARS-CoV S protein.
- *Subtask 1.3*: Construct and produce the monomeric MERS-CoV S_1 protein.
- *Subtask 1.4*: Construct and produce the monomeric MERS-CoV S_2 protein.
- *Subtask 1.5*: Construct and produce the oligomeric MERS N protein.
- *Subtask 1.6:* Construct and produce a monomeric Uganda MERS-related CoV S_1 protein
- *Subtask 1.7*: Use the completed MERS-CoV purified protein preparations to produce polyclonal rabbit serum for each individual glycoprotein as positive controls for assay validation.
- *Subtask 1.8*: Test the utility of each individual virus protein by Luminex bead-based platform, ELISA and Western blotting with matching rabbit polyclonal antisera and control camel and bat sera provided by supporting collaborators (Dr. Vincent Munster, RML, NIAID, NIH).

•*Milestone 2*: Establishing MERS-CoV multiplex microsphere immunoassays at partner labs in Jordan (JUST) and Georgia (R. Lugar Center) and training laboratory personnel to utilize Luminex bead-based platforms (MagPIX) to identify serological exposure to coronaviruses: FY19-20.

To enhance early detection and biosurveillance capacity at USCENTCOM and USEUCOM partner labs we will transfer MERS-CoV antigenic material and the multiplex microsphere immunoassay and conduct in-country staff training sessions at both JUST and R. Lugar Center in the fourth quarter of Year 1 and into Year 2. EcoHealth Alliance will procure necessary MagPIX machines and other equipment for transfer to partner countries. The individual subtasks linked to this specific *Milestone 2* for USU are:

- *Subtask 2.1*: Transfer MERS-CoV and SARS-CoV protein-coupled microspheres to JUST and R. Lugar Center
- *Subtask 2.2*: Transfer matching antigen reactive rabbit polyclonal antisera for use as assay positive controls to JUST and R. Lugar Center
- *Subtask 2.3*: Train staff in the theoretical development of the antigens, the Luminex bead-based platforms (MagPIX), advantages of this approach to standard ELISA and in the practical hands-on use and setup of the multiplex microsphere immunoassay in-country at JUST and R. Lugar Center. One 5-day training will take place in each Jordan and Georgia during FY19-20 including USU scientists and technicians and EcoHealth Alliance scientists.
- *Subtask 2.4*: Run positive control rabbit polyclonal antisera with the multiplex microsphere immunoassay in-country as part of trainings with in-country laboratory technicians at JUST and R. Lugar Center

•*Milestone 3*: Validation of the MERS-CoV multiplex microsphere immunoassay as a research and diagnostic tool: FY19-21.

Several commercial ELISA that use a single MERS-CoV antigens (e.g. S_1) have been used with varying degrees of success to detect serological evidence of exposure to MERS-CoV in livestock and human populations. We will validate a MERS-CoV multiplex serological assay and a more sensitive Luminex bead-based approach for virus surveillance. Details are provided in the Technical Proposal. The individual subtasks linked to this specific *Milestone 3* for USU are:

- *Subtask 3.1:* Obtain MERS-CoV infection control polyclonal sera from supporting partners at Rocky Mountain Laboratories, NIH and Colorado State University
- *Subtask 3.2:* Test the MERS-CoV multiplex immunoassay with camel and bat positive and negative infected control sera to determine assay specificity and sensitivity
- *Subtask 3.3*: Compare detection capabilities of the MERS-CoV S and N proteins multiplex microsphere immunoassay to a traditional ELISA assay
- *Subtask 3.4*: Statistically validate the MERS-CoV S and N proteins multiplex microsphere immunoassay as a surveillance research and diagnostic tool

•*Milestone 4*: Data analysis and analysis training to determine seroprevalence of MERS-CoV in targeted populations: FY20-21.

Sera samples will be tested at JUST and R. Lugar Center by in-country staff after training. The interpretation of serological data requires the appropriate controls and statistical methods to determine whether individual samples contain target MERS-CoV specific antibodies and to determine populations level seroprevalence. USU and EcoHealth Alliance scientists will train laboratory staff at JUST and R.

Lugar Center how to interpret positive and negative sera data generated by screening with the multiplex microsphere immunoassay. The individual subtasks linked to this specific *Milestone 4* are:

- *Subtask 4.1*: Use control rabbit polyclonal sera to analyze data for assay sensitivities and positive value cutoff thresholds
- *Subtask 4.2*: Use distribution analysis methods to determine positive and negative cutoffs with camel, bat and human sera tested at JUST and R. Lugar Center
- *Subtask 4.3*: Determine seroprevalence for MERS-CoV and SARS/SARS-like CoV in the camel, bat and human populations tested

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Laing, Eric D.

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6)

POSITION TITLE: Scientist

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of Maryland, College Park, MD	B.S.	05/2008	Biology
Uniformed Services University, Bethesda, MD	Ph.D.	10/2016	Emerging Infectious Diseases

A. Personal Statement

This project leverages ongoing DoD disease surveillance with partner nation laboratories in Jordan and Georgia and aligns with CENTCOM and EUCOM objectives to strengthen partner nation readiness and capabilities for early detection of emerging coronaviruses. MERS-coronavirus (CoV), like the related SARS-CoV, can be a fatal respiratory illness. Building surveillance capacity at partner labs will contribute to the development of informed risk-assessments and improvement of force health protection in the regions. I have had a collaborative research relationship with the PI, Kevin Olival (EcoHealth Alliance), dating back to 2014 when I was a co-chair of a symposium on bat ecology and emerging viruses where Dr. Olival was an invited speaker. This proposal will allow for an important extension of our collaboration and mutual research interests on emerging viruses and disease surveillance. Since 2017, I have been collaborating with EcoHealth Alliance, as a Co-PI, on a related DoD-funded virus biosurveillance project and am also involved in DoD-funded disease surveillance and capacity training projects in India, Thailand, and Cambodia that are aimed at understanding the geographic distribution/serological footprint of filoviruses and henipaviruses, the wild and domestic animals that are natural hosts of these viruses, and the at-risk human populations for virus spillover. I have led international capacity training with multiple collaborators including: 3+ years of collaboration at Duke-National University of Singapore, Singapore; 1+ year laboratory-based training at the National Wildlife and Forensic Laboratory and the National Public Health Laboratory, Kuala Lumpur, Malaysia; <1 year laboratory-based training at the National Centre for Biological Sciences, Bangalore, India. I have both the expertise in virology, biosurveillance and capacity training to successfully contribute to the aims of this proposal. In addition to serological biosurveillance, I have training in molecular virology techniques and optimized a reverse genetics approach to rescue a recombinant Cedar virus reporter virus, a non-pathogenic *Henipavirus* species that will be used as a model *Henipavirus* to explore host cell-pathogen interactions, cellular tropism, and test novel therapeutics against henipaviruses. Our laboratory is capable of producing native-like virus antigens that are used to capture conformationally-dependent antibodies and used in a serological multiplex assay as demonstrated by our successful biosurveillance for zoonotic select agents in the *Ebolavirus* and *Henipavirus* genera. We have piloted expression of select coronavirus antigens and this project will provide the opportunity to further develop our research into MERS-CoV disease surveillance.

B. Positions and Honors

Positions and Employment

2003-04 Howard Hughes Medical Institute student intern, Cellular and Developmental Neurobiology Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD.

2005-06 Undergraduate research assistant, Department of Animal and Avian Sciences, University of Maryland, College Park, MD.
2007-08 Undergraduate research assistant, Biology Departmental Honors research, Department of Biology, University of Maryland, College Park, MD.
2008-09 Lab technician, Department of Pharmacology, Uniformed Services University, Bethesda, MD.
2010 Lab technician, Department of Microbiology, Uniformed Services University, Bethesda, MD.
2010-16 Graduate research student, Department of Microbiology, Uniformed Services University, Bethesda, MD.
2016-17 Postdoctoral fellow, Henry M. Jackson Foundation, Department of Microbiology, Uniformed Services University, Bethesda, MD.
2017 - Junior scientist, Henry M. Jackson Foundation, Department of Microbiology, Uniformed Services University, Bethesda, MD.

Other Experience and Professional Memberships

2009 Mentor, At-Risk Student Mentoring, Bethesda Chevy Chase High School, Bethesda, MD.
2009-10 Mentor, EnvironMentors, Washington, D.C.
2013 Mentor, high school, undergraduate, and graduate students, Uniformed Services University, Bethesda MD.
2014 Participant, American Society of Microbiology Kadner Institute
2014-15 Volunteer, AAAS/Senior Scientists and Engineers STEM Volunteer Program
2014-17 Member, American Society of Tropical Medicine and Hygiene
2014-17 Member, American Society of Microbiology
2015-16 Member, USU Global Health Interest Group

Honors and Fellowships

2004-07 Maryland House of Delegates Scholarship
2005-07 Semester Academic Honors
2006 College Park Life Sciences Scholars Program Citation
2008 High Honors, Biology Departmental Honors Program
2015 USU Research Days Graduate Student Poster Presentation Finalist (Won)
2015 NSF East Asia and Pacific Summer Institutes (EAPSI) Fellowship
2015-16 Val G. Hemming Fellowship, Henry M. Jackson Foundation

C. Contribution to Science

1. Surveillance of emerging zoonotic viruses. My most recent research experiences have focused on the application of Luminex-based serological screening for virus surveillance. This research investigates the geographic distribution of ebolaviruses, marburgviruses, and henipaviruses through biosurveillance of wildlife, domestic animals, and human populations within Southeast Asia and India. These studies resulted in the expansion of a Luminex-based multiplex binding assay to include envelope glycoproteins from all known species of ebolaviruses, marburgviruses, and henipaviruses, and the identification of previously un-sampled bat populations that may contribute to the maintenance of these viruses in the environment. Present research efforts are focused on improving the interpretation of serological findings on individual and populations levels, antigenic-mapping, and building capacity for collaborating international laboratories to conduct screening in-country.

a. Dovih P, Low D.L.H, ChenY., **Laing E.D.**, Ansil B.R., Hitch A.T., Broder C.C, Smith G.J.D., Linster M., Ramakrishnan. *Manuscript in submission*
b. **Laing E**.**D**.*, Mendenhall I.H.*, Chen Y., Yan L., Wen D.L.H, Lynn J.L.S., Sterling S.L., Skiles M., Lee B.PY-H., Linster M., Wang L.F., Broder C.C., and G.J.D. Smith. Serologic evidence of fruit bat exposure to filoviruses, Singapore, 2011–2016. **Emerg Infect Dis**. 24(1):122-126, 2018.

2. Studies of virus-host cell interactions. My Ph.D. thesis research was to understand the bat as a host of zoonotic viruses. One aim of this work was focused on exploring the antiviral mechanisms that enable cellular persistence of viruses in bats. These findings revealed that the autophagy pathway is induced upon infection with Australian bat lyssavirus (ABLV), a Rabies-virus related virus carried by Australian *Pteropus* bats. The combined pharmacological and genetic studies of the autophagy pathway in the context of this virus-host

interaction indicated that autophagy functions as an antiviral defense. The study also revealed that bats have elevated levels of basal autophagy, which might help to explain the cellular mechanism that contribute to the ability of bats to act as host to these viruses. An additional finding from these studies was that activation of autophagy may have therapeutic benefits during neurotropic virus infection. A second aim of this work was to construct and propagate a recombinant, replication-component Cedar virus. Cedar virus is a non-pathogenic *Henipavirus* species closely related to Nipah virus that we use as a model virus species to understand differences in pathogenesis and cellular tropism.

a. Weir D. L., **Laing, E.D.**, Smith I.L., Wang L.F., and C. C. Broder**.** Host cell entry mediated by Australian bat lyssavirus G envelope glycoprotein occurs through a clathrin-mediated endocytic pathway that requires actin and Rab5. **Virology Journal**. 11:40, 2013.
b. **Laing E.D.***, Amaya M*, Navaratnarajah C.K., Cattaneo R, Wang L.F., Broder C.C. Rescue and characterization of recombinant Cedar virus, a non-pathogenic *Henipavirus* species. **Virology Journal**. 15(1):56, 2018.
c. Mastraccio K.E., Huaman C., Ho T., Vu B.K., Smith I., Weir D.L., **Laing E.D.**, Broder C.C., and B.C. Schaefer. Human monoclonal antibody therapy eliminates CNS-resident lyssavirus infection via a CD4 T-cell mediated mechanism. *Manuscript in submission*.
d. **Laing E.D.**, Weir D.L., Sterling S.L., Wang L.F., Schaefer B.S., and C.C. Broder. The antiviral role of autophagy in bats, reservoirs of emerging viruses. *Manuscript in prep.*
e. **Laing E.D.***, Navaratnarajah C.*, Silva S.*, Petzing S.R., Cattaneo R., Broder C.C., and Xu K. Alternative ephrin ligand entry receptor-usage and expanded tropism of *Cedar henipavirus. Manuscript in prep.*

D. Additional Information: Research Support and/or Scholastic Performance

Ongoing Research Support

GEISP0110-19 Hertz (PI) 10/01/18 – 09/30/19
Sero-survey of Nipah virus and other pathogenic bat borne paramyxoviruses in Cambodia
The goal of this study is to retrospectively investigate potential human exposure to Nipah virus and related paramyxoviruses in geographies with and without *Pteropus* colonies to inform risk assessments of Nipah virus spillover.
Role: Co-Investigator

HDTRA1-17-10037 Epstein (PI) 05/01/17 – 04/30/20
Serological Biosurveillance for Spillover of Henipaviruses and Filoviruses at Agricultural and Hunting Human-Animal Interfaces in Peninsular Malaysia
The overarching goal is to characterize the distribution and detect the spillover of henipaviruses and filoviruses among indigenous farming and hunting communities in Peninsular Malaysia. As part of this process, we will build capacity at key government labs in human and animal health sectors to enhance serological surveillance in animals and human populations for these high consequence pathogens.
Role: Co-Principal Investigator

Completed Research Support

USU T0-73-3274 Laing (PI) 11/06/14 – 11/05/15
The Role of Autophagy during *Lyssavirus* & *Henipavirus* infection of Bat Cells
The goal of this study was to investigate the role that the autophagy pathway has during replication of Australian bat lyssavirus and Cedar virus and whether differences exist between human and bat cells.
Role: Principal Investigator

NSF EAPSI 1515304 Laing (PI) 06/08/15 – 08/01/15
Utility of a Multiplex Assay to Examine the Presence of Bat Borne Viruses in Southeast Asia
The goals of this study were to 1) Investigate three bat populations in Southeast Asia for evidence of past exposure to ebolaviruses, marburgviruses, and henipaviruses, 2) Examine whether virus species included in this assay can be differentiated, and 3) Demonstrate that this assay can be transferred to international collaborators.
Role: Principal Investigator

From: Boxley, Kimberly on behalf of Boxley, Kimberly <kimberly.boxley.ctr@usuhs.edu>
To: hamel@ecohealthalliance.org; Kevin Olival
Cc: Christopher Broder; Laing, Eric
Subject: Sub-Award Documents on behalf of Drs Laing and Broder
Date: Wednesday, December 19, 2018 3:46:04 PM
Attachments: Budget Justification_Broder_CGHE_Final-CCB-EDL.docx
Research and Related Budget_Broder_CGHE.pdf
Performance Site_Broder.docx
CCBroder.Biosketch-NIH-EcoH-GHERI-120718.docx
CCBroder-USU-EcoH-LOC-Dec2018.pdf
SOW_Broder Lab-USU_VP-18-011 (GHERI FY18 FOA) v2.docx
LaingE_Biosketch_GHERI v2.docx

Hello all,

Please accept the following documents for Drs Broder and Laings' contribution to the CGHE GHERI award.

Once we hear back from USU-VPR with their review, we will follow up.

If you have any questions, please let me know.

Thanks much .... Kim

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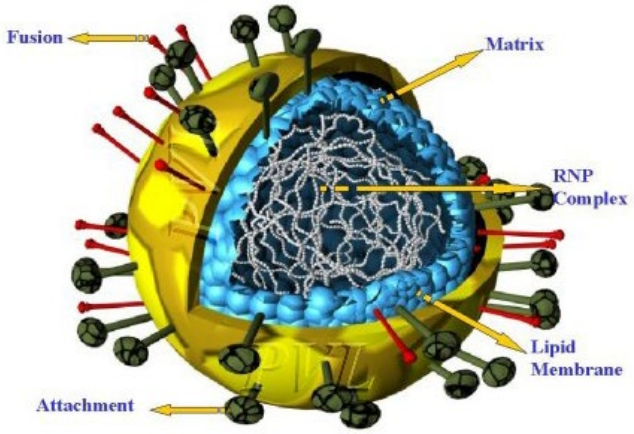
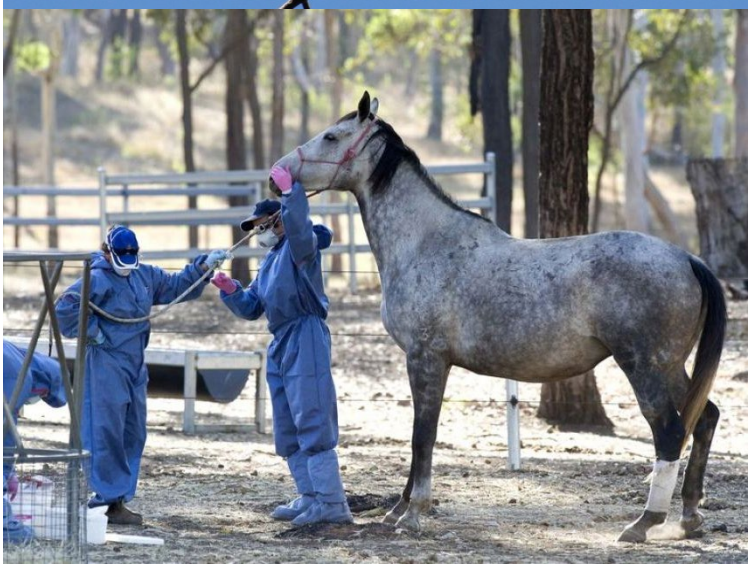
United States Department of Agriculture

Agricultural Research Service

November 2018

Henipavirus Gap Analysis
Workshop Report





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To cite this report:
Henipavirus Gap Analysis Workshop Report. 2018. U.S. Department of Agriculture, Agricultural Research Service, Washington, DC. http://go.usa.gov/xnHgR.

Contents

EXECUTIVE SUMMARY

Henipavirus is the taxonomic genus for a group of viruses in the family Paramyxoviridae that includes *Hendra virus* (HeV) and *Nipah virus* (NiV). These viruses are zoonotic agents that are highly pathogenic in humans with case fatality rates of 40% to 70%. As such, these viruses are classified as Biosafety Level 4 (BSL-4) agents, requiring the highest level of laboratory biocontainment. Importantly, they have many of the physical attributes to serve as potential agents of bioterrorism, and are also considered emerging zoonotic pathogens with increasing geographical distribution in Australia, New Caledonia, Southeast Asia, and Madagascar.

Hendra virus first emerged in 1994 in Australia spilling over from bats to horses to humans, causing several disease outbreaks since with significant fatality rates. Nipah virus emerged in Malaysia in 1999, resulting in nearly 300 human cases with over 100 deaths.

The Nipah virus outbreak in Malaysia was especially concerning, causing widespread panic and fear because of the high mortality rate in people and the inability to control the disease initially. There were also considerable social disruptions and tremendous economic loss to an important pig-rearing industry. This highly virulent virus, believed to be introduced into pig farms by fruit bats, spread easily and silently among pigs and was transmitted to humans who came into close contact with infected animals. A NiV outbreak in Bangladesh in 2001 resulted from direct bat to human transmission via contaminated date palm juice with further spread within the human population. From 2001 to 2012, the World Health Organization (WHO) reported a total of 209 cases, with 161 deaths due to of NiV infections. In 2014, the WHO reported a NiV outbreak in fourteen districts of Bangladesh, resulting in 24 cases and 21 deaths. In 2015, three fruit bats tested positive for NiV in New Caledonia at the Noumea National Park, including three bats at the Noumea Zoo.

This gap analysis report focuses primarily on NiV and its potential impact on agricultural swine production. However, information is also provided on the threat henipaviruses pose to public health, both as emerging zoonotic agents and as potential agents of bioterrorism. Included in this report is scientific information on *Henipavirus* virology, epidemiology, pathogenesis, immunology, and an assessment of the available veterinary medical countermeasures to detect, prevent, and control disease outbreaks. Importantly, gaps are provided to inform research needs and priorities. Some of the major gaps and obstacles for disease control can be summarized as follows:

Diagnostics

The availability of safe laboratory diagnostic tests are limited. Virus isolation and serum neutralization assays require live NiV; thus, BSL-4 containment laboratories are required. Nucleic acid-based assays, such as RT-PCR are available, but genetic variation amongst henipaviruses are reported to impact sensitivity and real time RT-PCR may not be able to detect all divergent and novel henipavirus strains. Serological assays are limited in their ability to differentiate between known and unknown henipaviruses, as cross-reactivity to one or more known viruses is possible. Commercial diagnostic test kits are not available. International standards for NiV assay validation are needed. Gaps include a lack of positive experimental and field samples for test validation (or even evaluation) and there are restrictions on material transfer (e.g., obtaining animal samples that could be used to validate tests) due to biosecurity concerns. Low biosafety level reference sera

against various isolates are not yet available. There is a need for high throughput antibody assays for disease outbreaks, recovery and surveillance purposes. There is also a need to develop operator-safe diagnostics tests and reagents that can be produced in low biocontainment facilities.

Vaccines

There is currently a commercial vaccine available for horses, but there are no vaccines for swine or humans. There are several experimental vaccine candidates that may be safe and effective in swine and other domestic animals. However, all these vaccine candidates will require further research to establish their efficacy, and they will need to be fully developed to be licensed and stockpiled for rapid use in an emergency disease outbreak in swine.

Surveillance

Surveillance is the first line of defense against a disease outbreak. Rapid and accurate detection affects the time when control measures can be implemented and affects the extent of the disease outbreak. Because of limitations with laboratory diagnosis, surveillance programs are dependent on the reporting of clinical signs in populations at risk. Diagnosis of NiV infections based on clinical presentation has a low positive predictive value as there are numerous etiologies for encephalitis in humans, and clinical signs in pigs are difficult to differentiate from many common endemic infectious diseases.

Depopulation

Depopulation is the primary countermeasure to reduce virus shedding and stop the spread of NiV in livestock. Disease outbreaks have shown that the control of NiV in pig populations through stamping out is complex due to the zoonotic nature of the agent. In addition, depopulation may be logistically difficult and may be impossible in a rapidly spreading outbreak in countries where there are pig dense regions with millions of pigs, such as the states of Iowa, North Carolina, and Minnesota in the United States, or South East China.

GROUP PICTURES

Henipavirus Gap Analysis Working Group, Winnipeg, Canada
November 14-17, 2017

The Nipah Virus Countermeasures Working Group, Geelong, Australia
March 17-19, 2009

GLOSSARY

APHIS: Animal and Plant Health Inspection Service, USDA, United States of America

ARS: Agricultural Research Service

AAHL: Australian Animal Health Laboratory

BSL-4: Biosafety Level 4

CDC: U.S. Centers for Disease Control and Prevention, HHS, United States of America

CFIA: Canadian Food Inspection Agency

DIVA: Differentiating between infected and vaccinated animals

ELISA: Enzyme-linked immunosorbent assay

FADDL: U.S Foreign Animal Disease Laboratory, Plum Island Animal Disease Center

FLI: Friedrich Loeffler Institute

GMP: good manufacturing practice

HeV: Hendra virus

HHS: Department of Human Health Services, United States of America

HSPD-9: Homeland Security Presidential Directive Nine

ICAR: Indian Council of Agricultural Research

Ig: Immunoglobulin

IEDCR: Institute of Epidemiology, Disease Control and Research in Bangladesh

MLV: Modified live virus vaccine

NAHLN: National Animal Health Laboratory Network, USA

NIHSAD: National Institute of High Security Animal Diseases, ICAR, India

NCFAD: National Center for Foreign Animal Disease, CFIA, Canada

NiV: Nipah virus

NiV-B: Nipah virus Bangladesh

NiV-M: Nipah virus Malaysia

NiV N: Nipah virus nucleoprotein

NVCWG: Nipah Virus Countermeasures Working Group

NVS: National Veterinary Stockpile

OIE: World Organisation for Animal Health

PCR: Polymerase Chain Reaction.

PPE: Personal Protective Equipment

RT-PCR: Reverse transcription-polymerase chain reaction

rRT-PCR: Real-time reverse transcription-polymerase chain reaction

sHeV G: recombinant soluble Hendra virus G protein

sNiV G: recombinant soluble Nipah virus G protein

USDA: United States Department of Agriculture, United States of America

INTRODUCTION

Nipah virus (NiV) is an emerging zoonotic virus. First isolated in pigs and people from an outbreak in Malaysia in 1998 (Ang *et al.* 2018), this emerging virus causes severe disease in humans. The source of transmission was determined to be from bats to pigs to humans, through close contact with infected animals. The virus is named after the location where it was first detected in Sungai Nipah, a village in the Malaysian Peninsula where exposed pig farmers became severely ill with encephalitis.

Nipah virus is closely related to another zoonotic virus called Hendra virus (HeV), formerly called Equine *Morbillivirus*, and named after the town where it first appeared in Australia. Hendra virus infection was first recognized in 1994, when it caused an outbreak of acute, fatal respiratory disease that killed 14 horses. Three human cases, leading to two deaths were recorded during the outbreak. The precise mode of virus transmission to the three Australian patients is not fully understood. All three individuals appear to have acquired their infection as a result of close contact with horses, which were ill and later died.

Although members of this group of viruses have only caused a few focal outbreaks, their ability to infect a wide range of animal hosts and to produce a high mortality rate in humans has made this emerging zoonotic viral disease a significant public health threat.

Certain species of bats of the genus *Pteropus* (fruit bats, also called flying foxes) are the principal natural reservoir hosts for NiV and HeV – see Table I. Bats are susceptible to infection with these viruses but do not develop disease. Fruit bats are distributed across an area encompassing Australia, Southeast Asia; including Indonesia, Malaysia, the Philippines and some of the Pacific Islands, the Indian subcontinent, and Madagascar (See Fig. 1). There is also growing evidence that viruses related to NiV and HeV circulate in non-pteropid fruit bats across the globe (Clayton, 2017).

The exact mode of transmission of henipaviruses is uncertain, but appears to require close contact with contaminated tissue or body fluids from infected animals. The role of domestic species other than pigs in transmitting NiV infection to other animals has not yet been determined. In 2014, an outbreak was reported in the Philippines involving the consumption of meat from NiV-infected horses, further expanding the potential routes of transmission for henipaviruses.

Despite frequent contact between fruit bats and humans there is no serological evidence of human infection among persons that are in contact with bats. Pigs were the apparent source of infection among most human cases in the Malaysian outbreak of NiV in 1998-1999. Nipah virus has continued to spillover over from animals with at least six outbreaks resulting in human fatalities in Bangladesh in 2013, one in India in 2014, and two in Bangladesh in 2015. The World Health Organization (WHO) had not reported any NIV cases 2016-2017, but in 2018 twenty three new cases and 21 deaths were reported in Kerala, India - See Table II.

The spread of henipaviruses to new geographical areas is a concern. In 2014, the Philippines reported an outbreak with a zoonotic paramyxovirus in horses and people. There is further evidence for broader distribution of NiV in pteropid fruit bats species. There is also growing evidence that viruses related to NiV and HeV also circulate in non-pteropid fruit bats worldwide.

BACKGROUND

Organization of the Gap Analysis Working Groups on Nipah Virus (2009 and 2017)

The United States Department of Agriculture (USDA) organized the first Nipah virus gap analysis workshop in Australia in 2009 with the support of the Australian Animal Health Laboratory (AAHL). The working group was charged by the USDA National Veterinary Stockpile Steering Committee with making recommendations on specific materials, commercially available and in the pipeline, which will ensure the United States has an arsenal of highly efficacious countermeasures to control and mitigate the impact of an outbreak of Nipah virus. Nipah virus experts representing laboratories in South East Asia, Australia, Canada, and the United States were invited to participate and contributed to this report. The second workshop was organized in 2017 by the Special Pathogens Unit, National Centre for Foreign Animal Disease, Canadian Food Inspection Agency (CFIA), in collaboration with BSL4ZNet and DISCONTOOLS (http://www.discontools.eu/). The participants were charged with assessing available veterinary medical countermeasures to control and respond to a Nipah virus disease outbreak. In addition, the workshop participants agreed to update the gap analysis conducted at the AAHL in Geelong, Australia, in 2009.

Report Updates

This report will be updated periodically with new scientific information, research breakthroughs, and/or the successful development of veterinary medical countermeasures. This report was last updated with the support of Henipavirus experts November 2018.

Reference Material

The following reports and websites are recommended:

OIE – World Organisation for Animal Health - Nipah in Animals
http://www.oie.int/en/animal-health-in-the-world/animal-diseases/Nipah-Virus/
Accessed July 22, 2018

FAO – Food and Agriculture Organization
Manual on the diagnosis of Nipah virus infection in animals
www.fao.org/DOCREP/005/AC449E/AC449E00.htm
Accessed July 22, 2018

CDC – Center for Disease Control and Prevention - Special Pathogens Branch
https://www.cdc.gov/vhf/nipah/index.html
Accessed July 22, 2018

WHO - World Health Organization
http://www.who.int/news-room/fact-sheets/detail/nipah-virus
Accessed July 22, 2018

Guidelines for Veterinarians Handling potential Hendra Virus infection in Horses (QDPI)
https://www.daf.qld.gov.au/__data/assets/pdf_file/0005/126770/2913_-Guidelines-for-veterinarians-handling-potential-Hendra-virus-infection-in-horses-V5.1.pdf

Accessed July 22, 2018

CFSPH – Center for Food Security and Public Health
Nipah Virus Infection
http://www.cfsph.iastate.edu/Factsheets/pdfs/nipah.pdf
Accessed July22, 2018

DEFINITION OF THE THREAT

The threat for a natural introduction of henipaviruses in the United States is low, but there is significant concern that henipaviruses could be used for nefarious purposes to harm agriculture and people. Both Hendra virus and Nipah virus are on the HHS and USDA list of overlap Select Agents and Toxins. Henipaviruses are listed as APHIS Tier 3 high-consequence foreign animal diseases and pests. Henipaviruses are promiscuous in their ability to cause severe morbidity in several animal species, including people, and human infections result in a very high mortality rate. The mortality rate in pigs is actually reported as about 2.5% in adult pigs – high morbidity, but low mortality. Mortality rates in humans range from 40% (Malaysia) to 75% (up to 100%) in Bangladesh. The animal reservoir includes several species of bats, and henipaviruses may thus be readily available in these wildlife reservoirs.

INFECTION IN PEOPLE

Between September 1998 and June 1999, a NiV outbreak in Malaysia resulted in severe viral encephalitis in 105 patients (Goh *et al.,* 2000; Epstein *et al.,* 2006). Ninety-three percent had had direct contact with pigs, usually within two weeks prior to the onset of illness, suggesting that there was direct viral transmission from pigs to humans and a short incubation period. The main presenting features were fever, headache, dizziness, and vomiting. Fifty-two patients (55%) had a reduced level of consciousness and prominent brain-stem dysfunction. Distinctive clinical signs included segmental myoclonus, areflexia and hypotonia, hypertension, and tachycardia. The initial cerebrospinal fluid findings were abnormal in 75% of patients. Antibodies against Hendra virus were detected in serum or cerebrospinal fluid in 76 percent of 83 patients tested. Thirty patients (32%) died after rapid deterioration in their condition. An abnormal doll's-eye reflex and tachycardia were factors associated with a poor prognosis. Death was probably due to severe brain-stem involvement. Neurologic relapse occurred after initially mild disease in three patients. Fifty patients (53%) recovered fully, and 14 (15%) had persistent neurologic deficits.

Unlike Malaysia, the NiV outbreaks in Bangladesh were strictly confined to human populations with significantly higher mortality rate (Hossain *et al.,* 2008). NiV outbreaks in Bangladesh have continued annually since 2008 resulting in a total of 207 reported cases, 152 of which were fatal resulting in a 70% mortality rate (Clayton,. 2017). In 2018, NiV infection was confirmed in Kerala, India, where 23 confirmed cases were reported and case fatality rates were 90% (Arunkumar *et al.,* 2018).

INFECTION IN PIGS

The NiV outbreak in Maylasia in 1999 was facilitated by the rapid spread of the virus in pig populations. Although some pigs demonstrated a febrile respiratory illness with epistaxis, dyspnoea, and cough, few animals exhibit neurological signs, and the majority of pigs had subclinical infections. There are no clinical signs in pigs that are specific for NiV infection. Both, apparently healthy pigs and pigs showing clinical signs shed significant amount of virus.

ECONOMIC IMPACT

The NiV outbreak in Malaysia in 1999 destroyed the main market for Malaysian hogs in Singapore. The Malaysia outbreak resulted in an 80% drop in pork consumption in the domestic market. Over half the standing pig population in the country was culled to halt the outbreak. Half the pig farms

went out of business. The cumulative economic losses based on government figures was estimated to be approximately $217 million USD.

BIOTERRORISM

NiV has many of the physical attributes needed for a biological weapon, including easy access to virus resulting from its wide distribution in nature and laboratories, easy to produce, easy to disseminate, and the potential for high morbidity and mortality in people.

GAP ANALYSIS

The following section summarizes what we know about henipaviruses, gaps in our knowledge, and the threat of bioterrorism.

VIROLOGY

The following summarizes our current knowledge of viral strains, taxonomy, reservoir, genome, morphology, determinants of virulence, host range, and tissue tropism.

Virus species

Nipah virus (NiV) was first isolated in 1999 from samples collected during an outbreak of encephalitis and respiratory illness among pig farmers. The name Nipah originated from Sungai Nipah, a village in the Malaysian Peninsula where pig farmers became sick. There are currently two genotypes identified: NiV-Malaysia and NiV-Bangladesh. Different strains/genotypes of NiV have emerged: Malaysia, Bangladesh, and Cambodia. NiV Malaysia resulted in the culling of a million pigs and 250 human cases (106 fatal). NiV Bangladesh is associated with outbreaks in people (Clayton, 2017).

Hendra virus (HeV) was first isolated in 1994 from specimens obtained during an outbreak of respiratory and neurologic disease in horses and humans in Hendra, a suburb of Brisbane, Australia.

Cedar virus (CedV) is a novel *Henipavirus* isolated from Australian bats, which appears to be non-pathogenic in lab animal experiments (Marsh et al. 2012).

Ghanaian bat henipavirus (GhV) is a species of henipaviruses assembled from sequences collected from *Eidolon helvum*, a bat species in the family Pteropodidae (Drexler *et al.* 2009; Drexler *et al*. 2012). No isolates have been reported, and both pathogenicity and the cross-species transmission remain unknown. Partial sequences of 19 phylogenetically novel African henipaviruses have also been discovered, suggestive of a further diversity of African henipaviruses.

Mòjiāng henipavirus (MojV) was discovered during retrospective surveillance for the etiologic agent responsible for cases of fatal respiratory illness in cave-miners, China. A full-genome was assembled from sequences detected from a cave-dwelling rodent species (Wu Z. *et al.* 2014). MojV is circumstantially associated with the fatal respiratory illness, however, pathogenicity studies have not been completed.

Taxonomy

NiV and HeV are members of the family Paramyxoviridae, order *Mononegavirales*. Comparison of nucleic acid and deduced amino acid sequences with other members of the family confirms that NiV and HeV are members of the family Paramyxoviridae, but with limited homology with members of the *Morbillivirus*, *Rubulavirus* and *Respirovirus* genera (See Fig. 2). The name *henipavirus* was recommended for the genus of both HeV and NiV (Wang *et al.,* 2000). HeV appear to be less diverse that NiV but molecular epidemiology studies are needed to identify new isolates that may bridge the gap between HeV and NiV.

Reservoir

The natural reservoir, or primary animal host, of the henipaviruses are fruit bats mainly from the genus *Pteropus* (flying foxes). Nucleic acid and antibody signatures of exposure to NiV or NiV-like viruses has been documented in a diversity of bat species across the globe (Table 1).

Genome

The complete genomes of both HeV and NiV have been sequenced (Wang *et al.,* 2001). Henipaviruses have a large non-segmented genome comprised of single-stranded negative-sense RNA. Their genomes are 18.2 kb in size and contain six genes corresponding to six structural proteins. All genes are of similar size to homologues in the respirovirus and morbillivirus genera, with the exception of P which is 100-200 amino acids longer (See Fig. 3). Most of the increase in genome length is due to longer untranslated regions between genes, mainly at the 3' end of each gene. The role of these long untranslated regions are not understood. Henipaviruses employ an unusual process called RNA editing to generate multiple proteins from a single gene. The process involves the insertion of extra guanosine residues into the P gene mRNA prior to translation. The number of residues added determines whether the P, V or W proteins are synthesized. The C protein is made via an alternative translational initiation mechanism. The functions of the V, W, and C proteins are unknown, but they may be involved in disrupting host antiviral mechanisms (see Immunology below). The function of the G protein is to attach the virus to the surface of a host cell via the major receptors ephrin-B2 and ephrin-B3 ligands, highly conserved proteins present in many mammals. G glycoprotein is the major neutralizing antigen and the target protein for vaccine development. X-ray crystal structure for NiV G complex with ephrin-B3 has been determined. This interaction is highly conserved between NiV and HeV. This interaction is a prime candidate for developing henipavirus specific therapeutics. The F protein fuses the viral membrane with the host cell membrane, releasing the virion contents into the cell. It also causes infected cells to fuse with neighboring cells to form large multinucleated syncytia.

The genome size and organization of CedPV is very similar to that of HeV and NiV. The nucleocapsid protein displays antigenic cross-reactivity with henipaviruses and CedPV uses the same receptor molecule (ephrin-B2) for entry during infection. Clinical studies with CedPV in *Henipavirus* susceptible laboratory animals confirmed virus replication and production of neutralizing antibodies although clinical disease was not observed. In this context, it is interesting to note that the major genetic difference between CedPV and HeV or NiV lies within the coding strategy of the P gene, which is known to play an important role in evading the host innate immune system. Unlike NiV and HeV, and almost all known paramyxoviruses, the CedPV P gene lacks both RNA editing and also the coding capacity for the highly conserved, interferon pathway antagonists, V or W proteins (Marsh *et al.* 2012).

Although, GhV and MojV have not yet been isolated from hosts, sequence constructed genomes are similar in size, organization, and coding capacity to HeV, NiV, and CedV (Wu Z *et al.* 2014, Drexler *et al*. 2012). Like HeV and NiV, both GhV and MojV are predicted to possess the RNA editing site in the P gene and presumably coding capacity for V and W proteins. Receptor-usage studies with recombinant GhV G glycoprotein demonstrated that like CedV, GhV G was capable of binding to

ephrin-B2, but not ephirn-B3 (Lee B *et al.* 2015). A receptor remains undiscovered for MojV; however, ephrin-B2, -B3 appear to be unlikely candidates (Rissanen I *et al.* 2017).

Morphology

Henipaviruses are pleomorphic ranging in size from 40 to 600 nm in diameter. They possess a lipid membrane overlying a shell of viral matrix protein. At the core is a single helical strand of genomic RNA tightly bound to the nucleocapsid (N) protein and associated with the large (L) and phosphoprotein (P) proteins, which provide RNA polymerase activity during replication. Embedded within the lipid membrane are spikes of fusion (F) protein trimers and attachment (G) protein tetramers.

Determinants of virulence, host range, and tissue tropism

Molecular determinants of virulence, host range and cell tropism have been extensively studied and are well understood for many paramyxoviruses. Infectivity is determined by the cell-attachment and fusion glycoproteins and the presence of appropriate P gene products modulate virulence by antagonizing the cellular interferon response.

Henipaviruses have a large host range, unlike other members of the Paramyxoviridae, which generally have a very narrow host range. The cell attachment protein, unlike many other members for the paramyxovirus subfamily, does not have haemagglutinating activity and as a consequence does not bind sialic acid on the surface of cells.

The receptor for henipavirus is present on many different cultured cell types from many different species. The receptors for HeV and NiV are the same and have been identified as ephrin-B2 and ephrin-B3. Ephrin-B2 or -B3 are highly conserved across vertebrate species and are members of a family of receptor tyrosine kinase ligands. Ephrin-B2 is highly expressed on neurons, smooth muscle, arterial endothelial cells and capillaries, which closely parallels the known tissue tropism of HeV and NiV *in vivo*. Ephrin-B3 is also widely expressed but particularly in specific regions of the central nervous system and may facilitate pathogenesis in certain neural subsets.

Virology Research Priorities

- Molecular epidemiology and determinants of strain variation
- Need sequencing of henipaviruses from bats, especially Bangladesh
- Determine molecular basis for virulence

PATHOGENESIS

The following summarizes our current knowledge of viral pathogenesis, including routes of infection, tissue tropism, pathogenesis, clinical signs, and clinical pathology reported in naturally acquired infections. It should be noted that experimental infection in other animal models have been developed. NiV and HeV (henipaviruses) are distinguished from all other paramyxoviruses particularly by their broad species tropism and ability to cause fatal disease in multiple vertebrate hosts including humans, monkeys, pigs, horses, cats, dogs, ferrets, hamsters and guinea pigs, spanning 6 mammalian Orders (Broder CC *et al.,* 2012; Geisbert TW *et al.,* 2012).

NiV infections in humans and pigs are linked to contact with bats. Clinical signs in human cases indicate primarily involvement of the central nervous system with 40% of the patients in the Malaysian outbreak having also respiratory syndromes, while in pigs the respiratory system is considered the primary virus target, with only rare involvement of the central nervous system.

Humans

The main histopathological findings include a systemic vasculitis with extensive thrombosis and parenchymal necrosis, particularly in the central nervous system (Wong *et al.,* 2002). Endothelial cell damage, necrosis, and syncytial giant cell formation are seen in affected vessels. Characteristic viral inclusions are seen by light and electron microscopy. Immunohistochemistry (IHC) analysis shows the widespread presence of NiV antigens in endothelial and smooth muscle cells of blood vessels (Hooper *et al*., 2001). Abundant viral antigens are also seen in various parenchymal cells, particularly in neurons. The brain appears to be invaded via the hematogenous route and virus has been isolated from the cerebrospinal fluid of patients with NiV encephalitis (Wong *et al*., 2002). Infection of endothelial cells and neurons as well as vasculitis and thrombosis seem to be critical to the pathogenesis of this new human disease.

NiV infection can rarely cause a late-onset encephalitis up to a couple of years following a non-encephalitic or asymptomatic infection, or a relapsed encephalitis in patients who had previously recovered from acute encephalitis (Wong *et al*., 2001; Goh *et al*., 2000; Tan *et al*., 2002).

The most recent NiV outbreak, and first reported in South India, resulted in 23 human cases with a case-fatality rate of 91% (Arunkumar *et al*., 2018). The clinical manifestations and high fatality rate among people were similar to those of earlier NiV outbreaks in India and Bangladesh, and the NiV isolate from this outbreak showed a 97% genetic similarity to the NiV-B lineage. All human cases, following the index case, were due to nosocomial transmission in three different hospitals. Although it was not possible to establish the exact NiV transmission event to the identified index case, the most likely zoonotic route was from *P.* giganteus (Indian flying fox). It was noted that in Kerala, date palms are not used for obtaining sap, and the narrow-mouthed vessels used to collect sap from coconut and Asian Palmyra palm do not allow access by bats. The human-to-human transmission rate was very high in this recent outbreak, and the index case transmitted NiV to 19 contacts (primary cases), while three cases were reported as secondary (Arunkumar *et al*., 2018). These nosocomial transmissions to the primary cases were concomitant with the index case presenting with a persistent cough and near the terminal stage of NiV illness. Of the 23 cases, 20 (87%) had respiratory symptoms presumably increasing the possibility of human-to-human transmission by droplet, and it was reported that only those with direct exposure to the patient's coughing appeared to have acquired NiV infection.

Pigs

Experimental challenge studies in piglets conducted at the National Centre for Foreign Animal Diseases, Winnipeg, Canada, demonstrated neurological signs in several inoculated pigs (Weingartl *et al.,* 2005; Berhane et al., 2008; Weingartl, H.M., personal communication of unpublished data). The rest of the pigs remained clinically healthy. NiV was detected in the respiratory system (turbinates, nasopharynx, trachea, bronchus, and lung), the lymphoreticular system (endothelial cells of blood and lymphatic vessels), submandibular and bronchiolar lymph nodes, tonsil, and spleen, with observed necrosis or lymphocyte depletion in lymphoid tissues, most importantly in lymph nodes (Hooper et al.,

2001, Weingartl et al., 2006; Berhane et al., 2008). NiV presence was confirmed in the nervous system of both sick and apparently healthy animals (cranial nerves, trigeminal ganglion, brain, and cerebrospinal fluid). No virus was detected urine, although NiV antigen was found in kidneys of field swine cases (Tanimura et al., 2004). This study suggests NiV invaded the porcine host central nervous system via cranial nerves after initial virus replication in the upper respiratory tract, and later in the infection also by crossing the blood-brain barrier as a result of viremia. Additional information on NiV and HeV pathogenesis in pigs are summarized in Middleton and Weingartl, 2012.

Cats

Cats were recognized as a naturally susceptible host for NiV during the 1998-99 Malaysian outbreak (Hooper *et al.*, 2001). Experimental infections of cats revealed they are highly susceptible to productive infection by both HeV and NiV and disease is severe. HeV infected cats develop fever and elevated respiratory rates, and there is rapid progression to severe illness and death within 24 hours of the onset of clinical signs (Westbury *et al.,* 1996). HeV disease in cats is similar to that observed in horses, with wide-spread vasculitis and parenchymal lesions in a wide range of organ systems particularly the lungs (Hooper *et al.,* 2001; Hooper *et al.,* 1997). Experimental NiV infection in the cat is essentially identical in outcome as compared to HeV infection and closely resembles most of the pathogenic processes seen in cases of henipavirus infection of people (Broder *et al.,* 2012).

Dogs

Middleton *et al*., 2017, conducted experimental infections with HeV in dogs and determined that the virus is not highly pathogenic in dogs but their oral secretions pose a potential transmission risk to people. The time window for potential oral transmission corresponded to the period of acute infection.

Horses

The pathology caused by HeV or NiV in horses (natural or experimental infection with HeV or natural infection with NiV) is more severe than that caused by either virus in pigs. Naturally acquired HeV infection in horses is often associated with severe disease, and experimental infections are essentially uniformly fatal. Animals initially become anorexic and depressed with general uneasiness and ataxia, with a developing fever with sweating. Respiration becomes rapid, shallow and labored with pulmonary edema and congestion with nasal discharge being a common terminal feature 1 to 3 days following the onset of clinical signs. Neurologic disease is also present but less frequent and noted in both terminally ill horses and in those that recovered from respiratory infection (Rogers *et al.,* 1996; Williamson *et al.,* 1998). Infection is wide-spread with an endothelial cell tropism with syncytia (Hooper *et al.,* 2001; Hooper *et al.,* 1997; Marsh *et al.,* 2011; Murray *et al.,* 1995; Williamson *et al.,* 1998). Experimental infection of horses with NiV has not been carried out, but the brain and spinal cord of one naturally infected horse was confirmed and revealed non-suppurative meningitis (Hooper *et al.,* 2001).

Bats

Fruit bats in the *Pteropus* genus have been identified as the reservoir hosts for HeV, NiV, and CedV. Henipaviruses have been isolated to date in *Pteropus* spp. from Australia (HeV, CedV) and Malaysia/Bangladesh/Cambodia/Thailand (NiV). Serological evidence of NiV or NiV-like exposure was detected in bats sampled in Madagascar and Ghana (Iehle C., *et al.,* 2007, Hayman *et al.,* 2008). Subsequently, 19 novel henipavirus sequences and one full-length genome of an African henipavirus,

GhV, were identified from related pteropodid bats, *Eidolon helvum*, sampled in Ghana (Drexler *et al.*, 2009; Drexler *et al.*, 2012). Nucleic acid and antibody signatures of henipaviruses have been detected serologically and by PCR in non-*Pteropus*, but related pteropodid bats in Central and West Africa, China, and Southeast Asia (Table 1); however the role that these non-*Pteropus* spp. play in the maintenance and transmission of henipaviruses remains unclear. The genome of MojV was constructed from sequences collected from a rodent, *Rattus flavipectus*, but comprehensive surveys have not been performed to rule out whether bats also host MojV.

There is no significant pathology in bats, and the frequency of viral shedding from wild bats is rare, with prevalence ranging from (1-3%) with temporal variation of infection and viral shedding observed among different bat populations (Gurley *et al.,* 2017 and Wacharaplusadee *et al.* 2010, 2016). Henipavirus isolation from bat excreta is challenging, potentially due to low viral load.

Pathogenesis Research Priorities

- Identify determinants of virulence in pigs
- Develop experimental infection models in bats to study shedding
- Comparative genomic studies of contemporaneous NiV strains collected from bats and humans during outbreaks.
- Expand knowledge of spectrum of henipaviruses in bat hosts in NiV hotspots (e.g. western Bangladesh & West Bengal India)
- Determine whether the innate immune system in bats is responsible for limiting viral replication
- Determine how the net reproductive value of henipaviruses are sustained in bats
- Determine how transmission effected within bats, and between bats and other species

IMMUNOLOGY

The following summarizes our current knowledge of NIV immunology, including innate and adaptive immune responses to wild-type virus, immune evasion mechanisms, and protective immunity.

Innate and adaptive immune responses to wild-type NiV

Viral RNA can be detected by both cytoplasmic and endosomal pattern recognition receptors (PRRs), resulting in innate immune Type I IFN induction/ and signaling pathways:

- Retinoic Acid-inducible Gene I (RIG- I)- recognizes 5' triphosphorylated RNA
- Melanoma Differentiation Antigen 5 (Mda-5)-recognizes cytosolic dsRNA
- RNA-dependent Protein Kinase (PKR)- recognizes cytosolic dsRNA
- Toll-like Receptor (TLR) 3- recognizes endosomal dsRNA
- TLR 7-8- recognizes endosomal ssRNA

Immune evasion mechanisms

The NiV uses unusual processes called RNA editing and internal translational initiation to generate multiple proteins from the phosphoprotein (P) gene, resulting in 4 proteins (P, C, V, and W) that function in inhibiting Type I interferon pathways:

- NiV P, V, and W have individually been shown to bind STAT1 and STAT2, effectively preventing STAT1 phosphorylation in type I IFN-stimulated cells.
- The V protein localizes to the cytoplasm, while the W protein localizes to the nucleus.

- The C protein can partially rescue replication of an IFN-sensitive virus, but the mechanism is unknown.
- Nuclear localization of W enables it to inhibit both dsRNA and TLR 3 (IRF-3 dependent) IFN-β induction pathways.
- A single point mutation in the V protein abrogates its ability to inhibit of IFN signaling.
- The V proteins of paramyxoviruses interact with the intracellular helicase Mda-5, and inhibits its IFN-β induction, but not with RIG-I.
- NiV V, W, and P bind polo-like kinase (PLK) via the STAT1 binding domain (Ludlow *et al.,* 2008).
- The P, V, and W proteins of NiV Malaysia and NiV Bangladesh inhibit IFN-stimulated response element (ISRE), which have a role in inducing transcription of IFN-stimulated genes (ISGs). Some of these ISGs include IRF-7, 2'5' Oligoadenylate Synthetase (OAS), RnaseL, p56, and double-stranded RNA-induced protein kinase (PKR). These ISGs all contribute to the generation of an 'antiviral state' in the cell.

Protective immunity

The G and F protein induce neutralizing antibodies that protect against challenge. Recent evidence from vaccination challenge studies indicates that both serum neutralizing antibody and T cell-mediated immunity are needed for protection from NiV infection in pigs (Pickering *et al.,* 2016).

Research needs

- Innate immunity and immunosuppression
  - Need studies in NiV infected cells and animal models
  - Need to study infection in various cell types, including cells of the immune system and bat cells
  - Use infectious clone to study virulence determinants
  - Identify targets for antiviral agents
  - Cytokine response to infection in human and bat cell lines
  - Need to study the potential for type 1 interferon or other cytokines to provide early protection from Nipah virus infection, transmission and/or clinical signs.

- Protective Immunity
  - Need to better define correlates of protection
  - Study T lymphocyte subset responses and cellular targets (e.g., N)

EPIDEMIOLOGY

Certain species of fruit bats of the genus *Pteropus* are the principal natural reservoir hosts for NiV and HeV. Bats are susceptible to infection with these viruses but do not develop disease. Other zoonotic viruses like Ebola, Marburg, and SARS virus, have also been identified in various bats (Leroy *et al*. 2005; Towner *et al*. 2009; Li W *et al*. 2005). Fruit bats are distributed across an area encompassing Australia, Southeast Asia; including Indonesia, Malaysia, the Philippines and some of the Pacific Islands, the Indian subcontinent, and Madagascar (See Fig. 1). There is further evidence for broader

distribution of NiV in pteropid fruit bats species across their range (Wacharapluesadee S. and Hemachudha T., 2007). There is also growing evidence that viruses related to NiV and HeV also circulate in non-pteropid fruit bats worldwide.

Hendra Virus

Hendra virus infection was first recognized in 1994 in Australia, when it caused an outbreak of acute, fatal respiratory disease that killed 14 horses. Three human cases, leading to two deaths were recorded during the outbreak. In 1995, a horse was infected with associated human cases. The precise mode of virus transmission to the three Australian patients is not fully understood. All three individuals appear to have acquired their infection as a result of close contact with horses, which were ill and later died.

There have been several recognized outbreaks in Australia since 1994. Hendra virus reemerged in 1999, 2004, and 2006-2010. All known HeV cases have occurred in Queensland or northern New South Wales. From 1994 to 2010, HeV was confirmed on 11 premises in Queensland and one premise in northern New South Wales. In Australia, GlobalincidentMap.com reported: 21 cases in 2011; 12 cases in 2012; 10 cases in 2013; four cases in 2014; three cases in 2015; one case in 2016; and four cases in 2017. Al cases have involved horses as an intermediate host along with some additional human infections, resulting in several fatalities. The Australian Veterinary Association's national president, Dr. Ben Gardiner, was quoted as stating "no state or territory was immune from the virus."

The natural reservoirs for HeV are flying foxes found in Australia. Bats are susceptible to infection with these viruses but do not develop disease.

Hendra virus infection has also been detected in two dogs that were in close contact with infected horses. Both dogs remained clinically normal with no history of related illness.

Updated statistics on HeV outbreaks, including locations, dates and confirmed human and animal cases may be found on the Australian Veterinary Association website (Assessed July 22, 2018).

Nipah Virus

Nipah virus is a recently-recognized, zoonotic paramyxovirus that causes severe disease and high fatality rates in people. Outbreaks have occurred in Malaysia, Singapore, India and Bangladesh, and a putative Nipah virus was also recently associated with human disease in the Philippines (Clayton, 2017). The following summarizes our current knowledge of NiV epidemiology taking into account disease outbreaks in Malaysia and Bangladesh.

Malaysia

Nipah virus was first described in 1999 in Malaysia. The outbreak in Malaysia resulted in over a million pigs culled, 800 pig farms demolished, 36,000 jobs lost, $120+ million exports lost, and over 300 human cases (106 fatal, ~35% mortality) in pig farmers (Chinese) and Singapore abattoir workers (Field *et al.,* 2001). The NiV outbreak in pigs was described as highly infectious, frequently asymptomatic, low mortality rate (~5%), with respiratory and neurological syndromes. The pig farm pattern of disease included 30% morbidity and 5% mortality with sows first affected, followed by weaners, growers and finishers. The duration of clinical disease on a farm lasted ~ 2 weeks with a sero-prevalence approaching 100% in some cases. The outbreak in Malaysian pigs was associated

with an increased incidence of human viral encephalitis cases, strongly associated with pig farm workers, with temporal and spatial links to disease in pigs.

During the outbreak, evidence of NiV infection was found in domestic animals such as goats and cats, but especially dogs (Field *et al.,* 2001). After pig populations were destroyed, but before residents were allowed to return to their homes, studies were undertaken in the epidemic area to determine whether domestic animal populations maintained active infection in the absence of infected pigs (Mills *et al.,* 2009). Dogs were especially suspected because they live commensally with both pigs and humans. However, serologic screening showed that in the absence of infected pigs, dogs were not a secondary reservoir for NiV.

Although human-to-human transmission of NiV during the 1998-1999 outbreak in Malaysia was not reported, a small number of infected people had no history of contact with pigs, suggesting human-to-human transmission occurred in these cases (Clayton, 2017).

The reservoir and natural host of NiV was determined to be fruit bats. Fruit bats have a wide geographic distribution, high antibody prevalence (17-30%), but no apparent clinical disease. A NiV neutralizing antibody study (Yob *et al.,* 2001) from 237 wild-caught bats surveyed on Peninsular Malaysia April 1–May 7, 1999, found four different species of fruit bats, and one species of insectivorous bats, tested positive for NiV (see Table I).

The routes of NiV excretion in bats include urine, saliva, and foetal tissues and fluids but the exact modes of transmission have yet to be determined.

The drivers of the emergence of NiV in Malaysia were determined to be large piggery (30,000+) adjacent to primary forest/fruit bat habitat and a network of other large farms close by. The stages of emergence associated with the outbreak included a spillover from flying foxes to domestic pigs near Ipoh (see Fig. 4), where farming practices and high pig densities facilitated the dissemination of the infection. Transportation of pigs for commerce led to the southern spread of the outbreak with the amplifying pig host facilitating the transmission of the virus to humans.

The epidemic enhancement of the outbreak included the initial introduction of infection in a naive pig population resulting in a rapid epidemic peak, followed by burn-out and localized human infections. Subsequent introduction(s) into partially immune pig populations resulted in a lower epidemic peak but prolonged duration and increased total number of infectious pigs, increasing the chances of spread to surrounding farms.

Bangladesh

Bangladesh experienced its first reported NiV outbreak in Siliguri and Naogaon in 2001 (Fig. 5). Unlike Malaysia, outbreaks in Bangladesh appeared to be strictly confined to human populations and significantly higher mortality rate. From 2001 to 2018, the WHO reported a total of 261 cases, with 198 deaths (76% mortality) due to NiV infection (see Table II).

The transmission of NiV to humans in Bangladesh was determined to be associated with drinking date palm juice, considered a delicacy in this region of the world. In the Tangail outbreak of 2005, it was

estimated that persons drinking raw date palm sap had a 7.0 odds ratio of developing a NiV infection when compared to controls (95% confidence level, 1.6).

NiV cases in Bangladesh have been seasonal, with human cases reported between the months of January and April. This coincides with the season for collecting date palm sap, late November through April. However, there is significant heterogeneity in the number of spillovers detected by district and year that remains unexplained. Cortes et al., in 2018 analyzed data from all 57 spillovers occurring during 2007–2013 and found that temperature differences explained 36% of the year-to-year variation in the total number of spillovers each winter, and that distance to surveillance hospitals explained 45% of spatial heterogeneity. January, when 40% of the spillover events occurred, was the month with the lowest mean temperature during every year of the study.

Bats are recognized as a nuisance and frequently drink the juice, defecate into juice, and occasionally drown in the palm sap collecting pot. Measures have been put in place to prevent bats access to the sap collecting pot, which has been very effective in reducing the spread of NiV from bats to humans in Bangladesh.

India

In 2001, an outbreak occurred within a hospital in Siliguri, West Bengal. Nosocomial transmission likely occurred, though it is unknown how primary cases were infected. Another outbreak in 2007 was reported in Nadia, West Bengal. Consumption of date palm sap was identified as the likely route of infection of primary cases there. In May of 2018, another outbreak was reported in Kerala. A total of 85 cases were reported in these three outbreaks in 2001, 2007, and 2018, with 62 deaths (73% mortality) due to NiV infection (see Table II).

In 2012, Yadav et al. surveyed the Indian states of Maharashtra and West Bengal to evaluate the presence of viral RNA and IgG against NiV in different bat populations belonging to the species Pteropus giganteus, Cynopterus sphinx and Megaderma lyra. The authors found NiV RNA in Pteropus bat thus suggesting it may be a reservoir for NiV in India.

In 2018, an outbreak of 23 cases of NiV disease was reported in Kerala, India. This was the first spillover in NiV in South India. 18 cases were lab-confirmed and the case fatality rate during this outbreak was 91% (Arunkumar G *et al*. 2018).

Philippines

In 2014, the Philippines reported an outbreak with a zoonotic paramyxovirus in horses and people that is very closely related to NiV based on sequence analysis. Virus isolation was unsuccessful so it was impossible to confirm that there was transmission from presumably bats to horses, from horses to people, and also human to human (Ching P.K., *et al.*, 2015; Clayton, 2017).

New Caledonia

In 2015, three fruit bats tested positive for NiV in New Caledonia at the Noumea National Park, including three bats at the Noumea Zoo.

Research needs

- Improved understanding of infection dynamics in flying foxes: modes of transmission, immune response, evidence of disease, and the implications of co-infection with NiV and other henipaviruses
- Better understanding of co-circulation of different strains / species of henipaviruses within Pteropus populations and the effect of waning herd immunity on outbreaks.
- Other animals such as infected dogs and cats need to be further studied to determine their potential role in the transmission of NiV.
- Improved understanding of infection dynamics in humans: modes of transmission, implications of genetic diversity of the virus for infection, transmission & pathogenicity
- Research into bat populations: additional research regarding bat distributions & ecological impacts
- Research aimed at improving the capacity to diagnose henipavirus infections and improve human health outcomes
- Research into infection and clinical signs in pigs in Bangladesh and potential for pig to human and human to pig transmission.

BIOTERRORISM

The following summarizes the rationale for considering NiV as a potential agent of bioterrorism.

NiV is classified by CDC as a Category C pathogen – emerging pathogens that could be engineered for mass dissemination in the future. Category C include pathogens are readily available, easy to produce, easy to disseminate, and have the potential for high morbidity and mortality with major health impact.

NiV has many of the physical attributes to serve as a potential agent of bioterrorism. The outbreak in Malaysia caused widespread panic and fear because of its high mortality and the inability to control the disease initially. There were considerable social disruptions and tremendous economic loss to an important pig-rearing industry. This highly virulent virus, believed to be introduced into pig farms by fruit bats, spread easily among pigs and was transmitted to humans who came into close contact with infected animals. From pigs, the virus was also transmitted to other animals such as dogs, cats, and horses.

Nipah Virus Bioterrorism Threat Assessment

Virology

- Reverse genetic methods are available for negative strand RNA viruses, including Nipah, and all genomic sequence data is available.
- Many laboratories are actively engaged in research programs on the cell biological properties of the henipaviruses.

- Virus can be amplified to reasonably high unconcentrated titers (>10^7). Several cell culture lines can be used, Vero cell use most often reported, and wild-type virus can be grown and harvested from cell cultures.
- A major constraint in handling Nipah is the requirement for BSL4 facilities; , however, potential terrorists may not respect this need.
- Inactivation of virus can be achieved with a variety of agents typically used for envelope viruses; but extensive environmental stability testing not reported.
- Vaccines and passively-delivered countermeasures are under development both for human and veterinary use. A commercial Hendra virus vaccine is available for horses, and the soluble G protein based vaccine has shown experimental efficacy against Nipah virus in nonhuman primates.
- Bats are sold (often live) in markets throughout their range, providing a potential source of virus; and serological tests are available for identifying henipaviruses

Economic Impact
- Destroyed the main market for Malaysian hogs in Singapore
- ~80% drop in pork consumption in the domestic market.
- Over half the standing pig population in the country was culled to halt the outbreak.
- Half the pig farms went out of business.
- During the outbreak cumulative economic losses based on government figures >$217 million USD.
- Cumulative government costs in operations and lost revenues >$298 million USD.
- Other countries in South East Asia often have a higher pig density than Malaysia. China, with approximately half of the pigs in the world, is especially vulnerable to an economic and public health disaster if NiV were to emerge and be rapidly transmitted between pigs and from pigs to people.

Epidemiology and Clinical Disease
- In outbreaks to date henipaviruses do not appear to be highly infectious. Infection requires close contact with secretions of diseased animals. Many infections can be mild to asymptomatic.
- In the initial 1998-99 outbreak the virus was *initially misdiagnosed* as Japanese Encephalitis; amplification occurred from veterinary reuse of needles in immunization programs to control JE, increasing outbreak severity and extent.
- Time from exposure to signs of infection averages ~2 weeks for humans and seroconversion occurs within a month of onset (dose / route dependent).
- Transmission directly to the vascular system could occur through bites from infected animals or broken skin exposed to secretions of infected animals.
- It is quite likely that an outbreak in animals would result in transmissions to humans.
- An outbreak of Nipah pneumonia or ARDs-like disease with human-to-human transmission as demonstrated in the Bangladesh outbreak could cause significant mortality. Nipah could cause more severe or different disease presentations in older or sick populations.

Viral Transmission
- Deliberate release of virus in some manner is possible.

- Aerosol delivery might transmit the disease effectively to domestic animals, but the environmental requirements for maintaining virus stability are not well known.
- Transmission to humans through consumption of contaminated food has been documented.
- The veterinary reuse of needles in the Japanese Encephalitis immunization campaign and in artificial insemination may have been a factor in the near 100% infection level of Nipah in pigs observed on affected farms.
- Deliberate contamination of veterinary needles could initiate an outbreak in susceptible domestic animals.
- Human-to-human transmission through travel has not been documented, but is possible.
- Transport of infected pigs on trucks was a transmission route in the Malaysian outbreak. Generalizing-- transportation of infected humans on crowded airplanes, buses or trains could also transmit the disease. Human cases have been transported to highly populous cities (e.g. Dhaka) where risk of exposure and spread among the public is increased.

Summary

- Nipah (henipaviruses) can be isolated from animal hosts.
- Several species of fruit bats, including *Pteropus spp.* widely distributed throughout Southeast Asia. The live animals are sold in food markets.
- A Nipah outbreak in swine producing areas can cause an economic crisis, even if human cases do not occur.
- Nipah virus can be amplified in permissive cell cultures (e.g., Vero cells) providing adequate laboratory facilities are available (Biosafety Level 4), although a bioterrorist group would not be restricted from growing the virus because of the lack of BSL-4 facilities.
- Effective aerosol delivery is likely possible but unpredictable on the basis of publicly available information. General unknowns are-- titers necessary for infection, virion stability in vitro, and how infectious the virus would be with this delivery.
- Effective surveillance programs, particularly in pig farming areas, are the best defense for early detection and containment of infection, whatever the source.

SUMMARY OF OBSTACLES TO PREVENTION AND CONTROL

The 2017 gap analysis working group determined that the following countermeasures were important but several weaknesses were identified.

DIAGNOSIS

NiV and HeV are zoonotic paramyxoviruses capable of causing severe disease in humans and animals. These viruses require biosafety level 4 (BSL-4) containment. The availability of safe laboratory diagnostic tests is limited. Sequence variation effects molecular diagnostics; both Clifton Beach (2007) and Redlands (2008) reported that Hendra virus strains failed in AAHL Hendra virus specific real-time PCR. Most published diagnostic PCRs only detect HeV or NiV, but not both. There is a need for a more general PCR to detect divergent and novel strains. Pan-paramyxovirus PCR assays exist and are in use to detect henipaviruses, but limitations in sensitivity limit diagnostic value. The USAID PREDICT program previously used its pan-paramyxovirus PCR assay for surveillance in more than 20 countries in Africa and Asia. Virus isolation and serum neutralization assays require live NiV. There is a need for diagnostics that can be used safely in the laboratory. There is a need for rapid nucleic acid-based assays that can detect all henipaviruses. There is also a critical need for improved antibody-based assays for disease outbreaks and disease surveillance. Importantly, there is a need to develop operator-safe diagnostic tests for which reagents can be produced without requiring high containment facilities.

Currently there are no expectations that validated tests will become available for livestock (or other species) in the near future. Nothing has been done in terms of test harmonization since 2009; however, a number of technology transfers have occurred: from AAHL to laboratories in Asia (Malaysia mainly); limited transfer from NCFAD to India (Bhopal High Containment Animal Health Laboratory); limited transfer from AAHL to the FLI and bilateral transfers between NCFAD and FLI.

VACCINATION

There is currently a commercially available vaccine for horses but no vaccines for swine or human vaccines. The goal for a HeV vaccine for horses is to vaccinate horses in areas at risk for transmission from bats to horses in order to prevent bat to horse transmission and subsequent horse to human transmission. The goal for a NiV vaccine for swine is to have a large stockpile of vaccine available for rapid use in an outbreak situation to prevent swine to swine, swine to human, and perhaps human to swine transmission to control the outbreak. A large stockpile of NiV vaccine, or vaccine antigen concentrate, for rapid emergency use in swine to control a potential outbreak that spreads too quickly to be stamped out in swine dense areas is needed. The vaccine should be licensed in the U.S., E.U or Australia for stockpiling as well as in the countries where NiV is endemic in bats. The stockpile should be available for use internationally where ever it may be needed.

SURVEILLANCE

Passive surveillance is the primary and most economical method used. Passive surveillance in commercial swine herds based on clinical signs has many weaknesses due to the difficulty of differentiating NiV from many common endemic infectious diseases of pigs; e.g., classical swine

fever, porcine reproductive and respiratory syndrome, pseudorabies, swine enzootic pneumoniae, and porcine pleuropneumonia.

In the case of infections in swine where recognition of Nipah symptoms is less likely, surveillance activities must be based on diagnostic testing to supplement surveillance based on clinical signs.

Active surveillance programs are expensive and would have to rely on direct diagnostic tests such as viral isolation and nucleic acid-based assays but available tests have significant weaknesses and have not been validated.

Rapid confirmation of cases is essential. Knowledge on serological cross-reactions with other henipaviruses and/or morbilliviruses in bats is improving. There is an urgent need to establish diagnostic capacity for Nipah virus in countries that are most likely to experience spillovers from the bat reservoirs.

DEPOPULATION

Depopulation is the primary countermeasure to reduce virus shedding and stop the spread of Nipah virus in swine. Recent outbreaks have shown that the control of Nipah virus in pig populations through stamping out is complex due to the zoonotic nature of the agent and may be very expensive, particularly in areas with high pig densities. Because Nipah virus spreads rapidly and silently in pigs, a large number of animals would need to be pre-emptively culled if an outbreak occurred in the U.S, or in other swine dense countries in order to minimize the virus spread in the vicinity of infected herds. Thus, this method of control would have significant financial implications due to the culling of thousands or millions of animals.

COUNTERMEASURES ASSESSMENT

ASSUMPTIONS

The following captures assumptions made by the gap analysis working group to assess potential countermeasures to enhance our ability to contain and eradicate an outbreak of NiV.

Situation
Countermeasures assessed for worst case scenario: A coordinated intentional distribution of NiV-contaminated material in a high density highly populated pig region of the United States.

Target Population
Countermeasures assessed for target pig production segments in priority order:

1. Backyard pigs
2. Comprehensive commercial swine operations (farrowing, nursery, and finishing)
3. Commercial indoor farrowing operations
4. Large intensive indoor pig farms
5. Valuable commercial genetic swine stock

Scope of Outbreak
Countermeasures assessed for multiple outbreaks occurring simultaneously in backyard pigs, three farrowing commercial operations, a finishing pig commercial operation, a sow replacement operation, and evidence of infection in feral swine.

DECISION MODEL

The quantitative Kemper-Trego (KT) decision model was used to assess available vaccines and diagnostics. For the criteria and weights used to assess NiV vaccines and diagnostics (See Appendices II, III).

Criteria
The following critical criteria were selected to enable the comparison of countermeasures using a pertinent and valid analysis, as follows:

<u>Vaccines</u>

- Efficacy
- Safety
- One dose
- Manufacturing safety
- DIVA compatible
- Manufacturing yield
- Rapid production
- Reasonable cost
- Short withdrawal period
- Long shelf life

Diagnostics

- Sensitivity
- Specificity
- DIVA detection
- Multispecies
- Validation to purpose
- Speed of scale-up
- Throughput
- Pen-side test
- Rapid result
- No need for a confirmatory test
- Easy to perform
- Safe to operate
- Availability
- Storage/Distribution
- Low cost to implement
- Perform at BSL-2
- Does not require use of live virus to prepare reagents

Weight

Each criterion was weighted to allow a quantitative comparison of the impact of the selected interventions (See Appendices II and III).

Product profile

To ensure a consistent and meaningful assessment, the desired product profile (i.e., the benchmark) was identified for each countermeasure:

Desired Vaccine Profile

1. Highly efficacious: prevent transmission; efficacy in all age animal target hosts, including maternal antibody override; cross protection across all henipavirus strains; quick onset of immunity; multiple animal target hosts; one year duration of immunity
2. Safe in all age animal target hosts; no reversion to virulence for live vaccines
3. One dose
4. Safe vaccine strain for manufacturing
4. DIVA compatible
5. Manufacturing method yields high number of doses
6. Rapid speed of production and scale-up
7. Reasonable cost
8. Short withdrawal period for food consumption
9. Long shelf life

Desired Diagnostic Test Profile
1. Detect all henipavirus
2. Identify Nipah virus specific strains
3. Direct tests for control and eradication
4. Indirect tests for post-control monitoring
5. Rapid test
6. >95% specificity
7. >95% sensitivity
8. Pen-side test
9. DIVA Compatible
10. Field validated
11. Easy to perform/easily train NAHLN's personnel
12. Scalable
13. Reasonable cost
14. Operator safe
15. Reagents can be produced in low containment

Values
The values assigned for each of the interventions reflect the collective best judgment of members of the gap analysis working groups (See Appendices I and II)

VACCINES
The human infections in the 1999 outbreak in Malaysia were linked to transmission of NiV from pigs. Accordingly, a swine vaccine able to prevent virus transmission would be an important tool to safeguard commercial swine operations and people at risk. In addition, since henipaviruses have a very broad host range, a vaccine that is efficacious in multiple susceptible animal species would be desirable. Although the 2017 gap analysis working group determined that there are still no NiV commercial vaccines available, there are several vaccine candidates that may be safe and effective in swine and other domestic animals that were recently reviewed in: (Weingartl H.M., 2015; Broder, C.C., *et al, 2016*; and Satterfield, B.A., *et al.,* 2016). After these reviews were published, a manuscript was published demonstrating the efficacy of a virus-like-particle (VLP) Nipah virus vaccine in hamsters for inducing virus neutralizing antibodies and protection from challenge (Walpita P., *et al.,* 2017). Another manuscript was published that concluded that an adjuvanted Hendra soluble G vaccine in pigs induced neutralizing antibody titers considered to be protective against Nipah virus without detectable T cell-mediated immunity to Nipah, which did not protect from challenge with Nipah virus. However, pigs that had been previously challenged with a low dose of NiV developed neutralizing antibodies and cell-mediated immune memory and were protected from a high challenge dose of NiV. The conclusion of this manuscript was that both virus neutralizing antibodies and cell-mediated immunity were necessary for protection from NiV challenge (Pickering B.S., *et al.,* 2016). Subsequent unpublished research demonstrated that a different adjuvant used with the soluble Hendra G vaccine caused the induction of both high tittered virus neutralizing antibody and detectable T cell-mediated immunity in pigs to NiV. Challenge studies were not conducted (J.A. Roth, personal communication). All of these vaccine candidates would need further research and development to be licensed, and would need to be made available as a stockpile for rapid use in an emergency if an outbreak in swine were to occur that could not be effectively stamped out. A swine vaccine would

also be needed if the Nipah virus were to mutate to be efficiently transmitted between people and between people and pigs.

Summary

- Vaccination against NiV has been successfully demonstrated
- Experimental henipavirus vaccines can prevent clinical disease
- Experimental henipavirus vaccines elicit systemic and mucosal immunity
- Experimental henipavirus vaccines prevent viral replication in target tissues
- HeV commercial vaccine Equivac® HeV does not cross protect against NiV infection in swine
- Henipavirus vaccines appear to be effective in several mammalian animal species

Assessment of Commercial Vaccines

A commercial vaccine (Equivac® HeV) against Hendra virus approved for use in horses (Middleton D.J. *et al.,* 2014) was registered by Zoetis in Australia in 2015. A six month booster dose is required for full protection, followed by annual vaccination. The vaccine is also approved for pregnant mares. There is currently no NiV vaccine approved for swine. Likewise, there is no vaccine against HeV or NiV approved for human use.

Assessment of Experimental Vaccines

The working group felt that limited information was available to assess and contrast experimental vaccines that have been reported in the literature. Experimental animal vaccines under investigation are summarized in Table I. Experimental vaccines for humans are summarized in Table II. Several of the working group members have directly or indirectly been involved in the research associated with these vaccines so that an assessment could be made (See Appendix I). The following describes some of the most promising experimental vaccine technologies.

1) Canarypox-vectored NiV Vaccines

The ALVAC canarypox virus-based recombinant vaccine vector (Taylor *et al.,* 1994) was used to construct two experimental NiV vaccines (Weingartl *et al.,* 2006). These experimental vaccines were engineered by Merial.

The first construct carries the gene for NiV attachment glycoprotein G (ALVAC-G). The second construct carries the NiV fusion protein F (ALVAC-F).

The efficacy of both the ALVAC-G and ALVAC-F were tested in pigs either as monovalent vaccine or in combination (ALVAC-G/F). The vaccine dose used was 10(8) PFU. The vaccine regimen was two doses administered 14 days apart. Both non-vaccinated controls and vaccinated pigs were challenged with 2.5 x 10(5) PFU of NiV two weeks later.

The combined ALVAC-F/G vaccine induced the highest levels of neutralization antibodies. Despite the low neutralizing antibody levels induced by ALVAC-F all vaccinated animals were protected against challenge. Virus was not isolated from the tissues of any of the vaccinated pigs post-challenge, and a real-time reverse transcription (RT)-PCR assay detected only small amounts of viral

RNA in several samples. In challenge control pigs, virus was isolated from a number of tissues or detected by real-time RT-PCR. Vaccination of pigs with the ALVAC-F/G stimulated both type 1 and type 2 cytokine responses. No virus shedding was detected in vaccinated animals, in contrast to challenge control pigs, from which virus was isolated from the throat and nose.

Based on the data generated in this one study, both the ALVAC-G or the combined ALVAC-F/G vaccine appears to be a very promising vaccine candidate for swine.

2) Soluble G Henipavirus Vaccine

HeV and NiV infect cells by a pH-independent membrane fusion event mediated by their attachment (G) and fusion (F) glycoproteins. Scientists at the Uniformed Services University of the Health Sciences in Bethesda, Maryland, in collaboration with the Australian Animal Health Laboratory characterized HeV- and NiV-mediated fusion activities and detailed their host-cell tropism characteristics. These studies suggested that a common cell surface receptor was utilized by both viruses. To further characterize the G glycoprotein and its unknown receptor, soluble forms of HeV G (sG) were constructed by replacing its cytoplasmic tail and transmembrane domains with an immunoglobulin kappa leader sequence coupled with an S-peptide tag (sG) to facilitate purification and detection. Expression of sG was verified in cell lysates and culture supernatants by specific affinity precipitation. Analysis of sG by size exclusion chromatography and sucrose gradient centrifugation demonstrated tetrameric, dimeric, and monomeric species, with the majority of the sG released as a disulfide-linked dimer. Immunofluorescence staining revealed that sG specifically bound to HeV and NiV infection-permissive cells. The scientists further reported that administration of sG to rabbits can elicit a potent cross-reactive neutralizing antibody response against infectious HeV and NiV (Bossart *et al.* 2005). The HeV sG subunit vaccine has been the most extensively studied NiV/HeV vaccine platform because of its ability to elicit a potent cross-protective immune response to NiV and has been shown to induce potent cross-reactive neutralizing antibody responses in a variety of animals including mice, rabbits, cats, ferrets, monkeys and horses.

Experimental subunit vaccine formulations containing either HeV sG or NiV sG were first evaluated as potential NiV vaccines in the cat model. Two cats were immunized with HeV sG and two cats were immunized with NiV sG. Immunized animals and two additional naïve controls were then challenged subcutaneously with 500 TCID50 of NiV. Naive animals developed clinical disease 6 to 13 days post-infection, whereas none of the immunized animals showed any sign of disease (Mungall *et al.,* 2006).

In a subsequent experiment, an experimental subunit formulation containing HeV sG and CpG adjuvant was evaluated as a potential NiV vaccine in the cat model. Vaccinated animals demonstrated varying levels of NiV-specific Ig systemically and importantly, all vaccinated cats possessed antigen-specific IgA on the mucosa. Upon oronasal challenge with NiV (50,000 TCID50), all vaccinated animals were protected from disease although virus was detected on day 21 post-challenge in one animal. (McEachern *et al.,* 2008).

Additional studies with the HeV-sG vaccine in the ferret model formulated in CpG and Allhydrogel™ and could provide complete protection from a 5,000 TCID50 dose of HeV (100 times the minimal lethal dose) with no disease or evidence of virus or viral genome in any tissues or body fluids and only a low level of HeV genome detected in the nasal washes from 1 of 4 animals in a low-dose vaccine

group, and no infectious HeV could be recovered from any immunized ferrets (Pallister J, et al. A recombinant Hendra virus G glycoprotein-based subunit vaccine protects ferrets from lethal Hendra virus challenge. Vaccine. 2011;29:5623-30) In a similar study with NiV-B, vaccinated ferrets remained disease free, and virus or viral genome was undetectable in all tissues and fluids with no observed pathology in examined tissues. The study also revealed good durable immunity with other ferrets challenged 434 days post-vaccination, with 5 of 5 animals were disease free following challenge and viral genome was detected only from the nasal secretions of one ferret and the bronchial lymph nodes of another ferret that were given an intermediate vaccine dose (Pallister JA, et al. Vaccination of ferrets with a recombinant G glycoprotein subunit vaccine provides protection against Nipah virus disease for over 12 months. Virol J. 2013;10:237).

The HeV-sG subunit vaccine has also been evaluated in the African green monkey (AGM), which is the only nonhuman primate model that has uniformly recapitulated human disease for both NiV and HeV infection (Rockx B, et al. A novel model of lethal Hendra virus infection in African green monkeys and the effectiveness of ribavirin treatment. J Virol. 2010;84:9831-9; Geisbert TW, et al. Development of an acute and highly pathogenic nonhuman primate model of Nipah virus infection. PLoS One. 2010;5:e10690). HeV-sG was initially tested by formulation in Allhydrogel^{TM} and CpG and animals were challenged by intratracheal administration with a 10-fold lethal dose of NiV (1×10^5 TCID50). Complete protection was observed in all vaccinated animals with no evidence of clinical disease, virus replication, or pathology in any vaccinated subject with some having pre-challenge NiV neutralizing titers as low as 1:28. A second study demonstrated HeV-sG vaccination and protection from a HeV in the AGM model and also showed that HeV-sG in Allhydrogel^{TM} alone was suffient to confer complete protection from infection and disease (Mire CE, et al. A recombinant Hendra virus G glycoprotein subunit vaccine protects nonhuman primates against Hendra virus challenge. J Virol. 2014;88:4624-31). The HeV-sG subunit vaccine is now being evaluated as a NiV/HeV vaccine for human use with support from the Coalition for Epidemic Preparedness Innovations (CEPI) (Hum Vaccin Immunother. 2017 Dec 2;13(12):2755-2762. doi: 10.1080/21645515.2017.1306615. Vaccines for epidemic infections and the role of CEPI. Plotkin SA)

A recent publication demonstrated that an adjuvanted HeV-sG vaccine in pigs induced SN antibody titers considered to be protective against NiV without detectable T cell-mediated immunity to NiV which did not protect from challenge with NiV. Pigs which had been previously challenged with a low dose of NiV developed SN antibodies and cell-mediated immune memory and were protected from a high challenge dose of NiV. The conclusion of this manuscript was that both SN antibodies and cell-mediated immunity were necessary for protection from NiV challenge (Protection against henipaviruses in swine requires both, cell-mediated and humoral immune response, B.S. Pickering, J.M. Hardham, G. Smith, E.T. Weingartl, P.J. Dominowski, D.L. Foss, D. Mwangi, C.C. Broder, J.A. Roth, H.M. Weingartl, Vaccine 34(40): 4777-4786, 2016). Subsequent unpublished research demonstrated that a different adjuvant used with the soluble HeV-sG vaccine caused the induction of both high tittered SN antibody and detectable T cell-mediated immunity in pigs to NiV. Challenge studies were not conducted (J.A. Roth, personal communication).

3) Vaccinia-vectored NiV Vaccine

The NYVAC vaccinia virus-based recombinant vaccine vector (Tartaglia *et al.,* 1992) was used to construct an experimental NiV vaccine where the vaccinia virus expresses both the NiV glycoproteins G and F (Guillaume *et al.,* 2004). This experimental vaccine was engineered by the Pasteur Institute.

Scientists at the Pasteur Institute in collaboration with University of Malaysia scientists showed that both of the NiV glycoproteins G and F when expressed as vaccinia virus recombinants induced an immune response in hamsters that protected against a lethal challenge with NiV. Furthermore, this team of scientists demonstrated passive transfer of antibody induced by either of the glycoproteins protected the animals.

DIAGNOSTICS

The gap analysis working group determined that the availability of validated diagnostic tests for surveillance, early detection, and recovery during a NiV outbreak were critical to minimize the spread of disease and reduce the economic and public impact.

Currently the diagnosis of NiV infection is by virus isolation, detection of viral RNA, or demonstration of viral antigen in tissue collected at necropsy. Specific antibody detection can also be useful, particularly in pigs where NiV infection may go unnoticed. Demonstration of specific antibody to NiV in either animals or humans is of diagnostic significance because of the rarity of infection and the serious zoonotic implication of NiV transmission.

Summary

- Antibody responses to NiV take at least 14 days and therefore early diagnosis based on serology will be less reliable than antigen or molecular tests
- Recombinant N-ELISA will likely not pick up all infected pigs
- The concept of a pen-side test is attractive, but the development and regulation of such a test will be extremely challenging

Assessment of Laboratory Diagnostic Tests (See Appendix II)

Details in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2015, Chapter 2.1.14 Hendra and Nipah Virus Diseases, provides recommendations for the following tests.

Identification of the agent

1. Virus isolation and characterization
    1.1. sampling and submission of specimens
    1.2. isolation in cultured cells
    1.3. Identification: immunostaining and Immuno EM
2. Viral identification: differentiation of HeV and NiV
    2.1 comparative immunostaining
    2.2. immunofluorescence
    2.3. microtiter neutralization

3. Molecular methods
    3.1. real-time RT-PCR
    3.2. Conventional RT-PCR and Sanger sequencing

4. Immunohistochemistry

Serological tests
1. Virus neutralization tests
2. Enzyme-linked immunosorbent assay
3. Bead-based assays

Histopathology
1. Veterinary diagnostic labs might use histopathology to make the first diagnosis
2. NiV does not produce pathognomonic lesions, but a generalized vasculitis with fibrinoid necrosis in several tissues (e.g. lung and kidneys) is characteristic; NiV might be considered in the initial differential diagnosis by experienced veterinary pathologists.

Assessment of Available Diagnostic Tests
Australia, Canada, and Germany have diagnostic capability for henipaviruses in livestock; India (e.g. NIHSAD) is building its veterinary diagnostic capability; U.S. veterinary diagnostic laboratories do not have diagnostic capability to detect NiV in livestock, although the Center for Disease Control (CDC) in Atlanta, Georgia, is an OIE collaborating center for NiV.

Currently, there are no expectations of validated tests for livestock (or other species). Nothing has been done in terms of test harmonization for serological, antigen, or nucleic acid detection assays; however, successful technology transfers have taken place, as follows: from AAHL to laboratories in Asia (Malaysia mainly); limited transfer from NCFAD to India (Bhopal High Containment Animal Health Laboratory); limited transfer from AAHL to FLI and bilateral transfers between NCFAD and FLI.

Serologic testing plays an important role in the diagnosis and detection of NiV infections. Serologic tests are the most straightforward and practical means to confirm acute cases of disease and serologic evidence of infection is used in screening programs for reservoir hosts and domestic animals. However, serological assays are limited in their ability to differentiate between known and unknown henipaviruses, as cross-reactivity to one or more known viruses is possible. Both serum neutralization and Luminex assays have shown positive reactivity to both NiV and HeV in bats where the presence of a yet-to-be characterized henipavirus could not be ruled out.

Several standard and new experimental technologies that are currently being used or considered for the detection of NiV in the laboratory or as pen-side tests for field use. Shedding of NiV in oral fluids starts early post-infection and rope sampling could prove convenient for collecting samples that could be used to test larger numbers (i.e., pen tests) of pigs. Suitability of oral fluid samples for various test platforms should be investigated. There is a need to develop a formalized worldwide structure for test validation and ring trials (i.e., inter-laboratory comparisons).

The following describes some of the most promising diagnostic platforms with potential application for NiV detection.

1) *Quantitative (q) real-time PCR*

Real-time PCR is a sensitive and useful approach to the detection of henipavirus genome in specimens. Due to its nature, rRT-PCR may not be able to detect all divergent and novel henapivirus strains, although adaptation of molecular tests to new virus variants could be rapid. Test methods and primers used depend on the technology platform and associated chemistry being used in individual laboratories. Test procedures have been described by different laboratories (Mungall *et al.,* 2006; Wacharapluesadee and Hemachudha, 2007; Guillaume *et al*., 2004; Chang *et al*., 2006; Feldman *et al*., 2009).

The AAHL has developed a quantitative real-time PCR to detect NiV or HeV RNA synthesis. The most commonly targeted amplification regions are directed against the N gene (Feldman et al., 2009).

RT-PCR targeting the N gene of NiV will detect both, NiV-M and NiV-B, with somewhat lower sensitivity for NiV-B. Confirmatory RT-PCR targeting the F gene specific only for NiV-B has therefore been developed (publication in preparation; H.M. Weingartl, personal communication).

2) *Conventional PCR*

Classical RT-PCR followed by sequencing may be more successful in detecting novel henipavirus strains. Combination of both approaches may need to be considered. Genomic RNA detection can be performed on blood or serum samples collected from live animals as well as tissues from dead animals. RNA is extracted using an RNA extraction kit [e.g., RNeasy Mini Kit (Qiagen)]. Extracted total cellular RNA is first subjected to first-stand cDNA synthesis using a reverse transcriptase kit [e.g., SensiScript (Qiagen)] and a reverse transcriptase primer. The resulting cDNA is amplified using a Master Mix PCR kit (Qiagen) and primers that are designed to target HeV and NiV positive-sense mRNA from the N, M and G genes and negative-sense genomic viral RNA (vRNA) at the N/P, M/F and F/G gene junctions.

3) *Field PCR*

Not available. Isothermal real-time RT-PCR is promising as a field deployable assay.

While this will be costly and not be practical to have in large numbers, it is worth considering having the capabilities to establish in several strategically located regions across the nation to response rapidly in an emergency situation. Technically it will not be difficult to achieve if there is the will and financial support.

4) *Virus isolation (VI)*

Virus isolation in permissive cell culture is considered the "gold standard" for isolating all strains of henipaviruses. Virus isolation greatly facilitates identification procedures and definitive diagnosis should be undertaken where operator safety can be guaranteed. Isolation is especially relevant in any new case or outbreak, particularly in countries or geographical areas where infection by NiV or HeV has not been previously documented. Implication of wildlife species as natural hosts of the viruses requires positive serology, PCR or virus isolation from wild-caught animals (Daniels *et al.,* 2007). The range of tissues yielding virus in natural and experimental cases include the brain, lung, kidney and spleen (Crameri G., *et al.* 2002).

Henipaviruses grow rapidly to high titers in a large number of cell lines. African green monkey kidney (Vero) and rabbit kidney (RK-13) cells have been found to be particularly susceptible. A CPE usually develops within 3 days, but two 5-day passages are recommended before judging the attempt unsuccessful. After low multiplicity of infection, the CPE is characterised by formation of syncytia that may, after 24–48 hours, contain over 60 or more nuclei. Syncytia formed by NiV in Vero cell monolayers are significantly larger than those created by HeV in the same time period. Although the distribution of nuclei in NiV-induced syncytia early in infection resembles that induced by HeV, with nuclei aggregated in the middle of the syncytia, nuclei in mature NiV-induced syncytia are distributed around the outside of the giant cell (Hyatt *et al.*, 2001).

Very low virus load in bats makes isolation very difficult. Linfa Wang and colleagues at the AAHL have increased sensitivity of cell lines by "rational engineering," consisting of a single point mutation in ephrinB2 resulting in enhanced affinity for NiV.

5) *Pen-side test*

Not yet developed.

While the concept is attractive, it is a huge challenge technically and in regulatory sense, especially considering how presumable false positive results would be handled.

6) *N and G ELISA*

Indirect recombinant N- ELISA and G-ELISA have been developed, and are now in the stage of diagnostic evaluation (Fisher K., *et al.*, 2018). The N-ELISA protocol was transferred to HSADDL (India) and validated and used for surveillance (Kulkarni *et al.,* 2016).

Problems with specificity (i.e., false positives) could arise. For example, swine sero-surveillance in West Bengal, India, appears to be negative; however, 8/328 samples tested positive (i.e., presumably false positive) using the anti-N antigen ELISA antibody detection test. Evaluation of the indirect IgG ELISA based on the recombinant NiV-N antigen using swine samples from Canada yielded similar results, including an indirect IgG ELISA based on the G glycoprotein. In Canadian context, the problem is the diagnostic specificity, with 5% false positives, resulting in the decision to complement with the G-ELISA. Only sera positive on both tests are considered

positive. Confirmatory testing may be required, if this was to be the first case reported in non-endemic area.

A diagnostic test for differentiating infected from vaccinated animals (DIVA) would have to most likely target the N antigen, or alternatively P gene coded products depending on the level of expression and antigenicity in animals, and the number of reactors in non-endemic areas.

The N ELISA assay could fulfill DIVA requirements if the canarypox vectored NiV-G-NiV-F vaccine is used because antibodies to N would only occur after NiV infection.

7) *IgM ELISA*

The U.S Center for Disease Control and Prevention (CDC) developed an IgM ELISA for human serology. Detection of IgM was used to confirm recent infection with NiV in both Malaysia and Bangladesh. NiV-infected cells that have been inactivated by gamma irradiation are used as antigens.

In theory the same can be done for different animal species as long as we have the right anti-species antibodies. For bats, that is still a challenge.

8) *Virus neutralization test (VNT)*

VNT serves as the traditional gold standard of serological investigations. The VNT requires live virus and thus BSL-4 containment facilities are required (Crameri *et al.,* 2002). It has proven to be a very valuable specific and sensitive tool in the diagnosis of NiV.

VNT rely on quantification methods. Three different procedures are available to titer HeV and NiV. In the traditional plaque and microtiter assay procedures, the titer is calculated as plaque forming units (PFU) or the tissue culture infectious dose capable of causing CPE in 50% of replicate wells ($TCID_{50}$), respectively.

In an alternative procedure, the viruses are titrated on Vero cell monolayers in 96-well plates and after 18–24 hours, foci of infection are detected immunologically in acetone-fixed cells using anti-viral antiserum (Crameri G., *et al.* 2002). The virus titre is expressed as focus-forming units (FFU)/ml.

Neutralisation assays using these three methods are described in the OIE Manuel of Diagnostic Tests and Vaccines for Terrestrial Animals.

Virus quantification procedures should be conducted at BSL4. A new version of the differential neutralisation test has been recently described, which avoids the use of infectious virus by the use of ephrin-B2-bound biospheres (Bossart *et al.,* 2007). Although the test has yet to be formally validated, it appears to have the potential to be a screening tool for use in countries without BSL4 facilities.

9) Pseudotype virus plaque reduction neutralization test (PRNT)

The standard plaque reduction neutralization assay (PRNT) used to detect NiV and HeV must be performed in BSL-4 containment and takes several days to complete. The CDC and the AAHL have modified the PRNT by using recombinant Vesicular Stomatitis Virus (VSV) derived from the cDNA of VSV Indiana to construct pseudotype particles expressing the F and G proteins of NiV (pVSV-NiV-F/G) as target antigens (Chang *et al.,* 2006; Tamin *et al.,* 2009; Kaku *et al*., 2009). This rapid assay can be performed at BSL-2. The PRNT was evaluated using serum samples from outbreak investigations and more than 300 serum samples from an experimental NiV vaccination study in swine. The results of the neutralization assays with pVSV-NiV-F/G as antigen showed a good correlation with those of standard PRNT. The PRNT titers give an indication of protective immunity. Therefore, this new method has the potential to be a rapid and cost-effective diagnostic method, especially in locations that lack high containment facilities, and will provide a valuable tool for basic research and vaccine development. A similar assay has been developed by the Japanese-Australian group (Kaku et al., 2009), which proved to be as specific as the VNT and much more sensitive than VNT.

10) Serological Binding Assay

Currently, a Luminex®-based (e.g. Bio-Rad Bio-Plex) multiplex microsphere immunoassay for the detection of antibodies specific to HeV and NiV G glycoproteins is used for bat surveillance at the AAHL, and by other research investigators. This multiplex microsphere immunoassay detects antibodies to recombinant soluble G (sG) proteins from NiV and HeV in a multiplexed assay. In contrast to traditional ELISAs, these Luminex-based platforms are more sensitive and require less sample sera to generate results with multiple analytes. The sG proteins retain their ability to bind the cellular receptor molecule, indicating their native conformation is maintained, which is important for the detection of neutralizing antibodies. Since the G specific antibody response to both NiV and HeV can be measured simultaneously, this assay can differentiate between the serologic responses to NiV and HeV. A variety of statistical models have been developed to determine thresholds to determine the cutoff value between negative and positives median fluorescence intensities (MFI). Instances when negative control sera is available, a MFI value three standard deviations above the z score can be used to interpret the cutoff for positive valuves.

11) Luminex® multiplexed nucleic acid detection assay

Foord et al, 2012, reported microsphere suspension array systems enable the simultaneous fluorescent identification of multiple separate nucleotide targets in a single reaction using commercially available oligo-tagged microspheres (Luminex® MagPlex-TAG) to construct and evaluate multiplexed assays for the detection and differentiation of HeV and NiV. Assays were developed to target multiple sites within the nucleoprotein (N) and phosphoprotein (P) encoding genes. The relative specificities and sensitivities of the assays were determined using reference isolates of each virus type, samples from experimentally infected horses, and archival veterinary diagnostic submissions. Results were assessed in direct comparison with an established qPCR. Foord reported the microsphere array assays achieved unequivocal differentiation of HeV and NiV

and the sensitivity of HeV detection was comparable to qPCR, indicating high analytical and diagnostic specificity and sensitivity.

12) Blocking Luminex® Assay

This is an extension of the Binding Luminex Assay, developed as a surrogate VNT in the sense that it measures antibodies that block the binding of the soluble henipavirus G protein to the ephrin-B2 receptor molecule. It is highly specific, but needs further validation with field samples.

DEPOPULATION

Preemptive culling of herds in the neighborhood of an infected herd is an effective and even indispensable measure in the control of a NiV epidemic in areas with high pig densities. The purpose of this measure is to prevent infection of new herds, which would generate massive infectious virus production, and thus to reduce the virus infection load in an area. This reduced infection load subsequently results in a reduction of the between-herd virus transmission. However, recent outbreaks have shown that the control of Nipah virus in pig populations through stamping out is complex due to the zoonotic nature of the agent. In addition, depopulation may be logistically difficult and very expensive in swine dense area, and would not be effective if the Nipah virus mutates to become easily transmitted between people and from people to pigs. Depopulation will not be possible in situation like those that occurred in Bangladesh in which NiV was transmitted from bats to humans without an amplifying host. Depopulation of swine may be impossible in a rapidly spreading outbreak in a pig dense region with hundreds of millions of swine, such as in southeast China (Vergne T. *et. al*. 2017).

SURVEILLANCE

The initial expression of NiV in U.S swine would be variable and unpredictable due to the myriad of host factors and the broad diversity of virulence among strains of henipaviruses. Different surveillance strategies will be required to detect the different clinical manifestations.

For acute infection, surveillance activities can be based on clinical signs, but signs are unlikely to be noticed by producers and practitioners. It would be prudent to develop surveillance activities based on diagnostic testing to supplement surveillance based on clinical signs.

The following surveillance programs are in place to meet the objective of rapid detection of henipaviruses in Malaysia and Australia:

1. Population-based passive reporting of suspicious NiV cases. Efforts to enhance reporting will be focused on high risk areas.
2. Laboratory-based surveillance of serum and tissue submitted from sick pigs.

There is no diagnostic capability for henipaviruses in United States veterinary diagnostic laboratories due to the lack of BSL-4 laboratory space. The only diagnostic capability for henipaviruses in the U.S is the Center for Disease Control and Prevention (CDC). There are no active or passive surveillance

programs. Henipavirus suspect samples would be sent to the CDC, the OIE reference laboratory at the Australian Animal Health Laboratory, or the National Canadian Foreign Animal Disease Center, in Winnipeg, Canada.

DRUGS

There are no licensed anti-viral drugs available to treat people or animals against Henipaviruses.

DISINFECTANTS

People: Soaps and detergents.

Fomite disinfection: Sodium hypochlorite to supply 10,000 ppm chlorine or Virkon.

PERSONAL PROTECTIVE EQUIPMENT (PPE)

PPE should be suitable to prevent farm-to-farm virus spread by diagnostic or vaccination teams.

RECOMMENDATIONS

RESEARCH

The 2017 gap analysis working group recommended the implementation of the following research priorities.

Viral Pathogenesis

- Determine early events of NiV infection, immune evasion and identify determinants for virulence and host susceptibility

Immunology

- Characterize the antibody and cell-mediated immune response to NiV infection and vaccination
- Develop the basic knowledge of the mechanisms NiV uses to evade the innate immune response
- Characterize the ability of interferons to inhibit virus replication and shedding early in infection.

Vaccine Discovery and Development Research.

- Implement comprehensive vaccine research program to deliver next generation NiV vaccines (e.g., DIVA [differentiate infected from vaccinated animals] capable), and specifically design strategies for control in priority susceptible hosts
- Investment in Nipah vaccine development needs to include conducting studies to demonstrate safety and efficacy necessary for licensure by authorities in countries that may have an emergency need for vaccine in swine.

Diagnostics

- Develop a panel of reference standards for both molecular and serologic tests that can be made available to all of the laboratories performing diagnostic tests for henipaviruses. This panel should also include monoclonal antibodies and recombinant antigens that would be readily available as low biosecurity BSL-2 reagents.
- Develop a formalized structured worldwide network for reference panel development and assay validation and harmonization.
- Develop and validate broadly reactive PCR assays targeting highly conserved genetic targets within the henipaviruses. Evaluate the relative sensitivity and specificity of the currently used PCR assays.
- Develop and validate field tests (both protein- and nucleic acid-based) to detect henipaviruses.
- Explore new antigen detection assays, including antigen capture, Loop Mediated Isothermal Amplification Protocol (LAMP) suitable for resource limited situations, and nanotechnology.
- Develop species specific reagents to improve the quality of serologic assays.
- Evaluate the relative sensitivity and specificity of molecular and serologic tests, especially new serologic tests that could replace serum neutralization titers (SNT) and meet DIVA requirements.
- Explore the use of serological assays based on recombinant antigens that could be produced at BSL-2. Classical serological tests using low biosecurity (recombinant) reagents produced at BSL-2 facilities could be developed reasonably quickly and at a reasonable cost.
- Develop species independent serologic assays using recombinant antigens.

Epidemiology

- The epidemiology of NiV in disease outbreaks needs to be assessed and modeled on the level of the individual pig, the herd, and the demographics of the region.
- Epidemiological investigations should be performed on the implementation of emergency vaccination and the use of 'DIVA' and other diagnostic tests to detect infected pigs in vaccinated populations
- Risk assessments need to be performed with regard to control or spread of henipaviruses
- The epidemiological evaluation of wildlife needs to be carried out in order to improve the risk estimates of outbreaks in domestic animal and human populations

PREPAREDNESS

Many of the countermeasures discussed in this report will require preparation and integration in a coordinated disease control program and funding for a stockpile for use in an emergency response plan for an outbreak of NiV infection. The Henipavirus gap analysis working group recommends investing in the implementation of the following preparedness plan to ensure the effective use of the countermeasures in the NVS:

- See the Ausvetplan: https://www.animalhealthaustralia.com.au/our-publications/ausvetplan-manuals-and-documents/ Assessed July 22, 2018
- See Guidelines for Veterinarians Handling potential Hendra Virus infection in Horses (QDPI): https://www.daf.qld.gov.au/__data/assets/pdf_file/0005/126770/2913_-Guidelines-for-veterinarians-handling-potential-Hendra-virus-infection-in-horses-V5.1.pdf Accessed July 22, 2018

Surveillance

Routine surveillance for NiV is now limited to serologic screening of pigs in several Southeast Asian countries.

- Develop a regional surveillance strategy, including laboratory, to detect spillovers of NiV into domestic and agricultural animals.
- Determine the optimal surveillance strategy to detect circulation of NiV in the bats reservoirs and other wild life.
- Improve surveillance capacity to detect henipaviruses in high risk countries.
- Establish a formal laboratory network for henipavirus surveillance that includes standardized specimen collection, laboratory testing scheme, quality control, specimen referral and accreditation.

Biosecurity

Design NiV-specific on-farm biosecurity programs to implement in a disease outbreak situation.

Personal Protective Equipment and Decontamination

- See Australian procedures https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0003/494202/Hendra-virus-ppe-procedures.pdf Assessed July 22, 2018

- FAO (Food and Agriculture Organization) – Manual on the Diagnosis of Nipah Virus in Animals: Chapter 2: Working safely with Nipah Virus
  http://www.fao.org/docrep/005/AC449E/ac449e05.htm#bm05
  Assessed July 22, 2018

Depopulation and Disposal

Develop plans for handling disposal of animals infected with a zoonotic agent, including an emergency plan to dispose of infected swine and decontaminate facilities and equipment determined to be infected.

- FAO (Food and Agriculture Organization) – Manual on the Diagnosis of Nipah Virus in Animals: Chapter 5: Control and eradication
  http://www.fao.org/docrep/005/AC449E/ac449e08.htm#bm08

CONCLUSION

The threat of an outbreak with a henipavirus in the United States due to a natural transmission from a reservoir host is very low since the reservoirs are known to be bats in South East Asia, South Asia, and Asia. However, an outbreak that is not controlled in swine or in people in Asia could result in infection being introduced accidentally into North America or Europe. There is considerable concern that henipaviruses could be used as a weapon of mass destruction (WMD) because they have many of the characteristics of the ideal biological weapon, including causing one of the highest mortality rate in people known for an infectious disease. The possibility of an intentional criminal spread at least in short clusters of terrorist attacks is a distinct possibility, for example by aerosolization in confined public spaces, or through infection of pigs. Surveillance brings challenges and weaknesses of diagnostic methods may impede the early detection of an outbreak in the United States. There are no commercially available diagnostic tests and although laboratory tests are available they have not been field validated. Depopulation is the primary method to eradicate NiV but present very high risks since henipaviruses are BSL-4 zoonotic agents. There are commercially available vaccines for horses, but none for swine and people. Accordingly, the gap analysis working group recommends investing in the research and development of countermeasures and ensure their use and integration in planning for preparedness and future control campaigns. Priority should be given to funding research to improve surveillance, diagnostics, and vaccines. Specific goals include 1) improving diagnostic tests to rapidly identify new disease outbreaks; 2) epidemiological research to better understand virus transmission in wildlife and maintain a passive surveillance program in high risk commercial livestock operations; and 3) develop safe and effective vaccines specifically designed for control and eradication. The United States should stockpile NiV vaccines when they become available for use in contact herds to create a buffer zone as an additional control measure to prevent the spread of henipaviruses should an outbreak ever occur.

FIGURES

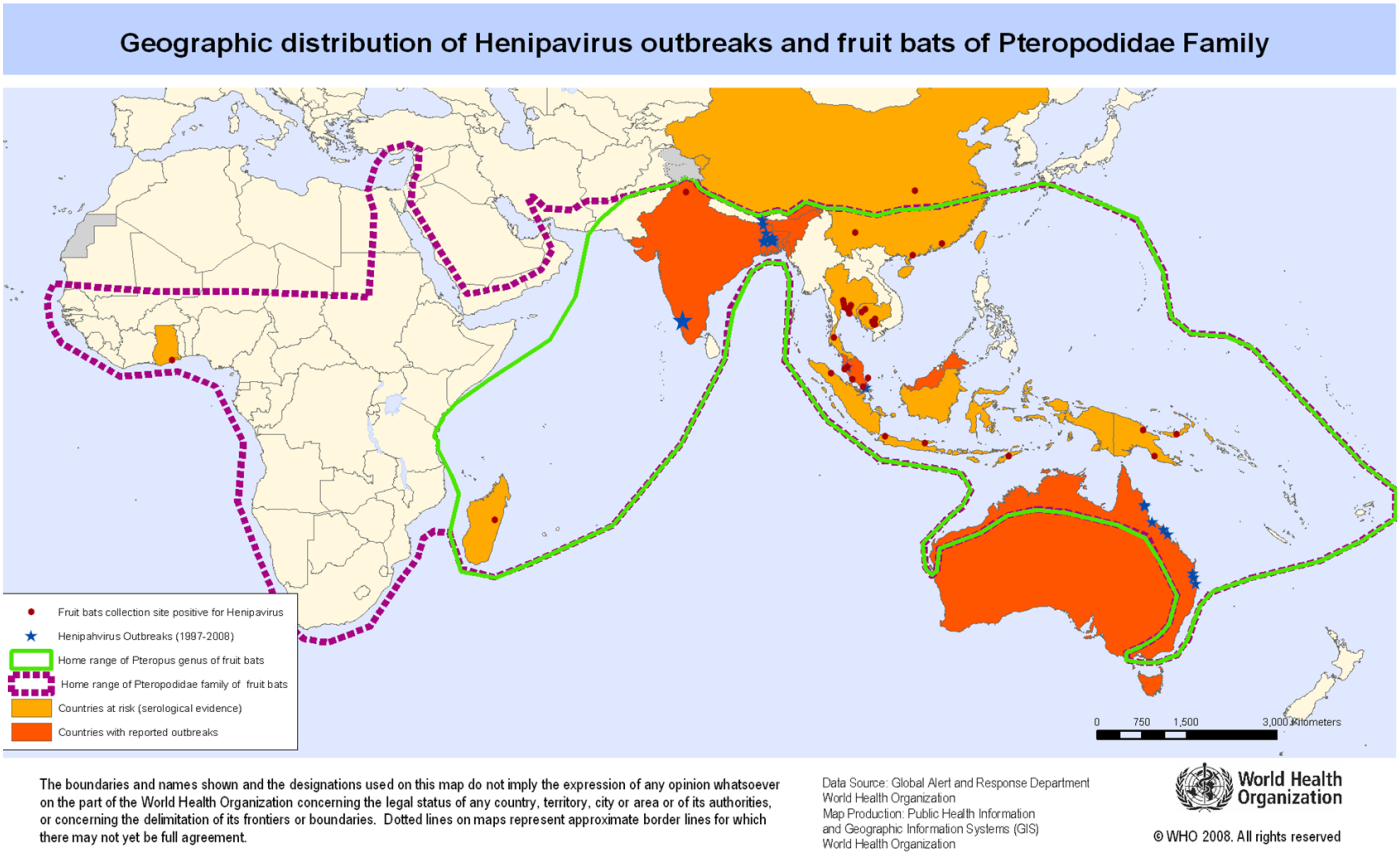

Figure 1: Geographic distribution of fruit bats of the Pteropodidae family. WHO: Nipah virus infections: http://www.who.int/csr/disease/nipah/en/ (Assessed and modified November 26, 2018).

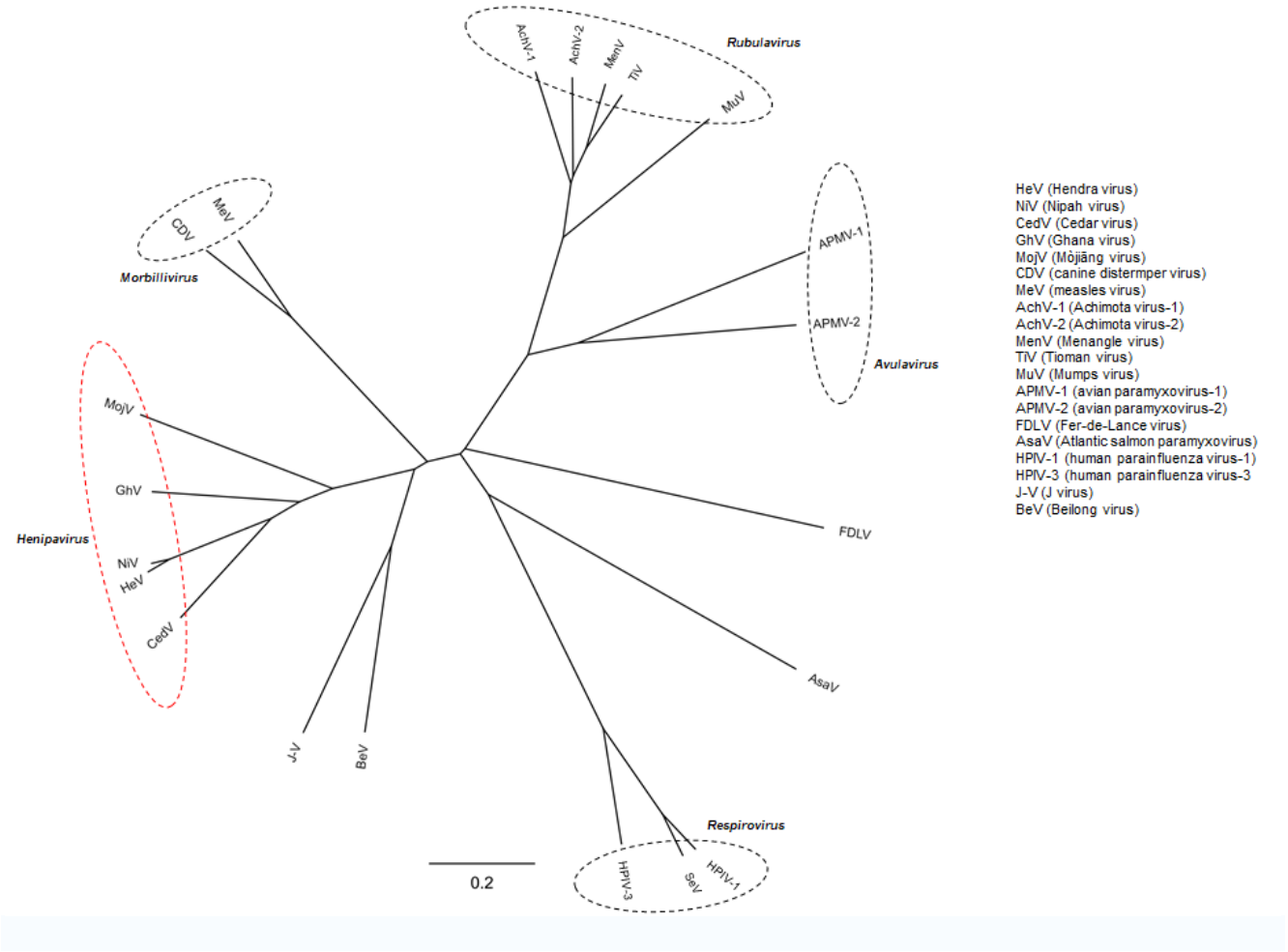

Figure 2: Phylogenetic tree based on alignment of amino acid sequence of the N-gene of selected *Paramyxovirinae* subfamily members.

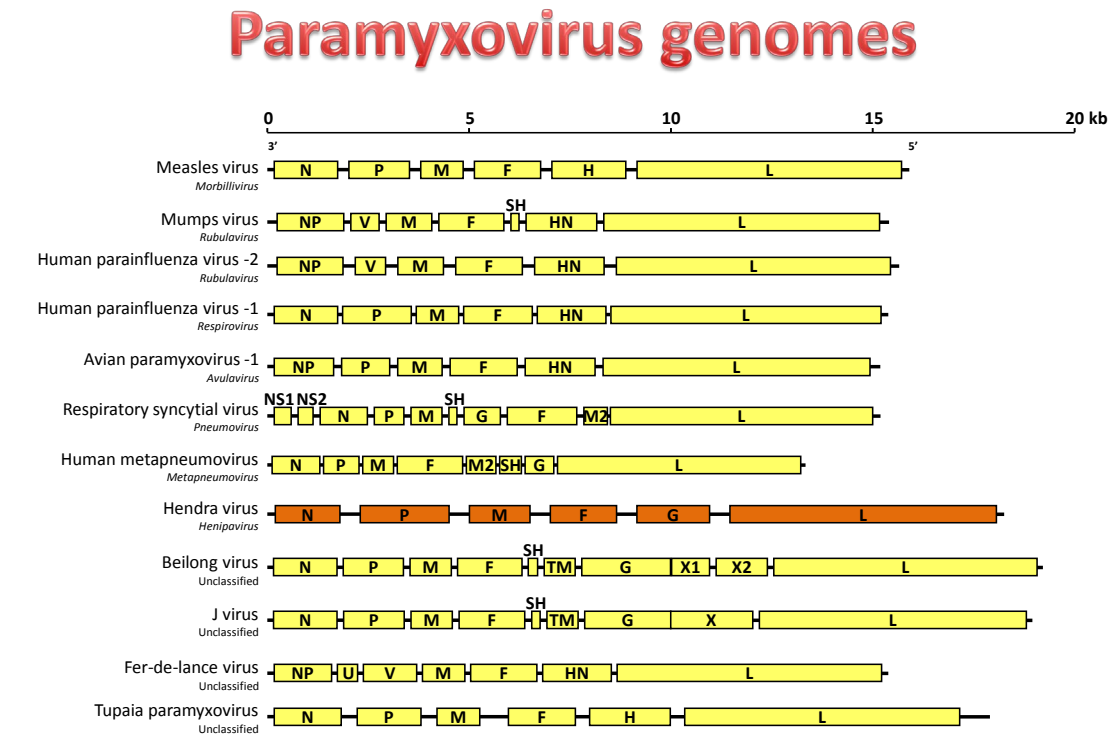

Figure 3: Comparison of *Paramyxoviridae* viruses genomes (Provided by Glen Marsh, AAHL)

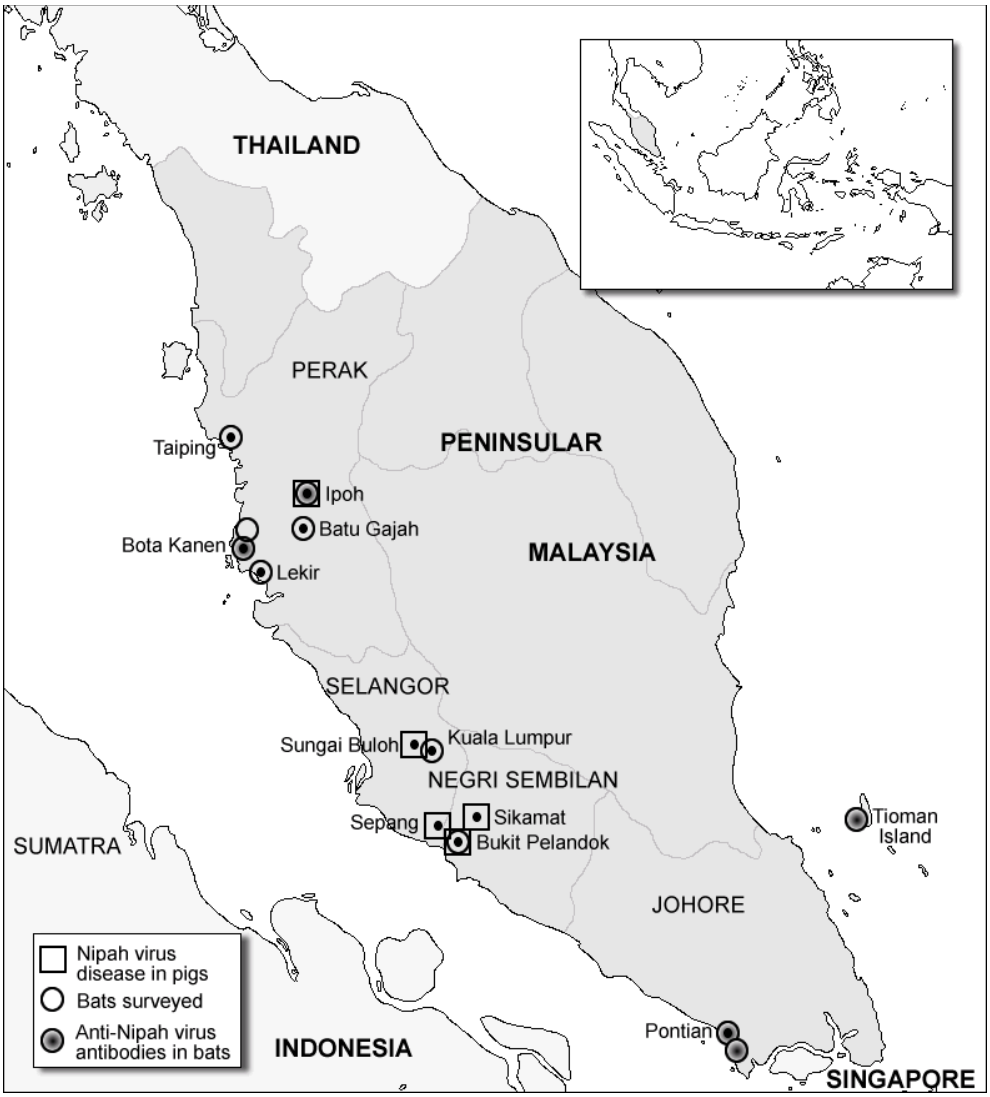

Figure 4: Descriptive map of NiV in Malaysia (Yob *et al.,* 2001)

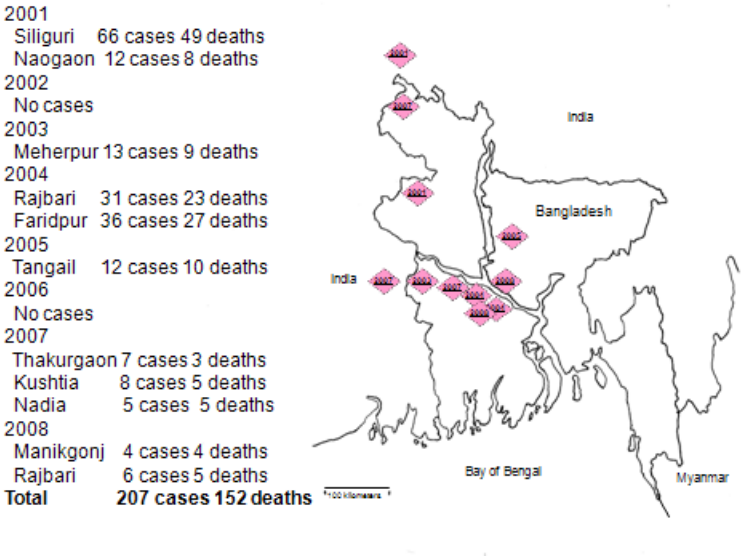

Figure 5: Epidemiology of Nipah Virus Infections in Bangladesh (Source: Steve Luby, icddr,b)

TABLE I: NIPAH VIRUS INFECTION IN BATS

Suborder	Species	No. of bats	No. Positive
Pteropodiformes	*Balionycterus macul*	4[*]	0
	Cynopterus brachyotis	56[*], 1[†]	2[*], 0[†]
	C. horsfieldi	24[*]	0
	C. sphinx	2[ǂ], 34[§], 68[†]	0[ǂ], 0[§], 0[†]
	Eidolon dupreanum	73[#]	14
	E. helvum	59[¶], 215[Δ]	23[¶], 3[Δ]
	Eonycteris spelaea	38[*], 64[§]	2[*], 0[§]
	Epomophorus gambianus	89[¶]	1
	Epomops buettikoferi	7[¶]	0
	E. franqueti	29[¶]	0
	Hipposideros armiger	63[*], 88[§], 1[†]	2[*], 0[§], 0[†]
	H. bicolor	1[*]	0
	H. larvatus	21[ǂ], 95[§], 81[†]	0[ǂ], 2[§], 0[†]
	H. pomona	60[*], 2[†]	1[*], 0[†]
	Hypsignathus monstrosus	18[¶]	1
	Macroglossus sobrinus	4[*], 1[†]	0, 0[†]
	Megaderma lyra	1[ǂ]	0
	Megaderma spasma	13[§]	0
	Megaerops ecaudatus	1[*]	0
	Nanonycteris veldkampii	4[¶]	0
	Rhinolophus acuminatus	2[†]	0
	R. affinis	6[*], 94[ǂ]	0[*], 1[ǂ]
	R. ferrumequinum	3[ǂ]	0
	R. luctus	11[ǂ], 1[†]	0[ǂ], 0[†]
	R. macrotis	3[ǂ]	0
	R. pearsoni	35[ǂ]	0
	R. pusillus	35[ǂ]	0
	R. refulgens	1[*]	0
	R. rex	1[ǂ]	0
	R. sinicus	51[*]	1
	Rousettus leschenaulti	52[*], 11[§], 15[†]	5[*], 0[§], 0[†]
	R. madagascariensis	5[#]	0
	Pteropus hypomelanus	35[*], 36[§]	11[*], 4[§]
	P. lylei	857[§], 408[†]	83[§], 50[†]
	P. medius	2790[«]	100
	P. rufus	349[#]	6
	P. vampyrus	29[*] 39[§]	5[*], 1[§]
Vespertilioniformes	*Chaerephon plicatus*	153[†]	0
	Emballonura monticola	14[§]	0
	Ia io	7[ǂ]	0
	Miniopterus spp.	32[ǂ]	5
	Myotis altarium	2[ǂ]	0
	M. daubentoni	89[ǂ]	9
	M. ricketti	84[ǂ]	8
	Murina cyclotis	1[†]	0
	Nyctalus velutinus	1[ǂ]	0
	Scotophilus heathi	3[§]	0
	Scotophilus kuhlii	33[*], 20[ǂ], 98[†]	1[*], 0[ǂ], 0[†]
	Tadarida plicata	50[§]	0
	Taphozous melanopogon	4[*], 69[†]	0[*], 0[†]
	T. saccolaimus	1[*]	0
	T. theobaldi	121[†]	0

[*]Yob JM, et al. 2001; , [ǂ]Yan L, et al. 2008; [§]Wacharapluesadee S, et al. 2005; [†]Reynes JM, et al. 2005; [¶]Hayman DTS, et al. 2008; [Δ]Drexler JF, et al. 2009; [#]Ihele C, et al. 2007; [«]Epstein JH, et al. 2016

TABLE II – NIPAH VIRUS CASES 2001-2018

Morbidity and mortality due to Nipah or Nipah-like virus encephalitis in WHO South-East Asia Region, 2001-2018

Country: Bangladesh

Month/Year	Location	No. cases	No. deaths	Case Fatality Rate
April, May 2001	Meherpur	13	9	69%
January 2003	Naogaon	12	8	67%
Jan 2004 Apr 2004	Rajbari Faridpur	31 36	23 27	74% 75%
Jan- Mar 2005	Tangail	12	11	92%
Jan-Feb 2007 Mar 2007 Apr 2007	Thakurgaon Kushtia Pabna, Natore and Naogaon	7 8 3	3 5 1	43% 63% 33%
Feb 2008 Apr 2008	Manikgonj Rajbari	4 7	4 5	100% 71%
Jan 2009	Gaibandha, Rangpur and Nilphamari Rajbari	3 1	0 1	0% 100%
Feb-Mar 2010	Faridpur Faridpur, Rajbari, Gopalgonj, Kurigram,	8 8 1	7 7 1	87.50% 87.50% 100%
Jan-Feb 2011	Lalmohirhat, Dinajpur, Comilla Nilphamari, Faridpur, Rajbari	44	40	91%
Jan 2012	Joypurhat	12	10	83%
Jan- Apr 2013	Pabna, Natore, Naogaon, Gaibandha, Manikganj	24	21	88%
Jan-Feb 2014	13 districts	18	9	50%
Jan-Feb 2015	Nilphamari, Ponchoghor, Faridpur, Magura, Naugaon, Rajbari	9	6	67%

Country: India

Month/Year	Location	No. cases	No. deaths	Case Fatality Rate
Feb 2001	Siliguri	66	45	68%
Apr 2007	Nadia	5	5	100%
May 2018	Kerala	23	21	91%

WHO (World Health Organization). Morbidity and mortality due to Nipah or Nipah-like virus encephalitis in WHO South-East Asia Region, 2001-2018. Available at: http://www.who.int/csr/disease/nipah/en/.

TABLE III – VACCINE PLATFORMS

C.C. Broder et al. / Vaccine 34 (2016) 3525–353

Table 1
Advanced active vaccination and passive immunization platforms tested in Hendra virus and/or Nipah virus animal challenge models.

Platform	Viral antigen target or immunogen	Animal challenge model
Active vaccination		
Recombinant vaccinia virus	Nipah F and/or G glycoprotein	Hamster[a] (NiV)
Recombinant canarypox virus	Nipah F and/or G glycoprotein	Pig[b] (NiV)
Recombinant VSV	Nipah F and/or G glycoprotein	Ferret[c] (NiV), Hamster[d] (NiV), nonhuman primate[e] (NiV)
Recombinant AAV	Nipah G glycoprotein	Hamster[f] (NiV, HeV)
Recombinant measles virus	Nipah G glycoprotein	Hamster and nonhuman primate[g] (NiV)
Recombinant subunit	Hendra soluble G glycoprotein	Cat[h] (NiV), Ferret[i] (HeV, NiV), nonhuman primate[j] (HeV, NiV), horse[k] (HeV)
Passive immunization		
Human monoclonal antibody m102.4	Hendra/Nipah G glycoprotein	Ferret[l] (NiV) Nonhuman primate[m] (HeV, NiV)

[a] Hamsters immunized with NiV F and/or G glycoprotein encoding recombinant vaccinia viruses were protected against disease following intraperitoneal challenge with 10^3 PFU of NiV [137].
[b] Pigs immunized with NiV F and/or G glycoprotein encoding recombinant canarypox viruses were protected against intranasal challenge with 2.5×10^5 PFU of NiV [138].
[c] Ferrets immunized with NiV F and/or G glycoprotein encoding recombinant vesicular stomatitis virus (VSV) vectors were protected against lethal intranasal challenge with 5×10^3 PFU of NiV [141]
[d] Hamsters immunized with NiV F and/or G glycoprotein encoding recombinant vesicular stomatitis virus (VSV) vectors were protected against lethal intraperitoneal challenge with 10^5 $TCID_{50}$ of NiV [143]; or 6.8×10^4 $TCID_{50}$ of NiV [142].
[e] African green monkeys immunized with a NiV G encoding recombinant VSV vector were protected against lethal intratracheal challenge with 10^5 $TCID_{50}$ of NiV [156].
[f] Hamsters immunized with a NiV G encoding recombinant adeno-associated virus (AAV) vector were protected against lethal intraperitoneal with 10^4 PFU of NiV [139].
[g] Hamsters and African green monkeys immunized with a NiV G encoding recombinant measles virus vector were protected against lethal intraperitoneal challenge with 10^3 $TCID_{50}$ of NiV (hamsters) or 10^5 $TCID_{50}$ of NiV (monkeys) [140].
[h] Hendra virus soluble G glycoprotein (HeV-sG) used to immunize cats protects against lethal subcutaneous (500 $TCID_{50}$) [120] or oronasal (5×10^4 $TCID_{50}$) NiV challenge [145].
[i] HeV-sG used to immunize ferrets protects against lethal oronasal challenge with 5×10^3 $TCID_{50}$ of HeV [124] or 5×10^3 $TCID_{50}$ of NiV challenge [146].
[j] HeV-sG used to immunize African green monkeys protects against lethal intratracheal challenge with 10^5 $TCID_{50}$ of NiV [157] or 5×10^5 PFU of HeV [147].
[k] HeV-sG used to immunize horses protects against lethal oronasal challenge with 2×10^6 $TCID_{50}$ of HeV [15].
[l] A NiV and HeV cross-reactive G glycoprotein specific neutralizing human mAb (m102.4) protects ferrets against lethal oronasal challenge with 5×10^3 $TCID_{50}$ of NiV [125] or 5×10^3 $TCID_{50}$ of HeV (J. Pallister and C. Broder, unpublished) by post-exposure infusion.
[m] Human mAb m102.4 protects African green monkeys by post-exposure infusion following lethal intratracheal challenge with 4×10^5 $TCID_{50}$ of HeV [153] or lethal intratracheal challenge with 5×10^5 PFU of NiV [154].

TABLE IV – CURRENT VACCINE CANDIDATES

B.A. Satterfield et al. / Vaccine 34 (2016) 2971–2975

Table 1
Development status of current vaccine candidates.

Candidate name/ identifier: institution	Preclinical	Developers	Ref
Subunit vaccine			
HeV sG	X	Zoetis, Inc./USU	[16,18,34,39]
Vectored vaccines			
VSV-NiV_B F and/or G	X	UTMB	[17]
VSV-NiV_M G	X	CDC	[15]
VSV-NiV_M G	X	RML	[14,19]
VSV-NiV_M F and/or G	X	Yale University	[40]
VSV-HeV G:	X	TJU/RML	[41]
RABV-HeV G:	X	TJU/RML	[41]
ALVAC-F/G	X	CFIA-NCFAD	[20,42]
AAV-NiV_M G	X	INSERM	[43]
rMV-Ed-G	X	UoT	[44]
V-NiVG	X	USU	[45]
rLa-NiVG and/or rLa-NiVF	X	CAAS-SKLVB	[21]
Passive antibody transfer			
Polyclonal serum NiV F or G	X	INSERM	[46]
Mouse mAbs NiV F or G	X	INSERM	[47]
Human mAb m102.4 Henipah G	X	USU	[35,48]

Abbreviations: USU (Uniformed Services University of the Health Sciences); UTMB (University of Texas Medical Branch); CDC (Centers for Disease Control and Prevention); RML (Rocky Mountain Laboratories); TJU (Thomas Jefferson University); CFIA-NCFAD (Canadian Food Inspection Agency – Centre for Foreign Animal Diseases); Institut national de la santé et de la recherché médicale (INSERM); UoT (University of Tokyo); CAAS-SKLVB (Chinese Academy of Agricultural Sciences (CAAS) – State Key Laboratory of Veterinary Biotechnology (SKLVB)).

APPENDIX I: COUNTERMEASURES WORKING GROUP INSTRUCTIONS

Decision Model

We will use a decision model to assess potential countermeasures to stockpile. These countermeasures must significantly improve our ability to control and eradicate an outbreak of Nipah virus in a disease-free country. The decision model is a simple tool that will allow us to focus on critical criteria for the National Veterinary Stockpile, and rank the available interventions relative to each other. The decision model is available as a Microsoft Excel spread sheet, which has been prepared to quantitatively assess the rankings we assign to a set of selected criteria that will lead to the selection of the highest cumulative option. We can use as many criteria as we want but the objective is to get down to the ones that will make or break success. The criteria for each intervention will be selected by the Countermeasures Working Group, but a preliminary set has been identified to expedite the process. You are encouraged to review the criteria prior to coming to the meeting and be prepared to modify the criteria as needed with the working group. The following provides an example of criteria and assumptions for assessing vaccines.

Criteria

If a vaccine is going to be used as an emergency outbreak control tool for Nipah virus, then we need to know: 1) is it efficacious (does it effectively eliminate shedding or just reduce shed by a known log scale); 2) does it work rapidly with one dose (probably do not have time for a second dose); 3) whether it is available today from the perspective of having a reliable & rapid manufacturing process (need to know it can be up & running rapidly and will yield a predictable amount of vaccine; 4) can we get the product to the outbreak site rapidly & safely; 5) once at the site, can we get it into the target population rapidly; 6) type of administration- mass or injected, people and equipment to do the job become important); and 7) are diagnostics available to monitor success and or DIVA compliant. While cost is important, the cost of the vaccine in an outbreak will be small in comparison to the other costs. In addition, how fast the product can be made is important because that will have a big impact on how big a stockpile will be needed. Accordingly, you will see from the Excel sheets that have been prepared for vaccines that the following critical criteria and assignment of weights for each criterion are proposed.

Weight	Critical Criteria
10	Efficacy
6	Safety
8	One dose
6	Speed of Scale up
2	Shelf life
2	Distribution/storage
10	Quick Onset of Immunity
8	DIVA Compatible
2	Withdrawal
2	Cost to Implement

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APPENDIX II – VACCINES ASSESSMENT

Experimental Veterinary Vaccines For Nipah Virus - USDA/ARS, 03-19-09								
	Rank each Intervention (2,4,6,8, or 10) as to its importance to making a decision, only one "10" rankings allowed							
Weight	**Critical Criteria**	CPV-G	CPV-F	VV-G	Soluble G			
10	Efficacy	6	4	2	6			
6	Safety	10	10	2	10			
8	One dose	4	4	4	2			
8	Manufacturing safety	8	8	6	8			
10	DIVA Compatible	8	8	8	8			
8	Manufacturing yield	8	8	8	6			
6	Rapid production	8	8	4	4			
4	Reasonable cost	6	6	4	2			
2	Short withdrawal	8	8	2	4			
8	Long shelflife	8	8	8	4			
Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed								
	Critical Criteria	CPV-G	CPV-F	VV-G	Soluble G	0	0	0
	Efficacy	60	40	20	60	0	0	0
	Safety	60	60	12	60	0	0	0
	One dose	32	32	32	16	0	0	0
	Manufacturing safety	64	64	48	64	0	0	0
	DIVA Compatible	80	80	80	80	0	0	0
	Manufacturing yield	64	64	64	48	0	0	0
	Rapid production	48	48	24	24	0	0	0
	Reasonable cost	24	24	16	8	0	0	0
	Short withdrawal	16	16	4	8	0	0	0
	Long shelflife	64	64	64	32	0	0	0
	0	0	0	0	0	0	0	0
	Value	512	492	364	400	0	0	0

APPENDIX III – DIAGNOSTICS ASSESSMENT

Experimental Diagnostics For Nipah Virus - USDA/ARS, 03-19-09

Rank each Intervention (2,4,6,8, or 10) as to its importance to you in making a decision, no more than one "10" rankings allowed

Weight	Critical Criteria	qPCR	conv PCR	field PCR	VI	penside	v ELISA	N ELISA	IgM ELISA	VNT	ps VNT	bind lum	block lum
10	Sensitivity	10	10	8	8	4	10	4	8	8	8	8	8
8	Specificity	8	6	8	10	6	6	6	8	10	8	8	8
2	DIVA	8	8	8	8	8	2	10	6	2	2	8	2
6	multispecies	8	8	8	8	8	6	6	2	8	8	6	8
8	Validation to purpose	8	8	8	8	4	8	4	10	8	10	8	10
4	Speed of Scaleup	8	4	4	2	6	8	8	8	2	4	4	4
4	Throughput	8	2	2	2	4	8	8	8	2	4	6	6
4	Flock Side Test	2	2	10	2	10	2	2	2	2	2	2	2
10	Rapid Result	6	4	8	2	8	6	6	6	4	4	10	8
4	No need to Confirm	6	4	4	8	2	6	4	6	8	8	8	8
8	Easy to perform	8	6	6	4	8	8	8	6	6	6	8	8
8	safe to operate	8	8	6	2	6	8	8	8	2	8	8	8
8	Availability	8	8	2	2	2	6	8	4	2	6	4	4
6	Storage/Distribution	4	6	6	2	6	6	6	6	2	4	4	4
4	Low Cost to Implement	2	4	2	2	4	6	8	6	2	4	4	2

Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed

Critical Criteria	qPCR	conv PCR	field PCR	VI	penside	v ELISA	N ELISA	IgM ELISA	VNT	ps VNT	bind lum	block lum
Sensitivity	100	100	80	80	40	100	40	80	80	80	80	80
Specificity	64	48	64	80	48	48	48	64	80	64	64	64
DIVA	16	16	16	16	16	4	20	12	4	4	16	4
multispecies	48	48	48	48	48	36	36	12	48	48	36	48
Validation to purpose	64	64	64	64	32	64	32	80	64	80	64	80
Speed of Scaleup	32	16	16	8	24	32	32	32	8	16	16	16
Throughput	32	8	8	8	16	32	32	32	8	16	24	24
Flock Side Test	8	8	40	8	40	8	8	8	8	8	8	8
Rapid Result	60	40	80	20	80	60	60	60	40	40	100	80
No need to Confirm	24	16	16	32	8	24	16	24	32	32	32	32
Easy to perform	64	48	48	32	64	64	64	48	48	48	64	64
safe to operate	64	64	48	16	48	64	64	64	16	64	64	64
Availability	64	64	16	16	16	48	64	32	16	48	32	32
Storage/Distribution	24	36	36	12	36	36	36	36	12	24	24	24
Low Cost to Implement	8	16	8	8	16	24	32	24	8	16	16	8
Value	672	592	588	448	532	644	584	608	472	588	640	628

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To:	Laing, Eric; Broder, Christopher; Weingartl, Hana (CFIA/ACIA); jaroth@iastate.edu; "Balkema-Buschmann, Anne"; "Diederich, Sandra"; Pickering, Bradley (CFIA/ACIA); "Daniels Family"; "Jon Epstein"; "Glenn.Marsh@csiro.au"; Carter, Bruce A - APHIS
Subject:	Henipavirus Gap Analysis Report - Updated
Date:	Tuesday, December 18, 2018 6:12:32 AM
Attachments:	Henipavirus Gap Analysis Report, Updated November 2018.pdf image003.png image005.png

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Dear Colleagues,

Just a quick note to let you know that our gap analysis report was updated with new information on emerging henipaviruses and detection in bats in new geographical areas of Africa and Asia. Many thanks to Eric Laing and Chris Broder at USUHS for their help in updating the report. The updated report is attached for your use. The reference and URL remain the same:

To cite this report:
Henipavirus Gap Analysis Workshop Report. 2018. U.S. Department of Agriculture, Agricultural Research Service, Washington, DC. http://go.usa.gov/xnHgR.

Also, you may have seen this already but our report was highlighted in Bat News – see below.

Thanks again everyone for all your help with this report and please send me updates in the future as needed. Next steps will be to share the report with funders of research to support the implementation of the research priorities identified in our report.

Wishing everyone a Merry Christmas, Happy Holidays, and success in 2019!

Best regards,

Cyril

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---------- Forwarded message ---------
From: **Keren Cox-Witton** <kcox-witton@wildlifehealthaustralia.com.au>

Date: Mon, Dec 17, 2018, 10:00 PM
Subject: Bat News - WHA - December 2018
To:

Dear Bat News subscribers,

Please see below for recent news articles and publications relating to bat health.

Wishing everyone a happy holiday and all the very best for 2019!

Best regards,
Keren

Hendra virus

- Henipavirus Gap Analysis Workshop Report

White-nose syndrome

- How to vaccinate a wild bat
- Groundbreaking science at TRU aims to save bats
- Mammoth Cave scientists studying white-nose syndrome
- What secrets are hidden inside the call of a bat?
- White-nose syndrome – other publications

Other news

- Heat stress – media
- 'It's extremely cruel': backyard netting killing, maiming fruit bats
- Flying foxes detect new invaders
- MSU project to prevent bat-borne diseases wins $10 million grant
- 'Pest-controlling' bats could help save rainforests
- Betting on bats for genetic treasures
- National Wildlife Biosecurity Guidelines
- National Flying-Fox Forum – Presentations available

Publications

- *Polychromophilus melanipherus* and haemoplasma infections not associated with clinical signs

in southern bent-winged bats (*Miniopterus orianae bassanii*) and eastern bent-winged bats (*Miniopterus orianae oceanensis)*

- Alston virus, a novel paramyxovirus isolated from bats causes upper respiratory tract infection in experimentally challenged ferrets

- Slow growth and delayed maturation in a Critically Endangered insular flying fox (*Pteropus natalis*)

- Active and passive surveillance for bat lyssaviruses in Italy revealed serological evidence for their circulation in three bat species

- Lleida Bat Lyssavirus isolation in *Miniopterus schreibersii* in France

- Active sero-survey for European bat lyssavirus type-1 circulation in North African insectivorous bats

- Rabies & bats – publications

- Coronaviruses & bats – publications

- Bacterial resistance in bats from the Phyllostomidae family and its relationship with unique health

- Coordinated change at the colony level in fruit bat fur microbiomes through time

- Handling stress and sample storage are associated with weaker complement-mediated bactericidal ability in birds but not bats

- Mass-culling of a threatened island flying fox species failed to increase fruit growers' profits and revealed gaps to be addressed for effective conservation

- Publications – Other bat diseases

Hendra virus

Henipavirus Gap Analysis Workshop Report

Henipavirus Gap Analysis Workshop Report. 2018. U.S. Department of Agriculture, Agricultural

Research Service, Washington, DC. http://go.usa.gov/xnHgR

"…The United States Department of Agriculture (USDA) organized the first Nipah virus gap analysis workshop in Australia in 2009 with the support of the Australian Animal Health Laboratory (AAHL). The working group was charged by the USDA National Veterinary Stockpile Steering Committee with making recommendations on specific materials, commercially available and in the pipeline, which will ensure the United States has an arsenal of highly efficacious

countermeasures to control and mitigate the impact of an outbreak of Nipah virus. Nipah virus experts representing laboratories in South East Asia, Australia, Canada, and the United States were invited to participate and contributed to this report. The second workshop was organized in 2017 by the Special Pathogens Unit, National Centre for Foreign Animal Disease, Canadian Food Inspection Agency (CFIA)…The participants were charged with assessing available veterinary medical countermeasures to control and respond to a Nipah virus disease outbreak. In addition, the workshop participants agreed to update the gap analysis conducted at the AAHL in Geelong, Australia, in 2009." Download the report from the STAR-IDAZ website or at the link above.

White-nose syndrome

How to vaccinate a wild bat

22/11/2018 Scientific American Article: "This probably won't come as a surprise, but vaccinating wild bats is a difficult task. It's also an important one: many bat populations are now endangered by white-nose syndrome, a serious fungal disease that invades the skin of bats… There's a vaccine against the fungus, but this requires painstaking capture and manual application of the medicine. It would be far better to administer vaccine to many bats at once, if it were possible to spray the vaccine onto the bats as they enter and exit their dwellings. The vaccine would then be consumed by the animals as they groom the sprayed material from their fur. Which is why the National Wildlife Health Center, a unit of the U.S. Geological Survey, recently partnered with PARC, a Xerox company, to undertake a wildlife protection project in Madison, Wisconsin. The goal is to explore the use of new spraying technologies to treat wild bats with topical vaccines…"

Groundbreaking science at TRU aims to save bats

29/11/2018 BC Local News Article: "…Fontaine is working with a team of researchers… to stop White-Nose Syndrome (WNS) from decimating the western North American bat populations… They're using the principal of probiotics — the application of good bacteria — in order to prevent the fungus that causes WNS… The probiotic was developed in Dr. Xu's lab based on bacteria that Cheeptham and her team discovered on the wings of healthy bats. Lab tests have shown the probiotic is effective at inhibiting the growth of the deadly fungus… Results from this first small-scale study are expected within the next two weeks, and another larger study is expected to begin next spring…"

Mammoth Cave scientists studying white-nose syndrome

27/11/2018 Glasgow Daily Times Article: "Scientists at Mammoth Cave National Park are taking part in two studies regarding white-nose syndrome, or WNS… The study involves testing bacteria found on bats in caves in New Mexico and Arizona to see if the bacteria has antagonistic effects against WNS… MCNP scientists have been afraid the bacteria that could be used to suppress the growth of WNS could harm cave crickets, cave beetles and other creatures found inside Mammoth Cave, so they have been working with researchers in New Mexico and New York to conduct preliminary tests in petri dishes to see what effect the bacteria might have on cave organisms… The other study MCNP is involved with is using ultra-violet light to see how it might affect cave organisms…"

What secrets are hidden inside the call of a bat?

20/11/2018 Connecticut Public Radio Article: "The fungal disease white-nose syndrome has killed off millions of bats across America… Now, scientists are trying to learn more about the impact of this devastating disease, by listening to the calls of the bats left behind…"

Winter 2018/2019 bat submission guidelines and updates from the 2017/2018 white-nose syndrome surveillance season [USA]

29/12/2018 USGS National Wildlife Health Center Article : "Updated guidance from the USGS National Wildlife Health Center (NWHC) is now available for bat submissions for the 2018/2019 white-nose syndrome (WNS) surveillance season… Included are reference charts and an updated WNS Management Area map to assist submitters in identifying priority species and collecting appropriate samples for submission to a diagnostic laboratory. These guidelines support surveillance objectives of the WNS National Plan designed to identify new geographic locations and bat species impacted by *Pseudogymnoascus destructans* (*Pd*) and WNS… Surveillance conducted last season documented an expansion in the distribution of Pd… and an increase in the number of North American bat species on which the fungus has been detected. Specifically, WNS was confirmed in two new states (Kansas and South Dakota) and two additional Canadian provinces (Manitoba and Newfoundland)… Additionally, Pd in absence of clinical signs of WNS was detected on bats from Mississippi, Texas, and Wyoming…" The updated guidelines are available from the USGS website.

For Australian information on WNS including how to identify and report a suspect case of WNS and sample submission guidelines for veterinarians, go to the Wildlife Health Australia website.

White-nose syndrome – other publications

- Morisak K (2018). Variation of *Pseudogymnoascus destructans* spore loads and risk of human vectored transport. MSc thesis, University of Akron Abstract

- Bansal S. (2018). A bibliometric study of research output on white-nose syndrome. *Indian Journal of Information Sources and Services*, *8*(2), 95-98 Article [PDF]

- Martínková N et al (2018). Modelling invasive pathogen load from non-destructive sampling data. *bioRxiv*, 474817 Abstract [Pre-print, not peer reviewed]

Other news

Heat stress – media

To report flying-fox heat stress events, fill out the flying-fox heat-stress data form from the Lab of Animal Ecology, Western Sydney University. For alerts, go to the Flying-fox Heat Stress Forecaster.

A selection of media:

- 30/11/2018 The Guardian Article **Queensland flying fox species decimated by record heatwave:** "Thousands of threatened flying foxes have dropped dead due to heat stress brought on by extreme temperatures in far north Queensland this week. Conservationists and wildlife volunteers estimate more than 4,000 have perished this week during the record heatwave, which has seen temperatures in Cairns reach all-time highs of 42.6C. The species of flying fox affected is the spectacled flying fox, an endemic Queensland species found in north Queensland. It's currently listed as vulnerable under national environment laws but conservationists have been pushing to have the species up-listed to endangered because of declines in the population. Volunteer carers that have been counting dead animals and taking orphaned young into care say it is the first time the species has suffered mass deaths because of extreme heat…."

- 28/11/2018 The Australian Article **Qld heatwave decimates bat population:** "Thousands of heat-stressed bats are dropping from trees and creating a health hazard in far north Queensland, as a record-breaking heatwave blasts the region. About 3500 flying foxes are estimated to have perished since the furnace-like conditions began on Sunday. However, Trish Wimberley of the Australian Bat Clinic says that's a conservative estimate, with thousands more likely to perish before the heatwave ends…"

- 27/11/2018 Sunshine Coast Daily Article **Heatwave contributes to rise in bat bites:** "Cairns and Hinterland Hospital and Health Service have advised people to be wary of bats, as the heatwave sends them falling from trees and into biting range… He said some bats may be infected with the potentially deadly Australian bat lyssavirus (ABLV)…"

'It's extremely cruel': backyard netting killing, maiming fruit bats

5/12/2018 Sydney Morning Herald Article: "…Habitats and food sources of the fruit bat, also known as the grey-headed flying fox, have been so heavily encroached by urban sprawl and development, the animals are driven to backyard fruit trees. But the wide-holed nets used by backyard gardeners to protect their fruit trees are killing and maiming the native animals. And babies are dying because their injured, or dead, mothers are not returning to feed them… Wildlife Victoria has already responded to more than 600 bat rescue call-outs this year, with 77 of them being entanglement cases…"

Flying foxes detect new invaders

Biosecurity Queensland Article: "An innovative surveillance project is underway to see if flying fox camps can provide an early warning system for potentially invasive plant species. Biosecurity Queensland, in partnership with the City of Gold Coast and the Queensland Herbarium, are surveying vegetation around flying fox camps to establish an 'early warning surveillance system' for serious new weed species, particularly Miconia and Mexican bean trees… Flying foxes eat the fruit of a variety of plant species and digest seeds through their waste under their overnight camps. If we find high-risk new weeds growing under the camp, we know that the species is growing in gardens or bushland nearby. We can focus our public awareness and on-ground surveillance activity to a specific radius and find it before it has a chance to develop into a major problem…"

MSU project to prevent bat-borne diseases wins $10 million

grant

3/12/2018 Montana State University Article: “In an effort to prevent some of the world's most lethal diseases, an international research team spanning five continents and led by Montana State University will study bats in Australia, Bangladesh, Madagascar and Ghana. Raina Plowright, assistant professor in the Department of Microbiology and Immunology in MSU's College of Agriculture and College of Letters and Science, is leading a project to unravel the complex causes of bat-borne viruses that have recently made the jump to humans, causing concern among global health officials. The research team — which includes more than 20 scientists from Johns Hopkins, Cornell, Cambridge, UCLA, Penn State, Rocky Mountain Laboratories in Montana, Griffith University in Australia and five other universities and institutions — is supported by a $10 million cooperative agreement with the Defense Advanced Research Projects Agency…”

'Pest-controlling' bats could help save rainforests

11/12/2018 ScienceDaily Article: “A new study shows that several species of bats are giving Madagascar's rice farmers a vital pest control service by feasting on plagues of insects. And this, a zoologist at the University of Cambridge believes, can ease the financial pressure on farmers to turn forest into fields…”

Cited journal article: Kemp J et al (2019). Bats as potential suppressors of multiple agricultural pests: A case study from Madagascar. *Agriculture, Ecosystems & Environment*, 269, 88-96 Abstract

Betting on bats for genetic treasures

29/10/2018 Knowable Magazine Article: “Most of us think of bats only when it’s time to decorate for Halloween. But a large group of scientists finds them fascinating all year round — so much so that they’ve launched an ambitious research program, known as the Bat1K Project, to sequence the genomes of every one of the world's 1,300-odd bat species. And the payoffs could be surprisingly high…”

National Wildlife Biosecurity Guidelines

26/11/2018 WHA Article: “Wildlife Health Australia this week released an unprecedented and valuable resource to help Australians who work with wildlife; the National Wildlife Biosecurity Guidelines… CEO of Wildlife Health Australia, Rupert Woods said: “These new Guidelines draw together the latest information and insights on how wildlife workers in all fields and working across Australia can adopt best-practices in applying biosecurity controls to every aspect of their work. If everyone working with wildlife; from vets to government agencies, students to carers, adopt practices that protect biosecurity, this will be critical to protecting wild animal populations and communities, and Australia’s animal industries from new and emerging diseases.” The guidelines can be downloaded from the Wildlife Health Australia website (or with this direct link to the PDF). WHA has also a one-page information sheet.

National Flying-Fox Forum – Presentations available

The 3rd Annual National Flying-Fox Forum was held in Cairns on 8th November 2018. “This Forum follows on from successful events held in 2017 and 2016, bringing together over 100 dedicated individuals from all levels of government, non-government organisations, universities, environmental consultancies and community groups to explore the issues of flying-fox management

and conservation…" Presentations from the forum are now available to download from the Ecosure website.

Publications

Polychromophilus melanipherus and haemoplasma infections not associated with clinical signs in southern bent-winged bats (*Miniopterus orianae bassanii*) and eastern bent-winged bats (*Miniopterus orianae oceanensis)*

Holz PH et al (2018). *Polychromophilus melanipherus* and haemoplasma infections not associated with clinical signs in southern bent-winged bats (*Miniopterus orianae bassanii*) and eastern bent-winged bats (*Miniopterus orianae oceanensis*). *International Journal for Parasitology: Parasites and Wildlife,* 8, 10-18 Article [Open access]

Abstract: "…The southern bent-winged bat (*Miniopterus orianae bassanii*) is a critically endangered subspecies endemic to south-eastern Australia. As part of a larger study… southern bent-winged bats from several locations in Victoria and South Australia were captured and examined for the presence of the blood parasite, *Polychromophilus melanipherus*, and haemoplasmas (*Mycoplasma* sp.)... Both organisms were found in both subspecies… with no association between the probability of infection, body weight, abnormal blood parameters or any other indicators of ill health. However, Victorian southern bent-winged bats had heavier burdens of *P. melanipherus* than both the South Australian southern bent-winged bats and eastern bent-winged bats. Further investigations are required to determine if these differences are impacting population health."

Alston virus, a novel paramyxovirus isolated from bats causes upper respiratory tract infection in experimentally challenged ferrets

Johnson RI et al (2018). Alston Virus, a novel paramyxovirus isolated from bats causes upper respiratory tract infection in experimentally challenged ferrets. *Viruses*, 10(12), 675 Article[Open access]

Abstract: "Multiple viruses with zoonotic potential have been isolated from bats globally. Here we describe the isolation and characterization of a novel paramyxovirus, Alston virus (AlsPV), isolated from urine collected from an Australian pteropid bat colony in Alstonville, New South Wales. Characterization of AlsPV by whole-genome sequencing and analyzing antigenic relatedness revealed it is a rubulavirus that is closely related to parainfluenza virus 5 (PIV5)…. Oronasal challenge of ferrets resulted in subclinical upper respiratory tract infection, viral shedding in respiratory secretions, and detection of viral antigen in the olfactory bulb of the brain…."

Slow growth and delayed maturation in a Critically Endangered insular flying fox (*Pteropus natalis*)

Todd CM et al (2018). Slow growth and delayed maturation in a Critically Endangered insular flying fox (*Pteropus natalis*). *Journal of Mammalogy*, 99(6), 1510-1521 Abstract

Abstract: "Flying foxes (family Pteropodidae) have distinct life histories given their size, characterized by longevity, low reproductive output, and long gestation. However, they tend to decouple the age at which sexual maturity is reached from the age at which they reach adult dimensions. We examined growth, maturation, and reproduction in the Critically Endangered Christmas Island flying fox (*Pteropus natalis*) to determine the timing of sex-specific life cycle events and patterns of growth… Growth and maturation are even slower in *P. natalis* than in the few other *Pteropus* species studied to date. The slow growth and delayed maturation of *P. natalis* imply slower potential population growth rates, further complicating the recovery of this Critically Endangered single-island endemic."

Active and passive surveillance for bat lyssaviruses in Italy revealed serological evidence for their circulation in three bat species

Leopardi S et al (2018). Active and passive surveillance for bat lyssaviruses in Italy revealed serological evidence for their circulation in three bat species. *Epidemiology & Infection,* doi: 10.1017/S0950268818003072 Abstract [Open access]

Abstract: "The wide geographical distribution and genetic diversity of bat-associated lyssaviruses (LYSVs) across Europe suggest that similar viruses may also be harboured in Italian insectivorous bats. Indeed, bats were first included within the passive national surveillance programme for rabies in wildlife in the 1980s, while active surveillance has been performed since 2008. The active surveillance strategies implemented allowed us to detect neutralizing antibodies directed towards *European bat 1 lyssavirus* in six out of the nine maternity colonies object of the study across the whole country. Seropositive bats were *Myotis myotis*, *M. blythii* and *Tadarida teniotis*..."

Lleida Bat Lyssavirus isolation in *Miniopterus schreibersii* in France

Picard-Meyer E et al (2018). Lleida Bat Lyssavirus isolation in *Miniopterus schreibersii* in France. *Zoonoses and Public Health*, doi: 10.1111/zph.12535 Abstract

Abstract: "Bat rabies cases are attributed in Europe to five different Lyssavirus species of 16 recognized Lyssavirus species causing rabies. One of the most genetically divergent Lyssavirus spp. has been detected in a dead *Miniopterus schreibersii* bat in France… The analysis of the complete genome sequence confirmed the presence of Lleida bat lyssavirus (LLEBV) in bats in France with 99.7% of nucleotide identity with the Spanish LLEBV strain (KY006983)."

Active sero-survey for European bat lyssavirus type-1 circulation in North African insectivorous bats

Serra-Cobo J et al (2018). Active sero-survey for European bat lyssavirus type-1 circulation in North African insectivorous bats. *Emerging Microbes & Infections,* 7, 213 Article [Open access]

Article: "…In Africa, to date, three lyssaviruses have been identified in bats… Little is known about the circulation and distribution of insectivorous bat lyssaviruses in North Africa, as well as the impact such viruses may have on public health… The aim of this study was to assess the potential circulation of European bat lyssaviruses in Northern Africa from 2007 to 2012…"

Rabies & bats – publications

- Seetahal JF et al (2019). Of bats and livestock: The epidemiology of rabies in Trinidad, West Indies. *Veterinary Microbiology,* 228, 93-100 Abstract

- Reed M et al (2018). Novel mass spectrometry based detection and identification of variants of rabies virus nucleoprotein in infected brain tissues. *PLoS Neglected Tropical Diseases,* 12(12): e0006984 Article [Open access, uncorrected proof]

Coronaviruses & bats – publications

- Zheng Y et al (2018). Lysosomal proteases are a determinant of coronavirus tropism. *Journal of Virology*, *92*(24), e01504-18 Abstract

- Cui J et al (2018). Origin and evolution of pathogenic coronaviruses. *Nature Reviews: Microbiology,* doi: 10.1038/s41579-018-0118-9 Abstract

Bacterial resistance in bats from the Phyllostomidae family and its relationship with unique health

Sens-Junior H et al (2018). Bacterial resistance in bats from the Phyllostomidae family and its relationship with unique health. *Pesquisa Veterinária Brasileira,* 38(6), 1207-16 Abstract (English)

Abstract: "….The present paper has the purpose to identify the oral and perianal microbiota and to detect the bacterial resistance of frugivorous bats captured near communities inhabited by humans in the northwestern region of the state of Paraná…. All bat species studied had resistant strains, with a few of them presenting multi-resistance to antimicrobials… This is an issue and a future warning for unique health, since high percentages of resistance were found against antimicrobials broadly used, such as ampicillin, amoxicillin and amoxicillin+clavulonate."

Coordinated change at the colony level in fruit bat fur microbiomes through time

Kolodny O et al (2018). Coordinated change at the colony level in fruit bat fur microbiomes through

time. *Nature Ecology & Evolution*, 3, 116-124 Abstract

Abstract: “The host-associated microbiome affects individual health and behaviour, and may be influenced by local environmental conditions… Here, we investigate longitudinal changes in the fur microbiome of captive and free-living Egyptian fruit bats. We find that, in contrast to patterns described in humans and other mammals, the prominent dynamics is of change over time at the level of the colony as a whole...”

Handling stress and sample storage are associated with weaker complement-mediated bactericidal ability in birds but not bats

Becker D et al (2019). Handling stress and sample storage are associated with weaker complement-mediated bactericidal ability in birds but not bats. *Physiological and Biochemical Zoology,* 92(1), 37-48 Abstract

Abstract: “Variation in immune defense influences infectious disease dynamics within and among species. Understanding how variation in immunity drives pathogen transmission among species is especially important for animals that are reservoir hosts for zoonotic pathogens. Bats, in particular, have a propensity to host serious viral zoonoses without developing clinical disease themselves. The immunological adaptations that allow bats to host viruses without disease may be related to their adaptations for flight... A number of analyses report greater richness of zoonotic pathogens in bats than in other taxa, such as birds (i.e., mostly volant vertebrates) and rodents (i.e., nonvolant small mammals), but immunological comparisons between bats and these other taxa are rare. To examine interspecific differences in bacterial killing ability (BKA), a functional measure of overall constitutive innate immunity, we use a phylogenetic meta-analysis to compare how BKA responds to the acute stress of capture and to storage time of frozen samples across the orders Aves and Chiroptera...”

Mass-culling of a threatened island flying fox species failed to increase fruit growers’ profits and revealed gaps to be addressed for effective conservation

Florens FBV & Baider C (2018). Mass-culling of a threatened island flying fox species failed to increase fruit growers’ profits and revealed gaps to be addressed for effective conservation. *Journal for Nature Conservation,* doi: 10.1016/j.jnc.2018.11.008 Abstract

Abstract: “Human-wildlife conflicts (HWC) pose a growing threat to biodiversity worldwide and solutions can be as sound as the understanding of the HWC itself… In this context, Mauritius implemented what may be the first mass-culls of an already threatened native species when it culled the flying fox (*Pteropus niger*)… We synthesized the best literature available locally and also elsewhere in relevant situations, to critically appraise the setting, nature, timeline of events and outcome of both completed mass-culling campaigns to explore why and how they happened so as to help towards devising better approaches to such conflicts...”

Related news: 5/12/2018 Mongabay News Article: **Culls push endangered fruit bat closer to extinction in Mauritius**

Publications – Other bat diseases

- Nelson C (2018). New bat genome and immunity. *Lab Animal,* 47(7), p.185 Abstract

- Lau SKP et al (2018). Replication of MERS and SARS coronaviruses in bat cells offers insights to their ancestral origins. *Emerging Microbes & Infections,* 7, 209 Article [Open access]

- Balkema-Buschmann A et al (2018). Productive propagation of Rift Valley fever phlebovirus vaccine strain MP-12 in *Rousettus aegyptiacus* fruit bats. *Viruses*, *10*(12), 681 Abstract [Open access]

- Jacquet S et al (2018). Evolution of hepatitis B virus receptor NTCP reveals differential pathogenicity and species-specificities of hepadnaviruses in primates, rodents and bats. *Journal of Virology,* doi: 10.1128/JVI.01738-18 Abstract

- Xu Z et al (2018). Isolation and identification of a highly divergent Kaeng Khoi virus from bat flies (*Eucampsipoda sundaica*) in China. *Vector-Borne and Zoonotic Diseases*, doi: 10.1089/vbz.2018.2350 Abstract

- Ahmed W et al (2018). Marker genes of fecal indicator bacteria and potential pathogens in animal feces in a subtropical catchment. *Science of The Total Environment*, doi: 10.1016/j.scitotenv.2018.11.439 Abstract

- Nowak K (2018). African fruit bats as potential reservoir for zoonotic pathogens - the example of *Escherichia coli*. PhD thesis, Friei Universität Berlin Thesis

- Muñoz-Leal S et al (2018). New records of ticks infesting bats in Brazil, with observations on the first nymphal stage of *Ornithodoros hasei*. *Experimental and Applied Acarology,* doi: 10.1007/s10493-018-0330-3 Abstract

- Rosskopf SP et al (2019). *Nycteria* and *Polychromophilus* parasite infections of bats in Central Gabon. *Infection, Genetics and Evolution*, 68, 30-34 Abstract

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Wildlife Health Australia acknowledges the traditional owners of country throughout Australia and their continuing connection to land, sea and community. We pay our respects to them and their cultures and to their Elders past, present and future.

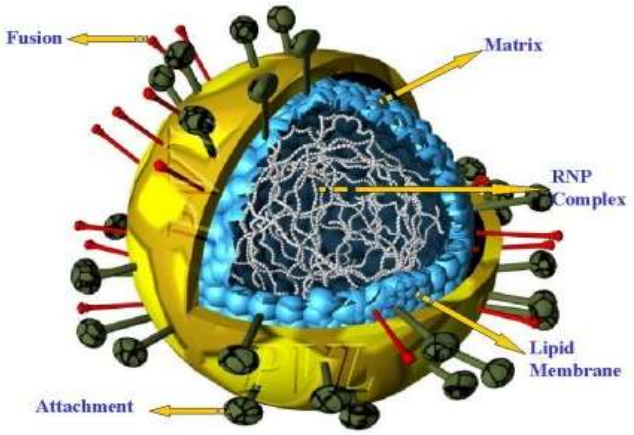
United States Department of Agriculture

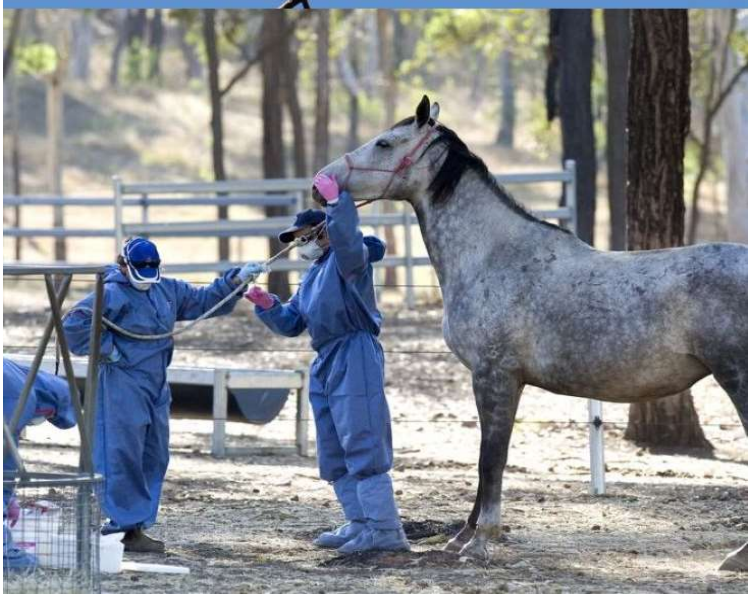
Agricultural Research Service

July 2018

Henipavirus Gap Analysis

Workshop Report





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Contents

EXECUTIVE SUMMARY

Henipavirus is the taxonomic genus for a group of viruses in the family Paramyxoviridae that includes *Hendra virus* (HeV) and *Nipah virus* (NiV). These viruses are zoonotic agents that are highly pathogenic in humans with case fatality rates of 40% to 70%. As such, these viruses are classified as Biosafety Level 4 (BSL-4) agents, requiring the highest level of laboratory biocontainment. Importantly, they have many of the physical attributes to serve as potential agents of bioterrorism, and are also considered emerging zoonotic pathogens with increasing geographical distribution in Australia, New Caledonia, Southeast Asia, and Madagascar.

Hendra virus first emerged in 1994 in Australia spilling over from bats to horses to humans, causing several disease outbreaks since with significant fatality rates. Nipah virus emerged in Malaysia in 1999, resulting in nearly 300 human cases with over 100 deaths.

The Nipah virus outbreak in Malaysia was especially concerning, causing widespread panic and fear because of the high mortality rate in people and the inability to control the disease initially. There were also considerable social disruptions and tremendous economic loss to an important pig-rearing industry. This highly virulent virus, believed to be introduced into pig farms by fruit bats, spread easily and silently among pigs and was transmitted to humans who came into close contact with infected animals. A NiV outbreak in Bangladesh in 2001 resulted from direct bat to human transmission via contaminated date palm juice with further spread within the human population. From 2001 to 2012, the World Health Organization (WHO) reported a total of 209 cases, with 161 deaths due to of NiV infections. In 2014, the WHO reported a NiV outbreak in fourteen districts of Bangladesh, resulting in 24 cases and 21 deaths. In 2015, three fruit bats tested positive for NiV in New Caledonia at the Noumea National Park, including three bats at the Noumea Zoo.

This gap analysis report focuses primarily on NiV and its potential impact on agricultural swine production. However, information is also provided on the threat henipaviruses pose to public health, both as emerging zoonotic agents and as potential agents of bioterrorism. Included in this report is scientific information on *Henipavirus* virology, epidemiology, pathogenesis, immunology, and an assessment of the available veterinary medical countermeasures to detect, prevent, and control disease outbreaks. Importantly, gaps are provided to inform research needs and priorities. Some of the major gaps and obstacles for disease control can be summarized as follows:

Diagnostics

The availability of safe laboratory diagnostic tests are limited. Virus isolation and serum neutralization assays require live NiV; thus, BSL-4 containment laboratories are required. Nucleic acid-based assays, such as RT-PCR are available, but genetic variation amongst henipaviruses are reported to impact sensitivity and real time RT-PCR may not be able to detect all divergent and novel henipavirus strains. Serological assays are limited in their ability to differentiate between known and unknown henipaviruses, as cross-reactivity to one or more known viruses is possible. Commercial diagnostic test kits are not available. International standards for NiV assay validation are needed. Gaps include a lack of positive experimental and field samples for test validation (or even evaluation) and there are restrictions on material transfer (e.g., obtaining animal samples that could be used to validate tests) due to biosecurity concerns. Low biosafety level reference sera

against various isolates are not yet available. There is a need for high throughput antibody assays for disease outbreaks, recovery and surveillance purposes. There is also a need to develop operator-safe diagnostics tests and reagents that can be produced in low biocontainment facilities.

Vaccines

There is currently a commercial vaccine available for horses, but there are no vaccines for swine or humans. There are several experimental vaccine candidates that may be safe and effective in swine and other domestic animals. However, all these vaccine candidates will require further research to establish their efficacy, and they will need to be fully developed to be licensed and stockpiled for rapid use in an emergency disease outbreak in swine.

Surveillance

Surveillance is the first line of defense against a disease outbreak. Rapid and accurate detection affects the time when control measures can be implemented and affects the extent of the disease outbreak. Because of limitations with laboratory diagnosis, surveillance programs are dependent on the reporting of clinical signs in populations at risk. Diagnosis of NiV infections based on clinical presentation has a low positive predictive value as there are numerous etiologies for encephalitis in humans, and clinical signs in pigs are difficult to differentiate from many common endemic infectious diseases.

Depopulation

Depopulation is the primary countermeasure to reduce virus shedding and stop the spread of NiV in livestock. Disease outbreaks have shown that the control of NiV in pig populations through stamping out is complex due to the zoonotic nature of the agent. In addition, depopulation may be logistically difficult and may be impossible in a rapidly spreading outbreak in countries where there are pig dense regions with millions of pigs, such as the states of Iowa, North Carolina, and Minnesota in the United States, or South East China.

GROUP PICTURE

Henipavirus Gap Analysis Working Group, Winnipeg, Canada
November 14-17, 2017

The Nipah Virus Countermeasures Working Group, Geelong, Australia
March 17-19, 2009

GLOSSARY

APHIS: Animal and Plant Health Inspection Service, USDA, United States of America

ARS: Agricultural Research Service

AAHL: Australian Animal Health Laboratory

BSL-4: Biosafety Level 4

CDC: U.S. Centers for Disease Control and Prevention, HHS, United States of America

CFIA: Canadian Food Inspection Agency

DIVA: Differentiating between infected and vaccinated animals

ELISA: Enzyme-linked immunosorbent assay

FADDL: U.S Foreign Animal Disease Laboratory, Plum Island Animal Disease Center

FLI: Friedrich Loeffler Institute

GMP: good manufacturing practice

HeV: Hendra virus

HHS: Department of Human Health Services, United States of America

HSPD-9: Homeland Security Presidential Directive Nine

ICAR: Indian Council of Agricultural Research

Ig: Immunoglobulin

IEDCR: Institute of Epidemiology, Disease Control and Research in Bangladesh

MLV: Modified live virus vaccine

NAHLN: National Animal Health Laboratory Network, USA

NIHSAD: National Institute of High Security Animal Diseases, ICAR, India

NCFAD: National Center for Foreign Animal Disease, CFIA, Canada

NiV: Nipah virus

NiV-B: Nipah virus Bangladesh

NiV-M: Nipah virus Malaysia

NiV N: Nipah virus nucleoprotein

NVCWG: Nipah Virus Countermeasures Working Group

NVS: National Veterinary Stockpile

OIE: World Organisation for Animal Health

PCR: Polymerase Chain Reaction.

PPE: Personal Protective Equipment

RT-PCR: Reverse transcription-polymerase chain reaction

rRT-PCR: Real-time reverse transcription-polymerase chain reaction

sHeV G: recombinant soluble Hendra virus G protein

sNiV G: recombinant soluble Nipah virus G protein

USDA: United States Department of Agriculture, United States of America

INTRODUCTION

Nipah virus (NiV) is an emerging zoonotic virus. First isolated in pigs and people from an outbreak in Malaysia in 1998 (Ang *et al.* 2018), this emerging virus causes severe disease in humans. The source of transmission was determined to be from bats to pigs to humans, through close contact with infected animals. The virus is named after the location where it was first detected in Sungai Nipah, a village in the Malaysian Peninsula where exposed pig farmers became severely ill with encephalitis.

Nipah virus is closely related to another zoonotic virus called Hendra virus (HeV), formerly called Equine *Morbillivirus*, and named after the town where it first appeared in Australia. Hendra virus infection was first recognized in 1994, when it caused an outbreak of acute, fatal respiratory disease that killed 14 horses. Three human cases, leading to two deaths were recorded during the outbreak. The precise mode of virus transmission to the three Australian patients is not fully understood. All three individuals appear to have acquired their infection as a result of close contact with horses, which were ill and later died.

Although members of this group of viruses have only caused a few focal outbreaks, their ability to infect a wide range of animal hosts and to produce a high mortality rate in humans has made this emerging zoonotic viral disease a significant public health threat.

Certain species of bats of the genus *Pteropus* (fruit bats, also called flying foxes) are the principal natural reservoir hosts for NiV and HeV – see Table I. Bats are susceptible to infection with these viruses but do not develop disease. Fruit bats are distributed across an area encompassing Australia, Southeast Asia; including Indonesia, Malaysia, the Philippines and some of the Pacific Islands, the Indian subcontinent, and Madagascar (See Fig. 1). There is also growing evidence that viruses related to NiV and HeV circulate in non-pteropid fruit bats across the globe (Clayton, 2017).

The exact mode of transmission of henipaviruses is uncertain, but appears to require close contact with contaminated tissue or body fluids from infected animals. The role of domestic species other than pigs in transmitting NiV infection to other animals has not yet been determined. In 2014, an outbreak was reported in the Philippines involving the consumption of meat from NiV-infected horses, further expanding the potential routes of transmission for henipaviruses.

Despite frequent contact between fruit bats and humans there is no serological evidence of human infection among persons that are in contact with bats. Pigs were the apparent source of infection among most human cases in the Malaysian outbreak of NiV in 1998-1999. Nipah virus has continued to spillover over from animals with at least six outbreaks resulting in human fatalities in Bangladesh in 2013, one in India in 2014, and two in Bangladesh in 2015. The World Health Organization (WHO) had not reported any NIV cases 2016-2017, but in 2018 fourteen new cases and 12 deaths were reported in Kerala, India - See Table II.

The spread of henipaviruses to new geographical areas is a concern. In 2014, the Philippines reported an outbreak with a zoonotic paramyxovirus in horses and people. There is further evidence for broader distribution of NiV in pteropid fruit bats species. There is also growing evidence that viruses related to NiV and HeV also circulate in non-pteropid fruit bats worldwide.

BACKGROUND

Organization of the Gap Analysis Working Groups on Nipah Virus (2009 and 2017)

The United States Department of Agriculture (USDA) organized the first Nipah virus gap analysis workshop in Australia in 2009 with the support of the Australian Animal Health Laboratory (AAHL). The working group was charged by the USDA National Veterinary Stockpile Steering Committee with making recommendations on specific materials, commercially available and in the pipeline, which will ensure the United States has an arsenal of highly efficacious countermeasures to control and mitigate the impact of an outbreak of Nipah virus. Nipah virus experts representing laboratories in South East Asia, Australia, Canada, and the United States were invited to participate and contributed to this report. The second workshop was organized in 2017 by the Special Pathogens Unit, National Centre for Foreign Animal Disease, Canadian Food Inspection Agency (CFIA), in collaboration with BSL4ZNet and DISCONTOOLS (http://www.discontools.eu/). The participants were charged with assessing available veterinary medical countermeasures to control and respond to a Nipah virus disease outbreak. In addition, the workshop participants agreed to update the gap analysis conducted at the AAHL in Geelong, Australia, in 2009.

Reference Material

The following reports and websites are recommended:

OIE – World Organisation for Animal Health - Nipah in Animals
http://www.oie.int/en/animal-health-in-the-world/animal-diseases/Nipah-Virus/
Accessed July 22, 2018

FAO – Food and Agriculture Organization
Manual on the diagnosis of Nipah virus infection in animals
www.fao.org/DOCREP/005/AC449E/AC449E00.htm
Accessed July 22, 2018

CDC – Center for Disease Control and Prevention - Special Pathogens Branch
https://www.cdc.gov/vhf/nipah/index.html
Accessed July 22, 2018

WHO - World Health Organization
http://www.who.int/news-room/fact-sheets/detail/nipah-virus
Accessed July 22, 2018

Guidelines for Veterinarians Handling potential Hendra Virus infection in Horses (QDPI)
https://www.daf.qld.gov.au/__data/assets/pdf_file/0005/126770/2913_-Guidelines-for-veterinarians-handling-potential-Hendra-virus-infection-in-horses-V5.1.pdf
Accessed July 22, 2018

CFSPH – Center for Food Security and Public Health
Nipah Virus Infection
http://www.cfsph.iastate.edu/Factsheets/pdfs/nipah.pdf
Accessed July22, 2018

DEFINITION OF THE THREAT

The threat for a natural introduction of henipaviruses in the United States is low, but there is significant concern that henipaviruses could be used for nefarious purposes to harm agriculture and people. Both Hendra virus and Nipah virus are on the HHS and USDA list of overlap Select Agents and Toxins. Henipaviruses are listed as APHIS Tier 3 high-consequence foreign animal diseases and pests. Henipaviruses are promiscuous in their ability to cause severe morbidity in several animal species, including people, and human infections result in a very high mortality rate. The mortality rate in pigs is actually reported as about 2.5% in adult pigs – high morbidity, but low mortality. Mortality rates in humans range from 40% (Malaysia) to 75% (up to 100%) in Bangladesh. The animal reservoir includes several species of bats, and henipaviruses may thus be readily available in these wildlife reservoirs.

Infection in people

Between September 1998 and June 1999, a NiV outbreak in Malaysia resulted in severe viral encephalitis in 105 patients (Goh *et al.,* 2000; Epstein *et al.,* 2006). Ninety-three percent had had direct contact with pigs, usually within two weeks prior to the onset of illness, suggesting that there was direct viral transmission from pigs to humans and a short incubation period. The main presenting features were fever, headache, dizziness, and vomiting. Fifty-two patients (55%) had a reduced level of consciousness and prominent brain-stem dysfunction. Distinctive clinical signs included segmental myoclonus, areflexia and hypotonia, hypertension, and tachycardia. The initial cerebrospinal fluid findings were abnormal in 75% of patients. Antibodies against Hendra virus were detected in serum or cerebrospinal fluid in 76 percent of 83 patients tested. Thirty patients (32%) died after rapid deterioration in their condition. An abnormal doll's-eye reflex and tachycardia were factors associated with a poor prognosis. Death was probably due to severe brain-stem involvement. Neurologic relapse occurred after initially mild disease in three patients. Fifty patients (53%) recovered fully, and 14 (15%) had persistent neurologic deficits.

Unlike Malaysia, the NiV outbreaks in Bangladesh were strictly confined to human populations with significantly higher mortality rate (Hossain *et al.,* 2008). NiV outbreaks in Bangladesh have continued annually since 2008 resulting in a total of 207 reported cases, 152 of which were fatal resulting in a 70% mortality rate (Clayton,. 2017).

Infection in pigs

The NiV outbreak in Maylasia in 1999 was facilitated by the rapid spread of the virus in pig populations. Although some pigs demonstrated a febrile respiratory illness with epistaxis, dyspnoea, and cough, few animals exhibit neurological signs, and the majority of pigs had subclinical infections. There are no clinical signs in pigs that are specific for NiV infection. Both, apparently healthy pigs and pigs showing clinical signs shed significant amount of virus.

Economic impact

The NiV outbreak in Malaysia in 1999 destroyed the main market for Malaysian hogs in Singapore. The Malaysia outbreak resulted in an 80% drop in pork consumption in the domestic market. Over half the standing pig population in the country was culled to halt the outbreak. Half the pig farms went out of business. The cumulative economic losses based on government figures was estimated to be approximately $217 million USD.

Bioterrorism

NiV has many of the physical attributes needed for a biological weapon, including easy access to virus resulting from its wide distribution in nature and laboratories, easy to produce, easy to disseminate, and the potential for high morbidity and mortality in people.

GAP ANALYSIS

The following section summarizes what we know about henipaviruses, gaps in our knowledge, and the threat of bioterrorism.

VIROLOGY

The following summarizes our current knowledge of viral strains, taxonomy, reservoir, genome, morphology, determinants of virulence, host range, and tissue tropism.

Virus species

Nipah virus (NiV) was first isolated in 1999 from samples collected during an outbreak of encephalitis and respiratory illness among pig farmers. The name Nipah originated from Sungai Nipah, a village in the Malaysian Peninsula where pig farmers became sick. There are currently two genotypes identified: NiV-Malasia and NiV-Bangladesh. Different strains/genotypes of NiV have emerged: Malaysia, Bangladesh, and Cambodia. NiV Malaysia resulted in the culling of a million pigs and 250 human cases (106 fatal). NiV Bangladesh is associated with outbreaks in people (Clayton, 2017).

Hendra virus (HeV) was first isolated in 1994 from specimens obtained during an outbreak of respiratory and neurologic disease in horses and humans in Hendra, a suburb of Brisbane, Australia.

Cedar virus (CedPV) is a novel *Henipavirus* isolated from Australian bats, which appears to be non-pathogenic in lab animal experiments (Marsh et al. 2012).

Taxonomy

NiV and HeV are members of the family Paramyxoviridae, order *Mononegavirales*. Comparison of nucleic acid and deduced amino acid sequences with other members of the family confirms that NiV and HeV are members of the family Paramyxoviridae, but with limited homology with members of the *Morbillivirus*, *Rubulavirus* and *Respirovirus* genera (See Fig. 2). The name *henipavirus* was recommended for the genus of both HeV and NiV (Wang *et al.,* 2000). HeV appear to be less diverse that NiV but molecular epidemiology studies are needed to identify new isolates that may bridge the gap between HeV and NiV.

Reservoir

The natural reservoir of the henipaviruses are fruit bats mainly from the genus *Pteropus* (flying foxes).

Genome

The complete genomes of both HeV and NiV have been sequenced (Wang *et al.,* 2001). Henipaviruses have a large non-segmented genome comprised of single-stranded negative-sense RNA. Their genomes are 18.2 kb in size and contain six genes corresponding to six structural proteins. All genes are of similar size to homologues in the respirovirus and morbillivirus genera, with the exception of P which is 100-200 amino acids longer (See Fig. 3). Most of the increase in genome length is due to longer untranslated regions between genes, mainly at the 3' end of each gene. The role of these long untranslated regions are not understood. Henipaviruses employ an unusual process called RNA editing to generate multiple proteins from a single gene. The process involves the

insertion of extra guanosine residues into the P gene mRNA prior to translation. The number of residues added determines whether the P, V or W proteins are synthesized. The C protein is made via an alternative translational initiation mechanism. The functions of the V, W, and C proteins are unknown, but they may be involved in disrupting host antiviral mechanisms (see Immunology below). The function of the G protein is to attach the virus to the surface of a host cell via the major receptor ephrin B2, a highly conserved protein present in many mammals. G glycoprotein is the major neutralizing antigen and the target protein for vaccine development. X-ray crystal structure for NiV G complex with ephrin-B3 has been determined. This interaction is highly conserved between NiV and HeV. This interaction is a prime candidate for developing henipavirus specific therapeutics. The F protein fuses the viral membrane with the host cell membrane, releasing the virion contents into the cell. It also causes infected cells to fuse with neighboring cells to form large multinucleated syncytia.

The genome size and organization of CedPV is very similar to that of HeV and NiV. The nucleocapsid protein displays antigenic cross-reactivity with henipaviruses and CedPV uses the same receptor molecule (ephrin- B2) for entry during infection. Clinical studies with CedPV in *Henipavirus* susceptible laboratory animals confirmed virus replication and production of neutralizing antibodies although clinical disease was not observed. In this context, it is interesting to note that the major genetic difference between CedPV and HeV or NiV lies within the coding strategy of the P gene, which is known to play an important role in evading the host innate immune system. Unlike NiV and HeV, and almost all known paramyxoviruses, the CedPV P gene lacks both RNA editing and also the coding capacity for the highly conserved V protein (Marsh *et al.* 2012).

Morphology

Henipaviruses are pleomorphic ranging in size from 40 to 600 nm in diameter. They possess a lipid membrane overlying a shell of viral matrix protein. At the core is a single helical strand of genomic RNA tightly bound to the nucleocapsid (N) protein and associated with the large (L) and phosphoprotein (P) proteins, which provide RNA polymerase activity during replication. Embedded within the lipid membrane are spikes of fusion (F) protein trimers and attachment (G) protein tetramers.

Determinants of virulence, host range, and tissue tropism

Molecular determinants of virulence, host range and cell tropism have been extensively studied and are well understood for many paramyxoviruses. Infectivity is determined by the cell-attachment and fusion glycoproteins and the presence of appropriate P gene products modulate virulence by antagonizing the cellular interferon response.

Henipaviruses have a large host range, unlike other members of the Paramyxoviridae, which generally have a very narrow host range. The cell attachment protein, unlike many other members for the paramyxovirus subfamily, does not have haemagglutinating activity and as a consequence does not bind sialic acid on the surface of cells.

The receptor for henipavirus is present on many different cultured cell types from many different species. The receptors for HeV and NiV are the same and have been identified as ephrin-B2 and ephrin-B3. Ephrin-B2 or -B3 are highly conserved across vertebrate species and are members of a family of receptor tyrosine kinase ligands. Ephrin-B2 is highly expressed on neurons, smooth muscle, arterial endothelial cells and capillaries, which closely parallels the known tissue tropism of

HeV and NiV *in vivo.* Ephrin-B3 is also widely expressed but particularly in specific regions of the central nervous system and may facilitate pathogenesis in certain neural subsets.

Virology Research Priorities

- Molecular epidemiology and determinants of strain variation
- Need sequencing of henipaviruses from bats, especially Bangladesh
- Determine molecular basis for virulence

PATHOGENESIS

The following summarizes our current knowledge of viral pathogenesis, including routes of infection, tissue tropism, pathogenesis, clinical signs, and clinical pathology."\

NiV infections in humans and pigs are linked to contact with bats. Clinical signs in human cases indicate primarily involvement of the central nervous system with 40% of the patients in the Malysian outbreak having also respiratory syndromes, while in pigs the respiratory system is considered the primary virus target, with only rare involvement of the central nervous system.

Humans

The main histopathological findings include a systemic vasculitis with extensive thrombosis and parenchymal necrosis, particularly in the central nervous system (Wong *et al.,* 2002). Endothelial cell damage, necrosis, and syncytial giant cell formation are seen in affected vessels. Characteristic viral inclusions are seen by light and electron microscopy. Immunohistochemistry (IHC) analysis shows the widespread presence of NiV antigens in endothelial and smooth muscle cells of blood vessels (Hooper *et al*., 2001). Abundant viral antigens are also seen in various parenchymal cells, particularly in neurons. The brain appears to be invaded via the hematogenous route and virus has been isolated from the cerebrospinal fluid of patients with NiV encephalitis (Wong *et al*., 2002). Infection of endothelial cells and neurons as well as vasculitis and thrombosis seem to be critical to the pathogenesis of this new human disease.

NiV infection can rarely cause a late-onset encephalitis up to a couple of years following a non-encephalitic or asymptomatic infection, or a relapsed encephalitis in patients who had previously recovered from acute encephalitis (Wong *et al*., 2001; Goh *et al*., 2000; Tan *et al*., 2002).

Pigs

Experimental challenge studies in piglets conducted at the National Centre for Foreign Animal Diseases, Winnipeg, Canada, demonstrated neurological signs in several inoculated pigs (Weingartl *et al.,* 2005; Berhane et al., 2008; Weingartl, H.M., personal communication of unpublished data). The rest of the pigs remained clinically healthy. NiV was detected in the respiratory system (turbinates, nasopharynx, trachea, bronchus, and lung), the lymphoreticular system (endothelial cells of blood and lymphatic vessels), submandibular and bronchiolar lymph nodes, tonsil, and spleen, with observed necrosis or lymphocyte depletion in lymphoid tissues, most importantly in lymph nodes (Hooper et al., 2001, Weingartl et al., 2006; Berhane et al., 2008). NiV presence was confirmed in the nervous system of both sick and apparently healthy animals (cranial nerves, trigeminal ganglion, brain, and cerebrospinal fluid). No virus was detected urine, although NiV antigen was found in kidneys of field swine cases (Tanimura et al., 2004). This study suggests NiV invaded the porcine host central nervous

system via cranial nerves after initial virus replication in the upper respiratory tract, and later in the infection also by crossing the blood-brain barrier as a result of viremia. Additional information on NiV and HeV pathogenesis in pigs are summarized in Middleton and Weingartl, 2012.

Dogs

Middleton *et al*., 2017, conducted experimental infections with HeV in dogs and determined that the virus is not highly pathogenic in dogs but their oral secretions pose a potential transmission risk to people. The time window for potential oral transmission corresponded to the period of acute infection.

Bats

Pteropus spp. fruit bats have been identified as the reservoir hosts for henipaviruses . Henipaviruses have been isolated to date in bats from Australia (HeV), Asia (NiV), and recently serological evidence of infection in bats in Madagascar (Hayman D.T.S., *et al.,* 2008). Related henipaviruses have been detected serologically and by PCR in non-*Pteropus*, but related pteropodid bats in Central and West Africa, and in insectivorous bats in China, expanding the host and geographical range beyond *Pteropus.*

There is no significant pathology in bats, and the frequency of viral shedding from wild bats is rare, with prevalence ranging from (1%-3%) with temporal variation of infection and viral shedding observed among different bat populations (Gurley *et al.,* 2017 and Wacharaplusadee *et al.* 2010, 2016). Henipavirus isolation from bat excreta is challenging, potentially due to low viral load.

Pathogenesis Research Priorities

- Identify determinants of virulence in pigs
- Develop experimental infection models in bats to study shedding
- Comparative genomic studies of contemporaneous NiV strains collected from bats and humans during outbreaks.
- Expand knowledge of spectrum of henipaviruses in bat hosts in NiV hotspots (e.g. western Bangladesh & West Bengal India)
- Determine whether the innate immune system in bats is responsible for limiting viral replication
- Determine how the net reproductive value of henipaviruses are sustained in bats
- Determine how transmission effected within bats, and between bats and other species

IMMUNOLOGY

The following summarizes our current knowledge of NIV immunology, including innate and adaptive immune responses to wild-type virus, immune evasion mechanisms, and protective immunity.

Innate and adaptive immune responses to wild-type NiV

Viral RNA can be detected by both cytoplasmic and endosomal pattern recognition receptors (PRRs), resulting in innate immune Type I IFN induction/ and signaling pathways:

- Retinoic Acid-inducible Gene I (RIG- I)- recognizes 5’ triphosphorylated RNA
- Melanoma Differentiation Antigen 5 (Mda-5)-recognizes cytosolic dsRNA
- RNA-dependent Protein Kinase (PKR)- recognizes cytosolic dsRNA

- Toll-like Receptor (TLR) 3- recognizes endosomal dsRNA
- TLR 7-8- recognizes endosomal ssRNA

Immune evasion mechanisms

The NiV uses unusual processes called RNA editing and internal translational initiation to generate multiple proteins from the phosphoprotein (P) gene, resulting in 4 proteins (P, C, V, and W) that function in inhibiting Type I interferon pathways:

- NiV P, V, and W have individually been shown to bind STAT1 and STAT2, effectively preventing STAT1 phosphorylation in type I IFN-stimulated cells.
- The V protein localizes to the cytoplasm, while the W protein localizes to the nucleus.
- The C protein can partially rescue replication of an IFN-sensitive virus, but the mechanism is unknown.
- Nuclear localization of W enables it to inhibit both dsRNA and TLR 3 (IRF-3 dependent) IFN-β induction pathways.
- A single point mutation in the V protein abrogates its ability to inhibit of IFN signaling.
- The V proteins of paramyxoviruses interact with the intracellular helicase Mda-5, and inhibits its IFN-β induction, but not with RIG-I.
- NiV V, W, and P bind polo-like kinase (PLK) via the STAT1 binding domain (Ludlow *et al.,* 2008).
- The P, V, and W proteins of NiV Malaysia and NiV Bangladesh inhibit IFN-stimulated response element (ISRE), which have a role in inducing transcription of IFN-stimulated genes (ISGs). Some of these ISGs include IRF-7, 2'5' Oligoadenylate Synthetase (OAS), RnaseL, p56, and double-stranded RNA-induced protein kinase (PKR). These ISGs all contribute to the generation of an 'antiviral state' in the cell.

Protective immunity

The G and F protein induce neutralizing antibodies that protect against challenge. Recent evidence from vaccination challenge studies indicates that both serum neutralizing antibody and T cell-mediated immunity are needed for protection from Nipah virus infection in pigs (Protection against henipaviruses in swine requires both, cell-mediated and humoral immune response, B.S. Pickering, J.M. Hardham, G. Smith, E.T. Weingartl, P.J. Dominowski, D.L. Foss, D. Mwangi, C.C. Broder, J.A. Roth, H.M. Weingartl, Vaccine 34(40): 4777-4786, 2016

Research needs

- Innate immunity and immunosuppression
  - Need studies in NiV infected cells and animal models
  - Need to study infection in various cell types, including cells of the immune system and bat cells
  - Use infectious clone to study virulence determinants
  - Identify targets for antiviral agents
  - Cytokine response to infection in human and bat cell lines
  - Need to study the potential for type 1 interferon or other cytokines to provide early protection from Nipah virus infection, transmission and/or clinical signs.

- Protective Immunity
  - Need to better define correlates of protection
  - Study T lymphocyte subset responses and cellular targets (e.g., N)

EPIDEMIOLOGY

Certain species of fruit bats of the genus *Pteropus* are the principal natural reservoir hosts for NiV and HeV (see Table I). Bats are susceptible to infection with these viruses but do not develop disease. Oher zoonotic viruses like Ebola, Marburg, and SARS virus, have also been identified in various Pteropus spp. fruit bats (Angeletti et al., 2016). Fruit bats are distributed across an area encompassing Australia, Southeast Asia; including Indonesia, Malaysia, the Philippines and some of the Pacific Islands, the Indian subcontinent, and Madagascar (See Fig. 1). There is further evidence for broader distribution of NiV in pteropid fruit bats species across their range (Wacharapluesadee S. and Hemachudha T., 2007). There is also growing evidence that viruses related to NiV and HeV also circulate in non-pteropid fruit bats worldwide.

Hendra Virus

Hendra virus infection was first recognized in 1994 in Australia, when it caused an outbreak of acute, fatal respiratory disease that killed 14 horses. Three human cases, leading to two deaths were recorded during the outbreak. In 1995, a horse was infected with associated human cases. The precise mode of virus transmission to the three Australian patients is not fully understood. All three individuals appear to have acquired their infection as a result of close contact with horses, which were ill and later died.

There have been several recognized outbreaks in Australia since 1994. Hendra virus reemerged in 1999, 2004, and 2006-2010. All known HeV cases have occurred in Queensland or northern New South Wales. From 1994 to 2010, HeV was confirmed on 11 premises in Queensland and one premise in northern New South Wales. In Australia, GlobalincidentMap.com reported: 21 cases in 2011; 12 cases in 2012; 10 cases in 2013; four cases in 2014; three cases in 2015; one case in 2016; and four cases in 2017. Al cases have involved horses as an intermediate host along with some additional human infections, resulting in several fatalities. The Australian Veterinary Association's national president, Dr. Ben Gardiner, was quoted as stating "no state or territory was immune from the virus."

The natural reservoirs for HeV are flying foxes found in Australia. Bats are susceptible to infection with these viruses but do not develop disease.

Hendra virus infection has also been detected in two dogs that were in close contact with infected horses. Both dogs remained clinically normal with no history of related illness.

Updated statistics on HeV outbreaks, including locations, dates and confirmed human and animal cases may be found on the Australian Veterinary Association website (Assessed July 22, 2018).

Nipah Virus

Nipah virus is a recently-recognized, zoonotic paramyxovirus that causes severe disease and high fatality rates in people. Outbreaks have occurred in Malaysia, Singapore, India and Bangladesh, and a putative Nipah virus was also recently associated with human disease in the Philippines (Clayton,

2017). The following summarizes our current knowledge of NiV epidemiology taking into account disease outbreaks in Malaysia and Bangladesh.

Malaysia

Nipah virus was first described in 1999 in Malaysia. The outbreak in Malaysia resulted in over a million pigs culled, 800 pig farms demolished, 36,000 jobs lost, $120+ million exports lost, and over 300 human cases (106 fatal, ~35% mortality) in pig farmers (Chinese) and Singapore abattoir workers (Field *et al.,* 2001). The NiV outbreak in pigs was described as highly infectious, frequently asymptomatic, low mortality rate (~5%), with respiratory and neurological syndromes. The pig farm pattern of disease included 30% morbidity and 5% mortality with sows first affected, followed by weaners, growers and finishers. The duration of clinical disease on a farm lasted ~ 2 weeks with a sero-prevalence approaching 100% in some cases. The outbreak in Malaysian pigs was associated with an increased incidence of human viral encephalitis cases, strongly associated with pig farm workers, with temporal and spatial links to disease in pigs.

During the outbreak, evidence of NiV infection was found in domestic animals such as goats and cats, but especially dogs (Field *et al.,* 2001). After pig populations were destroyed, but before residents were allowed to return to their homes, studies were undertaken in the epidemic area to determine whether domestic animal populations maintained active infection in the absence of infected pigs (Mills *et al.,* 2009). Dogs were especially suspected because they live commensally with both pigs and humans. However, serologic screening showed that in the absence of infected pigs, dogs were not a secondary reservoir for NiV.

Although human-to-human transmission of NiV during the 1998-1999 outbreak in Malaysia was not reported, a small number of infected people had no history of contact with pigs, suggesting human-to-human transmission occurred in these cases (Clayton, 2017).

The reservoir and natural host of NiV was determined to be fruit bats. Fruit bats have a wide geographic distribution, high antibody prevalence (17-30%), but no apparent clinical disease. A NiV neutralizing antibody study (Yob *et al.,* 2001) from 237 wild-caught bats surveyed on Peninsular Malaysia April 1–May 7, 1999, found four different species of fruit bats, and one species of insectivorous bats, tested positive for NiV (see Table I).

The routes of NiV excretion in bats include urine, saliva, and foetal tissues and fluids but the exact modes of transmission have yet to be determined.

The drivers of the emergence of NiV in Malaysia were determined to be large piggery (30,000+) adjacent to primary forest/fruit bat habitat and a network of other large farms close by. The stages of emergence associated with the outbreak included a spillover from flying foxes to domestic pigs near Ipoh (see Fig. 4), where farming practices and high pig densities facilitated the dissemination of the infection. Transportation of pigs for commerce led to the southern spread of the outbreak with the amplifying pig host facilitating the transmission of the virus to humans.

The epidemic enhancement of the outbreak included the initial introduction of infection in a naive pig population resulting in a rapid epidemic peak, followed by burn-out and localized human infections. Subsequent introduction(s) into partially immune pig populations resulted in a lower epidemic peak

but prolonged duration and increased total number of infectious pigs, increasing the chances of spread to surrounding farms.

Bangladesh

Bangladesh experienced its first reported NiV outbreak in Siliguri and Naogaon in 2001 (Fig. 5). Unlike Malaysia, outbreaks in Bangladesh appeared to be strictly confined to human populations and significantly higher mortality rate. From 2001 to 2018, the WHO reported a total of 261 cases, with 198 deaths (76% mortality) due to NiV infection (see Table II).

The transmission of NiV to humans in Bangladesh was determined to be associated with drinking date palm juice, considered a delicacy in this region of the world. In the Tangail outbreak of 2005, it was estimated that persons drinking raw date palm sap had a 7.0 odds ratio of developing a NiV infection when compared to controls (95% confidence level, 1.6).

NiV cases in Bangladesh have been seasonal, with human cases reported between the months of January and April. This coincides with the season for collecting date palm sap, late November through April. However, there is significant heterogeneity in the number of spillovers detected by district and year that remains unexplained. Cortes et al., in 2018 analyzed data from all 57 spillovers occurring during 2007–2013 and found that temperature differences explained 36% of the year-to-year variation in the total number of spillovers each winter, and that distance to surveillance hospitals explained 45% of spatial heterogeneity. January, when 40% of the spillover events occurred, was the month with the lowest mean temperature during every year of the study.

Bats are recognized as a nuisance and frequently drink the juice, defecate into juice, and occasionally drown in the palm sap collecting pot. Measures have been put in place to prevent bats access to the sap collecting pot, which has been very effective in reducing the spread of NiV from bats to humans in Bangladesh.

India

In 2001, an outbreak occurred within a hospital in Siliguri, West Bengal. Nosocomial transmission likely occurred, though it is unknown how primary cases were infected. Another outbreak in 2007 was reported in Nadia, West Bengal. Consumption of date palm sap was identified as the likely route of infection of primary cases there. In May of 2018, another outbreak was reported in Kerala. A total of 85 cases were reported in these three outbreaks in 2001, 2007, and 2018, with 62 deaths (73% mortality) due to NiV infection (see Table II).

In 2012, Yadav et al. surveyed the Indian states of Maharashtra and West Bengal to evaluate the presence of viral RNA and IgG against NiV in different bat populations belonging to the species Pteropus giganteus, Cynopterus sphinx and Megaderma lyra. The authors found NiV RNA in Pteropus bat thus suggesting it may be a reservoir for NiV in India.

Philippines

In 2014, the Philippines reported an outbreak with a zoonotic paramyxovirus in horses and people that is very closely related to NiV based on sequence analysis. Virus isolation was unsuccessful so it was impossible to confirm that there was transmission from presumably bats to horses, from horses to people, and also human to human (Ching P.K., *et al.*, 2015; Clayton, 2017).

New Caledonia

In 2015, three fruit bats tested positive for NiV in New Caledonia at the Noumea National Park, including three bats at the Noumea Zoo.

Research needs

- Improved understanding of infection dynamics in flying foxes: modes of transmission, immune response, evidence of disease, and the implications of co-infection with NiV and other henipaviruses
- Better understanding of co-circulation of different strains / species of henipaviruses within Pteropus populations and the effect of waning herd immunity on outbreaks.
- Other animals such as infected dogs and cats need to be further studied to determine their potential role in the transmission of NiH .
- Improved understanding of infection dynamics in humans: modes of transmission, implications of genetic diversity of the virus for infection, transmission & pathogenicity
- Research into bat populations: additional research regarding bat distributions & ecological impacts
- Research aimed at improving the capacity to diagnose henipavirus infections and improve human health outcomes
- Research into infection and clinical signs in pigs in Bangladesh and potential for pig to human and human to pig transmission.

BIOTERRORISM

The following summarizes the rationale for considering NiV as a potential agent of bioterrorism.

NiV is classified by CDC as a Category C pathogen – emerging pathogens that could be engineered for mass dissemination in the future. Category C include pathogens are readily available, easy to produce, easy to disseminate, and have the potential for high morbidity and mortality with major health impact.

NiV has many of the physical attributes to serve as a potential agent of bioterrorism. The outbreak in Malaysia caused widespread panic and fear because of its high mortality and the inability to control the disease initially. There were considerable social disruptions and tremendous economic loss to an important pig-rearing industry. This highly virulent virus, believed to be introduced into pig farms by fruit bats, spread easily among pigs and was transmitted to humans who came into close contact with infected animals. From pigs, the virus was also transmitted to other animals such as dogs, cats, and horses.

Nipah Virus Bioterrorism Threat Assessment

Virology

- Reverse genetic methods are available for negative strand RNA viruses, including Nipah, and all genomic sequence data is available.
- Many laboratories are actively engaged in research programs on the cell biological properties of the henipaviruses.
- Virus can be amplified to reasonably high unconcentrated titers (>10^7). Several cell culture lines can be used, Vero cell use most often reported, and wild-type virus can be grown and harvested from cell cultures.
- A major constraint in handling Nipah is the requirement for BSL4 facilities; , however, potential terrorists may not respect this need.
- Inactivation of virus can be achieved with a variety of agents typically used for envelope viruses; but extensive environmental stability testing not reported.
- Vaccines and passively-delivered countermeasures are under development both for human and veterinary use. A commercial Hendra virus vaccine is available for horses, and the soluble G protein based vaccine has shown experimental efficacy against Nipah virus in nonhuman primates.
- Bats are sold (often live) in markets throughout their range, providing a potential source of virus; and serological tests are available for identifying henipaviruses

Economic Impact

- Destroyed the main market for Malaysian hogs in Singapore
- ~80% drop in pork consumption in the domestic market.
- Over half the standing pig population in the country was culled to halt the outbreak.
- Half the pig farms went out of business.
- During the outbreak cumulative economic losses based on government figures >$217 million USD.
- Cumulative government costs in operations and lost revenues >$298 million USD.
- Other countries in South East Asia often have a higher pig density than Malaysia. China, with approximately half of the pigs in the world, is especially vulnerable to an economic and public health disaster if NiV were to emerge and be rapidly transmitted between pigs and from pigs to people.

Epidemiology and Clinical Disease

- In outbreaks to date henipaviruses do not appear to be highly infectious. Infection requires close contact with secretions of diseased animals. Many infections can be mild to asymptomatic.
- In the initial 1998-99 outbreak the virus was *initially misdiagnosed* as Japanese Encephalitis; amplification occurred from veterinary reuse of needles in immunization programs to control JE, increasing outbreak severity and extent.
- Time from exposure to signs of infection averages ~2 weeks for humans and seroconversion occurs within a month of onset (dose / route dependent).
- Transmission directly to the vascular system could occur through bites from infected animals or broken skin exposed to secretions of infected animals.

- It is quite likely that an outbreak in animals would result in transmissions to humans.
- An outbreak of Nipah pneumonia or ARDs-like disease with human-to-human transmission as demonstrated in the Bangladesh outbreak could cause significant mortality. Nipah could cause more severe or different disease presentations in older or sick populations.

Viral Transmission

- Deliberate release of virus in some manner is possible.
- Aerosol delivery might transmit the disease effectively to domestic animals, but the environmental requirements for maintaining virus stability are not well known.
- Transmission to humans through consumption of contaminated food has been documented.
- The veterinary reuse of needles in the Japanese Encephalitis immunization campaign and in artificial insemination may have been a factor in the near 100% infection level of Nipah in pigs observed on affected farms.
- Deliberate contamination of veterinary needles could initiate an outbreak in susceptible domestic animals.
- Human-to-human transmission through travel has not been documented, but is possible.
- Transport of infected pigs on trucks was a transmission route in the Malaysian outbreak. Generalizing-- transportation of infected humans on crowded airplanes, buses or trains could also transmit the disease. Human cases have been transported to highly populous cities (e.g. Dhaka) where risk of exposure and spread among the public is increased.

Summary

- Nipah (henipaviruses) can be isolated from animal hosts.
- Several species of fruit bats, including *Pteropus spp.* widely distributed throughout Southeast Asia. The live animals are sold in food markets.
- A Nipah outbreak in swine producing areas can cause an economic crisis, even if human cases do not occur.
- Nipah virus can be amplified in permissive cell cultures (e.g., Vero cells) providing adequate laboratory facilities are available (Biosafety Level 4), although a bioterrorist group would not be restricted from growing the virus because of the lack of BSL-4 facilities.
- Effective aerosol delivery is likely possible but unpredictable on the basis of publicly available information. General unknowns are-- titers necessary for infection, virion stability in vitro, and how infectious the virus would be with this delivery.
- Effective surveillance programs, particularly in pig farming areas, are the best defense for early detection and containment of infection, whatever the source.

SUMMARY OF OBSTACLES TO PREVENTION AND CONTROL

The 2017 gap analysis working group determined that the following countermeasures were important but several weaknesses were identified.

DIAGNOSIS

NiV and HeV are zoonotic paramyxoviruses capable of causing severe disease in humans and animals. These viruses require biosafety level 4 (BSL-4) containment. The availability of safe laboratory diagnostic tests is limited. Sequence variation effects molecular diagnostics; both Clifton Beach (2007) and Redlands (2008) reported that Hendra virus strains failed in AAHL Hendra virus specific real-time PCR. Most published diagnostic PCRs only detect HeV or NiV, but not both. There is a need for a more general PCR to detect divergent and novel strains. Pan-paramyxovirus PCR assays exist and are in use to detect henipaviruses, but limitations in sensitivity limit diagnostic value. The USAID PREDICT program previously used its pan-paramyxovirus PCR assay for surveillance in more than 20 countries in Africa and Asia. Virus isolation and serum neutralization assays require live NiV. There is a need for diagnostics that can be used safely in the laboratory. There is a need for rapid nucleic acid-based assays that can detect all henipaviruses. There is also a critical need for improved antibody-based assays for disease outbreaks and disease surveillance. Importantly, there is a need to develop operator-safe diagnostic tests for which reagents can be produced without requiring high containment facilities.

Currently there is no expectations that validated tests will become available for livestock (or other species) in the near future. Nothing has been done in terms of test harmonization since 2009; however, a number of technology transfers have occurred: from AAHL to laboratories in Asia (Malaysia mainly); limited transfer from NCFAD to India (Bhopal High Containment Animal Health Laboratory); limited transfer from AAHL to the FLI and bilateral transfers between NCFAD and FLI.

VACCINATION

There is currently a commercially available vaccine for horses but no vaccines for swine or human vaccines. The goal for a HeV vaccine for horses is to vaccinate horses in areas at risk for transmission from bats to horses in order to prevent bat to horse transmission and subsequent horse to human transmission. The goal for a NiV vaccine for swine is to have a large stockpile of vaccine available for rapid use in an outbreak situation to prevent swine to swine, swine to human, and perhaps human to swine transmission to control the outbreak. A large stockpile of NiV vaccine, or vaccine antigen concentrate, for rapid emergency use in swine to control a potential outbreak that spreads too quickly to be stamped out in swine dense areas is needed. The vaccine should be licensed in the U.S., E.U or Australia for stockpiling as well as in the countries where NiV is endemic in bats. The stockpile should be available for use internationally where ever it may be needed.

SURVEILLANCE

Passive surveillance is the primary and most economical method used. Passive surveillance in commercial swine herds based on clinical signs has many weaknesses due to the difficulty of differentiating NiV from many common endemic infectious diseases of pigs; e.g., classical swine

fever, porcine reproductive and respiratory syndrome, pseudorabies, swine enzootic pneumoniae, and porcine pleuropneumonia.

In the case of infections in swine where recognition of Nipah symptoms is less likely, surveillance activities must be based on diagnostic testing to supplement surveillance based on clinical signs.

Active surveillance programs are expensive and would have to rely on direct diagnostic tests such as viral isolation and nucleic acid-based assays but available tests have significant weaknesses and have not been validated.

Rapid confirmation of cases is essential. Knowledge on serological cross-reactions with other henipaviruses and/or morbilliviruses in bats is improving. There is an urgent need to establish diagnostic capacity for Nipah virus in countries that are most likely to experience spillovers from the bat reservoirs.

DEPOPULATION

Depopulation is the primary countermeasure to reduce virus shedding and stop the spread of Nipah virus in swine. Recent outbreaks have shown that the control of Nipah virus in pig populations through stamping out is complex due to the zoonotic nature of the agent and may be very expensive, particularly in areas with high pig densities. Because Nipah virus spreads rapidly and silently in pigs, a large number of animals would need to be pre-emptively culled if an outbreak occurred in the U.S, or in other swine dense countries in order to minimize the virus spread in the vicinity of infected herds. Thus, this method of control would have significant financial implications due to the culling of thousands or millions of animals.

COUNTERMEASURES ASSESSMENT

ASSUMPTIONS

The following captures assumptions made by the gap analysis working group to assess potential countermeasures to enhance our ability to contain and eradicate an outbreak of NiV.

Situation
Countermeasures assessed for worst case scenario: A coordinated intentional distribution of NiV-contaminated material in a high density highly populated pig region of the United States.

Target Population
Countermeasures assessed for target pig production segments in priority order:

1. Backyard pigs
2. Comprehensive commercial swine operations (farrowing, nursery, and finishing)
3. Commercial indoor farrowing operations
4. Large intensive indoor pig farms
5. Valuable commercial genetic swine stock

Scope of Outbreak
Countermeasures assessed for multiple outbreaks occurring simultaneously in backyard pigs, three farrowing commercial operations, a finishing pig commercial operation, a sow replacement operation, and evidence of infection in feral swine.

DECISION MODEL

The quantitative Kemper-Trego (KT) decision model was used to assess available vaccines and diagnostics. For the criteria and weights used to assess NiV vaccines and diagnostics (See Appendices II, III).

Criteria
The following critical criteria were selected to enable the comparison of countermeasures using a pertinent and valid analysis, as follows:

<u>Vaccines</u>

- Efficacy
- Safety
- One dose
- Manufacturing safety
- DIVA compatible
- Manufacturing yield
- Rapid production
- Reasonable cost
- Short withdrawal period

- Long shelf life

Diagnostics
- Sensitivity
- Specificity
- DIVA detection
- Multispecies
- Validation to purpose
- Speed of scale-up
- Throughput
- Pen-side test
- Rapid result
- No need for a confirmatory test
- Easy to perform
- Safe to operate
- Availability
- Storage/Distribution
- Low cost to implement
- Perform at BSL-2
- Does not require use of live virus to prepare reagents

Weight

Each criterion was weighted to allow a quantitative comparison of the impact of the selected interventions (See Appendices II and III).

Product profile

To ensure a consistent and meaningful assessment, the desired product profile (i.e., the benchmark) was identified for each countermeasure:

Desired Vaccine Profile

1. Highly efficacious: prevent transmission; efficacy in all age animal target hosts, including maternal antibody override; cross protection across all henipavirus strains; quick onset of immunity; multiple animal target hosts; one year duration of immunity
2. Safe in all age animal target hosts; no reversion to virulence for live vaccines
3. One dose
4. Safe vaccine strain for manufacturing
4. DIVA compatible
5. Manufacturing method yields high number of doses
6. Rapid speed of production and scale-up
7. Reasonable cost
8. Short withdrawal period for food consumption
9. Long shelf life

Desired Diagnostic Test Profile

1. Detect all henipavirus
2. Identify Nipah virus specific strains
3. Direct tests for control and eradication
4. Indirect tests for post-control monitoring
5. Rapid test
6. >95% specificity
7. >95% sensitivity
8. Pen-side test
9. DIVA Compatible
10. Field validated
11. Easy to perform/easily train NAHML's personnel
12. Scalable
13. Reasonable cost
14. Operator safe
15. Reagents can be produced in low containment

Values

The values assigned for each of the interventions reflect the collective best judgment of members of the gap analysis working groups (See Appendices I and II)

VACCINES

The human infections in the 1999 outbreak in Malaysia were linked to transmission of NiV from pigs. Accordingly, a swine vaccine able to prevent virus transmission would be an important tool to safeguard commercial swine operations and people at risk. In addition, since henipaviruses have a very broad host range, a vaccine that is efficacious in multiple susceptible animal species would be desirable. Although the 2017 gap analysis working group determined that there are still no NiV commercial vaccines available, there are several vaccine candidates that may be safe and effective in swine and other domestic animals that were recently reviewed in: (Weingartl H.M., 2015; Broder, C.C., *et al, 2016*; and Satterfield, B.A., *et al.,* 2016). After these reviews were published, a manuscript was published demonstrating the efficacy of a virus-like-particle (VLP) Nipah virus vaccine in hamsters for inducing virus neutralizing antibodies and protection from challenge (Walpita P., *et al.,* 2017). Another manuscript was published that concluded that an adjuvanted Hendra soluble G vaccine in pigs induced neutralizing antibody titers considered to be protective against Nipah virus without detectable T cell-mediated immunity to Nipah, which did not protect from challenge with Nipah virus. However, pigs that had been previously challenged with a low dose of NiV developed neutralizing antibodies and cell-mediated immune memory and were protected from a high challenge dose of NiV. The conclusion of this manuscript was that both virus neutralizing antibodies and cell-mediated immunity were necessary for protection from NiV challenge (Pickering B.S., *et al.,* 2016). Subsequent unpublished research demonstrated that a different adjuvant used with the soluble Hendra G vaccine caused the induction of both high tittered virus neutralizing antibody and detectable T cell-mediated immunity in pigs to NiV. Challenge studies were not conducted (J.A. Roth, personal communication). All of these vaccine candidates would need further research and development to be licensed, and would need to be made available as a stockpile for rapid use in an emergency if an outbreak in swine were to occur that could not be effectively stamped out. A swine vaccine would

also be needed if the Nipah virus were to mutate to be efficiently transmitted between people and between people and pigs.

Summary

- Vaccination against NiV has been successfully demonstrated
- Experimental henipavirus vaccines can prevent clinical disease
- Experimental henipavirus vaccines elicit systemic and mucosal immunity
- Experimental henipavirus vaccines prevent viral replication in target tissues
- HeV commercial vaccine Equivac® HeV does not cross protect against NiV infection in swine
- Henipavirus vaccines appear to be effective in several mammalian animal species

Assessment of Commercial Vaccines

A commercial vaccine (Equivac® HeV) against Hendra virus approved for use in horses (Middleton D.J. *et al.,* 2014) was registered by Zoetis in Australia in 2015. A six month booster dose is required for full protection, followed by annual vaccination. The vaccine is also approved for pregnant mares. There is currently no Nipah virus vaccine approved for swine. Likewise, there is no vaccine against Hendra virus (or Nipah virus) approved for human use.

Assessment of Experimental Vaccines

The working group felt that limited information was available to assess and contrast experimental vaccines that have been reported in the literature. Experimental animal vaccines under investigation are summarized in Table I. Experimental vaccines for humans are summarized in Table II. Several of the working group members have directly or indirectly been involved in the research associated with these vaccines so that an assessment could be made (See Appendix I). The following describes some of the most promising experimental vaccine technologies.

1) Canarypox-vectored NiV Vaccines

The ALVAC canarypox virus-based recombinant vaccine vector (Taylor *et al.,* 1994) was used to construct two experimental NiV vaccines (Weingartl *et al.,* 2006). These experimental vaccines were engineered by Merial.

The first construct carries the gene for NiV attachment glycoprotein G (ALVAC-G). The second construct carries the NiV fusion protein F (ALVAC-F).

The efficacy of both the ALVAC-G and ALVAC-F were tested in pigs either as monovalent vaccine or in combination (ALVAC-G/F). The vaccine dose used was 10(8) PFU. The vaccine regimen was two doses administered 14 days apart. Both non-vaccinated controls and vaccinated pigs were challenged with 2.5 x 10(5) PFU of NiV two weeks later.

The combined ALVAC-F/G vaccine induced the highest levels of neutralization antibodies. Despite the low neutralizing antibody levels induced by ALVAC-F all vaccinated animals were protected against challenge. Virus was not isolated from the tissues of any of the vaccinated pigs post-challenge, and a real-time reverse transcription (RT)-PCR assay detected only small amounts of viral

RNA in several samples. In challenge control pigs, virus was isolated from a number of tissues or detected by real-time RT-PCR. Vaccination of pigs with the ALVAC-F/G stimulated both type 1 and type 2 cytokine responses. No virus shedding was detected in vaccinated animals, in contrast to challenge control pigs, from which virus was isolated from the throat and nose.

Based on the data generated in this one study, both the ALVAC-G or the combined ALVAC-F/G vaccine appears to be a very promising vaccine candidate for swine.

2) Soluble G Henipavirus Vaccine

HeV and NiV infect cells by a pH-independent membrane fusion event mediated by their attachment (G) and fusion (F) glycoproteins. Scientists at the Uniformed Services University of the Health Sciences in Bethesda, Maryland, in collaboration with the Australian Animal Health Laboratory characterized HeV- and NiV-mediated fusion activities and detailed their host-cell tropism characteristics. These studies suggested that a common cell surface receptor was utilized by both viruses. To further characterize the G glycoprotein and its unknown receptor, soluble forms of HeV G (sG) were constructed by replacing its cytoplasmic tail and transmembrane domains with an immunoglobulin kappa leader sequence coupled with an S-peptide tag (sG) to facilitate purification and detection. Expression of sG was verified in cell lysates and culture supernatants by specific affinity precipitation. Analysis of sG by size exclusion chromatography and sucrose gradient centrifugation demonstrated tetrameric, dimeric, and monomeric species, with the majority of the sG released as a disulfide-linked dimer. Immunofluorescence staining revealed that sG specifically bound to HeV and NiV infection-permissive cells. The scientists further reported that administration of sG to rabbits can elicit a potent cross-reactive neutralizing antibody response against infectious HeV and NiV (Bossart *et al.* 2005).

Experimental subunit vaccine formulation containing either HeV sG or NiV sG were evaluated as potential NiV vaccines in the cat model. Two cats were immunized with HeV sG and two cats were immunized with NiV sG. Immunized animals and two additional naïve controls were then challenged subcutaneously with 500 TCID50 of NiV. Naive animals developed clinical disease 6 to 13 days post-infection, whereas none of the immunized animals showed any sign of disease (Mungall *et al.,* 2006).

In a subsequent experiment, an experimental subunit formulation containing HeV sG and CpG adjuvant was evaluated as a potential NiV vaccine in the cat model. Vaccinated animals demonstrated varying levels of NiV-specific Ig systemically and importantly, all vaccinated cats possessed antigen-specific IgA on the mucosa. Upon oronasal challenge with NiV (50,000 TCID50), all vaccinated animals were protected from disease although virus was detected on day 21 post-challenge in one animal. (McEachern *et al.,* 2008).

A recent publication demonstrated that an adjuvanted Hendra soluble G vaccine in pigs induced SN antibody titers considered to be protective against Nipah virus without detectable T cell-mediated immunity to Nipah which did not protect from challenge with Nipah virus. Pigs which had been previously challenged with a low dose of Nipah developed SN antibodies and cell-mediated immune memory and were protected from a high challenge dose of Nipah virus. The conclusion of this manuscript was that both SN antibodies and cell-mediated immunity were necessary for protection from Nipah virus challenge (Protection against henipaviruses in swine requires both, cell-mediated and

humoral immune response, B.S. Pickering, J.M. Hardham, G. Smith, E.T. Weingartl, P.J. Dominowski, D.L. Foss, D. Mwangi, C.C. Broder, J.A. Roth, H.M. Weingartl, Vaccine 34(40): 4777-4786, 2016). Subsequent unpublished research demonstrated that a different adjuvant used with the soluble Hendra G vaccine caused the induction of both high tittered SN antibody and detectable T cell-mediated immunity in pigs to Nipah virus. Challenge studies were not conducted (J.A. Roth, personal communication).

3) Vaccinia-vectored NiV Vaccine

The NYVAC vaccinia virus-based recombinant vaccine vector (Tartaglia *et al.,* 1992) was used to construct an experimental NiV vaccine where the vaccinia virus expresses both the NiV glycoproteins G and F (Guillaume *et al.,* 2004). This experimental vaccine was engineered by the Pasteur Institute.

Scientists at the Pasteur Institute in collaboration with University of Malaysia scientists showed that both of the NiV glycoproteins G and F when expressed as vaccinia virus recombinants induced an immune response in hamsters that protected against a lethal challenge with NiV. Furthermore, this team of scientists demonstrated passive transfer of antibody induced by either of the glycoproteins protected the animals.

DIAGNOSTICS

The gap analysis working group determined that the availability of validated diagnostic tests for surveillance, early detection, and recovery during a NiV outbreak were critical to minimize the spread of disease and reduce the economic and public impact.

Currently the diagnosis of NiV infection is by virus isolation, detection of viral RNA, or demonstration of viral antigen in tissue collected at necropsy. Specific antibody detection can also be useful, particularly in pigs where NiV infection may go unnoticed. Demonstration of specific antibody to NiV in either animals or humans is of diagnostic significance because of the rarity of infection and the serious zoonotic implication of NiV transmission.

Summary

- Antibody response to NiV take at least 14 days and therefore early diagnosis based on serology will be less reliable than antigen or molecular tests
- Recombinant N-ELISA will likely not pick up all infected pigs
- The concept of a pen-side test is attractive, but the development and regulation of such a test will be extremely challenging

Assessment of Laboratory Diagnostic Tests (See Appendix II)

Details in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2015, Chapter 2.1.14 Hendra and Nipah Virus Diseases, provides recommendations for the following tests.

Identification of the agent

1. Virus isolation and characterization
    1.1. sampling and submission of specimens
    1.2. isolation in cultured cells
    1.3. Identification: immunostaining and Immuno EM

2. Viral identification: differentiation of HeV and NiV
    2.1 comparative immunostaining
    2.2. immunofluorescence
    2.3. microtiter neutralization

3. Molecular methods
    3.1. real-time RT-PCR
    3.2. Conventional RT-PCR and Sanger sequencing

4. Immunohistochemistry

Serological tests
1. Virus neutralization tests
2. Enzyme-linked immunosorbent assay
3. Bead-based assays

Histopathology
1. Veterinary diagnostic labs might use histopathology to make the first diagnosis
2. NiV does not produce pathognomonic lesions, but a generalized vasculitis with fibrinoid necrosis in several tissues (e.g. lung and kidneys) is characteristic; NiV might be considered in the initial differential diagnosis by experienced veterinary pathologists.

Assessment of Available Diagnostic Tests

Australia, Canada, and Germany have diagnostic capability for henipaviruses in livestock; India (e.g. NIHSAD) is building its veterinary diagnostic capability; U.S. veterinary diagnostic laboratories do not have diagnostic capability to detect NiV in livestock, although the Center for Disease Control (CDC) in Atlanta, Georgia, is an OIE collaborating center for NiV.

Currently, there are no expectations of validated tests for livestock (or other species). Nothing has been done in terms of test harmonization for serological, antigen, or nucleic acid detection assays; however, successful technology transfers have taken place, as follows: from AAHL to laboratories in Asia (Malaysia mainly); limited transfer from NCFAD to India (Bhopal High Containment Animal Health Laboratory); limited transfer from AAHL to FLI and bilateral transfers between NCFAD and FLI.

Serologic testing plays an important role in the diagnosis and detection of NiV infections. Serologic tests are the most straightforward and practical means to confirm acute cases of disease and serologic evidence of infection is used in screening programs for reservoir hosts and domestic animals. However, serological assays are limited in their ability to differentiate between known and unknown henipaviruses, as cross-reactivity to one or more known viruses is possible. Both serum neutralization and Luminex assays have shown positive reactivity to both NiV and HeV in bats where the presence of a yet-to-be characterized henipavirus could not be ruled out.

Several standard and new experimental technologies that are currently being used or considered for the detection of NiV in the laboratory or as pen-side tests for field use. Shedding of NiV in oral fluids starts early post-infection and rope sampling could prove convenient for collecting samples that could be used to test larger numbers (i.e., pen tests) of pigs. Suitability of oral fluid samples for various test

platforms should be investigated. There is a need to develop a formalized worldwide structure for test validation and ring trials (i.e., inter-laboratory comparisons).

The following describes some of the most promising diagnostic platforms with potential application for NiV detection.

1) Quantitative (q) real-time PCR

Real-time PCR is a sensitive and useful approach to the detection of henipavirus genome in specimens. Due to its nature, rRT-PCR may not be able to detect all divergent and novel henapivirus strains, although adaptation of molecular tests to new virus variants could be rapid. Test methods and primers used depend on the technology platform and associated chemistry being used in individual laboratories. Test procedures have been described by different laboratories (Mungall *et al.,* 2006; Wacharapluesadee and Hemachudha, 2007; Guillaume *et al*., 2004; Chang *et al*., 2006; Feldman *et al*., 2009).

The AAHL has developed a quantitative real-time PCR to detect NiV or HeV RNA synthesis. The most commonly targeted amplification regions are directed against the N gene (Feldman et al., 2009).

RT-PCR targeting the N gene of NiV will detect both, NiV-M and NiV-B, with somewhat lower sensitivity for NiV-B. Confirmatory RT-PCR targeting the F gene specific only for NiV-B has therefore been developed (publication in preparation; H.M. Weingartl, personal communication).

2) Conventional PCR

Classical RT-PCR followed by sequencing may be more successful in detecting novel henipavirus strains. Combination of both approaches may need to be considered. Genomic RNA detection can be performed on blood or serum samples collected from live animals as well as tissues from dead animals. RNA is extracted using an RNA extraction kit [e.g., RNeasy mini kit (Qiagen)]. Extracted total cellular RNA is first subjected to first-stand cDNA synthesis using a reverse transcriptase kit [e.g., SensiScript (Qiagen)] and a reverse transcriptase primer. The resulting cDNA is amplified using a Master Mix PCR kit (Qiagen) and primers that are designed to target HeV and NiV positive-sense mRNA from the N, M and G genes and negative-sense genomic viral RNA (vRNA) at the N/P, M/F and F/G gene junctions.

3) Field PCR

Not available. Isothermal real-time RT-PCR is promising as a field deployable assay.

While this will be costly and not be practical to have in large numbers, it is worth considering having the capabilities to establish in several strategically located regions across the nation to response rapidly in an emergency situation. Technically it will not be difficult to achieve if there is the will and financial support.

4) *Virus isolation (VI)*

Virus isolation in permissive cell culture is considered the "gold standard" for isolating all strains of henipaviruses. Virus isolation greatly facilitates identification procedures and definitive diagnosis should be undertaken where operator safety can be guaranteed. Isolation is especially relevant in any new case or outbreak, particularly in countries or geographical areas where infection by NiV or HeV has not been previously documented. Implication of wildlife species as natural hosts of the viruses requires positive serology, PCR or virus isolation from wild-caught animals (Daniels *et al.,* 2007). The range of tissues yielding virus in natural and experimental cases include the brain, lung, kidney and spleen (Crameri G., *et al.* 2002).

Henipaviruses grow rapidly to high titers in a large number of cell lines. African green monkey kidney (Vero) and rabbit kidney (RK-13) cells have been found to be particularly susceptible. A CPE usually develops within 3 days, but two 5-day passages are recommended before judging the attempt unsuccessful. After low multiplicity of infection, the CPE is characterised by formation of syncytia that may, after 24–48 hours, contain over 60 or more nuclei. Syncytia formed by NiV in Vero cell monolayers are significantly larger than those created by HeV in the same time period. Although the distribution of nuclei in NiV-induced syncytia early in infection resembles that induced by HeV, with nuclei aggregated in the middle of the syncytia, nuclei in mature NiV-induced syncytia are distributed around the outside of the giant cell (Hyatt *et al.*, 2001).

Very low virus load in bats makes isolation very difficult. Linfa Wang and colleagues at the AAHL have increased sensitivity of cell lines by "rational engineering," consisting of a single point mutation in ephrinB2 resulting in enhanced affinity for NiV.

5) *Pen-side test*

Not yet developed.

While the concept is attractive, it is a huge challenge technically and in regulatory sense, especially considering how presumable false positive results would be handled.

6) *N and G ELISA*

Indirect recombinant N- ELISA and G-ELISA have been developed, and are now in the stage of diagnostic evaluation (Fisher K., *et al.*, 2018). The N-ELISA protocol was transferred to HSADDL (India) and validated and used for surveillance (Kulkarni *et al.,* 2016).

Problems with specificity (i.e., false positives) could arise. For example, swine sero-surveillance in West Bengal, India, appears to be negative; however, 8/328 samples tested positive (i.e., presumably false positive) using the anti-N antigen ELISA antibody detection test. Evaluation of the indirect IgG ELISA based on the recombinant NiV-N antigen using swine samples from Canada yielded similar results, including an indirect IgG ELISA based on the G glycoprotein. In Canadian context, the problem is the diagnostic specificity, with 5% false positives, resulting in the decision to complement with the G-ELISA. Only sera positive on both tests are considered

positive. Confirmatory testing may be required, if this was to be the first case reported in non-endemic area.

A diagnostic test for differentiating infected from vaccinated animals (DIVA) would have to most likely target the N antigen, or alternatively P gene coded products depending on the level of expression and antigenicity in animals, and the number of reactors in non-endemic areas.

The N ELISA assay could fulfill DIVA requirements if the canarypox vectored NiV-G-NiV-F vaccine is used because antibodies to N would only occur after NiV infection.

7) *IgM ELISA*

The U.S Center for Disease Control and Prevention (CDC) developed an IgM ELISA for human serology. Detection of IgM was used to confirm recent infection with NiV in both Malaysia and Bangladesh. NiV-infected cells that have been inactivated by gamma irradiation are used as antigens.

In theory the same can be done for different animal species as long as we have the right anti-species antibodies. For bats, that is still a challenge.

8) *Virus neutralization test (VNT)*

VNT serves as the traditional gold standard of serological investigations. The VNT requires live virus and thus BSL-4 containment facilities are required (Crameri *et al.,* 2002). It has proven to be a very valuable specific and sensitive tool in the diagnosis of NiV.

VNT rely on quantification methods. Three different procedures are available to titer HeV and NiV. In the traditional plaque and microtiter assay procedures, the titer is calculated as plaque forming units (PFU) or the tissue culture infectious dose capable of causing CPE in 50% of replicate wells ($TCID_{50}$), respectively.

In an alternative procedure, the viruses are titrated on Vero cell monolayers in 96-well plates and after 18–24 hours, foci of infection are detected immunologically in acetone-fixed cells using anti-viral antiserum (Crameri G., *et al.* 2002). The virus titre is expressed as focus-forming units (FFU)/ml.

Neutralisation assays using these three methods are described in the OIE Manuel of Diagnostic Tests and Vaccines for Terrestrial Animals.

Virus quantification procedures should be conducted at BSL4. A new version of the differential neutralisation test has been recently described, which avoids the use of infectious virus by the use of ephrin-B2-bound biospheres (Bossart *et al.,* 2007). Although the test has yet to be formally validated, it appears to have the potential to be a screening tool for use in countries without BSL4 facilities.

9) Pseudotype virus plaque reduction neutralization test (PRNT)

The standard plaque reduction neutralization assay (PRNT) used to detect NiV and HeV must be performed in BSL-4 containment and takes several days to complete. The CDC and the AAHL have modified the PRNT by using recombinant Vesicular Stomatitis Virus (VSV) derived from the cDNA of VSV Indiana to construct pseudotype particles expressing the F and G proteins of NiV (pVSV-NiV-F/G) as target antigens (Chang *et al.,* 2006; Tamin *et al.,* 2009; Kaku *et al*., 2009). This rapid assay can be performed at BSL-2. The PRNT was evaluated using serum samples from outbreak investigations and more than 300 serum samples from an experimental NiV vaccination study in swine. The results of the neutralization assays with pVSV-NiV-F/G as antigen showed a good correlation with those of standard PRNT. The PRNT titers give an indication of protective immunity. Therefore, this new method has the potential to be a rapid and cost-effective diagnostic method, especially in locations that lack high containment facilities, and will provide a valuable tool for basic research and vaccine development. A similar assay has been developed by the Japanese-Australian group (Kaku et al., 2009), which proved to be as specific as the VNT and much more sensitive than VNT.

10) Binding Luminex Assay

Sera are tested for antibodies binding to recombinant soluble G (sG) proteins in a Luminex® multiplexed microsphere binding assay. The sG proteins retain their ability to bind the cellular receptor molecule, indicating their native conformation is maintained, which is important for the detection of neutralizing antibodies. For bat sera, median fluorescence intensities (MFI) readings of $\geq$200 are considered positive. Three times the average background reading of negative sera is was used as a cut-off for the binding assay.

11) Luminex® multiplexed nucleic acid detection assay

Foord et al, 2012, reported microsphere suspension array systems enable the simultaneous fluorescent identification of multiple separate nucleotide targets in a single reaction using commercially available oligo-tagged microspheres (Luminex® MagPlex-TAG) to construct and evaluate multiplexed assays for the detection and differentiation of HeV and NiV. Assays were developed to target multiple sites within the nucleoprotein (N) and phosphoprotein (P) encoding genes. The relative specificities and sensitivities of the assays were determined using reference isolates of each virus type, samples from experimentally infected horses, and archival veterinary diagnostic submissions. Results were assessed in direct comparison with an established qPCR. Foord reported the microsphere array assays achieved unequivocal differentiation of HeV and NiV and the sensitivity of HeV detection was comparable to qPCR, indicating high analytical and diagnostic specificity and sensitivity.

12) Luminex® proprietary multiplex bead-based immunoassay

Currently, the Luminex® proprietary multiplex bead-based immunoassay testing platform for the detection of anti-G antibodies is used for bat surveillance at the AAHL, and by other research investigators. Luminex® technology detects antibodies to recombinant soluble G (sG) proteins from NiV and HeV in a multiplexed microsphere binding assay. Since the glycoprotein specific

antibody response to both NiV and HeV can be measured simultaneously, this assay can differentiate between the serologic responses to NiV and HeV.

13) Blocking Luminex® Assay

This is an extension of the Binding Luminex Assay, developed as a surrogate VNT in the sense that it measures antibodies that block the binding of the soluble henipavirus G protein to the ephrin-B2 receptor molecule. It is highly specific, but needs further validation with field samples.

DEPOPULATION

Preemptive culling of herds in the neighborhood of an infected herd is an effective and even indispensable measure in the control of a NiV epidemic in areas with high pig densities. The purpose of this measure is to prevent infection of new herds, which would generate massive infectious virus production, and thus to reduce the virus infection load in an area. This reduced infection load subsequently results in a reduction of the between-herd virus transmission. However, recent outbreaks have shown that the control of Nipah virus in pig populations through stamping out is complex due to the zoonotic nature of the agent. In addition, depopulation may be logistically difficult and very expensive in swine dense area, and would not be effective if the Nipah virus mutates to become easily transmitted between people and from people to pigs. Depopulation will not be possible in situation like those that occurred in Bangladesh in which NiV was transmitted from bats to humans without an amplifying host. Depopulation of swine may be impossible in a rapidly spreading outbreak in a pig dense region with hundreds of millions of swine, such as in southeast China (Vergne T. *et. al*. 2017).

SURVEILLANCE

The initial expression of NiV in U.S swine would be variable and unpredictable due to the myriad of host factors and the broad diversity of virulence among strains of henipaviruses. Different surveillance strategies will be required to detect the different clinical manifestations.

For acute infection, surveillance activities can be based on clinical signs, but signs are unlikely to be noticed by producers and practitioners. It would be prudent to develop surveillance activities based on diagnostic testing to supplement surveillance based on clinical signs.

The following surveillance programs are in place to meet the objective of rapid detection of henipaviruses in Malaysia and Australia:

1. Population-based passive reporting of suspicious NiV cases. Efforts to enhance reporting will be focused on high risk areas.
2. Laboratory-based surveillance of serum and tissue submitted from sick pigs.

There is no diagnostic capability for henipaviruses in United States veterinary diagnostic laboratories due to the lack of BSL-4 laboratory space. The only diagnostic capability for henipaviruses in the U.S is the Center for Disease Control and Prevention (CDC). There are no active or passive surveillance programs. Henipavirus suspect samples would be sent to the CDC, the OIE reference laboratory at the

Australian Animal Health Laboratory, or the National Canadian Foreign Animal Disease Center, in Winnipeg, Canada.

DRUGS

There are no licensed anti-viral drugs available to treat people or animals against Henipaviruses.

DISINFECTANTS

People: Soaps and detergents.

Fomite disinfection: Sodium hypochlorite to supply 10,000 ppm chlorine or Virkon.

PERSONAL PROTECTIVE EQUIPMENT (PPE)

PPE should be suitable to prevent farm-to-farm virus spread by diagnostic or vaccination teams.

RECOMMENDATIONS

RESEARCH

The 2017 gap analysis working group recommends the implementation of the following research priorities.

Viral Pathogenesis

- Determine early events of NiV infection, immune evasion and identify determinants for virulence and host susceptibility

Immunology

- Characterize the antibody and cell-mediated immune response to NiV infection and vaccination
- Develop the basic knowledge of the mechanisms NiV uses to evade the innate immune response
- Characterize the ability of interferons to inhibit virus replication and shedding early in infection.

Vaccine Discovery and Development Research.

- Implement comprehensive vaccine research program to deliver next generation NiV vaccines and specifically design strategies for control in priority susceptible hosts
- Investment in Nipah vaccine development needs to include conducting studies to demonstrate safety and efficacy necessary for licensure by authorities in countries that may have an emergency need for vaccine in swine.

Diagnostics

- Develop a panel of reference standards for both molecular and serologic tests that can be made available to all of the laboratories performing diagnostic tests for henipaviruses. This panel should also include monoclonal antibodies and recombinant antigens that would be readily available as low biosecurity BSL-2 reagents.
- Develop a formalized structured worldwide network for reference panel development and assay validation and harmonization.
- Develop and validate broadly reactive PCR assays targeting highly conserved genetic targets within the henipaviruses. Evaluate the relative sensitivity and specificity of the currently used PCR assays.
- Develop and validate field tests (both protein- and nucleic acid-based) to detect henipaviruses.
- Explore new antigen detection assays, including antigen capture, Loop Mediated Isothermal Amplification Protocol (LAMP) suitable for resource limited situations, and nanotechnology.
- Develop species specific reagents to improve the quality of serologic assays.
- Evaluate the relative sensitivity and specificity of molecular and serologic tests, especially new serologic tests that could replace serum neutralization titers (SNT) and meet DIVA (differentiate infected from vaccinated animals) requirements.
- Explore the use of serological assays based on recombinant antigens that could be produced at BSL-2. Classical serological tests using low biosecurity (recombinant) reagents produced at BSL-2 facilities could be developed reasonably quickly and at a reasonable cost.
- Develop species independent serologic assays using recombinant antigens.

Epidemiology

- The epidemiology of NiV in disease outbreaks needs to be assessed and modeled on the level of the individual pig, the herd, and the demographics of the region.
- Epidemiological investigations should be performed on the implementation of emergency vaccination and the use of 'DIVA' and other diagnostic tests to detect infected pigs in vaccinated populations
- Risk assessments need to be performed with regard to control or spread of henipaviruses
- The epidemiological evaluation of wildlife needs to be carried out in order to improve the risk estimates of outbreaks in domestic animal and human populations

PREPAREDNESS

Many of the countermeasures discussed in this report will require preparation and integration in a coordinated disease control program and funding for a stockpile for use in an emergency response plan for an outbreak of NiV infection. The Henipavirus gap analysis working group recommends investing in the implementation of the following preparedness plan to ensure the effective use of the countermeasures in the NVS:

- See the Ausvetplan: https://www.animalhealthaustralia.com.au/our-publications/ausvetplan-manuals-and-documents/ Assessed July 22, 2018
- See Guidelines for Veterinarians Handling potential Hendra Virus infection in Horses (QDPI): https://www.daf.qld.gov.au/__data/assets/pdf_file/0005/126770/2913_-Guidelines-for-veterinarians-handling-potential-Hendra-virus-infection-in-horses-V5.1.pdf Accessed July 22, 2018

Surveillance

Routine surveillance for NiV is now limited to serologic screening of pigs in several Southeast Asian countries.

- Develop a regional surveillance strategy, including laboratory, to detect spillovers of NiV into domestic and agricultural animals.
- Determine the optimal surveillance strategy to detect circulation of NiV in the bats reservoirs and other wild life.
- Improve surveillance capacity to detect henipaviruses in high risk countries.
- Establish a formal laboratory network for henipavirus surveillance that includes standardized specimen collection, laboratory testing scheme, quality control, specimen referral and accreditation.

Biosecurity

Design NiV-specific on-farm biosecurity programs to implement in a disease outbreak situation.

Personal Protective Equipment and Decontamination

- See Australian procedures https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0003/494202/Hendra-virus-ppe-procedures.pdf Assessed July 22, 2018

- FAO (Food and Agriculture Organization) – Manual on the Diagnosis of Nipah Virus in Animals: Chapter 2: Working safely with Nipah Virus
  http://www.fao.org/docrep/005/AC449E/ac449e05.htm#bm05
  Assessed July 22, 2018

Depopulation and Disposal

Develop plans for handling disposal of animals infected with a zoonotic agent, including an emergency plan to dispose of infected swine and decontaminate facilities and equipment determined to be infected.

- FAO (Food and Agriculture Organization) – Manual on the Diagnosis of Nipah Virus in Animals: Chapter 5: Control and eradication
  http://www.fao.org/docrep/005/AC449E/ac449e08.htm#bm08

CONCLUSION

The threat of an outbreak with a henipavirus in the United States due to a natural transmission from a reservoir host is very low since the reservoirs are known to be bats in South East Asia, South Asia, and Asia. However, an outbreak that is not controlled in swine or in people in Asia could result in infection being introduced accidentally into North America or Europe. There is considerable concern that henipaviruses could be used as a weapon of mass destruction (WMD) because they have many of the characteristics of the ideal biological weapon, including causing one of the highest mortality rate in people known for an infectious disease. The possibility of an intentional criminal spread at least in short clusters of terrorist attacks is a distinct possibility, for example by aerosolization in confined public spaces, or through infection of pigs. Surveillance brings challenges and weaknesses of diagnostic methods may impede the early detection of an outbreak in the United States. There are no commercially available diagnostic tests and although laboratory tests are available they have not been field validated. Depopulation is the primary method to eradicate NiV but present very high risks since henipaviruses are BSL-4 zoonotic agents. There are commercially available vaccines for horses, but none for swine and people. Accordingly, the gap analysis working group recommends investing in the research and development of countermeasures and ensure their use and integration in planning for preparedness and future control campaigns. Priority should be given to funding research to improve surveillance, diagnostics, and vaccines. Specific goals include 1) improving diagnostic tests to rapidly identify new disease outbreaks; 2) epidemiological research to better understand virus transmission in wildlife and maintain a passive surveillance program in high risk commercial livestock operations; and 3) develop safe and effective vaccines specifically designed for control and eradication. The Unites States should stockpile NiV vaccines when they become available for use in contact herds to create a buffer zone as an additional control measure to prevent the spread of henipaviruses should an outbreak ever occur.

FIGURES

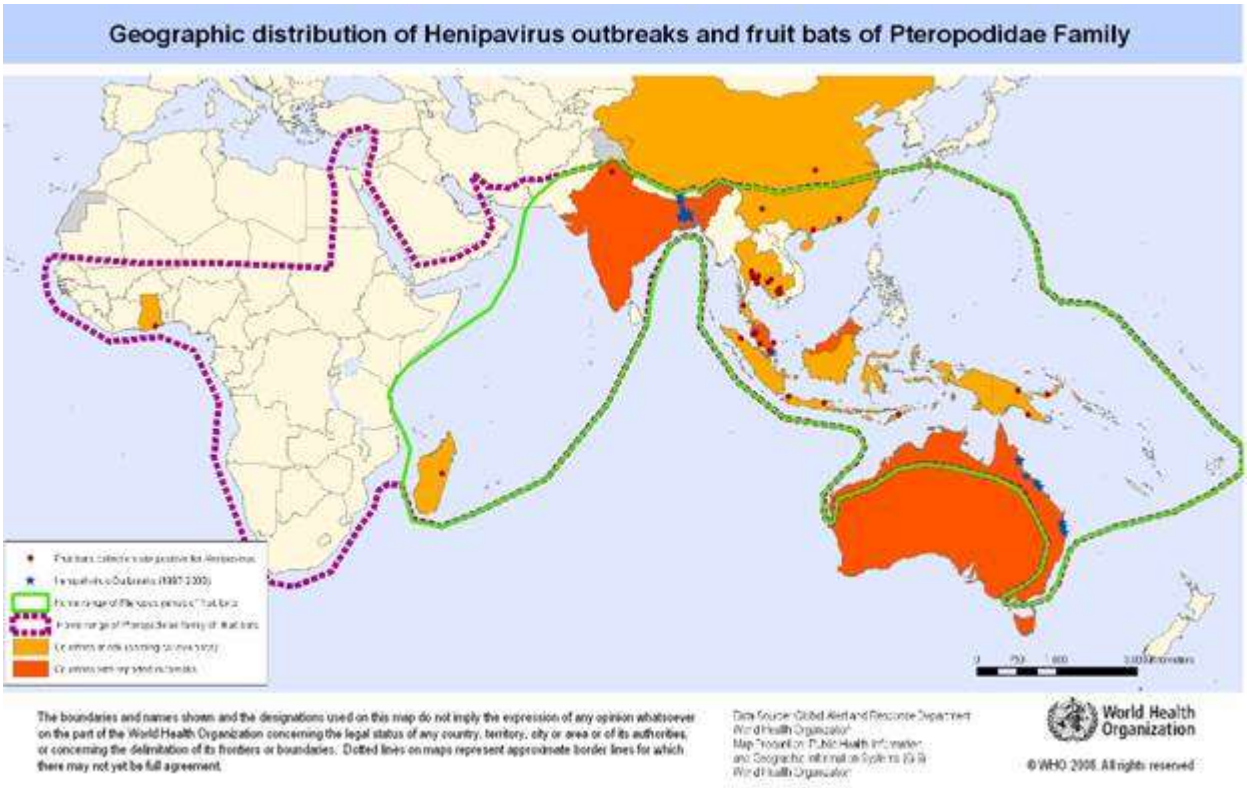

Figure 1: Geographic distribution of fruit bats of the Pteropoditae family. WHO: Nipah virus infections: http://www.who.int/csr/disease/nipah/en/ (Assessed July 22, 2018)

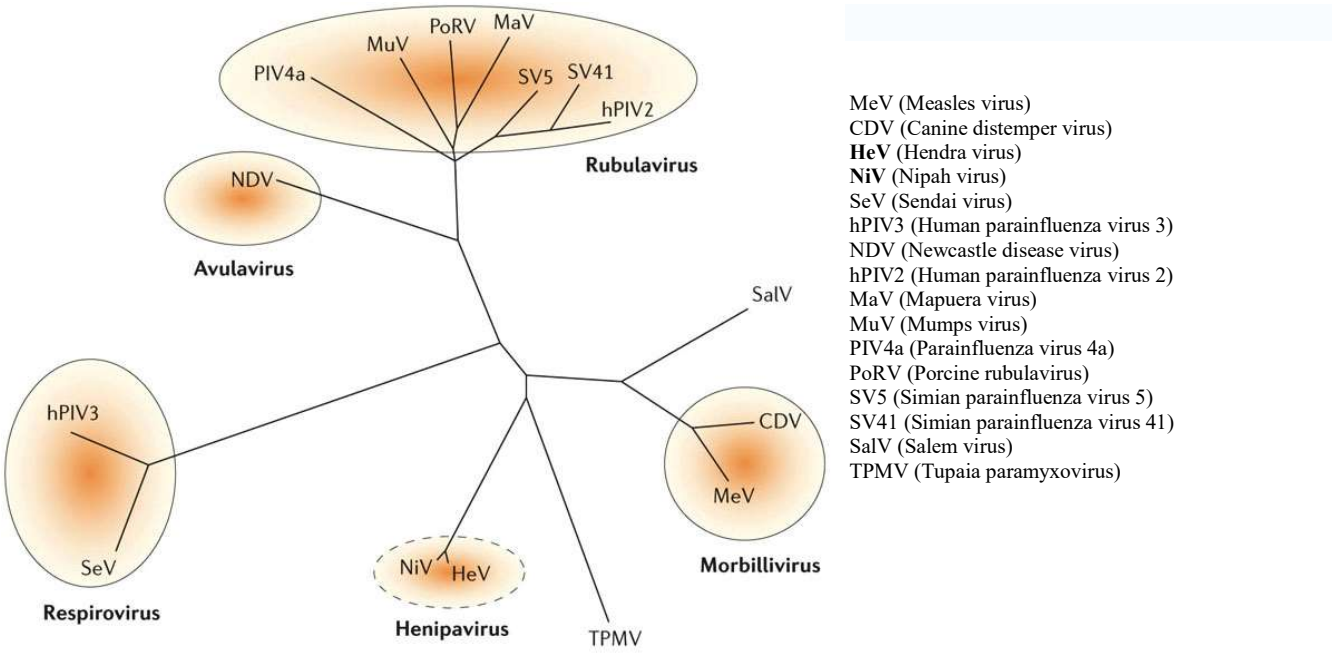

Figure 2: Phylogenetic tree based on alignment of deduced amino acid sequence of the N-gene of selected *Paramyxovirinae* subfamily members (Eaton *et al*, 2006. Nature Reviews Microbiology 4:25-35).

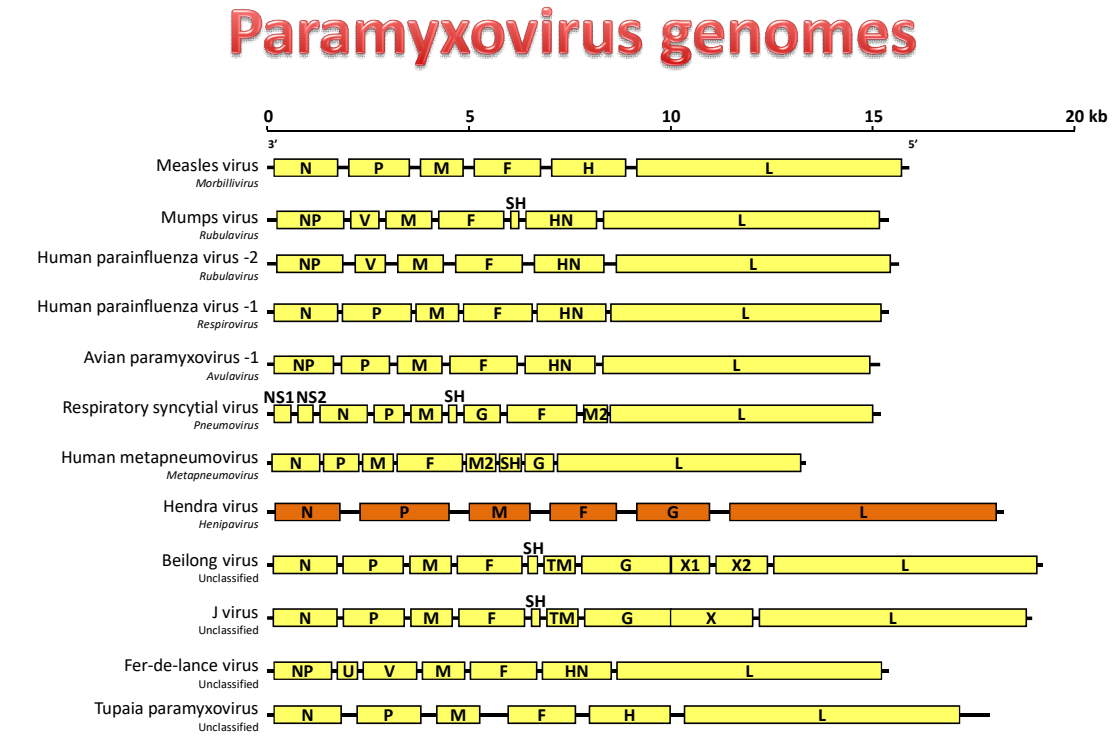

Figure 3: Comparison of *Paramyxoviridae* viruses genomes (Provided by Glen Marsh, AAHL)

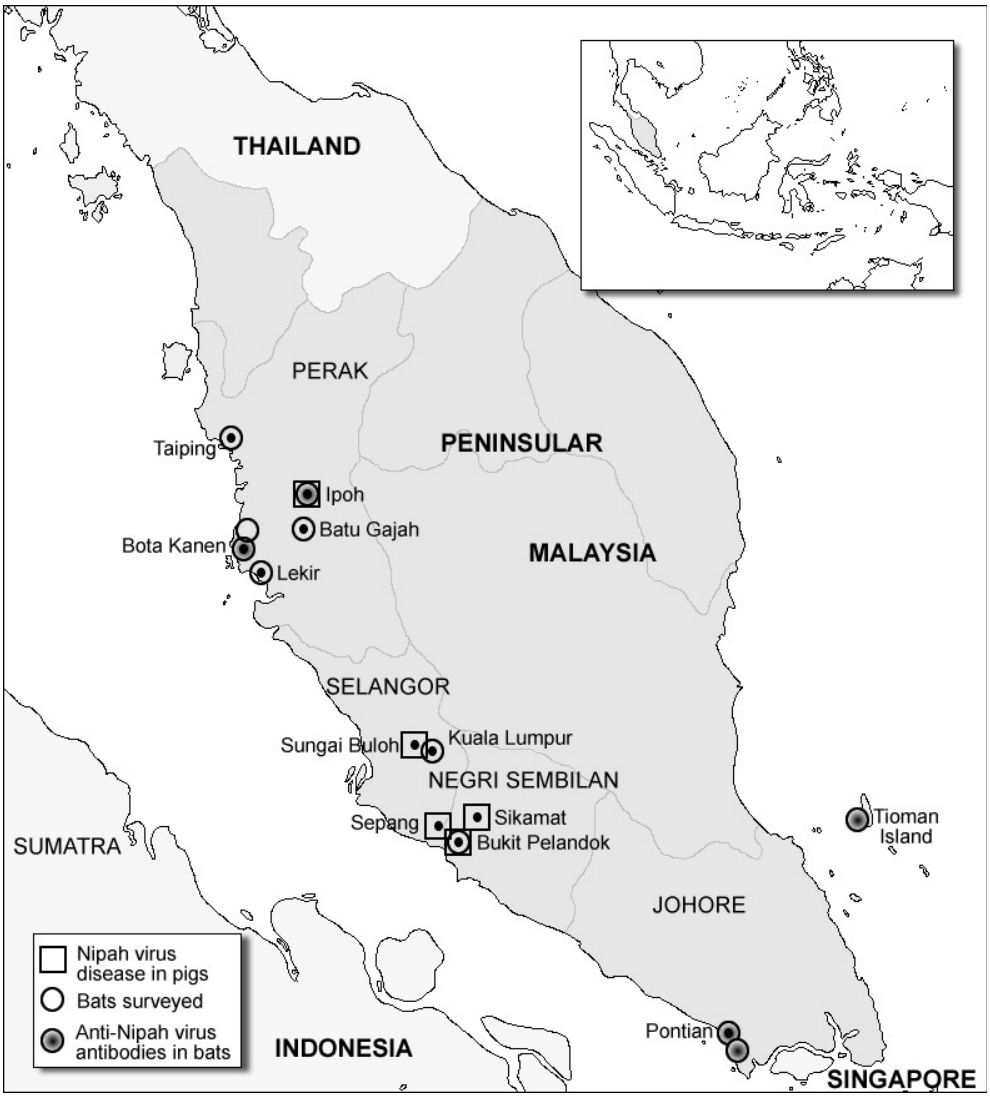

Figure 4: Descriptive map of NiV in Malaysia (Yob *et al.,* 2001)

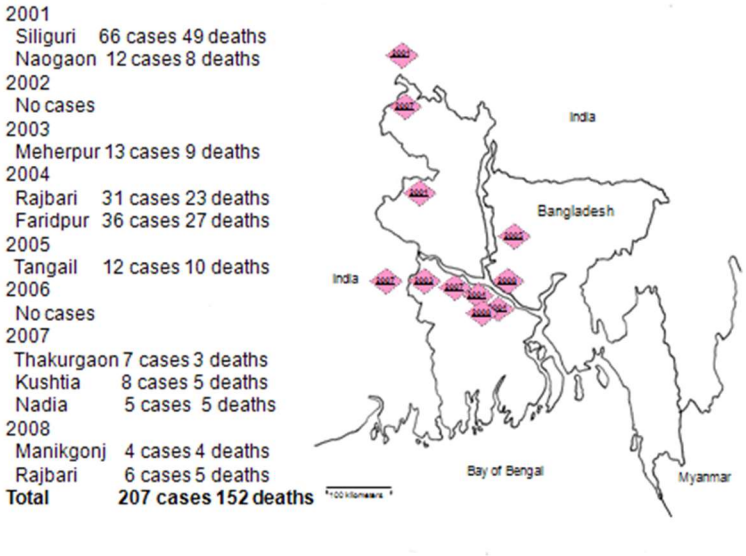

Figure 5: Epidemiology of Nipah Virus Infections in Bangladesh (Source: Steve Luby, icdd,b)

TABLE I: NIPAH VIRUS INFECTION IN BATS

Species	No. of bats	No. Positive (%)
Megachiroptera (fruit bats)		
Cynopterus brachyotis	56	2 (4)
Eonycteris spelaea	38	2 (5)
Pteropus hypomelanus	35	11 (31)
Pteropus vampyrus	29	5 (17)
Cynopterus horsfieldi	24	0
Ballionycterus maculata	4	0
Macroglossus sobrinus	4	0
Megaerops ecaudatus	1	0
Microchiroptera (Insectivorous bats)		
Scotophilus kuhlii	33	1 (3)
Rhinolophus affinis	6	0
Taphozous melanopogon	4	0
Taphozous saccolaimus	1	0
Hipperosiderus bicolor	1	0
Rhinolophus refulgens	1	0
Total	237	21

Source: Yob *et al.,* 2001

TABLE II – NIPAH VIRUS CASES 2001-2018

Morbidity and mortality due to Nipah or Nipah-like virus encephalitis in WHO South-East Asia Region, 2001-2018

Country: Bangladesh

Month/Year	Location	No. cases	No. deaths	Case Fatality Rate
April, May 2001	Meherpur	13	9	69%
January 2003	Naogaon	12	8	67%
Jan 2004 Apr 2004	Rajbari Faridpur	31 36	23 27	74% 75%
Jan- Mar 2005	Tangail	12	11	92%
Jan-Feb 2007 Mar 2007 Apr 2007	Thakurgaon Kushtia Pabna, Natore and Naogaon	7 8 3	3 5 1	43% 63% 33%
Feb 2008 Apr 2008	Manikgonj Rajbari	4 7	4 5	100% 71%
Jan 2009	Gaibandha, Rangpur and Nilphamari Rajbari	3 1	0 1	0% 100%
Feb-Mar 2010	Faridpur Faridpur, Rajbari, Gopalgonj, Kurigram,	8 8 1	7 7 1	87.50% 87.50% 100%
Jan-Feb 2011	Lalmohirhat, Dinajpur, Comilla Nilphamari, Faridpur, Rajbari	44	40	91%
Jan 2012	Joypurhat	12	10	83%
Jan- Apr 2013	Pabna, Natore, Naogaon, Gaibandha, Manikganj	24	21	88%
Jan-Feb 2014	13 districts	18	9	50%
Jan-Feb 2015	Nilphamari, Ponchoghor, Faridpur, Magura, Naugaon, Rajbari	9	6	67%

Country: India

Month/Year	Location	No. cases	No. deaths	Case Fatality Rate
Feb 2001	Siliguri	66	45	68%
Apr 2007	Nadia	5	5	100%
May 2018	Kerala	14	12	86%

WHO (World Health Organization). Morbidity and mortality due to Nipah or Nipah-like virus encephalitis in WHO South-East Asia Region, 2001-2018. Available at: http://www.who.int/csr/disease/nipah/en/. (Accessed on July 22, 2018).

TABLE III – VACCINE PLATFORMS

C.C. Broder et al. / Vaccine 34 (2016) 3525–353

Table 1
Advanced active vaccination and passive immunization platforms tested in Hendra virus and/or Nipah virus animal challenge models.

Platform	Viral antigen target or immunogen	Animal challenge model
Active vaccination		
Recombinant vaccinia virus	Nipah F and/or G glycoprotein	Hamster[a] (NiV)
Recombinant canarypox virus	Nipah F and/or G glycoprotein	Pig[b] (NiV)
Recombinant VSV	Nipah F and/or G glycoprotein	Ferret[c] (NiV), Hamster[d] (NiV), nonhuman primate[e] (NiV)
Recombinant AAV	Nipah G glycoprotein	Hamster[f] (NiV, HeV)
Recombinant measles virus	Nipah G glycoprotein	Hamster and nonhuman primate[g] (NiV)
Recombinant subunit	Hendra soluble G glycoprotein	Cat[h] (NiV), Ferret[i] (HeV, NiV), nonhuman primate[j] (HeV, NiV), horse[k] (HeV)
Passive immunization		
Human monoclonal antibody m102.4	Hendra/Nipah G glycoprotein	Ferret[l] (NiV) Nonhuman primate[m] (HeV, NiV)

[a] Hamsters immunized with NiV F and/or G glycoprotein encoding recombinant vaccinia viruses were protected against disease following intraperitoneal challenge with 10^3 PFU of NiV [137].
[b] Pigs immunized with NiV F and/or G glycoprotein encoding recombinant canarypox viruses were protected against intranasal challenge with 2.5×10^5 PFU of NiV [138].
[c] Ferrets immunized with NiV F and/or G glycoprotein encoding recombinant vesicular stomatitis virus (VSV) vectors were protected against lethal intranasal challenge with 5×10^3 PFU of NiV [141]
[d] Hamsters immunized with NiV F and/or G glycoprotein encoding recombinant vesicular stomatitis virus (VSV) vectors were protected against lethal intraperitoneal challenge with 10^5 $TCID_{50}$ of NiV [143]; or 6.8×10^4 $TCID_{50}$ of NiV [142].
[e] African green monkeys immunized with a NiV G encoding recombinant VSV vector were protected against lethal intratracheal challenge with 10^5 $TCID_{50}$ of NiV [156].
[f] Hamsters immunized with a NiV G encoding recombinant adeno-associated virus (AAV) vector were protected against lethal intraperitoneal with 10^4 PFU of NiV [139].
[g] Hamsters and African green monkeys immunized with a NiV G encoding recombinant measles virus vector were protected against lethal intraperitoneal challenge with 10^3 $TCID_{50}$ of NiV (hamsters) or 10^5 $TCID_{50}$ of NiV (monkeys) [140].
[h] Hendra virus soluble G glycoprotein (HeV-sG) used to immunize cats protects against lethal subcutaneous (500 $TCID_{50}$) [120] or oronasal (5×10^4 $TCID_{50}$) NiV challenge [145].
[i] HeV-sG used to immunize ferrets protects against lethal oronasal challenge with 5×10^3 $TCID_{50}$ of HeV [124] or 5×10^3 $TCID_{50}$ of NiV challenge [146].
[j] HeV-sG used to immunize African green monkeys protects against lethal intratracheal challenge with 10^5 $TCID_{50}$ of NiV [157] or 5×10^5 PFU of HeV [147].
[k] HeV-sG used to immunize horses protects against lethal oronasal challenge with 2×10^6 $TCID_{50}$ of HeV [15].
[l] A NiV and HeV cross-reactive G glycoprotein specific neutralizing human mAb (m102.4) protects ferrets against lethal oronasal challenge with 5×10^3 $TCID_{50}$ of NiV [125] or 5×10^3 $TCID_{50}$ of HeV (J. Pallister and C. Broder, unpublished) by post-exposure infusion.
[m] Human mAb m102.4 protects African green monkeys by post-exposure infusion following lethal intratracheal challenge with 4×10^5 $TCID_{50}$ of HeV [153] or lethal intratracheal challenge with 5×10^5 PFU of NiV [154].

TABLE IV – CURRENT VACCINE CANDIDATES

B.A. Satterfield et al. / Vaccine 34 (2016) 2971–2975

Table 1
Development status of current vaccine candidates.

Candidate name/ identifier: institution	Preclinical	Developers	Ref
Subunit vaccine			
HeV sG	X	Zoetis, Inc./USU	[16,18,34,39]
Vectored vaccines			
VSV-NiV_B F and/or G	X	UTMB	[17]
VSV-NiV_M G	X	CDC	[15]
VSV-NiV_M G	X	RML	[14,19]
VSV-NiV_M F and/or G	X	Yale University	[40]
VSV-HeV G:	X	TJU/RML	[41]
RABV-HeV G:	X	TJU/RML	[41]
ALVAC-F/G	X	CFIA-NCFAD	[20,42]
AAV-NiV_M G	X	INSERM	[43]
rMV-Ed-G	X	UoT	[44]
V-NiVG	X	USU	[45]
rLa-NiVG and/or rLa-NiVF	X	CAAS-SKLVB	[21]
Passive antibody transfer			
Polyclonal serum NiV F or G	X	INSERM	[46]
Mouse mAbs NiV F or G	X	INSERM	[47]
Human mAb m102.4 Henipah G	X	USU	[35,48]

Abbreviations: USU (Uniformed Services University of the Health Sciences); UTMB (University of Texas Medical Branch); CDC (Centers for Disease Control and Prevention); RML (Rocky Mountain Laboratories); TJU (Thomas Jefferson University); CFIA-NCFAD (Canadian Food Inspection Agency – Centre for Foreign Animal Diseases); Institut national de la santé et de la recherché médicale (INSERM); UoT (University of Tokyo); CAAS-SKLVB (Chinese Academy of Agricultural Sciences (CAAS) – State Key Laboratory of Veterinary Biotechnology (SKLVB)).

APPENDIX I – VACCINES ASSESSMENT

Experimental Veterinary Vaccines For Nipah Virus - USDA/ARS, 03-19-09

Rank each Intervention (2,4,6,8, or 10) as to its importance to making a decision, only one "10" rankings allowed

Weight	Critical Criteria	CPV-G	CPV-F	VV-G	Soluble G			
10	Efficacy	6	4	2	6			
6	Safety	10	10	2	10			
8	One dose	4	4	4	2			
8	Manufacturing safety	8	8	6	8			
10	DIVA Compatible	8	8	8	8			
8	Manufacturing yield	8	8	8	6			
6	Rapid production	8	8	4	4			
4	Reasonable cost	6	6	4	2			
2	Short withdrawal	8	8	2	4			
8	Long shelflife	8	8	8	4			

Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed

Critical Criteria	CPV-G	CPV-F	VV-G	Soluble G	0	0	0
Efficacy	60	40	20	60	0	0	0
Safety	60	60	12	60	0	0	0
One dose	32	32	32	16	0	0	0
Manufacturing safety	64	64	48	64	0	0	0
DIVA Compatible	80	80	80	80	0	0	0
Manufacturing yield	64	64	64	48	0	0	0
Rapid production	48	48	24	24	0	0	0
Reasonable cost	24	24	16	8	0	0	0
Short withdrawal	16	16	4	8	0	0	0
Long shelflife	64	64	64	32	0	0	0
0	0	0	0	0	0	0	0
Value	512	492	364	400	0	0	0

APPENDIX II – DIAGNOSTICS ASSESSMENT

Experimental Diagnostics For Nipah Virus - USDA/ARS, 03-19-09

Rank each Intervention (2,4,6,8, or 10) as to its importance to you in making a decision, no more than one "10" rankings allowed

Weight	Critical Criteria	qPCR	conv PCR	field PCR	VI	penside	v ELISA	N ELISA	IgM ELISA	VNT	ps VNT	bind lum	block lum
10	Sensitivity	10	10	8	8	4	10	4	8	8	8	8	8
8	Specificity	8	6	8	10	6	6	6	8	10	8	8	8
2	DIVA	8	8	8	8	8	2	10	6	2	2	8	2
6	multispecies	8	8	8	8	8	6	6	2	8	8	6	8
8	Validation to purpose	8	8	8	8	4	8	4	10	8	10	8	10
4	Speed of Scaleup	8	4	4	2	6	8	8	8	2	4	4	4
4	Throughput	8	2	2	2	4	8	8	8	2	4	6	6
4	Flock Side Test	2	2	10	2	10	2	2	2	2	2	2	2
10	Rapid Result	6	4	8	2	8	6	6	6	4	4	10	8
4	No need to Confirm	6	4	4	8	2	6	4	6	8	8	8	8
8	Easy to perform	8	6	6	4	8	8	8	6	6	6	8	8
8	safe to operate	8	8	6	2	6	8	8	8	2	8	8	8
8	Availability	8	8	2	2	2	6	8	4	2	6	4	4
6	Storage/Distribution	4	6	6	2	6	6	6	6	2	4	4	4
4	Low Cost to Implement	2	4	2	2	4	6	8	6	2	4	4	2

Rank each Criteria 2,4,6,8 or10 on each criterion -- no more than two "10" rankings allowed

Critical Criteria	qPCR	conv PCR	field PCR	VI	penside	v ELISA	N ELISA	IgM ELISA	VNT	ps VNT	bind lum	block lum
Sensitivity	100	100	80	80	40	100	40	80	80	80	80	80
Specificity	64	48	64	80	48	48	48	64	80	64	64	64
DIVA	16	16	16	16	16	4	20	12	4	4	16	4
multispecies	48	48	48	48	48	36	36	12	48	48	36	48
Validation to purpose	64	64	64	64	32	64	32	80	64	80	64	80
Speed of Scaleup	32	16	16	8	24	32	32	32	8	16	16	16
Throughput	32	8	8	8	16	32	32	32	8	16	24	24
Flock Side Test	8	8	40	8	40	8	8	8	8	8	8	8
Rapid Result	60	40	80	20	80	60	60	60	40	40	100	80
No need to Confirm	24	16	16	32	8	24	16	24	32	32	32	32
Easy to perform	64	48	48	32	64	64	64	48	48	48	64	64
safe to operate	64	64	48	16	48	64	64	64	16	64	64	64
Availability	64	64	16	16	16	48	64	32	16	48	32	32
Storage/Distribution	24	36	36	12	36	36	36	36	12	24	24	24
Low Cost to Implement	8	16	8	8	16	24	32	24	8	16	16	8
Value	672	592	588	448	532	644	584	608	472	588	640	628

APPENDIX III - CONTRIBUTORS

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Subject:	RE: Henipavirus Gap Analysis Report
Date:	Thursday, August 9, 2018 8:01:14 AM
Attachments:	Henipavirus Gap Analysis Report, August 2018.docx image003.png image005.png

Dear Colleagues,

Thanks to Hana for helping update Section 6 of the diagnostic section. I'm attaching here the report one more time for those of you that have not had a chance to look at it yet. I plan on posting the report on the USDA website next week so please take a quick look as indicated in my message below.

Thank you.

Cyril

From: Gay, Cyril
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Subject: Henipavirus Gap Analysis Report

Dear Colleagues,

Please find attached the subject report for your review and input. It's taken me a while to update the gap analysis report, but really appreciated the help received from Jim Roth, Jon Epstein, Bruce Carter, and Hanna Weingartl. I would like to post the report on the USDA website as soon as possible (a lot of people have been asking me for the report), so I would really appreciate if you could take a quick look in the sections that interest you and edit and supplement with new information and references where needed.

At a minimum, there are three areas that definitely need your attention.

1) The Recommendation section on "Preparedness" on Page 40 is weak as I had to delete all the reference materials previously provided by Peter Daniels because they have all been "deleted" from the AAHL website. I tried finding other relevant information on the internet, unsuccessfully. Perhaps this section should be deleted? Or you may be able to improve it?

2) The one area that requires special technical attention for sure is the diagnostic section, especially "6) *N and G ELISA" on Page 34.* I think this section needs to clearly differentiate antibody detection versus antigen detection ELISAs, which is not clear as currently written. I think we also need to add information on assays that have recently been developed that can differentiate NiV and HeV; e.g., publications from Chiang et al (2013), and Fisher et al (2018).

3) Lastly, I think the research recommendations on Page 39 could be improved with additional research priorities to fill the many gaps identified in the report.

As previously mentioned when we were all in Canada, this report will be distributed to stakeholders and funders of research (globally), as well as industry, and government agencies that have interest in stockpiling veterinary medical countermeasures – so your critical view of the report is essential as it reflects our collective expert opinion.

Thank you so much for your help and contributions.

Best regards,

Cyril

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EcoHealth Alliance BEP proposal 2018.

Activity #1 Project Title: Serological Biosurveillance for Henipaviruses and Filoviruses in Uttar Pradesh, India

Activity #1 Project Summary: EcoHealth Alliance (EHA) proposes to enhance early detection and surveillance capacity in India by: 1) transferring Luminex-based Bio-Plex technology and validated reagents to detect IgG antibodies against all henipaviruses (e.g. Nipah virus) and filoviruses (e.g. Ebola virus) to the premiere UP medical laboratory – Sanjay Gandhi Institute of Postgraduate Medical Sciences; 2) training laboratory personnel to develop and utilize Bio-Plex assays; 3) conducting biological surveillance in bats, domestic animals and people in rural communities where there are high levels of contact among people and animals. Activities will be coordinated with, and complementary to the USAID Emerging Pandemic Threats: PREDICT program and the Global Health Security Agenda, and surveillance data will be shared with the Government of India (GoI). Henipaviruses and filoviruses are highly pathogenic zoonotic viruses and select agents capable of causing public health emergencies of international concern. Henipaviruses are associated with acute encephalitis and filoviruses are associated with hemorrhagic fever in people. A disproportionate number of cases of acute encephalitis occur in Uttar Pradesh (UP), India, and most remain undiagnosed. Hemorrhagic fever has also recently been reported in UP. Bats are reservoirs for henipaviruses and filoviruses and zoonotic transmission of these viruses directly and via livestock has occurred in Asia.

Country of Impact: India

Nonproliferation Objective and the Project's Role in Meeting this Objective: In conjunction with activities included in this proposal for Indonesia, Jordan, and Malaysia, EcoHealth Alliance (EHA) will leverage its global expertise in biological threat reduction across the wildlife-livestock-human interface and existing relationships in India to strengthen the country's capacity to test, diagnose, and respond to natural and manmade threats from viral zoonoses and other dangerous pathogens. The proposed activities meet the outlined nonproliferation objectives as follows: promoting biorisk management practices through training workshops with partners from Ministries of Health, Agriculture, Environment, and Defense; securing life science institutions and dangerous pathogens in BEP second-highest priority countries through biosecurity, biosafety, and rapid agent/pathogen detection capacity-building; decreasing the risk that scientists with dual-use expertise will misuse pathogens through biosecurity/biosafety training workshops and introducing surveillance and detection duties that foster positive US relations; promoting the detection, disruption, mitigation, and investigation of biological terrorism plots through increased capacity for rapid agent detection and regular communication among government partners; promoting the adoption of and compliance with comprehensive international frameworks thatadvance U.S. biological nonproliferation objectives through training workshops.

Scope of Work: The Luminex-based Bio-Plex assay is a quantitative, multiplex, bead-based technology that allows the user to screen for antibodies against viruses using a relatively small volume of serum per test (~1µL) compared to ELISA (~20 µL) and avoids the need for working with live viruses. *This is ideal*

as reservoir species for select agents can be tiny and provide only a few μL of serum. Our group has developed and validated Bio-Plex reagents that will detect antibodies for each of the known henipavirus and filovirus species and we have previously screened bat and livestock sera using these reagents.

EHA will leverage its long-standing relationships with Indian government and non-governmental sector partners supported by current USAID and DoD DTRA CBEP projects to implement and scale up rapid and efficient diagnostic capacity for antibodies against any filovirus or henipavirus using BioPlex systems, which can be updated as more reagents become available. Through a series of training workshops and transfer of the Bio-plex technology and reagents, EHA and partners will conduct surveillance in animal reservoirs and at-risk human populations for these zoonotic viruses. Placing this technology in India's hotspot for acute encephalitis syndrome will provide significant benefit to India's public health system. EHA and local implementing partners will communicate with GoI stakeholders to ensure dissemination of information.

EHA has a long history of collaboration with the **Uniformed Services University of the Health Sciences**, which has developed the full panel of henipavirus and filovirus antigens and multiplexed platform using the Biorad Bio-plex technology. The lab will provide these reagents and deliver training to our implementing partner lab at **Sanjay Gandhi Institute of Postgraduate Medical Sciences (**SGIPGMS) in Lucknow, UP. Human and animal sample testing will be conducted at SGIPGMS. We will also partner with **DUVASU veterinary college** in UP for trainings and sample collection. SGIPGMS, DUVASU, and EHA are partners under the USAID PREDICT India project and the proposed activities will also allow PREDICT to screen sera that would not otherwise be tested.

Deliverables: EHA will complete the following tasks:

A. Procure all necessary Biorad Bio-Plex machines and transfer reagents;
B. Conduct two training workshops: Biosecurity, biosafety and One Health surveillance; and Bio- training workshop for core lab staff
C. Hold monthly webinars and check-ins with partners
D. Perform 3 quality control assessments of deliverables F, G, H
E. Distribute training curriculum to participants and train future trainers to ensure sustainability.

Demonstrated ability of partner labs to:

F. Conduct human & animal surveillance with safe specimen collection and transfer to partner lab
G. Screen serum using Bio-plex technologies & data analysis
H. Disseminate laboratory results to stakeholders

Schedule/Timeline: The project will be 12 months, beginning the month funding is received.

Month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Deliverable	AC	ACF	ABC	CDFG	BC	CF	CF	CDG	CG	CG	CG	CDEFGH

Sustainability: This project will provide enhanced capacity to detect exposure to biothreats in a BEP priority country. Labs will learn how to produce reagents for the Bio-Plex machine so work can continue beyond the performance period. This will allow our partner lab to serve a wider network of diagnostic labs and conduct enhanced serological detection of the aforementioned agents in an area of high risk for zoonotic disease emergence. The surveillance and training activities will be conducted by EHA and in-country partners who are established medical and veterinary teaching institutions, providing credibility and sustainability. Our partners have direct lines of communication to federal health agencies which will maximize this project's impact. Participants will include government officers from animal and human health sectors, who will be trained in enhanced biosecurity, biosafety, and biosurveillance, which will promote sustainable practices beyond the project period. Partner labs will be sufficiently trained to sustainably conduct frontline detection of biothreats, addressing nonproliferation objectives after the project's conclusion.

From:	Kevin Olival on behalf of Kevin Olival <olival@ecohealthalliance.org>
To:	Eric Laing
Cc:	Chris Broder
Subject:	Re: Georgia and GHERI
Date:	Thursday, July 26, 2018 5:41:16 PM
Attachments:	EcoHealth Alliance BEP proposal 2018.docx ATT00002.bin

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Hey Eric,

Thanks, sorry things have been crazy and I haven't gotten much in way of writing done yet.

The general plan for the Liberia/CIV project is to screen human and bat sera for Filos and Henipas only. Wondering if the assays will capture this one too? http://punchng.com/new-ebola-virus-found-in-sierra-leone-govt-says/ https://twitter.com/EcoHealthNYC/status/1022567864656699392 Late breaker, more to come.

As we discussed, we're hoping to use your existing Bio-plex panels, and to export samples to USU for analysis. Still a lot to figure out - e.g. if we should have any capacity building piece to this, and also adding in some "predictive risk mapping" with the results, but by tomorrow afternoon should have this scoped out more. Can you guys advise on the cost per specimen and labor costs, so I can think about the number of specimens and sampling design a little more?

Also, attached is the language I have from one of our previous (unfunded) BEP proposals that I'll modify. Please feel free to track changes and flesh out the methods a little more or change them if needed.

Cheers,
Kevin

CTR PROJECT PROPOSAL - Enhancing Serological Biosurveillance for Select Agents
EcoHealth Alliance

CTR Unique Identifier: EHA_GLO_18_001

Point of Contact:
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Activity #1 Project Title: Serological Biosurveillance for Henipaviruses and Filoviruses in Uttar Pradesh, India

Activity #1 Project Summary: EcoHealth Alliance (EHA) proposes to enhance early detection and surveillance capacity in India by: 1) transferring Luminex-based Bio-Plex technology and validated reagents to detect IgG antibodies against all henipaviruses (e.g. Nipah virus) and filoviruses (e.g. Ebola virus) to the premiere UP medical laboratory – Sanjay Gandhi Institute of Postgraduate Medical Sciences; 2) training laboratory personnel to develop and utilize Bio-Plex assays; 3) conducting biological surveillance in bats, domestic animals and people in rural communities where there are high levels of contact among people and animals. Activities will be coordinated with, and complementary to the USAID Emerging Pandemic Threats: PREDICT program and the Global Health Security Agenda, and surveillance data will be shared with the Government of India (GoI). Henipaviruses and filoviruses are highly pathogenic zoonotic viruses and select agents capable of causing public health emergencies of international concern. Henipaviruses are associated with acute encephalitis and filoviruses are associated with hemorrhagic fever in people. A disproportionate number of cases of acute encephalitis occur in Uttar Pradesh (UP), India, and most remain undiagnosed. Hemorrhagic fever has also recently been reported in UP. Bats are reservoirs for henipaviruses and filoviruses and zoonotic transmission of these viruses directly and via livestock has occurred in Asia.

Country of Impact: India

Nonproliferation Objective and the Project's Role in Meeting this Objective: In conjunction with activities included in this proposal for Indonesia, Jordan, and Malaysia, EcoHealth Alliance (EHA) will leverage its global expertise in biological threat reduction across the wildlife-livestock-human interface and existing relationships in India to strengthen the country's capacity to test, diagnose, and respond to natural and manmade threats from viral zoonoses and other dangerous pathogens. The proposed activities meet the outlined nonproliferation objectives as follows: promoting biorisk management practices through training workshops with partners from Ministries of Health, Agriculture, Environment, and Defense; securing life science institutions and dangerous pathogens in BEP second-highest priority countries through biosecurity, biosafety, and rapid agent/pathogen detection capacity-building; decreasing the risk that scientists with dual-use expertise will misuse pathogens through biosecurity/biosafety training workshops and introducing surveillance and detection duties that foster positive US relations; promoting the detection, disruption, mitigation, and investigation of biological terrorism plots through increased capacity for rapid agent detection and regular communication among government partners; promoting the adoption of and compliance with comprehensive international frameworks that advance U.S. biological nonproliferation objectives through training workshops.

Scope of Work: The Luminex-based Bio-Plex assay is a quantitative, multiplex, bead-based technology that allows the user to screen for antibodies against viruses using a relatively small volume of serum per test (~1µL) compared to ELISA (~20 µL) and avoids the need for working with live viruses. *This is ideal*

as reservoir species for select agents can be tiny and provide only a few μL of serum. Our group has developed and validated Bio-Plex reagents that will detect antibodies for each of the known henipavirus and filovirus species and we have previously screened bat and livestock sera using these reagents.

EHA will leverage its long-standing relationships with Indian government and non-governmental sector partners supported by current USAID and DoD DTRA CBEP projects to implement and scale up rapid and efficient diagnostic capacity for antibodies against any filovirus or henipavirus using BioPlex systems, which can be updated as more reagents become available. Through a series of training workshops and transfer of the Bio-plex technology and reagents, EHA and partners will conduct surveillance in animal reservoirs and at-risk human populations for these zoonotic viruses. Placing this technology in India's hotspot for acute encephalitis syndrome will provide significant benefit to India's public health system. EHA and local implementing partners will communicate with GoI stakeholders to ensure dissemination of information.

EHA has a long history of collaboration with the **Uniformed Services University of the Health Sciences**, which has developed the full panel of henipavirus and filovirus antigens and multiplexed platform using the Biorad Bio-plex technology. The lab will provide these reagents and deliver training to our implementing partner lab at **Sanjay Gandhi Institute of Postgraduate Medical Sciences (**SGIPGMS) in Lucknow, UP. Human and animal sample testing will be conducted at SGIPGMS. We will also partner with **DUVASU veterinary college** in UP for trainings and sample collection. SGIPGMS, DUVASU, and EHA are partners under the USAID PREDICT India project and the proposed activities will also allow PREDICT to screen sera that would not otherwise be tested.

Deliverables: EHA will complete the following tasks:

A. Procure all necessary Biorad Bio-Plex machines and transfer reagents;
B. Conduct two training workshops: Biosecurity, biosafety and One Health surveillance; and Bio- training workshop for core lab staff
C. Hold monthly webinars and check-ins with partners
D. Perform 3 quality control assessments of deliverables F, G, H
E. Distribute training curriculum to participants and train future trainers to ensure sustainability.

Demonstrated ability of partner labs to:

F. Conduct human & animal surveillance with safe specimen collection and transfer to partner lab
G. Screen serum using Bio-plex technologies & data analysis
H. Disseminate laboratory results to stakeholders

Schedule/Timeline: The project will be 12 months, beginning the month funding is received.

Month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Deliverable	AC	ACF	ABC	CDFG	BC	CF	CF	CDG	CG	CG	CG	CDEFGH

Sustainability: This project will provide enhanced capacity to detect exposure to biothreats in a BEP priority country. Labs will learn how to produce reagents for the Bio-Plex machine so work can continue beyond the performance period. This will allow our partner lab to serve a wider network of diagnostic labs and conduct enhanced serological detection of the aforementioned agents in an area of high risk for zoonotic disease emergence. The surveillance and training activities will be conducted by EHA and in-country partners who are established medical and veterinary teaching institutions, providing credibility and sustainability. Our partners have direct lines of communication to federal health agencies which will maximize this project's impact. Participants will include government officers from animal and human health sectors, who will be trained in enhanced biosecurity, biosafety, and biosurveillance, which will promote sustainable practices beyond the project period. Partner labs will be sufficiently trained to sustainably conduct frontline detection of biothreats, addressing nonproliferation objectives after the project's conclusion.

Activity #2 Project Title: Enhancing Serological Biosurveillance for Select Agents in Indonesia

Activity #2 Project Summary: EcoHealth Alliance (EHA) will leverage its global expertise in biological threat reduction across the wildlife-livestock-human interface and long-standing relationships with government and non-governmental sector partners in Indonesia to strengthen the country's capacity to test, diagnose, and respond to natural and manmade threats from dangerous pathogens known to occur in the region. We will establish affordable and efficient Bio-Plex (e.g. Luminex-based) technologies for serological biosurveillance, and implement wildlife and human testing for Henipaviruses (Nipah, Hendra, Cedar viruses) and Filoviruses (Ebola viruses and Marburg). EHA will train staff from academic and government partner organizations to optimize the technology, enhance biosecurity and biosafety practices, and conduct frontline detection of dangerous pathogens in humans and animals. We will leverage specimens collected under USAID PREDICT-2 for initial screening. Additional resources will support ongoing specimen collection and optimization of additional locally-relevant assays (e.g. melioidosis) to ensure sustainable and continued use of the platform for pre-emptive zoonotic biosurveillance. At project conclusion, local partners will have demonstrated ability to rapidly and accurately detect and differentiate exposure to select agents; implement One Health surveillance; respond to frontline dangerous pathogen detection requests and facilitate specimen collection and transfer; and rapidly disseminate laboratory results to government partners.

Country of Impact: Indonesia

Nonproliferation Objective and the Project's Role in Meeting this Objective: In conjunction with projects included in this proposal for India, Jordan, and Malaysia, the proposed activities meet the outlined nonproliferation objectives as follows: promoting biorisk management practices through training workshops for partners from academia and Ministries of Health, Agriculture, Environment, and Defense; securing life science institutions and dangerous pathogens in BEP second-highest priority countries through biosecurity, biosafety, and rapid agent/pathogen detection capacity-building; decreasing the risk that scientists with dual-use expertise will misuse pathogens through biosecurity/biosafety training workshops and introducing surveillance and detection duties that foster positive US relations; promoting the detection, disruption, mitigation, and investigation of biological terrorism plots through increased capacity for rapid agent detection and regular communication among government partners; promote biorisk management through training workshops inclusive of government partners; promoting the adoption of and compliance with frameworks that advance U.S. biological nonproliferation objectives through training workshops.

Scope of Work: EHA will leverage its existing disease surveillance and laboratory partners in Indonesia and experts in the US to implement and scale up rapid diagnostic capacity using bio-plex technologies to screen human and animal specimens for exposure to select agents (Filo- and Henipaviruses initially, and begin developing melioidosis (*Burkholderia pseudomallei*) assays. We will enhance biosecurity and biosafety at partner labs and institutions that handle high risk biological specimens through training events. We will procure and transfer Bio-Plex technology, equipment and reagents to **Institut Pertanian Bogor, Primate Research Center** (IPB-PRC), and prepare staff from IPB-PRC and **Eijkman Institut** (where Bio-Plex technology already exists) to conduct frontline detection of bioweapons and dangerous pathogens. Multiplex PCR Bio-Plex platforms will be used to detect potential bioweapons and dangerous pathogens from specimens collected from surveillance of wildlife populations and hospitalized patients with fevers of unknown origin, hemorrhagic fevers of unknown origin, severe acute respiratory illnesses of unknown origin, as well as from outbreaks, suspicious substances, and emergency scenarios reported to the team by government partners. EHA and local implementing partners will regularly communicate with Ministries of Health, Agriculture, Environment, and Defense to facilitate this collaboration. EHA has a long history of collaboration with the **Uniformed Services University of the Health Sciences** (USUHS), which has developed the full panel of henipavirus and filovirus antigens and multiplexed platform using

the Biorad Bio-plex technology. USUHS will produce these reagents, serve as experts in trainings, and participate in QA/QC and data analysis. EHA will strengthen local capacity with partners from the USAID PREDICT project including IPB-PRC for wildlife surveillance; Eijkman Institut for human surveillance; and personnel from the Ministry of Agriculture's Disease Investigation Centers for training.

Deliverables:

EHA will complete the following tasks:

A. Procure all necessary Biorad Bio-Plex machines and produce or procure reagents

B. Host two training workshops. 1 workshop on biosecurity and biosafety and One Health outbreak response for local partners; 1 Bio-Plex training workshop for lab staff from IPB-PRC and Eijkman

C. Hold monthly webinars and check-ins for partners

D. Perform 3 quality control assessments of deliverables F, G, H

E. Distribute training curriculum to participants for each workshop and train future trainers to ensure sustainable transfer of knowledge

Demonstrated ability of partner labs to:

F. Conduct One Health surveillance and facilitate specimen collection and transfer to reference labs

G. Screen wildlife and human serum using Bio-Plex technologies

H. Disseminate laboratory results to appropriate partner(s)

Schedule/Timeline: The project will be 12 months, beginning the month funding is received.

Month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Deliverable	AC	ABC	ACF	BCD	C	CF	C	CD	CF	C	C	CDEFGH

Sustainability: This project will result in sustainable frontline detection of bioweapons and dangerous pathogens in a BEP second-highest priority country. Partner labs will be fitted with Bio-Plex technologies for rapid detection of the aforementioned agents, and the work will be conducted by in-country scientists working at government institutions and universities. The project involves close collaboration among government ministries and research institutions, and promotes regional collaboration and skills sharing between nations. In-country personnel will be trained in enhanced biosecurity, biosafety, and rapid agent detection, so the work will continue past the 12-month performance period but under the leadership of in-country staff and government partners. Training workshops will be conducted such that they will be reproducible by in-country scientists for future training of scientists/partners following the completion of the project. Country staff at government labs and research facilities will be sufficiently trained to sustainably conduct frontline surveillance and detection of bioweapons and dangerous pathogens addressing nonproliferation objectives after the project's conclusion.

Activity #3 Project Title: Enhancing Serological Biosurveillance for Henipaviruses, Filoviruses, and MERS-CoV in Jordan

Activity #3 Project Summary: In conjunction with projects included in this proposal for India, Indonesia, and Malaysia, EcoHealth Alliance (EHA) will leverage its global expertise in biological threat reduction across the wildlife-livestock-human interface and long-standing relationships with the Jordanian government and non-governmental sector to strengthen the country's capacity to test, diagnose, and respond to natural and manmade threats from viral zoonoses and other dangerous pathogens. We will establish affordable and efficient Luminex-based technologies (Biorad Inc., Bio-Plex) for serological surveillance and implement rapid diagnostic capacity for Henipaviruses (e.g., Nipah virus) and Filoviruses (e.g., Ebola virus), and likewise serological surveillance for Middle East Respiratory Syndrome Coronavirus (MERS-CoV) through a commercially-available ELISA. EHA will train staff at existing partner organizations to utilize the technology and optimize assays, enhance biosecurity and biosafety practices, and conduct frontline detection of dangerous pathogens in humans and animals. We will leverage specimens collected under USAID PREDICT-2 from humans, livestock and wildlife for initial screening. At project conclusion, local partners will have demonstrated ability to rapidly and accurately detect and differentiate exposure to select agents; implement One Health surveillance; respond to dangerous pathogen detection requests and facilitate specimen collection and transfer; and rapidly disseminate laboratory results to government partners.

Country of Impact: Jordan

Nonproliferation Objective and the Project's Role in Meeting this Objective: The proposed activities meet the outlined nonproliferation objectives as follows: promoting biorisk management practices through training workshops partners from Ministries of Health, Agriculture, Environment, and Defense; securing life science institutions and dangerous pathogens in BEP second-highest priority countries through biosecurity, biosafety, and rapid agent/pathogen detection capacity-building; decreasing the risk that scientists with dual-use expertise will misuse pathogens through biosecurity/biosafety training workshops and introducing surveillance and detection duties that foster positive US relations; promoting the detection, disruption, mitigation, and investigation of biological terrorism plots through increased capacity for rapid agent detection and regular communication among government partners; partnering with non-scientific interlocutors such as policymakers, law enforcement, military, and diplomatic audiences to promote biorisk management through training workshops inclusive of government partners; promoting the adoption of and compliance with comprehensive international frameworks that advance U.S. biological nonproliferation objectives through training workshops.

Scope of Work: EHA will leverage its existing relationships supported by current USAID and DoD DTRA CBEP projects to implement and scale up rapid diagnostic capacity for Henipaviruses and Filoviruses (using Luminex-based technologies), and likewise for MERS-CoV using ELISA, in addition to enhancing biosecurity and biosafety at partner labs that handle high risk biological specimens.

Through a series of training workshops and transfer of the Bio-Plex technology and reagents assays and required laboratory equipment, EHA will prepare staff to conduct frontline detection of dangerous pathogens. Multiplex PCR Bio-Plex platforms will be used to detect exposure to these zoonotic viruses from specimens collected from surveillance in animal reservoirs and at-risk human populations for these zoonotic viruses. EHA and local implementing partners will regularly communicate with Ministries of Health, Agriculture, Environment, and Defense to facilitate this collaboration.

EHA has a long history of collaboration with the **Uniformed Services University of the Health Sciences** (USUHS), which has developed the full panel of henipavirus and filovirus antigens and multiplexed platform using the Biorad Bio-Plex technology. The lab will produce these reagents and deliver training

to each partner lab in country. They will also participate in QA/QC and data analysis. In Jordan, we will continue our partnerships with **Jordan University for Science and Technology** and the **Princess Haya Biotechnology Center**, to focus on Henipaviruses, Filoviruses, and MERS.

Deliverables:

EHA will complete the following tasks:

A. Procure all necessary Biorad Bio-Plex machines and produce or procure reagents

B. Host two training workshops. 1 workshop on biosecurity and biosafety and One Health outbreak response for local partners; 1 Bio-Plex training workshop for core lab staff

C. Hold monthly webinars and check-ins for partners

D. Perform 3 quality control assessments of deliverables F, G, H

E. Distribute training curriculum to participants and train future trainers to ensure sustainable transfer of knowledge.

Demonstrated ability of partner labs to:

F. Conduct One Health surveillance and facilitate specimen collection and transfer to reference labs

G. Screen serum using Bio-Plex technologies

H. Disseminate laboratory results to appropriate partner(s)

Schedule/Timeline: The project will be 12 months, beginning the month funding is received.

Month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Deliverable	AC	ABC	ACF	BCD	C	CF	C	CD	CF	C	C	CDEFGH

Sustainability: This project will result in sustainable frontline detection of dangerous pathogens in multiple BEP second-highest priority countries. Partner labs will be fitted with Bio-Plex technologies for rapid detection of the aforementioned agents, and the work will be conducted by in-country scientists working at government institutions and universities, trained by EHA scientists. The project involves close collaboration among government ministries and research institutions, and promotes regional collaboration and skills sharing between nations. In-country personnel will be trained in enhanced biosecurity, biosafety, and rapid agent detection, so the work will continue past the 12-month performance period but under the leadership of in-country staff and government partners. Training workshops will be conducted such that they will be reproducible by in-country scientists for future training of scientists/partners following the completion of the project. Country staff at government labs and research facilities will be sufficiently trained to sustainably conduct frontline detection of dangerous pathogens addressing nonproliferation objectives after the project's conclusion.

Activity #4 Project Title: Serological Biosurveillance for Henipaviruses and Filoviruses in Sabah, Malaysia

Activity #4 Project Summary: EcoHealth Alliance (EHA) proposes to build on our earlier work in Malaysia to enhance early detection and surveillance capacity by: 1) transferring Luminex-based Bio-Plex technology and validated reagents to detect IgG antibodies against all henipaviruses and filoviruses to the PREDICT lab at the Sabah Wildlife Department; 2) training laboratory personnel to develop and utilize Bio-Plex assays; 3) conducting biological surveillance in bats, rodents and primates in rural communities and ecotourism sites where there are high levels of contact among people and animals. Activities will be coordinated with, and complementary to USAID's Emerging Pandemic Threats: PREDICT program, the Global Health Security Agenda, and DTRA supported activities. Surveillance data will be shared with the Government of Sabah. Henipaviruses (e.g. Nipah virus) and filoviruses (e.g. Ebola virus) are highly pathogenic zoonotic viruses and select agents capable of causing public health emergencies of international concern. Bats in Malaysia are reservoirs for Nipah virus and possibly filoviruses. Zoonotic transmission of Nipah virus via livestock has previously occurred in Malaysia. Sabah, located in Borneo, has a large diversity of bats and areas where human contact is common.

Country of Impact: Malaysia

Nonproliferation Objective and the Project's Role in Meeting this Objective: In conjunction with projects included in this proposal for India, Jordan, and Indonesia, the proposed activities meet the outlined nonproliferation objectives as follows: promoting surveillance and biorisk management practices through training workshops with participants from departments of Health, livestock, and wildlife, and the University of Malaysia, Sabah Medical college in a BEP priority country. Activities under this project will also enhance the semi-autonomous state of Sabah's capabilities to detect spillover or exposure to select agents in animals and people. These activities support current priorities and One Health surveillance activities conducted by the PREDICT project in partnership with the government of Sabah. Sharing findings with state government policymakers will advance U.S. biological nonproliferation objectives through training workshops.

Scope of Work: EHA will leverage its long-standing relationships with Malaysian government and non-governmental sector partners supported by current USAID and DoD DTRA CBEP projects to implement and scale up rapid and efficient diagnostic capacity for antibodies against any filovirus or henipavirus using Bio-Plex systems, which can be updated as more reagents become available. The Luminex-based Bio-Plex assay is a quantitative, multiplex, bead-based technology that allows the user to screen for antibodies against viruses using a relatively small volume of serum per test (~1μL) compared to ELISA (~20 μL) and avoids the need for working with live viruses. *This is ideal as reservoir species for special agents are tiny and provide only a few μL of serum.* Our group has developed and validated Bio-Plex reagents that will detect antibodies for each of the known henipavirus and filovirus species and we have previously screened bat and livestock sera using these reagents. Through a series of training workshops and transfer of the Bio-plex technology and reagents, EHA and partners will conduct surveillance in animal reservoirs and at-risk human populations for these zoonotic viruses. As part of ongoing DTRA and PREDICT work BioPlex has been successfully installed and implemented in Peninsular Malaysia partner labs; this activity will leverage this partnership and expand this technology to Sabah, Malaysia (which is geographically and administratively distinct from Peninsular Malaysia), enhancing Malaysia's overall capacity to rapidly detect and respond to zoonotic viruses. This project will also strengthen collaboration and skills sharing between Peninsular Malaysia and Sabah through One Health biosecurity and biosafety trainings. EHA and local implementing partners will regularly communicate with state and federal stakeholders to ensure sharing of information and capacities.

EHA has a long history of collaboration with the Broder lab at the **Uniformed Services University of the Health Sciences** (USUHS), which has developed the full panel of henipavirus and filovirus antigens and multiplexed platform using the Biorad Bio-Plex technology. The lab will produce these reagents and deliver training to each partner lab in country. In Malaysia, we will continue our existing partnerships under USAID and DoD DTRA CBEP support with **Conservation Medicine, Ltd**., and the **Sabah Wildlife Department** (a state government agency). We will focus on screening wildlife sera for antibodies against henipaviruses and filoviruses at the PREDICT lab shared with Sabah Wildlife Department, with the potential to expand this capability to Sabah State Health Department labs in the future.

Deliverables:

EHA will complete the following tasks:

A. Procure all necessary Biorad Bio-Plex machines and produce or procure reagents
B. Host two training workshops. 1 workshop on biosecurity and biosafety and One Health outbreak response for local partners; 1 Bio-Plex training workshop for core lab staff
C. Hold monthly webinars and check-ins for partners
D. Perform 3 quality control assessments of deliverables F, G, H
E. Distribute training curriculum to participants and train future trainers to ensure sustainable transfer of knowledge.

Demonstrated ability of partner labs to:

F. Conduct One Health surveillance and facilitate specimen collection and transfer to reference labs
G. Screen serum using Bio-plex technologies
H. Disseminate laboratory results to appropriate partner(s)

Schedule/Timeline: The project will be 12 months, beginning the month funding is received.

Month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Deliverable	AC	ACF	ABC	CDFG	BC	CF	CF	CDG	CG	CG	CG	CDEGH

Sustainability: This project will result in sustainable frontline detection of bioweapons and dangerous pathogens in a BEP second-highest priority country. Partner labs will be fitted with Bio-Plex technologies for rapid detection of the aforementioned agents, and the work will be conducted by in-country scientists working at government institutions and universities, trained by EHA scientists. The project involves close collaboration among government ministries and research institutions, and promotes regional collaboration and skills sharing between nations. In-country personnel will be trained in enhanced biosecurity, biosafety, and rapid agent detection, so the work will continue past the 12-month performance period but under the leadership of in-country staff and government partners. Training workshops will be conducted such that they will be reproducible by in-country scientists for future training of scientists/partners following the completion of the project. Country staff at government labs and research facilities will be sufficiently trained to sustainably conduct frontline detection of dangerous pathogens addressing nonproliferation objectives after the project's conclusion.

From: Emma Lane on behalf of Emma Lane <lane@ecohealthalliance.org>
To: Broder, Christopher
Cc: Jon Epstein
Subject: Re:
Date: Friday, January 26, 2018 2:10:17 PM
Attachments: EHA_GLO_18_001_PROJECT SUMMARY.pdf

Hi Chris,

Please find the Project Summary from the submission. Please let me know if you need anything else.

Thanks,
Emma

Emma Lane
Program Coordinator
PREDICT 2 Country Liaison, Liberia

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www.ecohealthalliance.org

On Fri, Jan 26, 2018 at 1:44 PM, Broder, Christopher <christopher.broder@usuhs.edu> wrote:

> hi Emma
>
> Could you please send me the info from the state dept grant
> that went in last week?
>
> title, dates, number?
>
> thanks
> CB
>
> --
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> and Emerging Infectious Diseases
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(b) (6) [redacted]