ATTACHMENT A: Scope of Work Year 4

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 this contract, supervised and coordinated by the co-Investigators Dr. Supaporn Wacharapluesadee and Dr. Opass Putcharoen, the Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University will:

1. Project coordination, management and training

1.1 Increase the number of samples from human community, and hospital syndromic study. Either by new enrolment, or archived samples.

1.2 Work with EID-SEARCH global team to update protocols and questionnaires for community and hospital syndromic surveillance.

1.3 Facilitate and coordinate training on project data management and analysis (e.g., Airtable, serology result interpretation), biosafety, human subject research (CITI training) and complete relevant assessments.

1.4 Collaborating with project partner for training and analysis on bioinformatics (bioinformatics analysis and RBD/spike glycoprotein design), MMIA technique (bead design, coupling, optimization and data analysis), Field Biosafety training.

1.5 Maintain all research approval documents in Thailand, including Animal Care and Use Protocol (CU-ACUP) for animal investigations; Institutional Review Board (IRB) approval for human subject research; Institutional Biosafety Committee (IBC) approval for lab work; and other local permissions as needed.

2. Fieldwork

2.1 Enroll additional human participants from community sites in Thailand human-wildlife contact is common – to aim for a total of 500 people. – To add new study site in Chanthaburi province, where there are human-wildlife interfaces e.g., bat capture and bat consumption
129 people from Ratchaburi were enrolled.
Need 371 people.
a. Chanthaburi (eating bat): 120
b. Ratchaburi (bat guano collectors and Mojiang-positive villagers): 70*2 times=140
c. Chonburi (villagers from Nipah positive flying foxes): 120

2.2 Enroll additional participants at selected hospital/clinic sites for syndromic surveillance to aim for a total of 500 people. – To add a new hospital/clinic site in Surathani province
140 patients from 4 sites were enrolled, including 59 from the diarrhea outbreak.
Need 360 people.
a. Ratchaburi clinic to continue enrolling the Flu system patients: n=50
b. Mukdaharn to enroll severe flu-like illness, encephalitis, non-dengue hemorrhagic fever: n=100
c. Surat Thani to enroll severe flu-like illness, encephalitis, non-dengue hemorrhagic fever: n=100
d. Unidentified Flu-like illness and encephalitis patients from DDC Lab surveillance: n=100
e. Archived samples from Bacteria and Virus infection project, n=250; only serum samples are available. Test for serology only, if it is positive, PCR will be performed on a specific family.
a+b+c+d+e= 600

2.3 Identify archived samples that would also fit the criteria for 2.1 and 2.2 – We could use samples from other projects that fit criteria for this study??
2.1 Could we use PREDICT samples (>400) because we tested them?
If yes, we do not need to do 2.1b and 2.1c. However, we want to continue the human study at Ratchaburi.

2.4 Collect PBMCs from individuals with detectable antibodies against the Mojiang-related viruses for further characterization.
Collect PBMCs from 5 positive Moj and 5 control.

2.5 Conduct specimen collection from bats from different colonies to those already sampled (n=200), rodents/shrews (150 rodents, 150 shrews), and macaques (100 from diverse locations) at the selected sites, with increased sampling efforts for rodents/tree shrews and non-human primates. **(TOTAL animals=600)–** 500 macaques archived specimens from DNP, which we will use for screening by MMIA and/or sVNT
– adding a new study site, Khlong Saeng Wildlife Sanctuary in Surathani province, where there are flying foxes and other insectivore bats (waiting for more information about bat species from Nutthinee) and the viruses in bats at this study site have not been extensively studied
a. Bat at Surat Thani: colony 1=100
b. Bat at Petchaburi (archived samples from DNP): n=100
c. Rodents and shrew from Surat Thani: n=100
d. Rodents and livestock at Moj positive village: n= 120 (3 times collection, 40*3=120)
e. Macaques: archived samples from DNP: n=500, only 100 for PCR

TOTAL animals: a+b+c+d+e = 920 (520 for PCR)

2.6 Targeted investigation to identify the animal hosts and transmission of Mojiang-related viruses.
a. Collect neighboring villagers' blood for serology testing (2.1 b)
b. Collect rodents from Moj-positive house and working places bimonthly to test for antibodies and Moj PCR (2.5d).

Commented [SN1]: Yellow highlight = what we also planned to do

Blue text = adding more details from what we brainstormed in the last weekly meeting

Commented [SW2R1]: Red= detail of works

c. Investigate other pet/farm animals at the Moj positive lady, collect specimens for testing.

3. Laboratory analysis

3.1 Conduct viral family-level molecular screening of collected or archived wildlife samples for coronaviruses and paramyxoviruses. (*Filoviruses testing is suspended due to low detection rate in swabs*)
No. of animals = 520 * feces sample* 2 families = 1,040 PCRs

3.2 Improve primers to better identify diverse paramyxoviruses (e.g. Langya, other Mojiang-related viruses).
Primers and positive control design and synthesis

3.3 Conduct DNA barcoding to confirm field animal species identification as necessary on a subset of the specimens.
10% positive, n=104 samples

3.4 Conduct deep sequencing of the Spike protein of identified novel coronaviruses. – we might need to be more specific e.g., only do sequencing on sarbecovirus positive specimens
Need the protocol for Spike deep sequencing for all CoV genus.

3.5 Perform whole genome sequencing of identified CoVs from humans and animals.
a. Perform bat CoV WGS using pan-CoV enrichment kit
b. Perform human CoV WGS using RVOP enrichment kit.
c. Perform metagenomic (whole genome) sequencing of identified PMV using Oxford Nanopore Technologies

3.6 Perform family PCR testing of coronaviruses, filoviruses, and paramyxoviruses with swabs collected from humans enrolled at clinic/hospital-based and community sites.
No. of patients (2.2a-d) = 350*3 specimens (rectal, oral, and blood)*3 families = 3,150 PCRs

3.7 Perform serology testing on collected and archived wildlife and human samples.

- Perform serological testing with optimized multiplex assays for Coronaviruses, Filoviruses, and Henipaviruses.
  Animals No. total= 2,170 (animals: bat= 856+200, rodents=214+200, macaque=200+500), 100 rodents and 100 macaque samples were tested; 1,970 will be performed in year 4
  Humans= No. n= 739 (patients); n= 380 (community)
- Perform serological characterization of Sarbecovirus-positive samples using surrogate virus neutralization test (sVNT) RBD-based assay.
  All bats n = 1,056

3.8 Collaborate with EID-SEARCH partner for the validation of newly developed serological assays.
???

3.9 Collaborate with EID-SEARCH partner for further characterization of novel viruses including virus isolation, human cell infection experiments, and animal modeling for viral pathogenesis studies.
OK

4. Results and data sharing and analysis

4.1 Share or update sample collection, lab testing, questionnaire, notes, lab notebooks, or all other relevant data and results to the project central database in a timely manner.

4.2 Sharing of specimens will ONLY be possible for diagnostic purposes when capacity is not available in Thailand or when it's part of the capacity building and training program.

4.3 Participate in the CREID Working Group discussions and work with EID-SEARCH global team to develop data management and sharing policies.
4.4 Maintain current contacts with in-country government ministries responsible for human health, livestock/agriculture, and wildlife for project results debriefs.
4.5 Collaborate with the EID-SEARCH partners for data cleaning, analysis, interpretation, and contributing to scientific publications as agreed.
4.6 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**.
4.7 Complete the following programmatic and financial reporting by requested deadlines:

- Quarterly reporting to EcoHealth Alliance on programmatic activities
- Semi-annual and annual reports to NIH/NIAID.
- Annual reports to the CREID Network and other reports requested by NIAID.
- Invoices and financial reports to EcoHealth Alliance every two months, including 1-page or longer programmatic update, copies of receipts and/or other supporting document, and breakdown of the costs in each item as showed in **ATTACHMENT B** Project Budget.
- Submit to the requirements of the Federal Funding Accountability and Transparency Act (FFATA) included as **ATTACHMENT C,** with the signed contract.

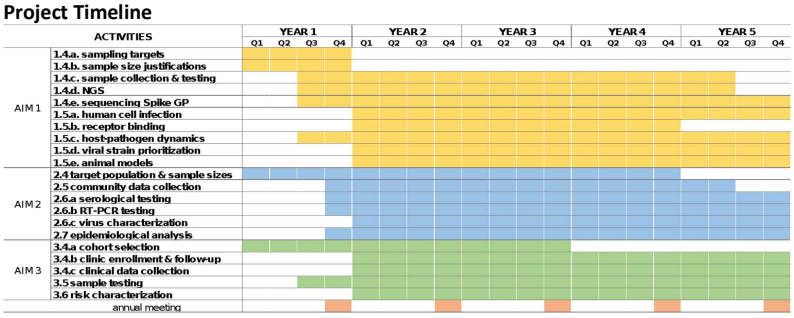
4.8 Reach out to communities and present appropriately available findings and public health information. – One Health Committee
~~4.9 Reach out to communities and present appropriately available findings and public health information.~~

5. Communication and reporting

5.1 Participate in monthly meetings with the EID-SEARCH global team and EcoHealth Alliance.
5.2 Participate in monthly meetings with all EID-SEARCH partners.
5.3 Participate in lab biosafety evaluation and regular research facilities inspection.
5.4 Represent EID-SEARCH on planning and other relevant meetings.
5.5 Complete the following programmatic and financial reporting by the requested deadlines:

- Semi-annual and annual reports to NIH/NIAID.
- Invoices and financial reports at least quarterly, including copies of receipts and/or other supporting documents, and a breakdown of the costs in each item as shown in Attachment C. A summary of programmatic updates accompanying all invoices or financial reports is required.
- Other reports requested by the CREID Network and NIH/NIAID.

6. Project Timeline



ATTACHMENT B: Project Budget

The project budget must correspond to the funder-approved proposal budget. Any and all modifications must be approved by EcoHealth Alliance before this contract is signed.

Items	Year 3
1. Personnel	$ 63,600.00
2. Fieldwork	
2.1 Human specimens sampling	$ 14,748.67
2.2 Animal specimens sampling	$ 20,500.00
3. Diagnosis	
3.1 Family PCR	$ 55,100.00
3.2 Barcoding PCR	$ 2,500.00
3.3 Spike gene and Next-generation sequencing	$ 15,000.00
3.4 Specific real-time PCR	$ 15,000.00
3.5 MMIA in human and animal samples	$ 3,500.00
3.6 Equipment maintenance	$ 5,000.00
4. Equipment (laptop and portable printer**)**	$ 5,000.00
Total direct cost	199,948.67
Indirect cost (8%)	$15,995.89
Total	$215,944.56

Items	Proposed Year 4	Year 3
1. Personnel	$ 63,600.00	$ 63,600.00
2. Fieldwork		
2.1 Human specimens sampling	**$ 47,074.17**	$ 14,748.67
2.2 Animal specimens sampling	$ 26,347.00	$ 20,500.00
3. Diagnosis		
3.1 Family PCR (3.1+3.6)	**$ 127,750.00**	$ 55,100.00
3.2 Improve Paramyxovirus primers	**$ 1,500.00**	0
3.3 Barcoding PCR	$ 3,120.00	$ 2,500.00
3.4 Spike gene and Next-generation sequencing	0	$ 15,000.00
3.4 Specific real-time PCR	**$ 37,500.00**	$ 15,000.00
3.5 MMIA in human and animal samples	**$ 14,507.50**	$ 3,500.00
3.6 Equipment maintenance	$ 5,000.00	$ 5,000.00
4. Equipment (camera trap)	$ 5,000.00	$ 5,000.00
Total direct cost	**$ 331,398.67**	199,948.67
Indirect cost (8%)	$ 26,511.89	$15,995.89
Total	**$ 357,910.56**	**$215,944.56**

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

Commented [HL3]: Copied pasted from submitted proposal, modified one place to remove previous co-workers' names, the project name also changed from EIDRC SEARCH to EID-SEARCH

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:	Supaporn Wacharapluesadee; Opass ID; Sasiprapa Ninwattana; Ananporn Jenny supataragul; Khwankamon Rattanatumhi; eric.laing_usuhs; Spencer Sterling; Kevin Olival; Cadhla Firth; Peter Daszak
Cc:	Aleksei Chmura
Subject:	Thailand EID-SEARCH Meeting this week August 2/3
Date:	Monday, July 31, 2023 9:28:36 AM
Attachments:	Thailand Y4 Scope of Work_v02_SN_SW.docx

Dear All,

This is a reminder of the Thailand EID-SEARCH Meeting **this Wednesday (9 PM NYC) and Thursday (8 AM BKK)** when we'll discuss the Y4 Scope of Work and check on a few items:

- Year 4 project Scope of Work in Thailand (please see the attached Word document with edits from both EHA and TRC-EIDCC teams)
- Update on the Mpox concept notes (budget)
- Update on papers
- Others

Look forward to the discussion.

Dear Dr. Supaporn - Thank you for sharing the edits. I didn't include the PPT slides in the email since there is budget information that you may want to present from your side at the meeting.

All the best,
Hongying

Hongying Li, MPH
Senior Program Manager & Senior Research Scientist

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SUMMARY STATEMENT

PROGRAM CONTACT:
Eun-Chung Park
240-627-3338
epark@niaid.nih.gov

(Privileged Communication)

Release Date: **07/12/2023**
Revised Date:

Application Number: **1R01AI179865-01**

Principal Investigators (Listed Alphabetically):

LAING, ERIC
ROSS, NOAM (Contact)

Applicant Organization: ECOHEALTH ALLIANCE, INC.

Review Group: **TVZ**
Transmission of Vector-Borne and Zoonotic Diseases Study Section

Meeting Date: **06/12/2023**
Council: **OCT 2023**
Requested Start: **09/01/2023**

Opportunity Number: **PA-20-185**
PCC: **M32A B**

Project Title: **Characterizing unknown emerging viruses through targeted serological profiling**

SRG Action: **++**
Next Steps: **Visit https://grants.nih.gov/grants/next_steps.htm**
Human Subjects: **X4-Human subjects involved - Exemption #4 designated**
Animal Subjects: **30-Vertebrate animals involved - no SRG concerns noted**

Project Year	Direct Costs Requested
1	492,733
2	489,677
3	499,863
4	498,388
5	499,507
TOTAL	2,480,168

++NOTE TO APPLICANT: Members of the Scientific Review Group (SRG) were asked to identify those applications with the highest scientific merit, generally the top half. Written comments, criterion scores, and preliminary impact scores were submitted by the assigned reviewers prior to the SRG meeting. At the meeting, the more meritorious applications were discussed and given final impact scores; by concurrence of the full SRG, the remaining applications, including this application, were not discussed or scored. The reviewers' comments (largely unedited by NIH staff) and criterion scores for this application are provided below. Because applications deemed by the SRG to have the highest scientific merit generally are considered for funding first, it is highly unlikely that an application with an ND recommendation will be funded. Each applicant should read the written critiques carefully and, if there are questions about the review or future options for the project, discuss them with the Program Contact listed above.
ADMINISTRATIVE NOTE
BIOHAZARD
NEW INVESTIGATOR

1R01AI179865-01 Ross, Noam

BIOHAZARD COMMENT
NEW INVESTIGATOR
SCIENTIFIC REVIEW OFFICER'S NOTES

DESCRIPTION (provided by applicant): Serology remains under-utilized as a reliable surveillance tool for the detection of emerging zoonotic viruses. Detection and estimation of Ebola virus spillovers is dependent on outbreak scale and thus may not be caught through early warning systems. The geographies at-risk for Nipah virus, and bat-borne and rodent-borne henipaviruses spill over across sub-Saharan Africa, Asia and Oceania remain under- surveyed. Limitations of serosurveillance in addressing virus emergence can be found at both the front- end in serological assay design and development, and back-end in the statistical frameworks used to analyze data. Both need to be adapted to the unique challenges associated with known emergent viruses and novel unknown viruses not yet genetically characterized. These challenges include cross-reactivity of antigens and antibodies and complex immune responses. The major goal of this proposal is to dramatically improve the utility of antigen-based serosurveillance for new viral discovery and epidemiology of emerging zoonotic viruses by addressing the challenges of cross-reaction and statistical rigor. We will create discovery panels - qualified and optimized panels of multiplexed immunoassays designed to identify a characterize previously unknown viruses only detected through the serologically profiling wildlife and human hosts. We will develop new statistical approaches to extract the signals of novel viruses from those of known viruses or mixtures of virus-specific antibodies from complex histories multiple previous viral exposures, as well as integrate these into models into population-scale epidemiological models. Finally, we will partner with international viral surveillance projects to apply these techniques to identifying and characterizing filoviruses and henipaviruses in bat populations in South Africa and workers at-risk for exposure to wildlife viruses in Thailand, and to modeling immune-epidemiological dynamics of Nipah virus circulation in bats and people in Bangladesh.

PUBLIC HEALTH RELEVANCE: This project develops new methods to identify emerging and zoonotic viruses by analyzing the virus- induced antibodies present in wildlife and human populations in contact with wildlife. It will create both new laboratory techniques to test for those antibodies as markers of infection and new mathematical models to analyze data to identify the trace of those viruses. It will test these methods in partnership with international virus surveillance projects in Bangladesh, Thailand, and South Africa.

CRITIQUE 1

Significance: 6
Investigator(s): 5
Innovation: 4
Approach: 8
Environment: 4

Overall Impact: The project seeks to develop a novel serological platform to identify emerging and zoonotic viruses by analyzing the virus induced antibody profiles present in wildlife and human populations at risk. The measurable outcomes would include new laboratory techniques for serodiagnostics and new mathematical models to analyze the seroprevalence data to for previously characterized and emerging viruses. Although investigative team is remarkably strong with a track record of independent and collaborative success, this specific project builds on a relatively weak

scientific premise that serological methods (as described in the proposal) can be applied to detect novel/emerging, previously uncharacterized viruses. This seems fundamentally wrong, because even current state-of-the-art serological techniques can only target KNOWN antigens. Another important issue is that the PI failed to emphasize the advantage and significance of the proposed approach compared with sequence based or comprehensive techniques for virus discovery/characterization. Further, the proposal refers to multiple limitations of the current serological techniques but fails to describe how those will be addressed. The diversity and complexity of the immune response in the target wildlife species (including bats) is poorly acknowledged. Preliminary data do not seem to be compelling. The model organism chosen for experimental studies (rabbit) does not seem appropriate, as it's not representative of the biology/immunology of wildlife reservoir species of the target viruses. The grant is written poorly and there is a lack of clarity in details throughout. There are other issues that will be detailed in individual sections. Overall enthusiasm for this proposal is very low.

1. Significance:

Strengths

- Ebola and Nipah viruses are a significant public health threat, and tools for their real-time monitoring/detection of new variant emergence are of high significance

Weaknesses

- The approach selected decreases the overall study significance
- The use of serology vs. molecular/sequence-based methods is not justified (other than it was mentioned that it's underutilized). Does not seem feasible or necessary
- The team acknowledged the challenges associated with serological methods that include cross-reactivity and complex/diverse immune responses. The team does not propose feasible strategies to circumvent those. Additionally, they do not acknowledge other issues associated with serological detection of viruses – you can only target known antigens/antibodies. Sensitivity of serological detection is way lower than that of molecular diagnostic methods. Duration and magnitude of immune responses vary per host and per virus, etc.
- The approach to identify shadow viruses by subtracting signals to known viruses does not seem feasible. Proof-of-concept experiments were not done, or results were not presented.
- Although, the PI states that the proposed approach will identify and CHARACTERIZE novel pathogens, the CHARACTERIZE component is not explain well, while the ability to identify novel pathogens using it is highly questionable.

2. Investigator(s):

Strengths

- A very strong team with highly complementary expertise. The team includes a mathematical and statistical disease ecologist, 2 virologists, 2 infectious disease epidemiologists/specialists, and 2 virologist-ecologists experienced with a variety of emerging and zoonotic viral diseases.
- Established collaborations are also beneficial.

Weaknesses

- No immunologist on the team shows in the lack of in-depth understanding of multiple challenges associated with species-specific immune responses, their diversity, complexity, etc.
- No wildlife (specifically bat) specialist on the team is also detrimental.

3. Innovation:

Strengths

- Using serology for virus discovery/characterization.

Weaknesses

- The use of microsphere-based multiplex immunoassay platforms is not particularly innovative.

4. Approach:

Strengths

- None noted.

Weaknesses

- The Approach has countless deficiencies including the main ones: flawed scientific premise, rabbit model to develop assays for bats and humans, extremely vague description of the proposed immunological experiments, the lack of a clearly presented rationale for detection of antibodies to unknown pathogens using specific reagents derived from previously characterized pathogens, and many others.
- Specifically, rabbits are highly immuno-reactive animals, they lack serological background against the target viruses. Neither they are the natural hosts of these viruses, which will affect the immune response characteristics. Targeted antibody isotypes (that vary between different hosts) are not mentioned.
- Immunization regimen is poorly and vaguely described. Not clear how the antigens will be derived, purified, processed. Not specified numbers of immunizations, timing between each, the time of antiserum harvest, etc.
- The team does not take in the account the immunological phenomena including antigenic sin, whereby exposure to the primary related antigen/pathogen affects subsequent immune responses against related antigens/pathogens.
- Preliminary data needed to prove feasibility of the proposed approach are not provided. At this point, the team should have had a proof that it is possible to detect a signal against an unknown (shadow) virus by simply subtracting the signal against known related viruses. The available preliminary data are not very compelling.
- The proposed statistical models do not seem to account for way too numerous biological variables across different species, pathogens, etc.
- Another factor that would greatly impact feasibility of the proposed research is the variable and sometimes unknown duration of antibody response against the target viruses. While it appears that in experimental rabbits the exposure to different antigens will be time-matched, and the magnitudes of the responses to the non-replicating antigens will be comparable, it is not the case in bats or humans, where time post-infection will vary greatly, and the magnitude/characteristics of the antibody response will be greatly affected by this and the fact.
- Besides the fact that bats are capable of reducing pro-inflammatory response, some studies reported that their antibody response to various test antigens may be lower compared to what is seen in conventional laboratory animals.
- While the team acknowledges that sex and age are important biological variables affecting antibody responses, they do not seem to account for it in their rabbit immunization experiments.

- At the genomic level, bats seem to possess a much larger repertoire of germline genes encoding immunoglobulin variable (V), diversity (D) and joining (J) segments than humans or rabbits, which may provide a much larger number of antigen specificities in their naive B cell receptor (BCR) repertoire. This suggests that antibody detection discovery matrix would not work for bats as it would work for rabbits or humans (even if it's possible for the latter species).

5. Environment:

Strengths

- The environment seems to be suitable for the proposed research with access to computer/bioinformatics and experimental lab resources.

Weaknesses

- Access to an advanced immunological lab focusing on human and bat adaptive immune responses is needed.

Study Timeline:

Strengths

- Sufficient time seem to be provided for Aim 2.

Weaknesses

- May be not feasible for the proposed experimental (Aim 1) and surveillance (Aim 3) studies.

Protections for Human Subjects:

Acceptable Risks and/or Adequate Protections

- The study will only access previously collected samples

Inclusion Plans:

- Sex/Gender: Distribution justified scientifically
- The sex is acknowledged as a biological variable throughout

Vertebrate Animals:

YES, all criteria addressed

Biohazards:

Unacceptable

- Not described, while it is perceivable that viruses will be used at some point to generate antigens for rabbit immunization

Applications from Foreign Organizations:

Justified

Select Agents:

Unacceptable

- Not discussed

Resource Sharing Plans:

Unacceptable

- Seems to be missing, while referred to multiple times.

Authentication of Key Biological and/or Chemical Resources:

Unacceptable

- Not sufficient detail regarding the sources of the antigens

Budget and Period of Support:

Recommend as Requested

CRITIQUE 2

Significance: 5
Investigator(s): 1
Innovation: 1
Approach: 5
Environment: 1

Overall Impact: This R01 proposal addresses questions related to serological cross reactivity between filoviruses and henipaviruses, which has confounded serosurveillance, virus discovery, and efforts to incriminate reservoir hosts for these viruses. The investigators propose to create innovative multiplex serological panels that are optimized for virus discovery and combine these with novel statistical methods to solve the cross-reactivity problem. As a result, this is a highly innovative approach from both a technical and conceptual standpoint. In addition, the investigative team is outstanding, and a major strength of the proposal is the ability to leverage several different disease surveillance networks to ground-truth the development of their assay and statistical approaches. However, there were some issues with the experimental design and presentation of the scientific premise that lowered my overall enthusiasm. One major weakness was that it is unclear how these tools would be used to identify putative reservoir hosts, when multiple animal species might be exposed and seroconvert. The other major weakness was the ambitious nature of the proposal which only provided limited depth to each study component. In addition, critical preliminary data were not included in the proposal. This made it difficult to evaluate everything that was being proposed. In conclusion, despite there being laudable aspects related to virus discovery and pandemic preparedness, the potential overall impact of this proposal is only moderate.

1. Significance:

Strengths

- It has been long recognized that cross-reactivity between closely related, antigenically variable viruses is a problem for serosurveillance. This has confounded diagnostics and has made it difficult to incriminate zoonotic reservoirs for emerging viruses.
- Incriminating zoonotic reservoirs for emerging viruses is important for prediction, prevention, and control efforts to mitigate the risk posed by these viruses.
- Improved serological assays have utility in diagnostics as well as for virus discovery, and if successful this approach would be useful for other virus families where serological cross-reactivity is a known problem.

Weaknesses

- It is unclear—even with improved serological approaches—how these data would be used to incriminate an animal species as a putative reservoir host without molecular (or some other) evidence of infection. Many animals that come into close contact with the reservoir may have abortive or asymptomatic infection where virus is not shed but still result in seroconversion. This does not mean that these animals are responsible for maintenance of the virus in nature.

2. Investigator(s):

Strengths

- The assembled team has a unique combination of laboratory, analytic, and field expertise and a history of successful collaboration in zoonotic disease surveillance.
- The mPI is very comprehensive.

Weaknesses

- None noted.

3. Innovation:

Strengths

- The approach to detect signatures of “shadow viruses” is conceptually and technically innovative. The use of discovery panels is an improvement on traditional serosurveillance techniques that rely on virus-specificity.
- Identifying putative reservoir hosts for some of these high consequence pathogens would represent a considerable shift in mitigation and prevention efforts for these viruses.
- The proposal has the added benefit on contributing to better understanding basic immune system responses to these viruses during sequential infections.

Weaknesses

- None noted.

4. Approach:

Strengths

- The ability to leverage specimens and infrastructure from the CREID network in Thailand, a DTRA program in South Africa, and a NIAD program in Bangladesh is a strength of the study.
- Preliminary data demonstrate feasibility of the approach.

- The combination of laboratory, field, and modeling studies addresses the hypotheses posed in complimentary but independent approaches. In addition, there is synergy amongst the aims.
- Aims 1 and 2 are developmental and Aim 3 provides proof-of-concept for the approach.

Weaknesses

- The primary weakness is that the proposal is expansive in scope, so only limited depth was provided for each study component. This made it difficult to evaluate the veracity of what is being proposed.
- Aim 1 references unpublished data on filovirus immunoreactivity in populations of hammer-headed fruit bats. These data need to be shown otherwise it is difficult to evaluate the rigor of the approach.
- For the immunization experiments in Aim 1, n=3 rabbits are not justified statistically or by some other method.
- In addition, better justification for the use of rabbits is warranted given the ultimate target will likely be bats and other wildlife species. Rabbits are highly immune-reactive compared to other species.

5. Environment:

Strengths

- The environments at all of the partner institutions are sufficient for the proposed work with the requisite infrastructure to conduct studies of this nature.

Weaknesses

- None noted.

Protections for Human Subjects:

Acceptable Risks and/or Adequate Protections

- Exempt under #4

Vertebrate Animals:

YES, all criteria addressed

- No concerns noted.

Biohazards:

Unacceptable

- Not described

Select Agents:

Unacceptable

- The investigators will not use authentic viruses but at a minimum there should be some description about how samples that might contain select agents are handled.

Resource Sharing Plans:

Unacceptable

- This was not included. They mention in the research strategy that they want to share their assay. This a critical resource that could be shared with the community.

Authentication of Key Biological and/or Chemical Resources:

Acceptable

Budget and Period of Support:

Recommend as Requested

CRITIQUE 3

Significance: 2
Investigator(s): 3
Innovation: 2
Approach: 5
Environment: 2

Overall Impact: This project aims to develop an ambitious and novel approach to viral discovery based on multiplexed serological panels. Such developments would have high significance for viral discovery and pandemic preparedness. The investigator team and environment is extremely strong. However, there are some concerns about the ultimate ability to resolve the identity of novel/shadow viruses and the lingering difficulties associated with poor detectability of viral sequence even when the target host species is known or highly suspected. Additionally, the data requirements and the intractability of the novel statistical methodology are likely extreme, and simulation studies that could characterize the potential pitfalls are needed. Overall, this project is high-risk, high-reward and has potentially critical methodological challenges.

1. Significance:

Strengths

- There is high potential for multiplex serological panels to enable definitive characterization of novel pathogens.
- The project is aimed squarely at a key bottleneck in viral discovery – the poor detection and characterization of novel pathogens.

Weaknesses

- Proposed viral identities based on multiplex serological panels would be able to yield a rough taxonomic identification of a “shadow virus,” but would not be paired with sequence information or any other capability of definitive identification. Definitive identification would remain hampered by its current plaguing issues, poor detectability of viral sequence.

2. Investigator(s):

Strengths

- The investigator team possesses a high degree of expertise across multiple disciplines relevant to achieve the proposed project.

Weaknesses

- None noted

3. Innovation:

Strengths

- Adapting methods from image processing to multivariate serological signatures is a novel approach to disambiguate sample identification

Weaknesses

- While the combination of multiple antigens can cover antigenic space, this coverage rests on the assumption that known/suspected space encompasses unknown space. It seems plausible that the identification of novel shadow viruses that extend antigenic space would require continuous updating of key estimated parameters, such as the reaction/cross reaction matrix.

4. Approach:

Strengths

- The discovery panel approach overcomes substantial limitations of prior serological approaches.

Weaknesses

- It is unclear if reaction/cross reaction is stable enough across age, exposure history, and other traits for an estimated cross reaction matrix to hold reliable and/or meaningful information to be used in disambiguating viral identity.
- Experiments in Aim 1 use extremely small sample size, n=3 per treatment, which may leave low power, high sensitivity to individual variation, and poor replicability.
- The methods developed in Aim 2 require large quantities of high-resolution data to parameterize. However, no simulation studies to establish the feasibility or data requirements of this approach are presented.

5. Environment:

Strengths

- The environment is highly supportive of this project’s goals.

Weaknesses

- None

Protections for Human Subjects:

Acceptable Risks and/or Adequate Protections

Vertebrate Animals:

YES, all criteria addressed

Biohazards:

Acceptable

Resource Sharing Plans:

Acceptable

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:

BIOHAZARD COMMENT:

From Critique 1:

- Not described, while it is perceivable that viruses will be used at some point to generate antigens for rabbit immunization

From Critique 2:

- Not described

SCIENTIFIC REVIEW OFFICER'S NOTES:

From Critique 1, regarding resource sharing plan:

- Seems to be missing, while referred to multiple times.

From Critique 2, regarding resource sharing plan:

- This was not included. They mention in the research strategy that they want to share their assay. This a critical resource that could be shared with the community.

From Critique 1, regarding authentication plan:

- Not sufficient detail regarding the sources of the antigens

From Critique 1, regarding select agents:

- Not discussed

From Critique 2, regarding select agents:

- The investigators will not use authentic viruses but at a minimum there should be some description about how samples that might contain select agents are handled.

Footnotes for 1R01AI179865-01; PI Name: Ross, Noam

NIH has modified its policy regarding the receipt of resubmissions (amended applications).See Guide Notice NOT-OD-18-197 at https://grants.nih.gov/grants/guide/notice-files/NOT-OD-18-197.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

Transmission of Vector-Borne and Zoonotic Diseases Study Section
Applied Immunology and Disease Control Integrated Review Group
CENTER FOR SCIENTIFIC REVIEW
TVZ
06/12/2023 - 06/13/2023

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-22-044 at https://grants.nih.gov/grants/guide/notice-files/NOT-OD-22-044.html, including removal of the application from immediate review.

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* Temporary Member. For grant applications, temporary members may participate in the entire meeting or may review only selected applications as needed.

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.

SUMMARY STATEMENT

PROGRAM CONTACT:
Eun-Chung Park
240-627-3338
epark@niaid.nih.gov

(Privileged Communication)

Release Date: **07/12/2023**
Revised Date:

Application Number: **1R01AI179865-01**

Principal Investigators (Listed Alphabetically):

LAING, ERIC
ROSS, NOAM (Contact)

Applicant Organization: ECOHEALTH ALLIANCE, INC.

Review Group: **TVZ**
Transmission of Vector-Borne and Zoonotic Diseases Study Section

Meeting Date: **06/12/2023**
Council: **OCT 2023**
Requested Start: **09/01/2023**

Opportunity Number: **PA-20-185**
PCC: **M32A B**

Project Title: **Characterizing unknown emerging viruses through targeted serological profiling**

SRG Action: **++**
Next Steps: **Visit https://grants.nih.gov/grants/next_steps.htm**
Human Subjects: **X4-Human subjects involved - Exemption #4 designated**
Animal Subjects: **30-Vertebrate animals involved - no SRG concerns noted**

Project Year	Direct Costs Requested
1	492,733
2	489,677
3	499,863
4	498,388
5	499,507
TOTAL	2,480,168

++NOTE TO APPLICANT: Members of the Scientific Review Group (SRG) were asked to identify those applications with the highest scientific merit, generally the top half. Written comments, criterion scores, and preliminary impact scores were submitted by the assigned reviewers prior to the SRG meeting. At the meeting, the more meritorious applications were discussed and given final impact scores; by concurrence of the full SRG, the remaining applications, including this application, were not discussed or scored. The reviewers' comments (largely unedited by NIH staff) and criterion scores for this application are provided below. Because applications deemed by the SRG to have the highest scientific merit generally are considered for funding first, it is highly unlikely that an application with an ND recommendation will be funded. Each applicant should read the written critiques carefully and, if there are questions about the review or future options for the project, discuss them with the Program Contact listed above.
ADMINISTRATIVE NOTE
BIOHAZARD
NEW INVESTIGATOR

1R01AI179865-01 Ross, Noam

BIOHAZARD COMMENT
NEW INVESTIGATOR
SCIENTIFIC REVIEW OFFICER'S NOTES

DESCRIPTION (provided by applicant): Serology remains under-utilized as a reliable surveillance tool for the detection of emerging zoonotic viruses. Detection and estimation of Ebola virus spillovers is dependent on outbreak scale and thus may not be caught through early warning systems. The geographies at-risk for Nipah virus, and bat-borne and rodent-borne henipaviruses spill over across sub-Saharan Africa, Asia and Oceania remain under- surveyed. Limitations of serosurveillance in addressing virus emergence can be found at both the front- end in serological assay design and development, and back-end in the statistical frameworks used to analyze data. Both need to be adapted to the unique challenges associated with known emergent viruses and novel unknown viruses not yet genetically characterized. These challenges include cross-reactivity of antigens and antibodies and complex immune responses. The major goal of this proposal is to dramatically improve the utility of antigen-based serosurveillance for new viral discovery and epidemiology of emerging zoonotic viruses by addressing the challenges of cross-reaction and statistical rigor. We will create discovery panels - qualified and optimized panels of multiplexed immunoassays designed to identify a characterize previously unknown viruses only detected through the serologically profiling wildlife and human hosts. We will develop new statistical approaches to extract the signals of novel viruses from those of known viruses or mixtures of virus-specific antibodies from complex histories multiple previous viral exposures, as well as integrate these into models into population-scale epidemiological models. Finally, we will partner with international viral surveillance projects to apply these techniques to identifying and characterizing filoviruses and henipaviruses in bat populations in South Africa and workers at-risk for exposure to wildlife viruses in Thailand, and to modeling immune-epidemiological dynamics of Nipah virus circulation in bats and people in Bangladesh.

PUBLIC HEALTH RELEVANCE: This project develops new methods to identify emerging and zoonotic viruses by analyzing the virus- induced antibodies present in wildlife and human populations in contact with wildlife. It will create both new laboratory techniques to test for those antibodies as markers of infection and new mathematical models to analyze data to identify the trace of those viruses. It will test these methods in partnership with international virus surveillance projects in Bangladesh, Thailand, and South Africa.

CRITIQUE 1

Significance: 6
Investigator(s): 5
Innovation: 4
Approach: 8
Environment: 4

Overall Impact: The project seeks to develop a novel serological platform to identify emerging and zoonotic viruses by analyzing the virus induced antibody profiles present in wildlife and human populations at risk. The measurable outcomes would include new laboratory techniques for serodiagnostics and new mathematical models to analyze the seroprevalence data to for previously characterized and emerging viruses. Although investigative team is remarkably strong with a track record of independent and collaborative success, this specific project builds on a relatively weak

scientific premise that serological methods (as described in the proposal) can be applied to detect novel/emerging, previously uncharacterized viruses. This seems fundamentally wrong, because even current state-of-the-art serological techniques can only target KNOWN antigens. Another important issue is that the PI failed to emphasize the advantage and significance of the proposed approach compared with sequence based or comprehensive techniques for virus discovery/characterization. Further, the proposal refers to multiple limitations of the current serological techniques but fails to describe how those will be addressed. The diversity and complexity of the immune response in the target wildlife species (including bats) is poorly acknowledged. Preliminary data do not seem to be compelling. The model organism chosen for experimental studies (rabbit) does not seem appropriate, as it's not representative of the biology/immunology of wildlife reservoir species of the target viruses. The grant is written poorly and there is a lack of clarity in details throughout. There are other issues that will be detailed in individual sections. Overall enthusiasm for this proposal is very low.

1. Significance:

Strengths

- Ebola and Nipah viruses are a significant public health threat, and tools for their real-time monitoring/detection of new variant emergence are of high significance

Weaknesses

- The approach selected decreases the overall study significance
- The use of serology vs. molecular/sequence-based methods is not justified (other than it was mentioned that it's underutilized). Does not seem feasible or necessary
- The team acknowledged the challenges associated with serological methods that include cross-reactivity and complex/diverse immune responses. The team does not propose feasible strategies to circumvent those. Additionally, they do not acknowledge other issues associated with serological detection of viruses – you can only target known antigens/antibodies. Sensitivity of serological detection is way lower than that of molecular diagnostic methods. Duration and magnitude of immune responses vary per host and per virus, etc.
- The approach to identify shadow viruses by subtracting signals to known viruses does not seem feasible. Proof-of-concept experiments were not done, or results were not presented.
- Although, the PI states that the proposed approach will identify and CHARACTERIZE novel pathogens, the CHARACTERIZE component is not explain well, while the ability to identify novel pathogens using it is highly questionable.

2. Investigator(s):

Strengths

- A very strong team with highly complementary expertise. The team includes a mathematical and statistical disease ecologist, 2 virologists, 2 infectious disease epidemiologists/specialists, and 2 virologist-ecologists experienced with a variety of emerging and zoonotic viral diseases.
- Established collaborations are also beneficial.

Weaknesses

- No immunologist on the team shows in the lack of in-depth understanding of multiple challenges associated with species-specific immune responses, their diversity, complexity, etc.
- No wildlife (specifically bat) specialist on the team is also detrimental.

3. Innovation:

Strengths

- Using serology for virus discovery/characterization.

Weaknesses

- The use of microsphere-based multiplex immunoassay platforms is not particularly innovative.

4. Approach:

Strengths

- None noted.

Weaknesses

- The Approach has countless deficiencies including the main ones: flawed scientific premise, rabbit model to develop assays for bats and humans, extremely vague description of the proposed immunological experiments, the lack of a clearly presented rationale for detection of antibodies to unknown pathogens using specific reagents derived from previously characterized pathogens, and many others.
- Specifically, rabbits are highly immuno-reactive animals, they lack serological background against the target viruses. Neither they are the natural hosts of these viruses, which will affect the immune response characteristics. Targeted antibody isotypes (that vary between different hosts) are not mentioned.
- Immunization regimen is poorly and vaguely described. Not clear how the antigens will be derived, purified, processed. Not specified numbers of immunizations, timing between each, the time of antiserum harvest, etc.
- The team does not take in the account the immunological phenomena including antigenic sin, whereby exposure to the primary related antigen/pathogen affects subsequent immune responses against related antigens/pathogens.
- Preliminary data needed to prove feasibility of the proposed approach are not provided. At this point, the team should have had a proof that it is possible to detect a signal against an unknown (shadow) virus by simply subtracting the signal against known related viruses. The available preliminary data are not very compelling.
- The proposed statistical models do not seem to account for way too numerous biological variables across different species, pathogens, etc.
- Another factor that would greatly impact feasibility of the proposed research is the variable and sometimes unknown duration of antibody response against the target viruses. While it appears that in experimental rabbits the exposure to different antigens will be time-matched, and the magnitudes of the responses to the non-replicating antigens will be comparable, it is not the case in bats or humans, where time post-infection will vary greatly, and the magnitude/characteristics of the antibody response will be greatly affected by this and the fact.
- Besides the fact that bats are capable of reducing pro-inflammatory response, some studies reported that their antibody response to various test antigens may be lower compared to what is seen in conventional laboratory animals.
- While the team acknowledges that sex and age are important biological variables affecting antibody responses, they do not seem to account for it in their rabbit immunization experiments.

- At the genomic level, bats seem to possess a much larger repertoire of germline genes encoding immunoglobulin variable (V), diversity (D) and joining (J) segments than humans or rabbits, which may provide a much larger number of antigen specificities in their naive B cell receptor (BCR) repertoire. This suggests that antibody detection discovery matrix would not work for bats as it would work for rabbits or humans (even if it's possible for the latter species).

5. Environment:

Strengths

- The environment seems to be suitable for the proposed research with access to computer/bioinformatics and experimental lab resources.

Weaknesses

- Access to an advanced immunological lab focusing on human and bat adaptive immune responses is needed.

Study Timeline:

Strengths

- Sufficient time seem to be provided for Aim 2.

Weaknesses

- May be not feasible for the proposed experimental (Aim 1) and surveillance (Aim 3) studies.

Protections for Human Subjects:

Acceptable Risks and/or Adequate Protections

- The study will only access previously collected samples

Inclusion Plans:

- Sex/Gender: Distribution justified scientifically
- The sex is acknowledged as a biological variable throughout

Vertebrate Animals:

YES, all criteria addressed

Biohazards:

Unacceptable

- Not described, while it is perceivable that viruses will be used at some point to generate antigens for rabbit immunization

Applications from Foreign Organizations:

Justified

Select Agents:

Unacceptable

- Not discussed

Resource Sharing Plans:

Unacceptable

- Seems to be missing, while referred to multiple times.

Authentication of Key Biological and/or Chemical Resources:

Unacceptable

- Not sufficient detail regarding the sources of the antigens

Budget and Period of Support:

Recommend as Requested

CRITIQUE 2

Significance: 5
Investigator(s): 1
Innovation: 1
Approach: 5
Environment: 1

Overall Impact: This R01 proposal addresses questions related to serological cross reactivity between filoviruses and henipaviruses, which has confounded serosurveillance, virus discovery, and efforts to incriminate reservoir hosts for these viruses. The investigators propose to create innovative multiplex serological panels that are optimized for virus discovery and combine these with novel statistical methods to solve the cross-reactivity problem. As a result, this is a highly innovative approach from both a technical and conceptual standpoint. In addition, the investigative team is outstanding, and a major strength of the proposal is the ability to leverage several different disease surveillance networks to ground-truth the development of their assay and statistical approaches. However, there were some issues with the experimental design and presentation of the scientific premise that lowered my overall enthusiasm. One major weakness was that it is unclear how these tools would be used to identify putative reservoir hosts, when multiple animal species might be exposed and seroconvert. The other major weakness was the ambitious nature of the proposal which only provided limited depth to each study component. In addition, critical preliminary data were not included in the proposal. This made it difficult to evaluate everything that was being proposed. In conclusion, despite there being laudable aspects related to virus discovery and pandemic preparedness, the potential overall impact of this proposal is only moderate.

1. Significance:

Strengths

- It has been long recognized that cross-reactivity between closely related, antigenically variable viruses is a problem for serosurveillance. This has confounded diagnostics and has made it difficult to incriminate zoonotic reservoirs for emerging viruses.
- Incriminating zoonotic reservoirs for emerging viruses is important for prediction, prevention, and control efforts to mitigate the risk posed by these viruses.
- Improved serological assays have utility in diagnostics as well as for virus discovery, and if successful this approach would be useful for other virus families where serological cross-reactivity is a known problem.

Weaknesses

- It is unclear—even with improved serological approaches—how these data would be used to incriminate an animal species as a putative reservoir host without molecular (or some other) evidence of infection. Many animals that come into close contact with the reservoir may have abortive or asymptomatic infection where virus is not shed but still result in seroconversion. This does not mean that these animals are responsible for maintenance of the virus in nature.

2. Investigator(s):

Strengths

- The assembled team has a unique combination of laboratory, analytic, and field expertise and a history of successful collaboration in zoonotic disease surveillance.
- The mPI is very comprehensive.

Weaknesses

- None noted.

3. Innovation:

Strengths

- The approach to detect signatures of “shadow viruses” is conceptually and technically innovative. The use of discovery panels is an improvement on traditional serosurveillance techniques that rely on virus-specificity.
- Identifying putative reservoir hosts for some of these high consequence pathogens would represent a considerable shift in mitigation and prevention efforts for these viruses.
- The proposal has the added benefit on contributing to better understanding basic immune system responses to these viruses during sequential infections.

Weaknesses

- None noted.

4. Approach:

Strengths

- The ability to leverage specimens and infrastructure from the CREID network in Thailand, a DTRA program in South Africa, and a NIAD program in Bangladesh is a strength of the study.
- Preliminary data demonstrate feasibility of the approach.

- The combination of laboratory, field, and modeling studies addresses the hypotheses posed in complimentary but independent approaches. In addition, there is synergy amongst the aims.
- Aims 1 and 2 are developmental and Aim 3 provides proof-of-concept for the approach.

Weaknesses

- The primary weakness is that the proposal is expansive in scope, so only limited depth was provided for each study component. This made it difficult to evaluate the veracity of what is being proposed.
- Aim 1 references unpublished data on filovirus immunoreactivity in populations of hammer-headed fruit bats. These data need to be shown otherwise it is difficult to evaluate the rigor of the approach.
- For the immunization experiments in Aim 1, n=3 rabbits are not justified statistically or by some other method.
- In addition, better justification for the use of rabbits is warranted given the ultimate target will likely be bats and other wildlife species. Rabbits are highly immune-reactive compared to other species.

5. Environment:

Strengths

- The environments at all of the partner institutions are sufficient for the proposed work with the requisite infrastructure to conduct studies of this nature.

Weaknesses

- None noted.

Protections for Human Subjects:

Acceptable Risks and/or Adequate Protections

- Exempt under #4

Vertebrate Animals:

YES, all criteria addressed

- No concerns noted.

Biohazards:

Unacceptable

- Not described

Select Agents:

Unacceptable

- The investigators will not use authentic viruses but at a minimum there should be some description about how samples that might contain select agents are handled.

Resource Sharing Plans:

Unacceptable

- This was not included. They mention in the research strategy that they want to share their assay. This a critical resource that could be shared with the community.

Authentication of Key Biological and/or Chemical Resources:

Acceptable

Budget and Period of Support:

Recommend as Requested

CRITIQUE 3

Significance: 2
Investigator(s): 3
Innovation: 2
Approach: 5
Environment: 2

Overall Impact: This project aims to develop an ambitious and novel approach to viral discovery based on multiplexed serological panels. Such developments would have high significance for viral discovery and pandemic preparedness. The investigator team and environment is extremely strong. However, there are some concerns about the ultimate ability to resolve the identity of novel/shadow viruses and the lingering difficulties associated with poor detectability of viral sequence even when the target host species is known or highly suspected. Additionally, the data requirements and the intractability of the novel statistical methodology are likely extreme, and simulation studies that could characterize the potential pitfalls are needed. Overall, this project is high-risk, high-reward and has potentially critical methodological challenges.

1. Significance:

Strengths

- There is high potential for multiplex serological panels to enable definitive characterization of novel pathogens.
- The project is aimed squarely at a key bottleneck in viral discovery – the poor detection and characterization of novel pathogens.

Weaknesses

- Proposed viral identities based on multiplex serological panels would be able to yield a rough taxonomic identification of a “shadow virus,” but would not be paired with sequence information or any other capability of definitive identification. Definitive identification would remain hampered by its current plaguing issues, poor detectability of viral sequence.

2. Investigator(s):

Strengths

- The investigator team possesses a high degree of expertise across multiple disciplines relevant to achieve the proposed project.

Weaknesses

- None noted

3. Innovation:

Strengths

- Adapting methods from image processing to multivariate serological signatures is a novel approach to disambiguate sample identification

Weaknesses

- While the combination of multiple antigens can cover antigenic space, this coverage rests on the assumption that known/suspected space encompasses unknown space. It seems plausible that the identification of novel shadow viruses that extend antigenic space would require continuous updating of key estimated parameters, such as the reaction/cross reaction matrix.

4. Approach:

Strengths

- The discovery panel approach overcomes substantial limitations of prior serological approaches.

Weaknesses

- It is unclear if reaction/cross reaction is stable enough across age, exposure history, and other traits for an estimated cross reaction matrix to hold reliable and/or meaningful information to be used in disambiguating viral identity.
- Experiments in Aim 1 use extremely small sample size, n=3 per treatment, which may leave low power, high sensitivity to individual variation, and poor replicability.
- The methods developed in Aim 2 require large quantities of high-resolution data to parameterize. However, no simulation studies to establish the feasibility or data requirements of this approach are presented.

5. Environment:

Strengths

- The environment is highly supportive of this project’s goals.

Weaknesses

- None

Protections for Human Subjects:

Acceptable Risks and/or Adequate Protections

Vertebrate Animals:

YES, all criteria addressed

Biohazards:

Acceptable

Resource Sharing Plans:

Acceptable

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:

BIOHAZARD COMMENT:

From Critique 1:

- Not described, while it is perceivable that viruses will be used at some point to generate antigens for rabbit immunization

From Critique 2:

- Not described

SCIENTIFIC REVIEW OFFICER'S NOTES:

From Critique 1, regarding resource sharing plan:

- Seems to be missing, while referred to multiple times.

From Critique 2, regarding resource sharing plan:

- This was not included. They mention in the research strategy that they want to share their assay. This a critical resource that could be shared with the community.

From Critique 1, regarding authentication plan:

- Not sufficient detail regarding the sources of the antigens

From Critique 1, regarding select agents:

- Not discussed

From Critique 2, regarding select agents:

- The investigators will not use authentic viruses but at a minimum there should be some description about how samples that might contain select agents are handled.

Footnotes for 1R01AI179865-01; PI Name: Ross, Noam

NIH has modified its policy regarding the receipt of resubmissions (amended applications).See Guide Notice NOT-OD-18-197 at https://grants.nih.gov/grants/guide/notice-files/NOT-OD-18-197.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

Transmission of Vector-Borne and Zoonotic Diseases Study Section
Applied Immunology and Disease Control Integrated Review Group
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* Temporary Member. For grant applications, temporary members may participate in the entire meeting or may review only selected applications as needed.

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: Noam Ross on behalf of Noam Ross <ross@ecohealthalliance.org>
To: Laing, Eric
Subject: R01 summary responses and follow-up
Date: Monday, July 17, 2023 11:36:02 AM
Attachments: 1R01AI179865-01.pdf
1R01AI179865-01.pdf

Hi Eric,

I have the summary response to R01 submission (attached). I'm not sure if you got it. I'm still processing it. One reviewer really hated it. I think the other critiques are addressable but fundamentally I think we need to be able to demonstrate the feasibility with a preliminary version and figure out how carve out the time in current/smaller projects to do so.

I have a very busy rest of the month, but propose that we regroup in August to think about plans for next steps/resubmission.

A couple of other things:
- On August 1 at 4PM, we have the team from NIH ImmPort giving a talk at EHA's regular "methods and models" meeting. I'll send along an invite if you or any of your lab are interested in attending.
- I'm also considering submitting a proposal to this NIH software development call: https://grants.nih.gov/grants/guide/rfa-files/RFA-AI-23-038.html, for creating R packages that would implement the both current and in-development serology analysis methods. It says "No wet-lab technologies or new data collection will be supported," but I'd include you and your team in it for data/testing/consultation.

Best,
Noam

--
Dr. Noam Ross
Principal Scientist, Computational Research

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Chapter 3

FILOVIRUSES AND HENIPAVIRUSES AT THE HUMAN-LIVESTOCK-WILDLIFE INTERFACE IN FOREST FRINGE CATTLE FARMS IN SELECTED STATES OF PENINSULAR MALAYSIA

3.1 Introduction

Filoviruses and henipaviruses are high consequence emerging zoonotic pathogens that have caused severe outbreaks in many parts of the world leading to high mortality and morbidity and grave economic losses in both human and animal health sectors (Knust et al., 2017; Looi & Chua, 2007). The economic losses due to Ebola has been estimated to be USD2.8 billion in the 2014 Ebola outbreak and USD446 million in 1998 Nipah outbreak (FAO and APHCA, 2002; World Bank, 2016).

The viruses within the family Filoviridae; Ebola (EBOV) and Marburg virus (MARV) cause haemorrhagic fever outbreaks in Africa, Germany and isolated cases in other parts of the world like Spain, United States of America, United Kingdom, Philippines, Italy, and Russia (CDC, 2018; C. E. Smith et al., 1967; WHO, 2014). The mortality from Ebola disease in Africa was 50-90% in humans and almost 100% mortality in non-human primates (Bowen et al., 1978; Jaax et al., 1996; Jahrling et al., 1996; K. M. Johnson, 1978; Simpson, 1978). While *Henipavirus*; Hendra virus (HeV) and Nipah virus (NiV) have cause respiratory and neurological signs in humans, pigs and horses in Malaysia, Australia, Bangladesh and

India (Chadha et al., 2006; Chua et al., 1999; Hsu et al., 2004; Nor, Gan, Ong, et al., 2000; O'Sullivan et al., 1997). The mortality from Nipah virus infection was 40-70% in humans in Malaysia and Bangladesh; and of 1-5% in adult pigs and 40% in suckling pigs in Malaysia (Chua et al., 2000; Goh et al., 2000; Hsu et al., 2004; Luby et al., 2009; Nor, Gan, & Ong, 2000).

With an exception of Nipah virus which emerged in 1998 (Fikri et al., 2011; Rahman et al., 2013a; Sohayati et al., 2011), limited information is available on both filoviruses and henipaviruses in Malaysia. The investigative work on Nipah has led to numerous works on bat ecology and bat-borne microbial agents. It is now understood that bats have co-evolved with multiple viruses that may be of public health significance such a Nipah (Hayman, 2016). Filoviruses have been detected in bats in Singapore, Bangladesh, China, and the Philippines (Laing et al., 2018; Olival et al., 2013; Taniguchi et al., 2011; Yuan et al., 2012). While henipaviruses widely circulate in bats in Malaysia, India, Vietnam, Cambodia, Indonesia and Australia (Breed et al., 2013; Epstein et al., 2008; Hasebe et al., 2012; Rahman et al., 2013a; Reynes et al., 2005; Sendow et al., 2006). Besides bats, filoviruses and henipaviruses can infect a broad range of mammals such as human primates, rodents, shrews, guinea pigs, bats, dogs, pigs, cattle, donkey, chicken; and rodents, horse, cats, pigs, dogs, goats, respectively (Chua et al., 2000; Edson et al., 2015; Gonzalez et al., 2005; Leroy et al., 2004; Morvan et al., 1999; Wu et al., 2014).

Commented [A1]: I would not use the Laing et al 2019 conference poster abstract as a reference for NiV emergence or really anywhere else.

Despite the apparently wide distribution and reported prevalence of these pathogens amongst the reservoir hosts in Southeast Asia, outbreak has been rare in both humans and domestic animals. Following the Nipah outbreak in 1998-1999 in Malaysia, no further cases have been reported in both humans and animals to date (Fikri et al., 2011; Muniandy et al., 2001; Naama et al., 2013; Sohayati et al., 2016). There has also been no human outbreaks in Cambodia despite serological and molecular evidence widely found in Cambodian bats (Olson et al., 2002; Reynes et al., 2005). However, the SEA region is considered a hotspot for emerging pathogens because several localized emerging zoonosis have occurred in the past two decades and because multiple factors that drives emerging infectious diseases are highly prevalent. For example, wide circulation and abundance of reservoir hosts, topographical or landscape changes and intensified systems of agriculture and animal production may increase contact opportunities for viral transmission at the wildlife-livestock-human interfaces (Breed et al., 2013; Horby et al., 2013; Jung & Threlfall, 2016; I. Smith & Wang, 2013). In additon,the global climatological changes may impact distribution and concentration of disease vectors which subsequently facilitates spatiotemporal changes of filovirus and henipavirus viral spillover events (Martin et al., 2018; McFarlane et al., 2011; Schmidt et al., 2017).

The one health approach of managing complex emerging diseases requires the understanding of all actors that take part at the environment-animal-human interface. For example, food animals may become infected with pathogens from wildlife that can then be transmitted to human such as Nipah, Highly Pathogenic Avian Influenza H5N1,

Pandemic Influenza H1N1 (2009) amongst others. Many livestock farms in this region are located in the rural or peri-urban areas bordering primary or secondary forests. In Malaysia, livestock especially cattle are commonly managed extensively or semi-intensively at forest fringes thus may have higher probability of contact with infectious materials from infected wildlife or domestic animals. Beef cattle are most commonly raised using the aforementioned systems where animals are let out to graze in a field, plantations or in a village located at forest fringes where wildlife and peri-domestic animals are plentiful. Such setting improves the chance and frequency for pathogen spillover events to livestock or humans (Hassell et al., 2017). The spillover occurrences varies given available drivers and rarely result in an epidemic, however frequent spillover events will increase the chance of an outbreak (Clayton et al., 2013; Hayman et al., 2011; Parrish et al., 2008; Plowright et al., 2017).

In this chapter, we targeted cattle farms located at forest fringe areas with high contact risk and probability of pathogen spillover events. We hypothesise that cattle, other domestic animals, wild animals and farmers are seropositive for filoviruses and henipaviruses. The objectives of this study are: 1) To determine the serological evidence of filoviruses and/or henipaviruses in cattle, farmers, and other domestic animals and wildlife in and at close proximity to the targeted cattle farm; 2) To determine risk factors of virus infection in those species.

3.2 Materials and Methods

3.2.1 Ethics statement

All human and animal samples from cattle farms were collected in accordance to an established USAID Emerging Pandemic Threats PREDICT Operating Procedures with permission from the local institutional and ministerial Institutional Animal Care and Use Committee from UPM (AUP-R064/2018), UC Davis (#19300) and Tufts (G2021-131) and the Institutional Review Board from the Medical Research and Ethics committee (MERC) from National Medical Research Register (NMRR), Malaysia (NMRR-17-3356-37135, IIR) and UC Davis (#804522) in collaboration with Ministry of Health (MOH), Malaysia, Department of Veterinary Services (DVS), Malaysia, and the Department of Wildlife and National Parks (PERHILITAN). The work performed for this project is part of a larger project in collaboration with the EcoHealth Alliance.

3.2.2 Study design and study sites

A cross-sectional survey was performed in 3 semi-intensive, 2 integrated and 1 intensive cattle farm. These farms were selected based on a defined selection criteria which include, 1) located in districts with existing NMRR and IRB approval for human study, 2) located 5 km from periphery of forests as approved by the Department of Veterinary Services, Putrajaya, 3) a minimum of 30 cattle in farm, and 4) has other domestic animals and wildlife in or around farm. The approved districts were Kuala Kangsar in the state of Perak, Gua Musang in Kelantan and Kuala Lipis in Pahang. In these districts, the district

health officers had worked with EcoHealth Alliance on previous projects and have agreed to assist the human sampling aspects in this project.

The identification of the cattle farms consisted of several steps as in Figure 3.1. The details of the steps in identifying location and farms are further described in Appendix X. All animals sampled in this study were within 5 km radius of a primary forest.

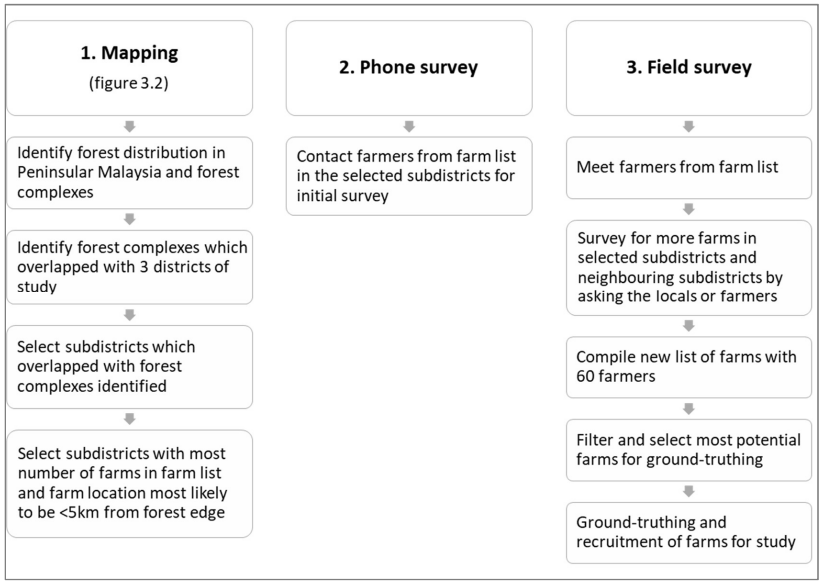

Figure 3. 1 Identification of forest-fringed cattle farm for the study on filovirus and henipavirus exposure at the wildlife-livestock-human interface

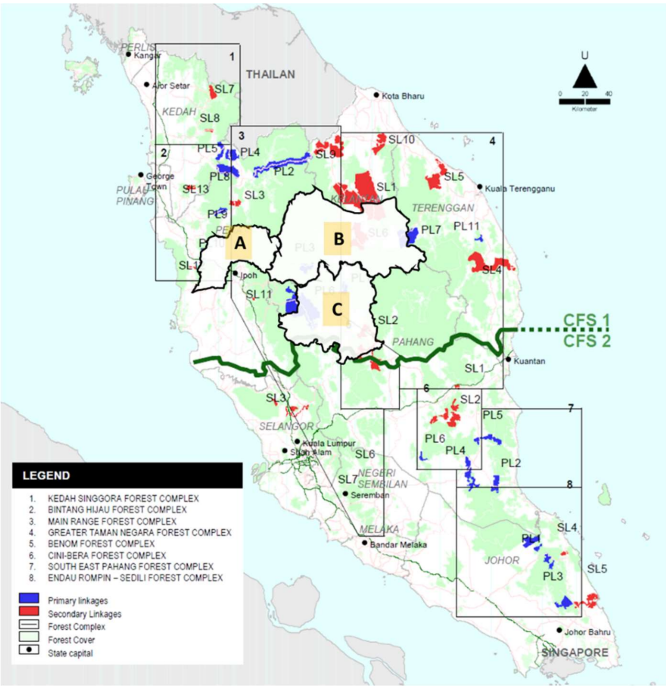

Figure 3. 2 Map of forest complexes in Peninsular Malaysia and the overlapping targeted districts in our study marked by A: Kuala Kangsar, B: Gua Musang, C: Lipis. (Source: Regional Planning Division, Department of Town and Country Planning, Peninsular Malaysia, 2009)

3.2.3 Sample size

Four cattle farms from Kuala Kangsar district, Perak and two cattle farms from Gua Musang district, Kelantan within 5 km radius of forests were sampled beginning 2019.

Targeted sample size for each animal species per farm were 30 cattle from participating target farms, 30 other domestic animals (eg, goat, sheep, dog, etc) and 60 wildlife (eg, bats and rodents). The sample size (n) for this study (Table 3.1) was determined using

the Open-Epi Version 3 calculator. The software program applies the formula of n = [DEFF*Np (1-p)]/[(d2/Z21-□/2 *(N-1)+p*(1-p)], where N = population size, p = prevalence, d = confidence limits = 10%, and DEFF = design effect for cluster surveys = 1. The estimated prevalence used in the current study was 5% based on prevalence studies of filoviruses and henipaviruses in the region which reported seroprevalence of 0-79% in domestic animals and 1-33% in bats (Table). Other domestic animals and wildlife were opportunistically captured and sampled with priority given to animals in and closest to the participating target farms.

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Table 3. 1 Sample size calculation for the sero-detection of filovirus and henipavirus in cattle, other domestic animals and wildlife in participating cattle farms located at the forest fringe in Perak and Kelantan, Malaysia

	Estimated Population Size, N per farm	Estimated Prevalence, p (% +/-5)[a]	Sample size, n per Farm	No. of farms	Total
Cattle : (Semi-intensive (n=3), Integrated oil palm (n=2), Intensive farm(n=1)	50	5	30	6	180
Other domestic animals : Goat, sheep, dog, etc.	50	5	30	6	180
Wildlife : Bat, rodent, other small mammal	1249 per roost for bats	5	70 (max of 60)[c]	6	360

[a]Prevalence of 5% was decided based on prevalence studies of filoviruses and henipaviruses in the region (see Table ???).
[b]Estimated population size for bats is based on the average roost size of bats. Total number of bats calculated is an overestimation as it will be highly dependent on the bats captured in nets and number of days the nets are placed in a location.
[c]Total number of wildlife are set to the maximum of 60 due to logistical and financial limitations.

3.2.4 Sampling of cattle and other domestic animals

Cattle and other domestic animals such as goat, sheep, dog and rabbit were conducted with the team from the EcoHealth Alliance in accordance to USAID Emerging Pandemic Threats PREDICT Operating Procedures (PREDICT One Health Consortium, 2016b, 2016e).

All domestic animals were manually restrained for the sampling procedures (Appendix B). Blood samples were obtained from the appropriate venepuncture sites according to species. In cattle, goat, and sheep, the jugular vein or coccygeal vein was used (Figure 3.3). In dogs, blood was collected from the cephalic vein, jugular vein, femoral vein, lateral or medial saphenous vein. In rabbits, blood was collected from the lateral saphenous vein. Three to five mL of blood were taken from these animals and distributed equally in a BD Vacutainer® serum tube and EDTA tube.

A basic physical examination was performed by veterinarian to assess the health status and identify the age and sex of the animal. Photographs of sampled animal was taken from the sides and front, including dentition, or presence of any lesions, abnormalities or identifying marks or characteristics for future reference.

All blood tubes were labelled appropriately and placed in cooler boxes and transported back to sampling processing base. Blood in BD Vacutainer® serum tubes were left to clot

for serum collection within 1 hour. After clot is form, serum tubes were centrifuged at 1300 r.p.m for 15 minutes and 1 mL of serum was transferred into 0.5 mL cryovial tubes to produce at least two aliquots. Cryovial tubes were labelled appropriately. Samples were then placed in MVE Doble 34 liquid nitrogen tanks in the field and later stored at -80 °C freezer in the lab according to proper cold chain methods.

Figure 3. 3 Locating the jugular vein in the jugular groove for blood sampling

3.2.5 Sampling of wildlife

Permission was obtained from the local authorities or landowners before bat and rodent trapping and sampling begun. Selected sites were first surveyed with priorities given to potential trapping locations in the farm and nearest to farm within 5 km radius. All personnel handling trapping and sampling of wildlife had to be immunized against rabies

virus, Japanese encephalitis virus, hepatitis B virus, tetanus and negative sputum tests for tuberculosis.

3.2.5.1 Sampling of bat

Bats were captured using mist nets suitable for capturing bats in open spaces as described by FAO Manual for Investigating the Role of Bats in Emerging Zoonoses (Food and Agriculture Organisation of the United Nations, 2011). Further description on the sampling procedure is in Appendix X. Briefly, bats were captured using mist nets placed at anticipated bat flyways in the farm, village and in nearby plantations such as across streams or trails. The nets were also positioned near roosting or feeding sites such as near flowering banana trees and flowering durian trees (Figure 3.4).

Figure 3. 4 A bat trapping site with banana and durian plantation in a village near a participating cattle farm.

The mist nets were raised around sunset at 7.30pm and remained opened until 12:00am for trapping to occur (Figure 3.5) and closed neatly after trapping period (Figure 3.6). Bat nets were repositioned to new locations when recapture rates were high, or captures were absent. The GPS coordinates for each mist nets were recorded.

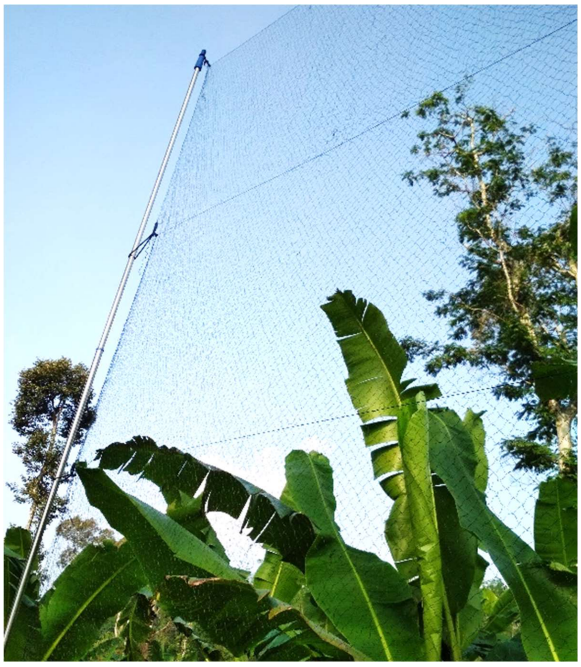

Figure 3. 5 Extended mist nets for capture of bats at sunset.

Figure 3. 6 Retracted mist nets that is secured after trapping period

Mist nets were checked for bats every 30 minutes to an hour. Captured bats were extracted from the nets carefully using leather gloves and forceps or a small stick to move the netting or bat mandibles. Bats are then placed into a porous cotton bag with a draw-string mouth and taken to a sampling processing site where sampling was performed immediately (Figure 3.7). Approximately 20-30 bats were sampled in a night. No bats should be kept for more than 6 hours.

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Figure 3. 7 Bags containing bats hung in draw-string cloth bags on a raffia string between two pillars

Bats were sampled and identified according to the USAID Emerging Pandemic Threats PREDICT Operating Procedures with some modifications (Hoffmann et al., 2010; PREDICT One Health Consortium, 2016a; C. Smith et al., 2010). A bat was removed from the cloth bag and manually restrained for sampling using leather gloves. In bats that were > 100 g, a non-heparinised syringe was used to collect blood from the cephalic vein, brachial vein or saphenous vein. Blood was then placed in a serum vacutainer tube containing serum-clotting factor and centrifuge. Extracted serum was placed into 2 cryovials of a minimum of 60 µL if volume. If bats were < 100 g, the tip of a sterile 25G needle was used to puncture the brachial or propatagial vein (C. Smith et al., 2010).

A 1:10 serum dilution (10 µL blood into 90 µL PBS) was centrifuged, and the supernatant was transferred into a new cryovial tube. Cryovial tubes were labelled appropriately and placed in MVE Doble 34 liquid nitrogen tanks in the field and later stored at -80 °C freezer in the lab according to proper cold chain methods.

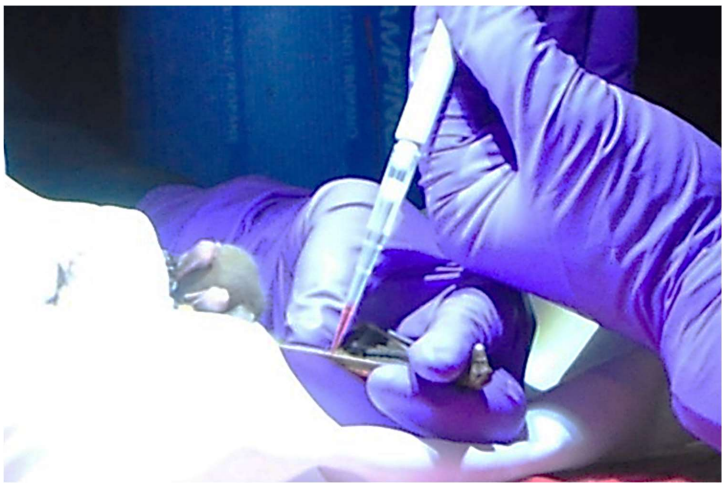

Figure 3. 8 Blood sampling from the cephalic vein of a bat by aspirating the drop of blood using a pipette.

Morphometric measurements and photographs of each bat were taken during the sampling procedure for the purpose of species identification. Identification of bat species was performed by an experienced rangers and field veterinarian based on the morphology and biometric measurements. Guide books were used for identification of bats in the field (Francis, 2008; Kingston et al., 2006; Payne et al., 1985; Phillipps & Phillipps, 2016).

Sexual characteristics and size were used to classify the age of bats as adult, subadult and juvenile. Males with descended testis and relative body size were considered adults while those with unfused epiphyses and undescended testis were considered subadults. Relative body size, prominent nipples, pregnant or lactating, and complete fusion of the phalangeal symphysis were morphological features used to classify female bats as adult. Whereas female bats that were fully grown with unfused epiphyses were considered subadults. Pregnancy status was identified by gently palpating the abdomen, and lactating status was identified by attempted milk expression from the teats. Female and male bats that were small, clinging to dam, and suckling were considered juveniles.

Typically, sampling per bat took less than 15 minutes with one person handling and performing the morphometric measurements, while a second person focused on the sampling. Bats nails were painted with a red nail polish as identification in case of recapture. Frugivorous or nectivorous bats were fed with approximately 1 mL of 100% fruit juice or honey water prior to release. Bats were released after sampling.

3.2.5.2 Sampling of rodent and other small mammal

Rodents and other small mammals were trapped and sampled according to the USAID Emerging Pandemic Threats PREDICT Operating Procedures with some modifications (Aplin et al., 2003; Hoffmann et al., 2010; Mills et al., 1995; PREDICT One Health Consortium, 2016d, 2016e). Further description on the sampling technique is in

Appendix X. A total of 100 traps were used to trap rodents, including 45 Sherman traps, 45 small Tomahawk for smaller mammals such as mice, rats, tree shrews, and 10 large Tomahawk traps for larger mammals, such as civets (Figure 3.9).

Figure 3. 9 Sorting of 100 rodent traps according to transect lines before deployment

Sites for traps were first surveyed and divided between inside farm or nearby plantation or forest. Transect lines were used for placement of traps as opposed to grid to reduce overlapping and increase sampling area coverage and increase chance of capturing more species through coverage of small mammal home ranges and microhabitats (Pearson et al., 2003).

Traps containing appropriate baits were placed at strategic pathways for rodents (Figure 3.10). Oil palm fruit was the preferred option as it does not attract insects. Traps were placed at the same sites for a minimum of three consecutive nights from 6:00pm to 8:00am the next day. If adverse weather such as heavy rain was expected, traps would not be opened for that night.

Figure 3. 10 Placement of rodent trap by the fences in a farm

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Figure 3. 11 Roasting of oil palm fruits that were used as baits for rodents

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Trapped small mammal were gently transferred into a draw-string cloth bag by covering the bag over the opening of the trap and slightly tilting the trap downwards. Animals that were too stressed were released, while animals found to be injured or weak were be brought back to sampling processing base for veterinary treatment.

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Smaller rodents were removed from the bag gently and placed in a modified container for anaesthetic induction. Whereas, in small mammal species like civets, a gas mask was used for induction without completely removing animal out from the bag to reduce stress (Figure 3.12). Once an animal was motionless and unresponsive, it was taken out

and placed on the table with an appropriate fitting face mask for continue induction or maintenance of anaesthesia. Monitoring of animal was performed by a veterinarian throughout during procedure along with a basic physical examination to access health status.

Figure 3. 12 Induction of general anesthesia using isoflurane in a small Indian civet (*Viverricula indica*)

Blood samples were collected from the lateral tail vein, ventral tail artery or lateral saphenous vein or lateral saphenous vein in rodents and other small mammals (Hem et al., 1998; G. Lee & Goosens, 2015; Mills et al., 1995; Office of the University Veterinarian, 2017a; PREDICT One Health Consortium, 2016d). In smaller rodents, approximately 1 mL of blood was collected, and in larger small mammals (~200 g), 3-5 mL of blood was collected. Whole blood was diluted 1:10 in PBS, left to stand for a minimum of 30 minutes

and centrifuged to remove red blood cells. Sera was collected and subsequent steps in processing and storage are as mentioned in 3.2.5.1.

After blood collection, fluid volume replacement was given subcutaneously using Lactated Ringer's or 0.9% NaCL solution to avoid hypovolemic shock. Markings on sampled rodents or small mammals was done by clipping 3-5 mm of hair at the right ventral thigh. Morphometric measurements and photographs of each animal were taken during the sampling procedure for the purpose of species identification.

Secondary sexual characteristics and size were used to classify animal ages as adult, subadult and juvenile. Adults were considered fully grown with adult pelage and sexually mature. Subadults were not fully grown, were developing pelage and were or were not sexually mature. Juveniles were smaller than subadults and not sexually mature with grey and soft pelage (Hoffmann et al., 2010). Animals were monitored in a small cage until fully recovered from anaesthesia before release at place of capture.

3.2.5.3 Sampling of Wild boar

Wild boar were sampled according to established protocols (Office of the University Veterinarian, 2017b, 2017c; PREDICT One Health Consortium, 2016b). Further description on the sampling technique is in Appendix X. Wild boar were trapped according to trapping protocol described for rodent and other small mammals, and transported in a cage to sampling processing base for sampling (Figure 3.13).

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Figure 3. 13 A subadult wild boar in a Tomahawk trap from an oil palm plantation

Wild boar were sedated with by creating an anaesthetic chamber for induction and maintained with a gas mask. Monitoring of anaesthesia was performed by a veterinarian throughout during procedure. Blood samples were collected from the anterior vena cava and is distributed equally in a BD Vacutainer® serum tube and an EDTA tube. Processing of blood for serum extraction and storage were similar to previously mentioned in 3.2.4. After blood collection, fluid volume replacement was given subcutaneously using Lactated Ringer's or 0.9% NaCL solution. A basic physical examination was performed by veterinarian to access health status. Morphometric measurements and photographs of each animal were taken during the sampling procedure for the purpose of species identification.

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Wild boar were classified into adult, subadult and juvenile based on secondary sexual characteristics and size. Adults were considered fully grown with adult pelage and is sexually mature. Subadults ~~are~~ were not fully grown, were ~~with~~ developing pelage and ~~may or may~~were or were not be sexually mature. Juveniles ~~are~~ were smaller than subadults and not sexually mature with grey and soft pelage (Hoffmann et al., 2010).

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3.2.5.4 Sampling of Non-human primate

Non-human primates (NHP) were kept as pets hence trapping was not required. NHP were anaesthetized and sampled upon the agreement of its owner according to established protocols (Hoffmann et al., 2010; PREDICT One Health Consortium, 2016c). Further description on the sampling technique is in Appendix X. Briefly, aesthesia drug used to sedate animals were the combination of: 1) 4mg/kg Ketamine and 0.15mg/kg Xylazine with Yohimbine 1:1 as reversal agent for Xylazine, or 2) Tiletamine-Zolazepam, Zoletil® 3-5mg/kg in more aggressive primates. A dart syringe and blowpipe were used to deliver anaesthetic drug intra-muscularly by an experienced and trained ranger. Anaesthetic monitoring was performed by a veterinarian. After immobilization, basic physical examination was performed. A sterile lubricant is placed into animal's eyes to prevent corneal drying or scratches during sedation.

NHP were placed in a supine position. Blood was withdrawn from femoral vein located in the inguinal region lateral and parallel to the femoral artery (Figure 3.14). Three to five

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mL of blood were taken from these animals and distributed equally in a BD Vacutainer® serum tube and EDTA tube. Processing of blood for serum extraction and storage are similar to previously mentioned in 3.2.4.

Figure 3. 14 Sampling of an anaesthetized pig-tailed macaque (*Macaca nemestrina*)

Photographs of the anterior and ventral view of the full body, anterior and lateral face, upper and lower dentition and genitalia were taken for future reference. Age was classified to 7 age class according to the PREDICT Operating Procedures for Non-Human Primates. Neonates are animals that show signs of being born within few days; infants are young that were still suckling and clinging on to mother; juveniles are mostly independent from mother, sexually immature and have not developed into adult size; immature primates are individual that have not sexually mature, subadults are fully independent, appears sexually mature but has not fully grown into adult size; adults are fully grown size with sexually mature sexual characteristics; old adults are adults with

signs of age degeneration. Monitoring is done from a safe distance until NHP is fully awake and ambulatory.

3.2.6 Sampling of Human

Human samples were collected from farmers in participating cattle farms. Sample size was limited to 10 farmers per farm due to logistical and budget constraints. Individuals were recruited based on their willingness to participate, were above 18 years old and have worked or assisted with handling of animals in participating farms. Individuals were recruited based on recommendations from the farm owner.

Human sampling was conducted by district health officers from Ministry of Health, Malaysia and staffs of EcoHealth Alliance in accordance to USAID Emerging Pandemic Threats PREDICT Operating Procedures (Huppenthal, 2009; C. K. Johnson & Saylors, 2016). A written informed consent from the participant was obtained for biological sampling collection and questionnaire. Participants were explained about the study objectives and that the biological testing was for research and not diagnostic for their health, the types of question asked, duration of questionnaire and potential risks of their participation. They were also informed that the study was voluntary and anonymous in nature.

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Biological sampling and questionnaire were conducted in a convenient, comfortable and private environment. Further description on the sampling is in Appendix X. Briefly, blood was taken from the median cubital vein on the upper limb or metacarpal plexus or the dorsal venous arch on the dorsal surface of the hand. Three mL of blood were taken from these animals and distributed equally in a BD Vacutainer® serum tube and EDTA tube. Processing of blood for serum extraction and storage were similar to previously mentioned in 3.2.4.

3.2.7 Data collection

Following biological sampling, interviews with farmers were conducted using questionnaire designed to gauge exposure or behavioural risks to zoonotic spillover events. The questionnaire has been approved for used by the Institutional Review Board for studies on human research subjects and has been verified in previous studies (Kamau et al., 2021; Li et al., 2019; The PREDICT Consortium, 2021). The questionnaire was designed into several parts to gather information on participants' demographics, exposure to different species of domestic animal and wildlife, forms of exposure to domestic animal and wildlife, reported unusual illnesses within the past year. The questionnaire took less than 30 minutes to be completed and were administered by trained EcoHealth Alliance staff~~s~~ in Malay language.

Data for livestock, other domestic animals and wildlife were recorded accordingly during the sampling process as described above (Table 3.2).

Table 3. 2 Types of data collected during sampling of animals and human from participating cattle farms located at the forest fringe in Perak and Kelantan, Malaysia

Species	Data collected
Livestock/Dogs	Date of sampling, weather (clear, cloudy, rain), moon (full, half, new moon), weight, animal classification (domestic, feral domestic, captive wild, wild), species scientific name, species' English name, breed, farm location GPS coordinates, husbandry, ecological setting, other animals at site, tag number, sex, pregnancy status, lactating status, descended testes, age class, age, age in captivity, health condition during sampling, presence of ectoparasite, notes (special characteristics/health status/injury/body condition), specimen type, specimen collected, GPS coordinates of sampling base
Rodent/Small mammal/Non-human primates/Wild boar	Date of capture, time of trap check, trap ID and type, weather (clear, cloudy, rain), moon (full, half, new moon), weight, animal taxa, species scientific name, species' English name, sex, pregnancy status, lactating status, descended testes, age class, health condition during sampling, presence of ectoparasite, morphometric measurements (body length, right ear height, right hind foot, tail length), recapture marking, notes (special characteristics/health status/injury/body condition), specimen type, specimen collected, GPS coordinates of traps, GPS coordinates of sampling base
Bats	Date of capture, time of capture, GPS coordinate of net, weather (clear, cloudy, rain), moon (full, half, new moon), weight, animal taxa, species scientific name, species' English name, sex, pregnancy status, lactating status, descended testes, age class, health condition during sampling, presence of ectoparasite, morphometric measurements (forearm or radius length, body length, ear length, hind foot length, tail length), recapture marking, notes (special characteristics/health status/injury/body condition), specimen type, specimen collected, volume of serum dilution, of traps, GPS coordinates of sampling base
Human	Participant ID, site location, GPS coordinates, date of sampling, body temperature, blood pressure, pulse, weight, height, obvious signs of illness, age, sex, ethnicity, education level, years of residence in village, frequency of entering forest, exposure to different species of domestic animal and wildlife (primate, bat, rodent, small mammal, wild boar, wild bird, swine, cattle, dog, cat, goat, chicken, others), forms of exposure to domestic animal and wildlife (1. General exposure: lived with pet, handled live animals, raised animals, share water source with animals, seen faeces in or near food, animal entered house, eaten food that were touched, disturbed or damaged by animal, scratched or bitten by animal; and 2. Exposure to wildlife: cook or handle recently slaughtered meat, offal or blood of wildlife, eaten raw or undercooked meat, offal or blood of wildlife, eaten wildlife of unknown health status, eaten or shared dead wildlife, collect and sell dead wildlife, slaughter or butcher wildlife), reported unusual illnesses within the past year (fever with headaches and lethargy @ encephalitis, haemorrhagic fever and rash, severe acute respiratory infection (SARI), Influenza-like illnesses (ILI), fever with diarrhoea and vomiting, fever with skin rash), interviewer's name

3.2.8 Multiplex Serology

Serum samples from all species of wildlife and domestic animals, and humans were analysed using an antigen-based multiplex microsphere immunoassay (MMIA) (Bossart et al., 2007). This antigen-based MMIA utilizes Luminex xMAP (xMAP = multianalyte profile) technology based on flow-cytometry principles which allows high-throughput, multiplex and simultaneous detection of up to 500 analytes in a 96-well microtiter plate. Since its invention in the late 1990s, this technology has been extensively used for detection of viral, bacterial, parasitical and fungal agents (Reslova et al., 2017). It has also been used increasingly for seroprevalence study of filoviruses and henipaviruses globally (Chowdhury et al., 2014; Dovih et al., 2019; Hayman et al., 2008; Laing et al., 2018; Peel et al., 2013b).

Filovirus envelope attachment glycoproteins (GP) and henipavirus envelope receptor-binding proteins (G) were designed as structurally native-like ectodomains for recombinant production, expressed in a mammalian cell-culture system, and purified by size exclusion and affinity chromatography (Bossart et al., 2005; Chan et al., 2009). Purified protein antigens were coupled to 5.6 µm diameter polystyrene magnetic beads for antigen-antibody binding. The list of filoviruses and henipaviruses tested are shown in Table 3.3.

Table 3. 3 Filovirus and henipavirus envelope glycoprotein antigen conjugated magnetic beads used in multiplex assay for antibody detection.

Filoviruses (GP)	Henipaviruses (G)
EBOV, Ebola virus	HeV, Hendra virus
BDBV, Bundibugyo virus	NiV, Nipah virus
BOMV, Bombali virus	CedV, Cedar virus
SUDV, Sudan virus	MojV, Mojiang virus
MLAV, Mengla virus	GhV, Ghana virus.
RESTVm, Reston virus monkey isolate	
RESTVp, Reston virus pig isolate	
LLOV, Lloviu virus	
MARV, Marburg virus	
RAVV, Ravn virus	

Prior to testing, individual serum samples were diluted 1:100 in ~~phosphate-buffered saline (~~PBS ~~)~~ in 1.5 mL Eppendorf tube and vortexed briefly to mix followed by heat inactivation at 56 °C for 30 minutes. Duplicates of 100 µl diluted sera were added into 96-wells plate containing 100 µL of bead master mix containing specific GP- and G-coated beads. Mixture is covered with plastic shield and aluminium foil and incubated on a shaker for 1200 r.p.m for 1 minute followed by 900 r.p.m for 45 minutes. Samples were washed to remove unbound antibodies three times and incubated at room temperature maintaining plate shaker agitation with 100 µL of 1:1 mixture of biotinylated-Protein A and biotinylated Protein G (Thermo Fisher Scientific, Waltham, MA, USA) diluted in 1:1000 in PBS-Tween 20 (0.05%). Mixture was washed three times and lastly incubated on a shaker with 100 µL 1:1000 streptavidin-phycoerythrin (PE) in into each well and agitated for 30 minutes. Antigen-antibody complexes were measured by a Bio-Rad Bio-

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Plex 200 HTF multiplexing system with at least 50 antigen-bead results per spectral region and antigen-bound IgG levels were reported as median fluorescence intensities (MFI). Cut-off values in the first round of testing at dilutions 1:100 were determined by outlier-based detection (3*Sigma, 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations) (Dovih et al., 2019).

Positive samples in the first round were rerun at the optimum dilutions of 1:400 and 1:500 with additional positive and negative controls. ~~Sera from~~ EBOV ~~infected bats~~ GP positive rabbit polyclonal sera at dilutions of 1:6400 were used as positive controls for EBOV which resulted in an average MFI value of 20359. Negative rabbit polysera and PBS were used as negative controls with a maximum MFI value of 1629 and 1851 respectively. Naïve livestock sera (N=46) comprising of cattle, goat, sheep and pigs tested with filovirus and henipavirus antigens by collaborators in Uniformed Services University of the Health Sciences, USA yield a maximum MFI value of less than 900. Antigen-antibody binding were measured and read using a Bio-Plex 200 system. Data were transformed to adjusted MFI values by taking the mean MFI values duplicates of each sample and subtracting average blank-PBS MFI per plate.

Cut-off were determined by using 3*Sigma and Latent cluster analysis (LCA). LCA was used in absence of gold standard assays and lack of positive and negative reference samples for the viral and animal species tested (Peel et al., 2013a). To improve cut-off

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determination, MFI values from local samples collected as part of the research programme were combined with this study dataset. Serum samples above the cut-off values were considered seropositive. The 3*Sigma cut-off values for filoviruses and henipaviruses were determined to be 4182 MFI and 4641 MFI respectively. LCA for filoviruses and henipaviruses were attempted but were not possible due to insufficient samples reactive samples, prohibiting the LCA models to converge on specific MFI values for threshold cut-off determination.

3.2.9 Data Analysis

Serodetection of filoviruses and henipavirus for different species were calculated based on the number of seropositive animals over the total number of sample and reported as percentage (%). The association between the serological evidence and determinants from animals (such as, farm, sex, age, age class, species, reproductive status and health status) and humans (such as, farm, husbandry, sex, age range, ethnicity, education level, years of residence in village, frequency of entering forest, exposure to different species of domestic animal and wildlife, forms of exposure to domestic animal and wildlife, reported unusual illnesses within the past year) were analysed for sero-epidemiological data using Pearson Chi-Square test, X^2 or Fisher's exact test (two-tailed), when X^2 is not appropriate (one of expected cell value is less than 5) at significance level $\alpha = 0.05$. Post-hoc test Bonferroni corrections and Bonferroni adjusted p-value were used for multiple comparison. Odds ratio (OR) and OR 95% Confidence intervals (CI) with Haldane-Anscombe correction were applied when contingency table has values of 0. The

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difference between seropositivity and hypothesized risk factors were tested using Kruskal-Wallis, KW (more than two groups) test at α=0.05 and post-hoc Dunn's test was applied if applicable. Variable was excluded from further analysis if data is insufficient or too small (n<10). All analysis were conducted using the Statistical Package for Social Sciences (SPSS 27) and Microsoft Excel.

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Commented [A37R36]: For eg, when values were 0 or 1 in 2x2 table (eg, sheep, dog, bat; table 3.15-3.18). Or when total of a variable was less than 10 (eg, Table 3.20 on exposure to animal species).

3.3 Results

3.3.1 Population characteristics

3.3.1.1 Domestic animals

Six forest-fringed cattle farms were enrolled in the study in 2018 from Kuala Kangsar district, Perak and Gua Musang district, Kelantan. Serum samples were collected from farmers (n=55), cattle (n=156), goats (n=158), sheep (n=11), dogs (n=23), bats (n=265), rodents (n=36), civet (n=1), wild boar (n=1), rabbits (n=3) and non-human primates (n=2). In total, 709 serum samples were collected.

Cattle were mostly sampled from integrated cattle farms (Table 3.13). Other domestic animals such as goats and sheep were mainly sampled from semi-intensive goat and sheep farms (Table 3.14 and 3.15). Dogs sampled were either tethered or free-roaming in the village or plantation (Table 3.16). Besides these animals, a pet rabbit was also sampled. Majority of the farm animals sampled were adults.

3.3.1.2 Wildlife

A total of 265 bats that were sampled during the study with an average of 44 (25-56) bats sampled per farm. Out of 9 species captured, 6 species were frugivores or nectarivores and 3 species were insectivores. Species that were most sampled were mostly adult male *C. brachyotis* (n=108), *E. spelaea* (n=70) and *C. horsfieldii* (n=52) (Table 3.17).

Table 3. 4 Bats based on sex, age class and species sampled from the surrounding targeted cattle farm at forest fringe

Bat	
	n
Sex	
Female	139
Male	126
	265
Age class	
Adult	242
Subadult	23
Juvenile	0
	265
Species	
Balionycteris maculata	3
Cynopterus brachyotis	108
Cynopterus horsfieldii	52
Eonycteris spelaea	70
Hipposideros galeritus	1
Macroglossus minimus	6
Macroglossus sobrinus	5
Nycteris tragata	1
Tylonycteris pachypus	19
	265

Overall, 36 rodents were sampled with an average of 6 (3-5) rodents per farm (Table 3.5). Twelve species of rodents mostly adult *Rattus exulans* (n=8) and *Rattus tiomanicus* (n=7) were captured.

Table 3. 5 Rodents based on sex, age class and species sampled from the surrounding targeted cattle farm at forest fringe.

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Rodent		
		n
Sex		
	Female	12
	Male	24
		36
Age class		
	Adult	29
	Subadult	7
	Juvenile	0
		36
Species		
	Chiropodomys gliroides	5
	Lariscus insignis	1
	Leopoldamys sabanus	5
	Maxomys rajah	1
	Maxomys surifer	3
	Niviventer cremoriventer	1
	Rattus argentiventer	2
	Rattus exulans	8
	Rattus rattus	1
	Rattus tiomanicus	7
	Sundasciurus lowii	1
	Tupaia glis	1
		36

Other wildlife sampled were pet macaques (n=2), a wild boar (n=1) and a civet, *Viverricula indica* (n=1).

3.3.1.3 Human

A total of 56 farmers from participating cattle farms were enrolled but only 55 samples were obtained. An average of 9 farmers were sampled from each farm. Majority sampled were male, between 20-29 years old, with mean age of 42 (18-68) of the Malay race.

Most have completed secondary school or above and have resided in the village for more than 10 years (51/55, 92.7%) (Table 3.19).

The most common domestic animals that farmers were exposed to within the past one year of sampling were cattle, cat and poultry. Whereas the most common wildlife exposed to were rodents and bats (Table 3.20). Farmers had general exposure to domesticated animals and wildlife in the past one year primarily through handling of live animals, animal entering living quarters and living with pet. Farmers were exposed to wildlife primarily through slaughter, butchering, cooking and handling of recently slaughtered meat, offal or blood of wildlife (Table 3.21).

Out of the 55 farmers, many farmers reported Influenza-like illnesses (ILI), followed by fever with diarrhoea and vomiting, Severe acute respiratory infection (SARI), fever with headaches and lethargy (3/55, 5.5%), and fever with skin rash (1/55, 1.8%) in the past year. No farmers reported haemorrhagic fever (Table 3.22).

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3.3.2 Serological result

3.1.1.1 Filovirus

A total of 45 out of 709 (6%) ~~sera~~ serum samples were reactive to filovirus. ~~(6%, 45/709??).~~ The serological detection for filovirus among species in descending order were goat (15.2%, 95% CI 10-22), sheep (9.1%, 95% CI 0.2-41.3), human (9.1%, 95% CI 3-35

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20), dog (8.7%, 95% CI 1.1-28), cattle (5.1%, 95% CI 2.2-9.9) and bat (1.9%, 95% CI 0.6-4.3) (Table 3.6, Figure 16-23). Bats species that were seropositive for filoviruses were *C. brachyotis*, *E. spelaea* and *C. horsfieldii* (Table 3.7).

Table 3. 6. Serological evidence of filovirus based on farm and species sampled using multiplex serology.

		Filovirus						
	Participating cattle farms	**1**	**2**	**3**	**4**	**5**	**6**	**Total**
Category	**Husbandry**	**SI**	**SI**	**SI**	**It**	**It**	**In**	
Human								
	Individual sampled	10	9	10	10	10	6	55
	Seropositive, n (%)	1 (10)	0	1 (10)	2 (20)	0	1 (16.7)	5 (9.1)
Cattle								
	Individual sampled	22	15	24	29	36	30	156
	Seropositive, n (%)	2 (9.1)	2 (13.3)	2 (8.3)	1 (3.4)	1 (2.8)	0	8 (5.1)
Goat								
	Individual sampled	20	44	31	22	22	19	158
	Seropositive, n (%)	0	7 (15.9)	8 (25.8)	5 (22.7)	0	4 (21.7)	24 (15.2)
Sheep								
	Individual sampled	0	0	0	9	0	2	11
	Seropositive, n (%)	0	0	0	0	0	1 (50)	1 (9.1)
Dog								
	Individual sampled	10	1	2	1	0	9	23
	Seropositive, n (%)	1 (10)	1 (100)	0	0	0	0	2 (8.7)
Bat								
	Individual sampled	45	49	47	43	25	56	265
	Seropositive, n (%)	0	3 (6.1)	1 (2.1)	0	1 (4)	0	5 (1.9)
Rodent								
	Individual sampled	10	5	8	3	7	3	36
	Seropositive, n (%)	0	0	0	0	0	0	0
Civet								
	Individual sampled	0	1	0	0	0	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Wild boar								
	Individual sampled	0	0	0	0	1	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Rabbit								
	Individual sampled	0	0	3	0	0	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Primate								
	Individual sampled	0	1	1	0	0	0	2
	Seropositive, n (%)	0	0	0	0	0	0	0
Total		4	13	12	8	2	6	45

Production system: SI, semi-intensive system; It, integrated system; In, intensive system

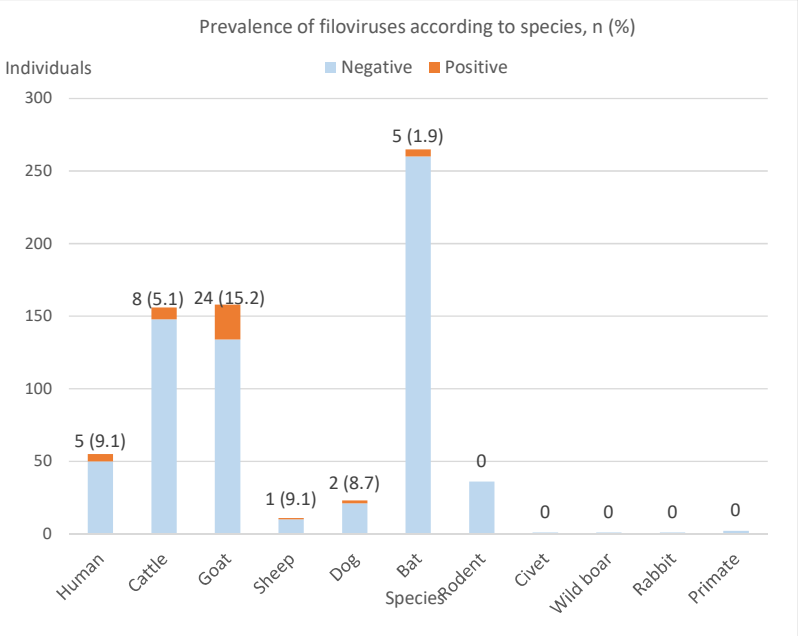

Figure 3. 15. Serological evidence of filovirus based on species sampled using multiplex serology.

Table 3. 7. Serological evidence of filovirus in bats using multiplex serology.

No	Species	Individuals sampled (n)	Filovirus positive (%)
1	*Balionycteris maculata*	3	0
2	*Cynopterus brachyotis*	108	2 (1)
3	*Cynopterus horsfieldii*	52	1 (2)
4	*Eonycteris spelaea*	70	2 (2)
5	*Hipposideros galeritus*	1	0
6	*Macroglossus minimus*	6	0
7	*Macroglossus sobrinus*	5	0
8	*Nycteris tragata*	1	0
9	*Tylonycteris pachypus*	19	0
	Total	265	5

Most filovirus reactive samples were reactive to BDBV (29/45) followed by SUDV (16/45) and RESTV (16/45) (Table 3.8). Twenty-three (23/45, 51%) samples were reactive to only one antigen, with the most being BDBV (16/23), followed by EBOV (4/23), RESTVp (2/23) and SUDV (1/23). Whereas twenty-two (22/45, 48.9%) samples were reactive to two to five filovirus antigens which involved BDBV, SUDV, RESTVp the most. Cross-reactivity were observed between EBOV, BDBV, BOMV, SUDV, RESTVm, RESTVp, MARV, and RAVV (Appendix).

Table 3. 8. Reactivity with filovirus across species using multiplex serology.

			Reactivity to filovirus, n (%)									
*Category	Total	Total reactive	EBOV	BDBV	BOMV	SUDV	RESTVm	RESTVp	LLOV	MLAV	MARV	RAVV
Human	55	5	2 (3.6)	3 (5.5)	0	0	0	0	0	0	0	1 (1.7)
Cattle	156	8	3 (1.9)	6 (3.8)	0	2 (1.3)	2 (1.3)	0	0	0	0	0
Goat	158	24	0	14 (8.9)	0	12 (7.6)	0	14 (8.9)	0	0	0	0
Sheep	11	1	0	1 (9.1)	1 (9.1)	1 (9.1)	0	1 (9.1)	0	0	1 (9.1)	0
Dog	23	2	1 (4.3)	2 (8.7)	0	0	0	0	0	0	0	0
Bat	265	5	2 (0.8)	3 (1.1)	1 (0.4)	1 (0.4)	0	1 (0.4)	0	0	0	0
Total		**45**	**8 (17.8)**	**29 (64.4)**	**2 (4.4)**	**16 (35.6)**	**2 (4.4)**	**16 (35.6)**	**0**	**0**	**1 (2.2)**	**4 (8.9)**

EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

*Non-reactive category not included

The range of MFI for filovirus positive sera was between 4183 and 24059, with the highest being 24059 MFI for BDBV in a human. There were 10 reactions which produced an MFI reading of 10000-14999 MFI (EBOV, BDBV, SUDV and RAVV) and 2 reactions with >20000 MFI (BDBV) (Table 3.9).

Table 3. 9. Filovirus range of MFI of positive sera, number of high reactivity and its antigen among different species using multiplex serology.

	High reactivity for filovirus							
	No. of Sera with 10000-14999 MFI	Type of Antigen reaction	No. of Sera with 15000-19999 MFI	Type of Antigen reaction	No. of Sera with >20000 MFI	Type pf antigen reaction	MFI range	Mean MFI
Bat	2	EBOV, BDBV	0	-	0	-	4382-10784	8248
Cattle	1	EBOV	0	-	0	-	4246-10205	6316
Dog	0	-	0	-	0	-	5429-7561	6785
Goat	3	BDBV, RAVV	0	-	1	BDBV	4183-21882	6806
Sheep	1	SUDV	0	-	0	-	4317-10890	7665
Human	3	EBOV, BDBV, RAVV	0	-	1	BDBV**	4928-24059	12752
Total	10		0		2			8095

**Among all reactivity to filoviruses, a human reported highest MFI for BDBV
EBOV, Ebola virus; BDBV, Bundibugyo virus; SUDV, Sudan virus; RAVV, Ravn virus

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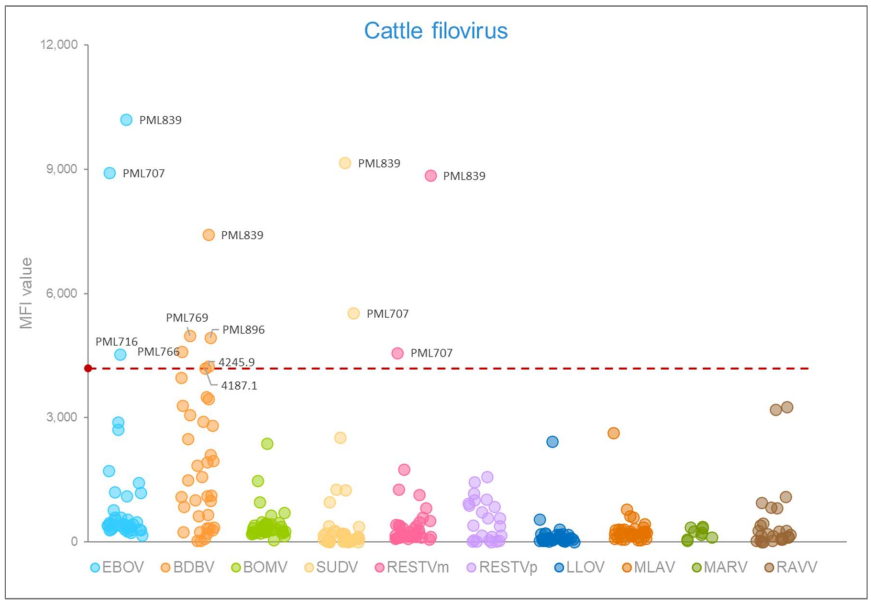

Figure 3. 16. Filovirus range of MFI of positive cattle sera using multiplex serology.

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

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Commented [A48R47]: Thanks for the suggestion, labels added

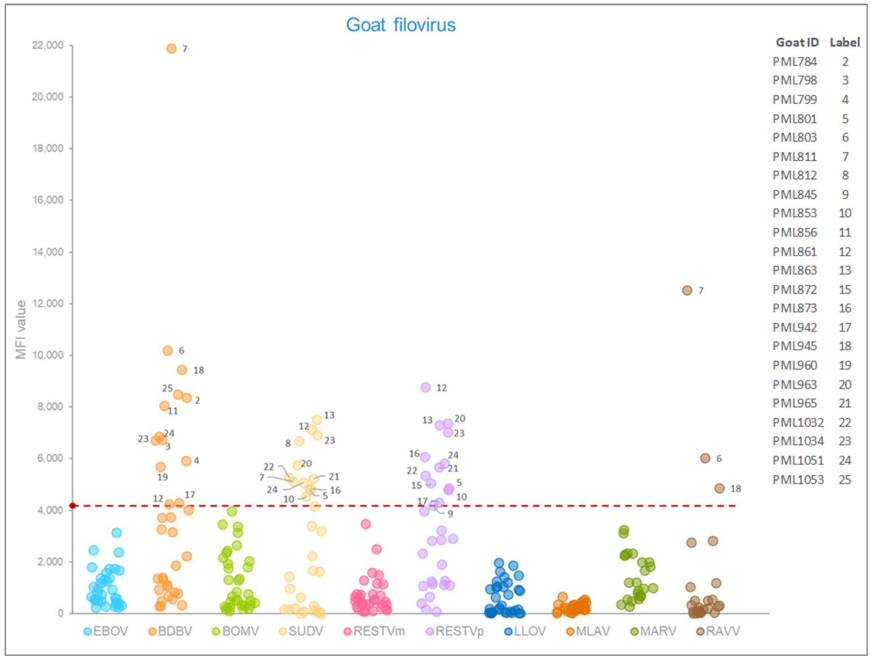

Figure 3. 17. Filovirus range of MFI of positive goat sera using multiplex serology.

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

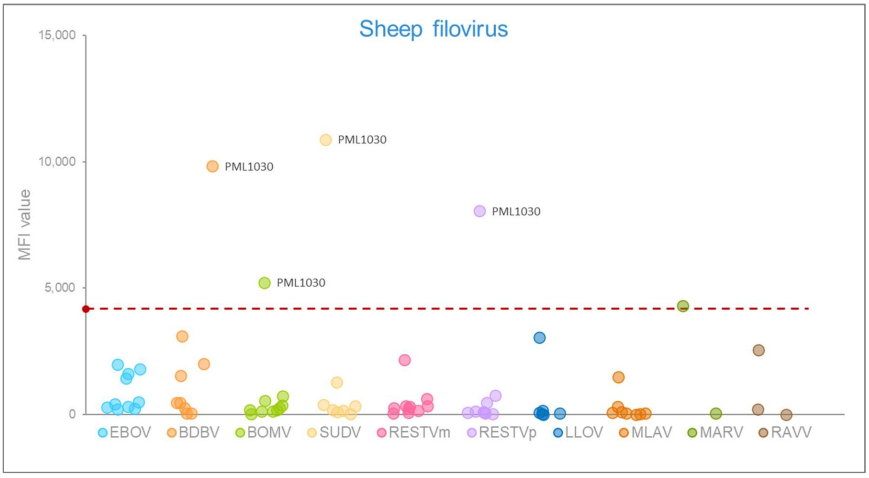

Figure 3. 18. Filovirus range of MFI of positive sheep sera using multiplex serology.

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

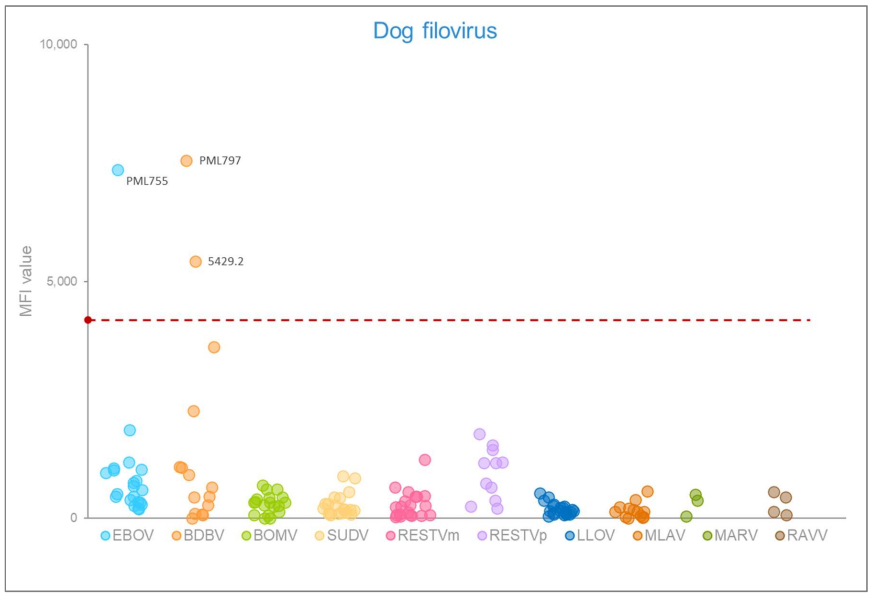

Figure 3. 19. Filovirus range of MFI of positive dog sera using multiplex serology.

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

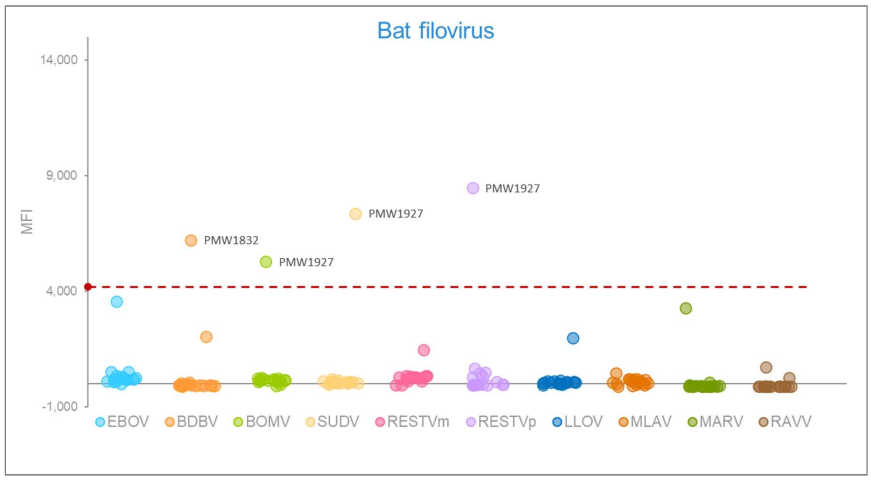

Figure 3. 20. Filovirus range of MFI of positive *Cynopterus brachyotis* bat using multiplex serology.

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

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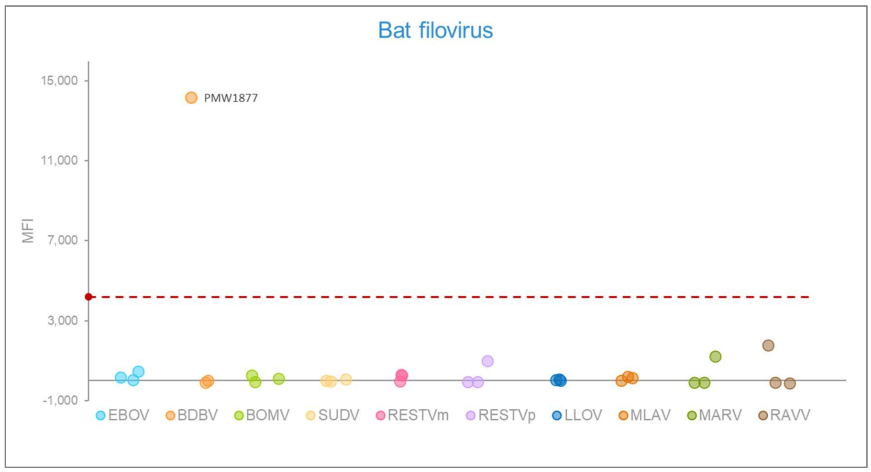

Figure 3. 21. Filovirus range of MFI of positive *Cynopterus horsfieldii* bat using multiplex serology.

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

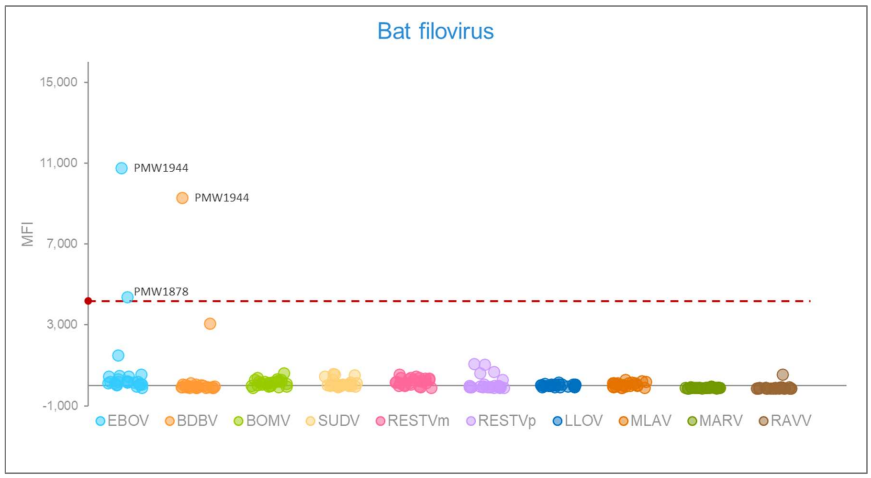

Figure 3. 22. Filovirus range of MFI of positive *Eonycteris spelaea* bat using multiplex serology.

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

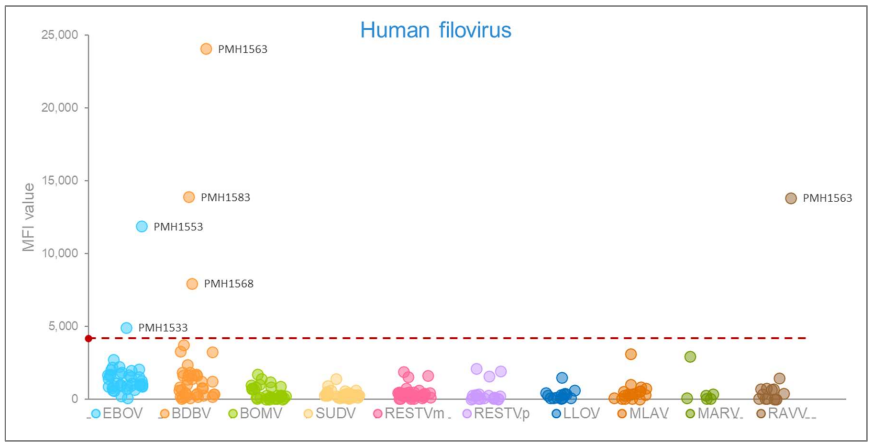

Figure 3. 23. Filovirus range of MFI of positive human sera using multiplex serology.

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

3.1.1.2 Henipavirus

A total of 36 out of 709 ~~sera~~ serum samples were reactive with henipavirus protein antigens. The reactive sera were in detected from goats (13.9%, 95% CI 8.9-20.3), sheep (9.1%, 95% CI 0.2-41.3), humans (7.3%, 95% CI 2-17.6), dogs (4.3%, 95% CI 0.1-21.9), cattle (3.2%, 95% CI 1.0-7.3) and bats (1.1%, 95% CI 0.2-3.3) (Table 3.10 and Figure 3.25-31). Bats seropositive for henipavirus were *C. brachyotis* and *C. horsfieldii* (Table 3.11).

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Table 3. 10. Serological evidence of henipavirus exposure based on farm and species using multiplex serology.

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Commented [A54R53]: There wasn't. Results in 3.3.3.1 and Table 3.14

		Henipavirus						
	Participating cattle farms	**1**	**2**	**3**	**4**	**5**	**6**	**Total**
	Husbandry	**SI**	**SI**	**SI**	**It**	**It**	**In**	
Human	Individual sampled	10	9	10	10	10	6	55
	Seropositive, n (%)	0	1 (10)	0	2 (20)	0	1 (16.7)	4 (7.3)
Cattle	Individual sampled	22	15	24	29	36	30	156
	Seropositive, n (%)	1 (4.5)	0	1 (4.2)	1 (3.4)	0	2 (6.7)	5 (3.2)
Goat	Individual sampled	20	44	31	22	22	19	158
	Seropositive, n (%)	0	7 (15.9)	7 (22.6)	5 (22.7)	0	3 (15.8)	22 (13.9)
Sheep	Individual sampled	0	0	0	9	0	2	11
	Seropositive, n (%)	0	0	0	0	0	1 (9.1)	1 (9.1)
Dog	Individual sampled	10	1	2	1	0	9	23
	Seropositive, n (%)	0	0	0	0	0	1 (11.1)	1 (4.3)
Bat	Individual sampled	45	49	47	43	25	56	265
	Seropositive, n (%)	0	2 (4.1)	1 (2.1)	0	0	0	3 (1.1)
Rodent	Individual sampled	10	5	8	3	7	3	36
	Seropositive, n (%)	0	0	0	0	0	0	0
Civet	Individual sampled	0	1	0	0	0	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Wild boar	Individual sampled	0	0	0	0	1	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Rabbit	Individual sampled	0	0	3	0	0	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Primate	Individual sampled	0	1	1	0	0	0	2
	Seropositive, n (%)	0	0	0	0	0	0	0
Total		1	10	9	8	0	8	

Production system: SI, semi-intensive system; It, integrated system; In, intensive system

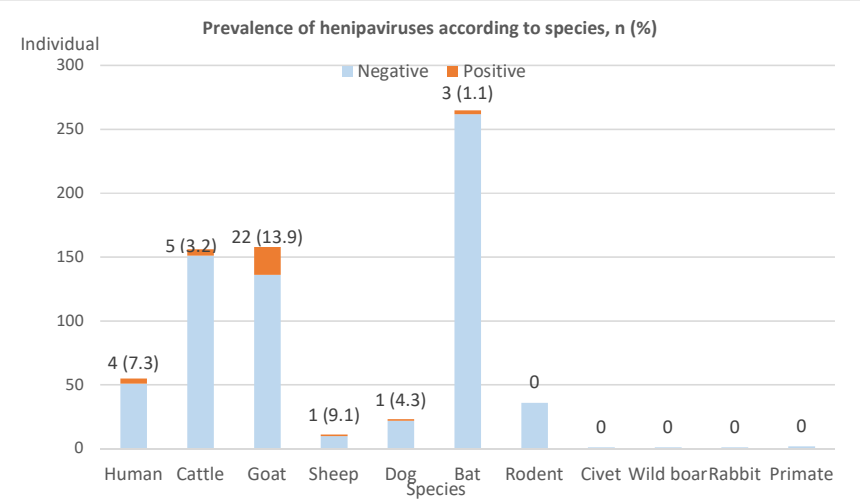

Figure 3. 24. Serological evidence of henipaviruses based on species sampled using multiplex serology.

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Table 3. 11. Serological evidence of henipavirus in bats using multiplex serology.

No	Species	Individuals sampled (n)	Henipavirus positive (%)
1	*Balionycteris maculata*	3	0
2	*Cynopterus brachyotis*	108	2 (1)
3	*Cynopterus horsfieldii*	52	1 (2)
4	*Eonycteris spelaea*	70	0
5	*Hipposideros galeritus*	1	0
6	*Macroglossus minimus*	6	0
7	*Macroglossus sobrinus*	5	0
8	*Nycteris tragata*	1	0
9	*Tylonycteris pachypus*	19	0
	Total	265	3

Most seropositive samples were reactive with GhV (32/36), HeV (22/36), NiV (19/36), CedV (9/36) and MojV (7/36) (Table 3.11). Eleven (11/36, 31%) samples were reactive with only one antigen, with the most being GhV (9/11), followed by MojV (2/11). Twenty-two (25/36, 69.4%) samples were reactive with two to five henipavirus antigens which

Commented [A57]: React with/bind to.

Commented [A58R57]: Apologies for my bad grammar

involved GhV, HeV and NiV the most. In these instances, cross-reactivity were observed between all five antigens tested (Appendix).

Table 3. 12 Reactivity with henipavirus across species using multiplex serology.

			Reactivity with henipavirus, n (%)				
	Total sampled	Total reactive	HeV	NiV	CedV	MojV	GhV
Human	55	4	1 (1.7)	0	1 (1.7)	4 (6.7)	4 (6.7)
Cattle	156	5	1 (0.6)	0	1 (0.6)	2 (1.3)	4 (2.6)
Goat	158	22	17 (10.8)	15 (9.5)	5 (3.2)	0	20 (12.7)
Sheep	11	1	1 (9)	1 (9.1)	1 (9.1)	0	1 (9)
Dog	23	1	0	0	0	1 (4.3)	0
Bat	265	3	2 (0.8)	2 (0.8)	1 (0.4)	0	3 (1.1)
Total		36	22 (61.1)	19 (52.8)	9 (25)	7 (19.4)	32 (88.9)

HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

The range of MFI for henipavirus positive sera was between 4182 and 24689 with the highest being 24689 MFI against GhV in a farmer. There were 39 reactions with an MFI reading of 10000-19999 MFI (HeV, NiV, CedV, and GhV), and 5 reactions of >20000 MFI (HeV, CedV, MojV and GhV). Goat sera displayed many high MFI readings of >10000. In general, almost all henipavirus reactive sera had reactivity with GhV G with many being >10000 MFI. (Table 3.13 and Appendix).

Commented [A59]: Do you mean that these are all cross-reactive, with the highest binding to GhV?

Commented [A60R59]: Yes, 25 sera were cross-reactive to more than 1 antigen. Among them, 23 were cross-reactive to GhV

Commented [A61R59]: This is the breakdown of cross-reaction
H, C, G . -1
H, C, Mo, G - 1
H, G . -1
H, N . -2
H, N, C, G . -6
H, N, C, Mo, G . -1
H, N, G . -10
Mo, G . -3
Total: 25

Commented [A62]: Were these values collected from dilutions of 1:100 (initial testing) or follow-up 1:400/500 dilutions?

Commented [A63R62]: 1/400/500 dilution

Table 3. 13. Henipavirus range of MFI of positive sera, number of high reactivity and its antigen among different species using multiplex serology.

	High reactivity for henipavirus							
	No. of Sera with 10000-14999 MFI	Type of Antigen reaction	No. of Sera with 15000-19999 MFI	Type of Antigen reaction	No. of Sera with >20000 MFI	Type pf antigen reaction	MFI range	Mean MFI
Bat	4	-	0	-	0	-	4776-12117	8248
Cattle	3		2	HeV, CedV	1	GhV	5000-20100	6316
Dog	0	-	0	-	0	-	5042[a]	6785
Goat	21	HeV, NiV, CedV, GhV	4	HeV, GhV	0	-	4834-19905	6806
Sheep	0		3	HeV, NiV, GhV	0	-	9894-17401	7665
Human	2	-	0	-	4	HeV, CedV, MojV, GhV	7170-24689	12752
Total	30	GhV	9		5			8095

[a]no upper MFI range as there was only 1 reactivity in 1 dog serum
HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

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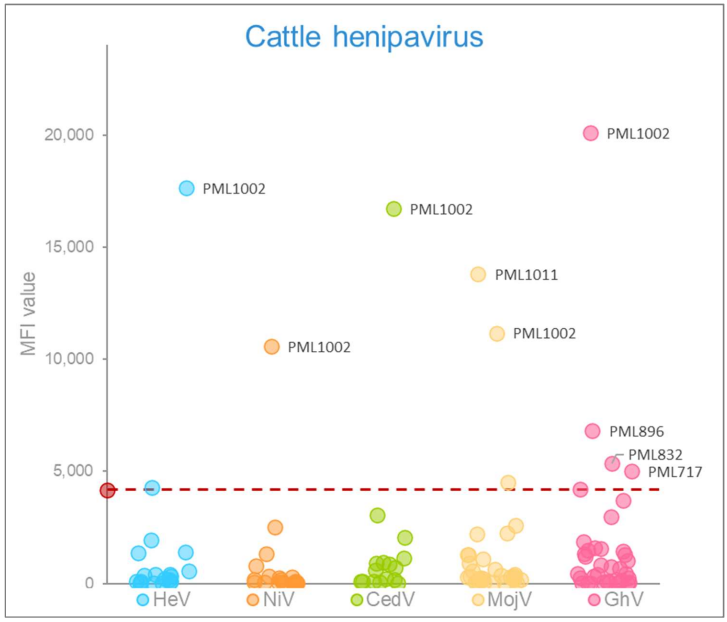

Figure 3. 25. Henipavirus range of MFI of positive cattle sera using multiplex serology.

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

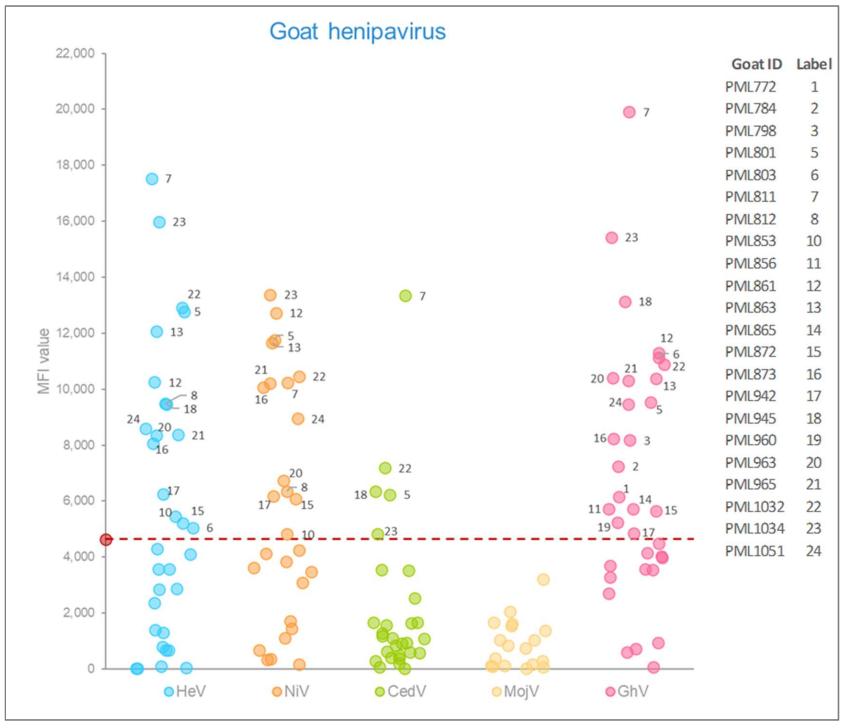

Figure 3. 26. Henipavirus range of MFI of positive goat sera using multiplex serology.

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

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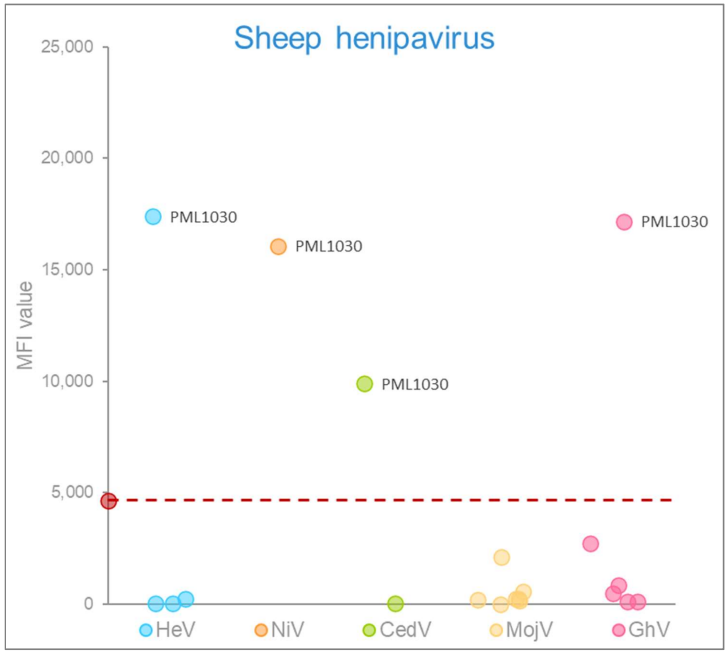

Figure 3. 27. Henipavirus range of MFI of positive sheep sera using multiplex serology.

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

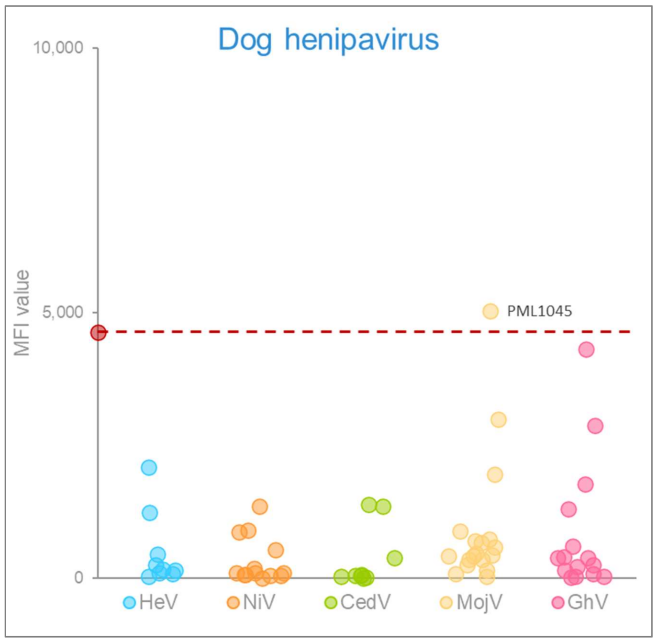

Figure 3. 28. Henipavirus range of MFI of positive dog sera using multiplex serology.

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

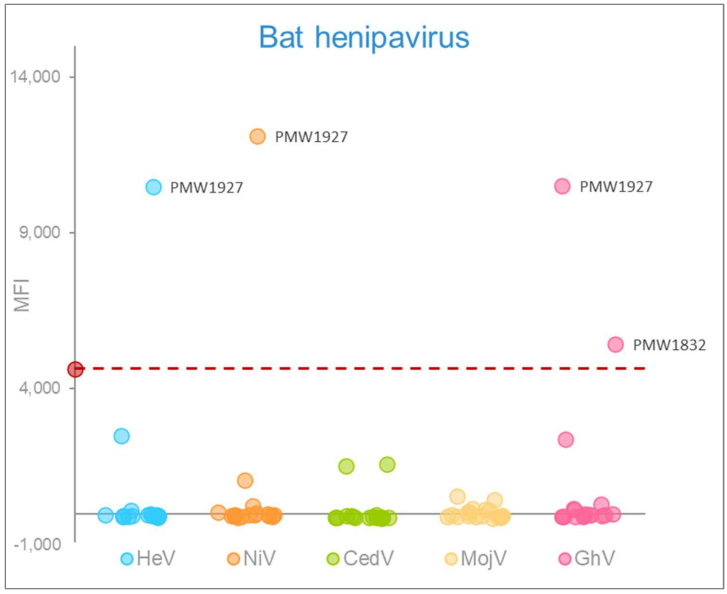

Figure 3. 29. Henipavirus range of MFI of positive *Cynopterus brachyotis* bat sera using multiplex serology.

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

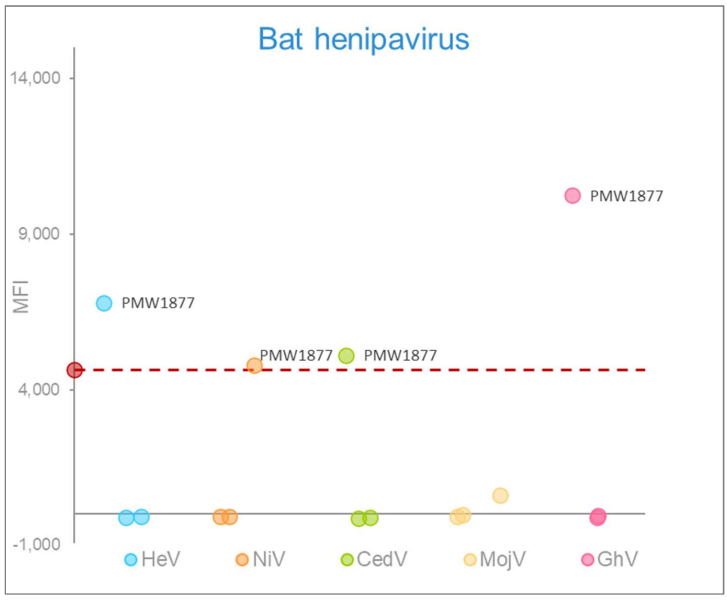

Figure 3. 30. Henipavirus range of MFI of positive *Cynopterus horsfieldii* bat sera using multiplex serology.

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

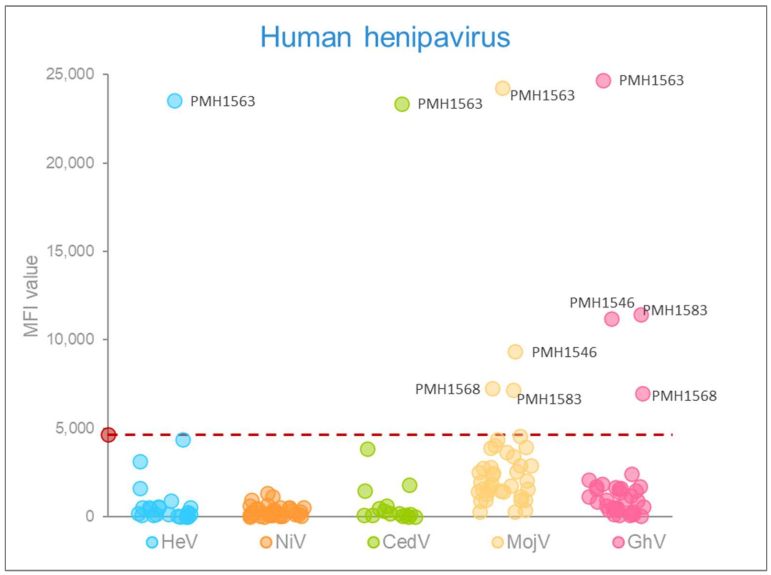

Figure 3. 31. Henipavirus range of MFI of positive human sera using multiplex serology.

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

3.3.3 Serological evidence and demographic distribution

3.3.3.1 Cattle

The serological evidence of filovirus and henipavirus in cattle were 5.1%, 8/156 (95% CI 2.2-9.9) and 3.2%, 5/156 (95% CI 1.0-7.3) respectively. Among the filovirus positive (8/156) and henipavirus positive (5/156) cattle sera, most were female, adults and apparently healthy and had no observable ectoparasite at the time of sampling. No

significant difference was observed between serological evidence of filovirus or henipavirus with husbandry, sex, age class and health status (Table 3.14).

Table 3. 14 Serological evidence of filovirus and henipavirus in cattle based on farm, sex, age class, reproduction status, health status and presence of ectoparasite

	Cattle				
	n	**Filovirus positive**	**P-value**	**Henipavirus positive**	**P-value**
Farm			a		a
1	22	2		1	
2	15	2		0	
3	24	2		1	
4	29	1		1	
5	36	1		0	
6	30	0		2	
	156	8		5	
Husbandry			0.108		0.343
Intensive	30	0		2	
Semi-intensive	61	6		2	
Integrated	65	2		1	
	156	8		5	
Sex			1.000		a
Female	101	5		5	
Male	55	3		0	
	156	8		5	
Age class			1.000		1.000
Adult (>2 yr)	121	7		4	
Subadult (6m-2yr)	28	1		1	
Juvenile (<6m)	7	0		0	
	156	8		5	
Reproduction status					
Pregnant	18	0	a	0	a
Lactating	33	1	a	2	0.285
		1		2	
Health status			0.192		0.123
Apparently healthy	153	7		4	
Sick	3	1		1	
	156	8		5	
Ectoparasite			a		a
No	147	8		5	
Yes	9	0		0	
	156	8		5	

[a]Variable is excluded from further analysis due to insufficient or too small data

3.3.3.2 Goat

The serological evidence of filovirus and henipavirus in goat were 15.2%, 24/158 (95% CI 10-22) and 13.9%, 22/158 (95% CI 8.9-20.3) respectively. Among the filovirus positive (24/158) and henipavirus positive (22/158) goat sera, most were semi-intensively reared, female, adults and apparently healthy. Subadult goats were 6.3 times less likely to be seropositive for filoviruses as compared to adults (P= 0.028, OR=6.3, 95% CI=1.149, 34.383). No significant difference was observed between serological evidence of henipavirus and the various factors (Table 3.15).

Commented [A64]: Stats significance?

Commented [A65R64]: It was not significant after Bonferroni correction

Table 3. 15 Serological evidence of filovirus and henipavirus in goat based on farm, sex, age class, reproduction status, health status and presence of ectoparasite sampled from goat farms surrounding participating targeted cattle farms

	Goat				
	n	Filovirus positive	P-value	Henipavirus positive	P-value
Targeted Cattle Farm			a		a
1	20	0		0	
2	44	7		7	
3	31	8		7	
4	22	5		5	
5	22	0		0	
6	19	4		3	
	158	24		22	
Husbandry of goat farm			0.009[b]		0.004[b]
Intensive	52	2		1	
Semi-intensive	101	21		20	
Extensive	5	1		1	
	158	24		22	
Sex			0.243		0.629
Female	104	13		13	
Male	54	11		9	
	158	24		22	
Age class			0.028		0.114
Adult (>1 yr)	106	21		19	
Subadult (6m-1yr)	38	1		2	
Juvenile (<6m)	14	2		1	
	158	24		22	
Reproduction status					
Pregnant	28	5	0.771	5	0.548
Lactating	28	4	1.000	4	1.000

Health status			0.148		0.403
Apparently healthy	122	21		18	
Sick	36	3		4	
	158	24		22	
Ectoparasite			0.604		0.788
No	120	17		16	
Yes	38	7		6	
	158	24		22	

[a]Variable is excluded from further analysis due to insufficient or too small data
[b]Not significant after Bonferroni correction

3.3.4 Sheep

The serological evidence of filovirus and henipavirus in sheep were 9.1%, 1/11 (95% CI 0.2-41.3) for both. Out of 11 sheep sampled, only one sheep was seropositive for filoviruses and henipaviruses. The sheep was a female pregnant and lactating adult that was raised extensively, and apparently healthy (Table 3.16). Further analysis with the variables were not performed due to insufficient or too small data.

Table 3. 16 Serological evidence of filovirus and henipavirus in sheep based on farm, sex, age class, reproduction status, health status and presence of ectoparasite sampled from sheep farms surrounding participating targeted cattle farm

	Sheep		
	n	**Filovirus positive**	**Henipavirus positive**
Targeted Cattle Farm			
1	0	0	0
2	0	0	0
3	0	0	0
4	9	0	0
5	0	0	0
6	2	1	1
	11	1	1
Husbandry of sheep farm			
Semi-intensive	9	0	0
Extensive	2	1	1
	11	1	1
Sex			
Female	6	1	1
Male	5	0	0
	11	1	1
Age class			

Adult (>1 yr)	7	1	1
Subadult (6m-1yr)	2	0	0
Juvenile (<6m)	2	0	0
	11	1	1
Reproduction status			
Pregnant	2	1	1
Lactating	2	1	1
Health status			
Apparently healthy	11	1	1
Sick	0	0	0
	11	1	1
Ectoparasite			
No	11	1	1
Yes	0	0	0
	11	1	1

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3.3.5 Dog

The serological evidence of filovirus and henipavirus in dog were 8.7%, 2/23 (95% CI 1.1-28) and 4.3%, 1/23 (95% CI 0.1-21.9) respectively. Filovirus positive dog (2/23) were adult and subadult tethered males. Whereas henipavirus positive dog was an adult male raised extensively. All filovirus and henipavirus positive dogs were apparently healthy with no observable ectoparasite at time of sampling. Further analysis with the variables were not performed due to insufficient or too small data (Table 3.17).

Table 3. 17 Serological evidence of filovirus and henipavirus in dog based on farm, sex, age class, reproduction status, health status and presence of ectoparasite sampled surrounding participating targeted cattle farm

	n	Dog Filovirus positive	Henipavirus positive
Targeted Cattle Farm			
1	10	1	0
2	1	1	0
3	2	0	0
4	1	0	0
5	0	0	0
6	9	0	1
	23	2	1

Husbandry of dog			
Extensive	18	0	1
Tethered	5	2	0
	23	2	1
Sex			
Female	14	0	0
Male	9	2	1
	23	2	1
Age class			
Adult (>1y)	12	1	1
Subadult (<1y)	6	1	0
Juvenile (<1m)	5	0	0
	23	2	1
Reproduction status			
Pregnant	0	0	0
Lactating	1	0	0
Health status			
Apparently healthy	23	2	1
Sick	0	0	0
Ectoparasite			
No	10	2	0
Yes	13	0	1
	23	2	1

3.3.6 Bat

The serological evidence of filovirus and henipavirus in bat were 1.9%, 5/265 (95% CI 0.6-4.3) and 1.1%, 3/265 (95% CI 0.2-3.3) respectively. Majority of the filovirus positive (5/265) and henipavirus positive (3/265), bat sera were adult females that were apparently healthy (Table 3.18). Seropositivity was detected in 3 bat species (*C. brachyotis*, *E. spelaea* and *C. horsfieldii).* Further analysis with the variables were not performed due to insufficient or too small data.

Table 3. 18 Serological evidence of filovirus and henipavirus in bat based on sex, age class, reproduction status, health status and presence of ectoparasite surrounding participating targeted cattle farms

	Bat		
	n	Filovirus positive	Henipavirus positive
Sex			
Female	139	3	2
Male	126	2	1
	265	5	3
Age class			
Adult	242	5	3
Subadult	23	0	0
Juvenile	0	0	0
	265	5	3
Species			
Balionycteris maculata	3	0	0
Cynopterus brachyotis	108	1	1
Cynopterus horsfieldii	52	2	2
Eonycteris spelaea	70	2	0
Hipposideros galeritus	1	0	0
Macroglossus minimus	6	0	0
Macroglossus sobrinus	5	0	0
Nycteris tragata	1	0	0
Tylonycteris pachypus	19	0	0
	265	5	3
Reproduction status			
Pregnant	14	0	0
Lactating	20	0	0
Health status			
Apparently healthy	263	5	3
Sick	2	0	0
	265	5	3
Ectoparasite			
No	209	4	3
Yes	56	1	0
	265	5	3

3.1.1.1 Human

The serological evidence of filovirus and henipavirus in human were 9.1%, 5/55 (95% CI 3-20) and 7.3%, 4/55 (95% CI 2-17.6) respectively. Humans positive for filovirus (5/55) and henipavirus (4/55) were mostly males of Malay ethnicity, completed secondary level education and have resided in village for more than 10 years. No significant difference was observed between serological evidence of filovirus or henipavirus with the various factors (Table 3.19).

Commented [A66]: Were seropositive humans residing on SI farms, and raising goats?

Commented [A67R66]: Yes, 2 seropositive humans were residing on SI farms. We didn't sample goat owners as they were not part of the study. Although two positive cattle farmer raised goats in the past year (1 extensive system cattle farmer which also had a goats which we sampled).

Table 3. 19 Serological evidence of filovirus and henipavirus in human based on demographics of participating farmers sampled from targeted cattle farms

	Human				
	n	Filovirus positive	P-value	Henipavirus positive	P-value
Targeted Cattle Farm			a		a
1	10	1		0	
2	9	0		1	
3	10	1		0	
4	10	2		2	
5	10	0		0	
6	6	1		1	
	55	5		4	
Husbandry of cattle farm			0.664		0.417
Intensive	6	1		1	
Semi-intensive	29	2		1	
Integrated	20	2		2	
	55	5		4	
Sex					
Male	45	3	0.220	3	0.563
Female	10	2		1	
	55	5		4	
Age range			0.069		0.291
10-29	16	1		2	
30-49	20	0		0	
50-69	19	4		2	
	55	5		4	
Years of residence in village			0.325		0.203
1 month - 1 year	1	0		0	
1-10 years	3	1		1	
>10 years	51	4		3	
	55			4	
Ethnic					

	Melayu	51	4	a	3	a
	Semai	1	0		0	
	Indian	1	0		0	
	Bugis	1	1		1	
	Indonesian	1	0		0	
		55	5		4	
Education level				0.433		0.406
	No education	3	0		0	
	Primary	16	0		0	
	Secondary	29	4		4	
	Tertiary	7	1		0	
		55	5		4	
Frequency of entering forest				0.205		0.900
	Never	8	2		1	
	Once a year	3	0		0	
	≥1 in a month	31	1		2	
	≥1 in a week	13	2		1	
		55	5		4	

[a]Variable is excluded from further analysis due to insufficient or too small data

Filovirus and henipavirus serologically positive humans reported exposure to cat, cattle, poultry, goat, rabbit, rodent, wild bird, bat and deer. One henipavirus positive human had exposure to non-human primates. No significant finding were found between serological evidence of filovirus and henipavirus and exposure to different animal species (Table 3.20).

Table 3. 20 Serological evidence of filovirus and henipavirus in human based on exposure to different species of domestic animals and wildlife in the past year

		Human, n=55					
No.	**Species**	**Exposure to animal species**	**%**	**Filovirus positive, n=5**	**P-value**	**Henipavirus positive, n=4**	**P-value**
					FE		**FE**
Domestic animal							
1	**Cattle**	50	90.9	4	0.391	3	0.325
2	**Cat**	49	89.1	5	a	4	a
3	**Poultry**	41	74.5	3	0.592	3	1.000
4	**Goat**	27	49.1	2	1.000	2	1.000
5	**Dog**	6	10.9	0	a	0	a
6	**Rabbit**	2	3.6	1	a	1	a
Wildlife							
1	**Rodent (rats/shrews/squirrel)**	34	61.8	5	a	4	a
2	**Wild bird**	19	34.5	2	1.000	2	0.602

3	**Non-human primates**	15	27.3	0	a	1	1.000
4	**Bat**	13	23.6	2	0.582	2	0.234
5	**Reptiles (snake, monitor lizard)**	9	16.4	0	a	0	a
6	**Deer**	7	12.7	1	0.508	1	0.429
7	**Porcupine**	6	10.9	0	a	0	a
8	**Mousedeer**	3	5.5	0	a	0	a
9	**Wild boar**	1	1.8	0	a	0	a
10	**Pangolin**	1	1.8	0	a	0	a
11	**Fox**	1	1.8	0	a	0	a
12	**Binturong**	1	1.8	0	a	0	a
13	**Bear**	1	1.8	0	a	0	a

[a]Variable is excluded from further analysis due to insufficient or too small data

A majority (26.7%, 4/15) filovirus seropositive humans have eaten food that were touched, disturbed or damaged by animals. These ~~animals~~ humans were 10 times more likely to be seropositive for filovirus compared to those who have not (P=0.017, OR=10.3, 95% CI=1.45, 73.24). For henipavirus, there was no significant association between seropositivity to the different forms of exposure to various animal species. However, there is a meaningful increase of risk for consuming food that were touched, disturbed or damaged by animal (P=0.057) and eating raw or undercooked meat, offal or blood of wildlife (0.097) (Table 3.21).

Commented [A68]: These humans?

Commented [A69R68]: Yes :D

Table 3. 21 Serological evidence of filovirus and henipavirus in human based on different forms of exposures to animals and wildlife in the past year

		Human, n=55					
	Forms of exposure	**Reported forms of exposure**	**%**	**Filovirus positive, n=5**	**Fischer Exact p-value**	**Henipavirus positive, n=4**	**Fischer Exact p-value**
General exposure to domesticated animals or wildlife							
1	**Handled live animals**	53	96.4	5	1.000	4	1.000
2	**Animal entered house**	49	89.1	5	1.000	4	1.000
3	**Lived with pet**	44	80	3	0.259	4	0.573

4	**Raised animals**	39	70.9	4	1.000	3	1.000
5	**Eaten food that were touched, disturbed or damaged by animal**	15	27.3	4	0.017*	3	0.057
6	**Share water source with animals**	14	25.5	0	a	0	a
7	**Scratched or bitten by animal**	13	23.6	1	a	2	0.234
8	**Seen feces in or near human food**	7	12.7	0	a	0	a
Specific exposure to wildlife							
1	**Cook or handle recently slaughtered meat, offal or blood of wildlife**	18	32.7	1	a	1	a
2	**Slaughter or butcher wildlife**	18	32.7	2	1.000	2	0.59
3	**Eaten raw or undercooked meat, offal or blood of wildlife**	8	14.5	1	a	2	0.097
4	**Hunt or trap wildlife**	6	10.9	0	a	0	a
5	**Eaten wildlife of unknown health status**	2	3.6	0	a	0	a
6	**Cut or injured oneself when butchering wildlife**	2	3.6	0	a	0	a
7	**Eaten or shared dead wildlife**	0	0	0	a	0	a
8	**Collect and sell dead wildlife**	0	0	0	a	0	a

[a]Variable is excluded from further analysis due to insufficient or too small data
*Humans that consumed food damaged by animal were more likely to be seropositive for filovirus (P=0.017, OR=10.3, 95% CI=1.45, 73.24).

Most farmers reported have had symptoms of Influenza-like illnesses (ILI) in the past one year. A few filoviruses and henipavirus seropositive farmers also reported other symptoms such as severe acute respiratory infection and fever with diarrhea and vomiting (Table 3.22). However, bivariate analysis between seropositivity and reported symptoms were not performed due to insufficient data for meaningful analysis.

Table 3. 22 Seroprevalence of filovirus and henipavirus in human based on symptoms experienced by farmers in participating cattle farms within the past year

		Human, n=55			
No.	**Symptoms**	**Individuals with reported symptoms**	**%**	**Filovirus positive, n=5**	**Henipavirus positive, n=4**
1	**Fever with headaches and lethargy**	3	5.5	0	0
2	**Haemorrhagic fever**	0	0	0	0
3	**Severe acute respiratory infection (SARI)**	3	5.5	1	0
4	**Influenza-like illnesses (ILI) symptoms**	10	18.2	0	1

5	**Fever with diarrhea and vomiting**	4	7.3	1[a]	1[a]
6	**Fever with skin rash**	1	1.8	0	0
	Total	21		2	2

*3/5 filovirus seropositive humans had no symptoms, 2/4 henipavirus seropositive humans had no symptoms. Two seropositive humans for both filovirus and henipavirus had no symptoms.
[a]The same individual

A comparison was made to determine if a particular exposure to animal species influenced reported ILI symptoms within the past year in farmers. ILI was most reported in farmers with exposure to cats and cattle, but no significant differences were observed (Table 3.23)

Table 3. 23 Influenza-like illnesses (ILI) symptoms and exposure to animals and wildlife species in human within the past year

		Human, n=55			
No.	**Species**	**Exposure to animal species**	**%**	**Influenza-like illnesses (ILI) symptoms, n=10**	**Fisher exact P-value**
Domestic animal					
1	**Cattle**	50	90.9	9	1.000
2	**Cat**	49	89.1	10	0.579
3	**Poultry**	41	74.5	6	0.255
4	**Goat**	27	49.1	6	0.340
5	**Dog**	6	10.9	1	1.000
Wildlife					
	Rodent				
1	**(rats/shrews/squirrel)**	34	61.8	7	0.725
2	**Wild bird**	19	34.5	4	0.723
3	**Primate**	15	27.3	4	0.434
4	**Bat**	13	23.6	1	0.421
	Reptiles				
5	**(snake, monitor lizard)**	9	16.4	3	0.340
6	**Deer**	7	12.7	2	0.599
7	**Porcupine**	6	10.9	1	0.702

*Only exposure to animal species with >2 reports were included in this comparison

ILI symptoms reported were also compared to forms of exposure to animals and wildlife within the past year of survey in farmers. All farmers that reported ILI symptoms have

reported handling live animals and most of them lived with a pet animal. No significant findings were found between ILI and forms of exposure to animals and wildlife within the past year of survey (Table 3.24).

Table 3. 24 Influenza-like illnesses (ILI) symptoms in human based on forms of exposure to animals and wildlife within the past year

		Human			
	Forms of exposure	n=55	%	Influenza-like illnesses (ILI) symptoms, n=10	Fisher exact P-value
General exposure					
1	**Lived with pet**	44	80	8	1.000
2	**Handled live animals**	53	96.4	10	1.000
3	**Raised animals**	39	70.9	6	0.453
4	**Share water source with animals**	14	25.5	2	0.503
5	**Seen feces in or near food**	7	12.7	0	0.328
6	**Animal entered house**	49	89.1	9	1.000
7	**Eaten food that were touched, disturbed or damaged by animal**	15	27.3	2	0.710
Exposure to wildlife					
1	**Cook or handle recently slaughtered meat, offal or blood of wildlife**	18	32.7	3	1.000
2	**Eaten raw or undercooked meat, offal or blood of wildlife**	8	14.5	2	0.627
3	**Scratched or bitten by animal**	13	23.6	3	0.685
4	**Slaughter or butcher wildlife**	18	32.7	2	0.470
5	**Hunt or trap wildlife**	6	10.9	0	0.347

*Only exposure to animal species with >2 reports were included in this comparison

3.4 Discussion

In this study, domestic animals and wildlife in and around forest-fringe cattle farms were tested for exposure to the viruses in family *Filoviridae* and the genus *Henipavirus* using multiplex serology. This technology has been developed and widely used for screening of viruses globally (Chowdhury et al., 2014; Dovih et al., 2019; Hayman et al., 2008, 2011; Laing et al., 2018; Peel et al., 2013b) but is not meant for diagnostic determinations. The findings can be used to target areas of surveillance for emerging pathogens but should not be used for policy decisions such as trade, quarantine or culling (MacNeil et al., 2011; Peel et al., 2013a). Serologically positive samples may indicate positivity to the tested virus or to antigenically related viruses. Antigenically related viruses share similar epitopes on its surface antigenic molecule that confers binding by antibodies or T cell receptor (Frank, 2020). Phylogenetically closely related viruses have higher epitope amino acid sequence similarity and binding to similar antibodies which result in greater degrees of antigenic-similarity and cross-reactivity of antibodies to antigens included in the test (Brangel et al., 2018; Marsh et al., 2012a; Schuh et al., 2019). Further testing and characterization are necessary using virus neutralisation test could improve virus specific interpretation (OIE, 2018).

<u>Filovirus</u>

This study found the highest serological evidence for filoviruses in goats (24/158, 15.2%), sheep (1/11, 9.1%), humans (5/55, 9.1%), cattle (5/156, 5.1%) and lowest in bats (5/265,

1.9%). Seropositivity indicates evidence of past exposure to specific viruses antigenically related to filoviruses. Studies on filoviruses in domestic animals have been scarce. Only dogs and pigs have been demonstrated to be infected with filoviruses (Allela et al., 2005; Chowdhury et al., 2014). In Gabon, 28% of dogs from an Ebola epidemic areas were found to be IgG positive for Ebola virus (Allela et al., 2005). Meanwhile, Reston ebolavirus (RESTV) was identified in a spillover event between pigs and human in the Philippines and later in sick pigs in China (Barrette et al., 2009; Pan et al., 2014). RESTV infection in humans are rare and occurs when there is close contact through occupational exposure (Barrette et al., 2009; Miranda & Miranda, 2011; Morris, 2009). To our knowledge, this study is the first to report evidence of exposure to filoviruses in cattle, goat and sheep. This is also the first evidence of exposure of filoviruses in dogs beyond Africa.

Commented [A70]: There is a Reston virus paper focused on abattoirs in the Philippines

Commented [A71R70]: Yes, tq. My mistake. The paper which I cited two sentence before reported RESTV seropositive animal handlers

Highest seropositivity was reported among goats in this study particularly in semi-intensively reared goats which were 4 times more compared to cattle. The reasons for this may need further exploration however we suggest a few reasons for this findings: 1) Goats are known to browse extensively relative to other species (Hofmann, 1989; Rutagwenda et al., 1989) and have the habit of sampling wide ranges of items (edible and non-edible), therefore may increase their risk of exposure to multiple disease agents; 2) Many of the seropositive goats reported in this study were managed semi-intensively to browse freely around village and raised in close proximity where flowering and fruiting trees are abundant which attracts bats such as *C. brachyotis* (V. C. Lim et al., 2017; Tan et al., 1998). Goats are very likely to pick up partially eaten fruits consistent

with their feeding behaviour therefore increases risk of contracting multiple disease agents.

Predictive modelling demonstrated that Southeast Asia is a hotspot for filovirus carriers, particularly in Old World fruit bats (family Pteropodidae) (Han et al., 2016). Our study results corroborate with the model and filovirus surveillance in the region. Seropositivity of bats ~~in this study~~was found to be ~~is~~ lower than ~~previous reports of bats in this region. Studies from~~ neighbouring country, Singapore which reported 9.1% (17/186) in *E. spelaea*, 8.5% (13/153) in *C. brachyotis* and 4.3% (3/70) in *P. lucasi*; and in the Philippines which reported 31% (5/16) in *Rousettus amplexicaudatus* bats, all of which belong to family Pteropodidae. Besides *E. spelaea* and *C. brachyotis*, *C. horsfieldii* was seropositive for filoviruses in our study. *Cynopterus sp*. and *E. spelaea* are adaptive species that occupy a range of habitats from orchards, plantations, primary and secondary forests and urban sites (Campbell et al., 2004; Fukuda et al., 2009; Heidenan & Heaney, 1989; Kitchener et al., 1990; V. Lim, 2018; Tan et al., 1998). Livestock farming in rural and sub-rural areas where fruit trees are plenty provides bat roosting and foraging areas (Fukuda et al., 2009). This overlapping spatial distribution of bats and livestock increase risk of viral spillover to livestock and agricultural workers (Mikail et al., 2017). Longitudinal spatial-temporal distribution studies will ascertain the level of such risk (Zhao et al., 2022).

Commented [A72]: This is a good reference. They used predictive traits to ID possible ebolavirus bat hosts. https://journals.plos.org/plosntds/article?id=10.1371/journal.pntd.0004815

Commented [A73R72]: Thank you, reference added.

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Antibody reactivity to specific filovirus antigen in our study suggests that circulating filoviruses in this region are predominantly more closely related to BDBV, SUDV, EBOV,

RESTV and less so with BOMV, MARV, and RAVV. Reactivity to filoviruses closely related to EBOV, BDBV and SUDV corresponds to a similar study in Singapore bats (Laing et al., 2018). However, in contrast to that study, we report a considerably high number of reactivity to antigenically related but not identical to RESTV-pig in a *C. brachyotis* and many goats. RESTV is an zoonotic Asiatic filovirus responsible for sporadic cases in non-human primates, pigs and human (Barrette et al., 2009; CDC, 1993; Demetria et al., 2018; Miranda et al., 1999). Bats may be natural reservoirs for RESTV in the Philippines, Bangladesh and China (Jayme et al., 2015; Olival et al., 2013; Taniguchi et al., 2011; Yang et al., 2017). Finding of our study suggests possible transmission of RESTV-related viruses from bats to goat. However, nucleic-acid detection of RESTV genome is necessary to confirm spillover.

Henipavirus

Seropositivity to henipaviruses was highest in goats (13.9%), sheep (9.1%), human (7.3%), dog (4.3%), cattle (3.2%) and bat (1.1%). Seropositivity indicates evidence of past exposure to viruses antigenically related to henipaviruses. Antibody reactivity to specific henipavirus antigen in our study suggests that circulating henipavirus in this region is predominantly more closely related to GhV, HeV, NiV, MojV and less so to CedV. The MFI values for GhV is high with many that cross-reacted with other henipaviruses and several that did not no cross-react with other henipaviruses. Presently, little is known about GhV which was first detected in African bats and is the only African henipavirus (Drexler et al., 2009, 2012). GhV is also antigenically closer to ancestral henipaviruses

(Pernet et al., 2014). Thus, reaction to GhV in our study indicates prevalence of an ancestral henipavirus more antigenically related to GhV than the other presently known Asiatic viruses.

A *C. brachyotis* bat and three goats were seropositive for NiV antigenically related viruses. All four reactions cross-reacted with GhV and HeV with MFI difference of less than 3000 between them. Besides that, sera that were seropositive for HeV antigenically related viruses cross-reacted with different combinations of NiV, GhV and CedV. These sera were all from seven goats. Typically, cross-reactivity between NiV and HeV is high while cross-reactivity between NiV/HeV and GhV is low due to the less sequence identity (<30%) between their G protein (B. Lee et al., 2015). Cross-reactivity MFI values generated using positive NiV positive rabbit polyclonal sera in Chapter 4 also supports this finding. Hence, the bats and goats were likely exposed to an unidentified henipavirus most closely related to NiV or NiV and HeV respectively.

Interestingly, our study found two reactions only to MojV with no cross-reactivity in a cattle and dog from the same intensive cattle farm. One MojV reaction in human also cross-reacted with GhV. MojV is a rodent henipavirus discovered in anal specimens of *Rattus flavipectus* in Southern China. This novel virus was identified in the same cave 6 months later where three patients died of respiratory illness of unidentified aetiology. MojV pathogenicity remains to be determined due to the absence of isolate (Wu et al.,

2014). Cattle and dog may have acquired MojV closely related virus through contact with rodent excrement. Previously, most henipaviruses were fruit-bat associated, such as HeV, NiV, CedV, GhV, including novel Angavokely virus (AngV)(Madera et al., 2022). In the recent years, more rodent-associated henipaviruses have been identified such as MojV, Gamak (GAKV), Daeryong viruses (DARV) and Langya henipavirus (LayV). Rodents are probable reservoir hosts or amplifying host for MojV (Cheliout et al., 2021). GAKV and DARV were identified Korea (Lee et al., 2021), whereas LayV was identified in shrews and rodents during an epidemiological investigation of febrile pneumonia outbreak in patients who were mostly farmers in China, indicative of a spillover event from rodents to humans (ECDC, 2022; Zhang et al., 2022). Increasing ~~the~~ surveillance efforts and sample size of rodents to detect henipavirus antibodies and genome of MojV or MojV-like virus will improve chances of detection and determine its circulation locally.

Commented [A74]: I suggest adding in Langya virus, and Gamak/Daeryong virus refernces. These are new rodent-associated henipaviruses. It may be worth to mention that HeV/NiV/CedV/GhV/AngV are fruit-bat associated and MojV/LayV/GamV/DarV are rodent-associated.

Commented [A75R74]: Thanks, added

Pteropus spp. (flying foxes) are reservoirs for NiV, HeV and CedV (Anderson et al., 2019; Boardman et al., 2020; Halpin et al., 2011; Marsh et al., 2012b). Previous survey of bats in Malaysia were mostly focused in the *P. vampyrus* and *P. hypomelanus* species with a reported seroprevalence of 1-33% (Rahman et al., 2013a; Yob et al., 2001). Those studies used indirect ELISA and serum neutralization test to detect the antibodies against Nipah virus in samples. ~~A recent survey using multiplex serological assay in the local *P. hypomelanus* bats samples found 25% reactivity to NiV (Laing et al., 2019). The reported seroprevalence in our study of 1% (2/108) in *C. brachyotis* and *C. horsfieldii* 2% (1/52) is much less than that of Laing et al. (2109) *Pteropus sp.* surveys~~ Although our study did

Commented [A76]: I'd mention that flying foxes have been confirmed as the reservoirs of NiV/HeV/CedV. That you did not sample flying foxes, and found little seroprevalence in the fruit bats, cynos/eonycteris, for NiV. Corroborating that flying foxes are the wildlife host.

Commented [A77R76]: Thank you for the suggestion, added them

Commented [A78]: Difference between types of bats – but again, I would not cite the conference abstract from IMED.

Commented [A79R78]: Okay

not sample *Pteropus sp*. bats, we determined that seropositivity in Cynopterus sp. and Eonycteris sp. bats to be low. This finding is comparable to the seroprevalence of 4% (2/56) in *C. brachyotis* and 5% (2/38) in *E. spelaea* in a separate study in 1999 (Yob et al., 2001) and corroborates that flying foxes are wildlife host for henipavirus.

Surveillance of NiV in human outbreak areas near *Pteropus sp*. bat roosts in Bangladesh using the a similar antigen-based multiplex test ~~via a Luminex platform~~ found that 6.5% (26/400) of cattle and 4.3% (17/400) of goats were positive for henipavirus (Chowdhury et al., 2014). During the Nipah virus outbreak in Malaysia, dogs sampled near infected pig farms had detectable antibodies for Nipah virus (Mills et al., 2009; Rahman et al., 2013b). Since 2001, NiV have not been reported in domestic animals and wildlife locally using ELISA (Fikri et al., 2011; Naama et al., 2013). This study is the first report that uses Luminex technology of henipavirus infection in dogs since 1999 and in local cattle, goat and sheep. Multiplex serology approach with recently described henipaviruses employed may be more sensitive to capture antibodies against antigenically related henipaviruses, thus, is an advantageous screening tool for epidemiological studies especially in new populations (Brook et al., 2019; Hayman et al., 2008; Peel et al., 2013b).

Reported seropositivity of henipaviruses in goats was high at 13.9% (22/158) particularly in semi-intensively reared goats and was 4 times more as compared to cattle 3.2% (5/156) for hypothetical reasons of risk of exposure to infectious materials as explained before.

Commented [A80]: I suggest reworking this paragraph. Are the henipavirus positive samples, GhV or NiV positive? The work done in Chowdhury et al did not run CedV, GhV, and MojV G so it's similar but needs to be compared and contrasted. I would pull out the NiV discussion and focus on your results, then bring up NiV more generally towards the end of the discussion. Highlighting, the ELISA data from 2001 that you cited, and that the multiplex approach with recently described henipaviruses may be more sensitive to capture antigenically-related henipavrisues.

Commented [A81R80]: Thank you for the suggestion!

Risk Factors

We detected exposure against viruses antigenically related to filoviruses, EBOV, BDBV and RAVV; and henipaviruses HeV, CedV, MojV in humans. Our study found those that consumed food that were touched, disturbed or damaged by animal were more 10 times more likely to be seropositive for filovirus. This is supported by the study by Adjemian et al., which established increased risk of filovirus infection amongst febrile tribesmen presented to healthcare facilities in Uganda that were exposed to activities related to ~~Increased risk of filovirus infection was observed in activities such as~~ hunting (37.5 times), touching (5.6 times) or eating wildlife (10.7 times) (Adjemian et al., 2011). Likewise, bushmeat slaughtering or working closely with infected pigs or horse enhanced risk of henipavirus infection (Chua, 2010; Pernet et al., 2014; Rogers et al., 1996). The finding is expected because the both viruses are transmitted via direct contact with body fluids (Amal et al., 2000; Bausch et al., 2007; O'Sullivan et al., 1997; Saéz et al., 2015; Williamson et al., 1998). Therefore, poor hygiene practices or improper personal protective equipment may expose farmers to infectious materials, especially when handling sick or infected animals, animal waste or carcasses.

Commented [A82]: unclear

Commented [A83R82]: Corrected

Approximately half of the humans sampled (21/55) reported Influenza-like illnesses (ILI), fever with diarrhoea and vomiting, fever with headaches and lethargy and severe acute respiratory infection (SARI) symptoms within the past year of survey. Half of the seropositive human for filovirus and/or henipavirus in our study had reported one of the symptoms in the past year, while the other half did not. The symptoms can be due to

several multitude of reasons, unfortunately causal relationship between viral infection and symptoms in our study cannot be ascertain without clinical and longitudinal data. Syndromic surveillance incorporating important pathogens at the family or genus level will improve early detection and appropriate intervention.

The catastrophic Nipah outbreak in 1998 is a turning point for cross-sectoral ministerial integration in managing zoonotic disease and recognition that the country is susceptible to spillover of pathogenic viruses (Chua, 2003). Unfortunately, at present, novel or emerging viral surveillance in animal population is sporadic and scant; usually linked to specific research endeavours. The ecological concerns and agricultural intensification in Malaysia strongly indicate the need for regular monitoring and surveillance of livestock and humans working in close proximity to animals especially those that are located in geographic areas with high potential for wildlife viral spillover events. A better understanding on the interaction and viral transmission between human, livestock, other domestic animals and wildlife will inform disease surveillance and control policy to reduce the risk of transmission and prevent undesirable animal and public health consequences.

Limitations

This study was performed on highly targeted populations to increase the chance of viral detection in high-risk populations at the wildlife-livestock-human interface. Therefore,

the findings should be interpreted in light of the sampling bias, limited sample size, non-random sampling, cross-reactions between antigenically related viruses and the diversity of uncharacterized filoviruses and henipaviruses that is missed from the test panel. Further characterization of filoviruses and henipaviruses in the region, larger data set, improved diagnostic assays and species-specific positive controls will influence cut-off values and reported sero-detection.

Additionally, infection status in bats fluctuates seasonally and are influenced by reproduction phases, nutritional stress, migrations or contacts with infected animals (Amman et al., 2012; Baker et al., 2014; Hayman, 2015; Plowright et al., 2008; Sohayati et al., 2011; Wang et al., 2013). This infection pattern in bats as a reservoir host would subsequently impact degree of spillover to other potential hosts like cattle, goats or humans in our case (Amman et al., 2012; Plowright et al., 2017). Antibody post-infection also wax and wanes over time (Boardman et al., 2020; Diallo et al., 2021). Thus, the sero-detection reported in this cross-sectional study only provides information at the time of sampling which may be an underestimation or overestimation and will differ depending on time of sampling.

In addition, maternal antibodies to NiV can last up to 14 months, hence without longitudinal data, some reactivity in our study may be misrepresented (Sohayati et al., 2011). As a result of lack of longitudinal data, serological findings only demonstrate past

exposure to filoviruses and henipaviruses. Despite these limitations, our study generated valuable information of filoviruses and henipaviruses in the multiple animal host species to inform future studies. Besides that, research on viral diversity characterization in this region will improve the depth of interpreting serological results.

3.5 Conclusion

This study provides the first evidence of exposure to viruses antigenically related to filoviruses and henipaviruses in domestic animals and humans in Malaysia, as well as viruses antigenically related to henipaviruses in previously unreported bat species. Results from our study supports more extensive and longitudinal viral surveillance in these populations to enhance disease preparedness and improve our understanding on the viral distribution, diversity and potential spillover at the human-livestock-wildlife interface.

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Table 3. 1 Sero-detection of filovirus and henipavirus from selected references of countries closest to Malaysia

Species	Year	Test used	Seroprevalence*	Location	Reference
Cattle	-	Luminex using NiV and HeV glycoproteins	NiV: 26/400 (6.5%)	Bangladesh	(Chowdhury et al., 2014)
Goat	-	Luminex using NiV and HeV glycoproteins	NiV: 17/400 (4.3%)	Bangladesh	(Chowdhury et al., 2014)
Pig	2009	Luminex with NiV and HeV glycoproteins	NiV: 138/312 (44.2%)	Bangladesh	(Chowdhury et al., 2014)
	2008 & 2010	Immunofluorescence and IgG-ELISA using RESTV-NP and RESTV-GP	RESTV- affected farms: 71-79% Non-RESTV-affected farms: 0-1%	Philippines	(Sayama et al., 2012)
Dog	2001-2013	NiV Indirect ELISA	NiV: 0/2184 (0%)	Malaysia	(Naama et al., 2013)
Bats	2004-2007	NiV serum neutralization test	Cross-sectional study : 82/253 (32.8%) in P. vampyrus : 13/117 (11.1%) in P. hypomelanus Longitudinal study : 64/650 (9.8%) in *P. hypomelanus* *Fluctuated between 1-20% in the span of 3 years	Malaysia	(Rahman et al., 2013a)
	1999	NiV Indirect ELISA	: 11/35 (31%) in *P. hypomelanus* : 5/29 (17%) in P. vampyrus : 2/38 (5%) in E. *spelaea* : 2/4 (4%) in *C. brachyotis* : 1/33 (3%) in *Scotophilus kuhli*	Malaysia	(Yob et al., 2001)
	2002-2004	NiV Indirect ELISA	: 82/1,304 (7.8%) from all bats : 4/26 (15.4%) in *P. hypomelanus* : 76/813 (9.3%) in *P. lylei* : 1/39 (2.6%) in *P. vampyrus* : 1/74 (1.3%) in *H. larvatus*	Thailand	(Wacharapluesadee et al., 2005)
	2011-2016	Luminex using EBOV, BDBV, SUDV, or TAFV glycoproteins	Filovirus seropositivity : 17/186 (9.1%) in *E. spelaea* : 13/153 (8.5%) in *C. brachyotis* : 3/70 (4.3%) in *P. lucasi*	Singapore	(Laing et al., 2018)
	2011-2013	EBOV ELISA	: 0/699 (0%) in all bats	Thailand	(Wacharapluesadee et al., 2015)
	2009	Luminex with NiV and HeV glycoproteins	NiV: 29/71 (42%)	Indonesia	(Sendow et al., 2013)
Rodents	1999	NiV Indirect ELISA	: 0/25 (0%) trapped on farms with NiV-infected pigs	Malaysia	(Rahman et al., 2013b)
Non-human primate	2013	EBOV ELISA	: 0/50 (0%) in *Macaca fascicularis*	Thailand	(Wacharapluesadee et al., 2015)
	2005-2006	ELISA (ZEBOV, SEBOV, CIEBOV, BEBOV, REBOV, and MARV), Year 2005-2006	: 18.4% (65/353) EBOV : 1.7% (6/353) MARV in *Pongo pygmaeus*	Indonesia	(Nidom et al., 2012)

*Sero-surveys during non-outbreak periods were prioritized

ID	Species	Farm	EBOV-PK	BDBV-PBG	BOMV-P	SUDV-PK	RESTVm-PK	RESTVa-PK	LUDV-PBG	MLAV-PK	MARV-PBG	RAVV-PBG	Gove
PMW1834	Cynopter	2	518	**6212**	239	139	291	473	124	48	49	269	
PMW1877	Cynopter	2	458	**14168**	282	64	312	995	25	208	1219	1776	
PMW1878	Cynopter	2	**4382**	63	287	65	384	1085	43	162	-132	-145	
PMW1927	Cynopter	2	3567	3027	**5284**	**7361**	1448	**8475**	1989	450	3286	716	
PMW1944	Cynopter	5	**10784**	**9312**	639	551	327	626	90	122	-106	-130	
PML707	Cattle	1	**8916**	3971	1475	**5527**	**4564**	1573	546	344	-97	-137	
PML716	Cattle	1	**4595**	2830	439	2526	819	1441	185	185	-96	-137	
PML717	Cattle	1	331	3455	277	59	124	-17	71	63	-52	900	
PML766	Cattle	2	2890	**4196**	475	1266	1263	892	180	319	-63	137	
PML769	Cattle	2	364	**4976**	329	19	185	11	52	259	-53	-65	
PML832	Cattle	3	766	**4187**	422	258	242	159	147	55	-17	-5	
PML839	Cattle	3	**10205**	**7417**	2369	**9159**	**8851**	571	2425	2637	176	247	
PML896	Cattle	4	301	**4927**	236	20	122	-2	43	42	301	3190	
PML927	Cattle	5	536	**4246**	437	190	400	1019	74	121	-76	-78	
PML1002	Cattle	6	408	235	270	25	122	1	10	229	-79	0	
PML1011	Cattle	6	151	20	69	184	24	-91	-42	-72	-60	-21	
PML755	Dog	1	**7364**	**5429**	347	311	457	1163	98	135	-83	-85	
PML797	Dog	2	1861	**7561**	618	887	1238	1776	442	565	372	453	
PML1045	Dog	6	1054	653	268	102	132	-20	90	145	-82	138	
PML772	Goat	2	518	3157	461	168	465	1109	72	258	-57	-93	
PML784	Goat	2	659	**8374**	344	167	524	1160	66	272	-47	86	
PML798	Goat	2	328	**6738**	281	46	174	167	65	131	-5	2835	
PML799	Goat	2	511	**5932**	431	206	500	1269	88	275	562	312	
PML801	Goat	2	1181	1196	2042	**4794**	2508	**4861**	1032	144	1673	270	
PML803	Goat	2	589	**10139**	266	8	179	-7	46	153	353	**6038**	
PML811	Goat	2	963	**21882**	1815	**5116**	3484	3975	1033	550	2336	**12138**	
PML812	Goat	2	1318	941	3150	**6679**	775	3233	1484	653	568	-10	
PML845	Goat	3	931	839	1301	3214	390	**4183**	630	149	889	8	
PML853	Goat	3	1596	1065	1320	**4546**	761	**4796**	954	355	1209	82	
PML856	Goat	3	287	**8040**	101	79	270	402	-12	19	-10	182	
PML861	Goat	3	3150	**4250**	3971	**7145**	1170	**8761**	1864	519	3248	502	
PML803	Goat	3	2460	4011	3456	**7519**	754	**7310**	1977	435	2342	539	
PML865	Goat	3	315	**4038**	243	86	271	542	71	88	-5	36	
PML872	Goat	3	1385	2223	1364	1990	668	**5954**	900	303	1219	98	
PML873	Goat	3	2378	1872	2656	**4850**	632	**6564**	1055	163	1830	298	
PML942	Goat	3	1737	**4230**	1756	4161	727	**4320**	1429	372	1000	-2	
PML945	Goat	4	456	**9440**	532	646	320	1250	174	288	979	**4835**	
PML960	Goat	4	1594	**12756**	1581	3703	949	**4875**	591	212	1722	3466	
PML963	Goat	4	1802	1362	2447	**5746**	556	**7375**	1629	332	2003	206	
PML965	Goat	4	1254	1310	2161	**5226**	1502	**5669**	936	450	2302	547	
PML1027	Goat	6	1378	3742	2163	**5275**	1145	**5353**	1314	393	2000	1199	
PML1034	Goat	6	1735	**6713**	3373	**6929**	1507	**7037**	1252	206	3120	2773	
PML1053	Goat	6	1042	**6853**	1942	**5067**	1510	**5819**	752	295	2245	338	
PML1054	Goat	6	451	**8690**	169	117	155	656	34	31	-84	-37	
PML1030	Sheep	6	1977	**9834**	**5236**	**10890**	2178	**8059**	3066	1481	**6317**	2572	
PMN1133	Human	1	**4928**	928	707	166	1876	2081	96	518	-144	-199	
PMH1546	Human	2	1274	756	1401	902	720	344	608	846	73	12	
PMH1553	Human	2	**11867**	1838	1025	288	1635	1906	99	506	-43	36	
PMH1563	Human	4	1649	**24050**	861	335	374	304	365	309	2646	**13816**	
PMH1568	Human	4	2053	**7949**	1686	570	625	218	1498	2125	261	1440	
PMH1583	Human	6	1948	**13891**	938	287	485	247	380	362	58	735	

No. of reactions	Rx1	Rx2	Rx3	Rx4	RxG	Rx
1	BDBV related					Bd
1	BDBV related					Bd
1	EBOV related					E
3	RESTVp	SUDV	BOMV			Ro-S-Bo
2	EBOV	BDBV				E-Bd
3	EBOV	SUDV	RESTVm			E-S-R
1	EBOV related					E
1	BDBV related					Bd
1	BDBV related					Bd
1	BDBV related					Bd
4	EBOV	SUDV	RESTVm	BDBV		E-S-Rm-Bd
1	BDBV related					Bd
1	BDBV related					Bd
2	EBOV	BDBV				E-Bd
1	BDBV related					Bd
1	BDBV related					Bd
2	BDBV	RAVV				Bd-R
3	BDBV	RAVV	SUDV			Bd-R-S
1	SUDV related					S
1	RESTVp related					Rp
2	SUDV	RESTVp				S-Rp
1	BDBV related					Bd
3	RESTVp	SUDV	BDBV			Rp-S-Bd
2	SUDV	RESTVp				S-Rp
1	BDBV related					Bd
1	RESTVp related					Rp
2	RESTVp	SUDV				Rp-S
2	BDBV	RAVV				Bd-R
2	BDBV	RESTVp				Bd-Rp
2	RESTVp	SUDV				Rp-S
2	RESTVp	SUDV				Rp-S
2	RESTVp	SUDV				Rp-S
3	RESTVp	SUDV	BDBV			Rp-S-Bd
3	BDBV	RESTVp	SUDV			Bd-Rp-S
1	BDBV related					Bd
5	SUDV	BDBV	RESTVp	BOMV	MARV	S-Bd-Rp-Bd
1	EBOV related					Bd
1	BDBV related					Bd
1	EBOV related					E
2	BDBV	RAVV				Bd-R
1	BDBV related					Bd
1	BDBV related					Bd

MFI range for positive reaction

Yellow	2	EBOV, BDBV
	4246	10205
Yellow	1	EBOV
	5429	7561
	4183	21882
Yellow	3	BDBV, RAVV
Dark ora	1	BDBV
Yellow	1	Yellow=1 SUDV
	4928	24050
Yellow	3	EBOV, BDBV, RAVV
Dark ora	1	BDBV

ID	Species	Farm	Hev-PBG	NiV-PBGove	CedPV-P	MoJV-PBGove	GHV-PBGove
PMW1832	Cynopter	2	2497	1058	1515	103	**5429**
PMW1877	Cynopter	2	**6797**	**4776**	**5113**	600	**10246**
PMW1878	Cynopter	2	-81	-51	-98	-141	-18
PMW1927	Cynopter	2	**10478**	**13117**	1593	128	**10509**
PMW1944	Cynopter	5	2	-34	-95	-95	-86
PML707	Cattle	1	-14	278	-50	-68	810
PML716	Cattle	1	-52	48	-91	-83	1030
PML717	Cattle	1	1420	115	1144	1111	**5000**
PML766	Cattle	2	602	2518	602	4527	1486
PML769	Cattle	2	-25	-9	-69	329	34
PML832	Cattle	3	580	173	178	78	**5354**
PML839	Cattle	3	65	71	-13	55	202
PML896	Cattle	4	4294	782	3072	2162	**6813**
PML927	Cattle	5	15	10	-38	-43	1501
PML1002	Cattle	6	**17641**	**10580**	**16712**	**11175**	**20100**
PML1011	Cattle	6	33	-54	-65	**13701**	-80
PML755	Dog	1	-15	59	-63	-61	207
PML797	Dog	2	1236	874	1360	1662	4123
PML1045	Dog	6	70	89	-28	**5042**	24
PML772	Goat	2	792	329	284	72	**6149**
PML784	Goat	2	1401	1437	580	758	**7249**
PML798	Goat	2	2372	1106	1080	1033	**8162**
PML799	Goat	2	684	674	381	-10	3508
PML801	Goat	2	**12778**	**11762**	**6234**	838	**9125**
PML803	Goat	2	**9054**	-30	3521	1668	**11118**
PML811	Goat	2	**17528**	**10245**	**13342**	2063	**19805**
PML812	Goat	2	**9403**	**6314**	3539	-89	4477
PML845	Goat	3	4302	4247	620	-84	4141
PML853	Goat	3	**5408**	**4828**	946	-9	4035
PML856	Goat	3	679	-22	408	-53	**5731**
PML861	Goat	3	**10255**	**12723**	1671	-95	**11295**
PML803	Goat	3	**12061**	**11660**	2527	-37	**10369**
PML865	Goat	3	3865	1880	1781	242	**7795**
PML872	Goat	3	**5202**	**6086**	910	-56	**5644**
PML873	Goat	3	**8066**	**10054**	855	-70	**8225**
PML942	Goat	3	**6248**	**6168**	1279	-101	**4814**
PML945	Goat	4	**9464**	3086	**6239**	1552	**13133**
PML960	Goat	4	**9464**	**8401**	3091	1869	**13062**
PML963	Goat	4	**8356**	**6726**	1630	-124	**10408**
PML965	Goat	4	**8376**	**10213**	1557	1087	**10302**
PML1027	Goat	6	**12918**	**10462**	**7191**	292	**10879**
PML1034	Goat	6	**11086**	**13179**	**6834**	1034	**15434**
PML1053	Goat	6	**8595**	**8913**	1104	3206	**9416**
PML1054	Goat	6	31	-11	-26	-74	2752
PML1030	Sheep	6	**17601**	**10271**	**9894**	575	**17170**
PMN1133	Human	1	143	231	61	288	496
PMH1546	Human	2	4366	530	3845	**9334**	**11225**
PMH1553	Human	2	248	1160	147	954	526
PMH1563	Human	4	**23110**	967	**21614**	**24157**	**24050**
PMH1568	Human	4	3133	468	1826	**7264**	**6949**
PMH1583	Human	6	1643	134	1477	**7170**	**13414**

No. of reactions	Rx1	Rx2	Rx3	Rx4	RxG	Rx
1	GHV related					G
4	GHV	HeV	CedV	NiV		G-H-C-N
3	NiV	GHV	HeV			N-G-H
1	GHV related					G
1	GHV related					G
1	GHV related					G
5	GHV	HeV	CedV	MoJV	NiV	G-H-C-M-N
1	MoJV related					M
1	GHV related					G
1	GHV related					G
1	GHV related					G
4	HeV	NiV	GHV	CedV		H-N-G-C
2	GHV	HeV				G
4	GHV	HeV	CedV	NiV		G-H-C-N
2	HeV	NiV				
2	HeV	NiV				H-N
1	GHV related					G
3	NiV	GHV	HeV			N-H
3	HeV	NiV	GHV			H-N-G
1	GHV related					G
3	NiV	GHV	HeV			N-G-H
3	NiV	GHV	HeV			N-G-H
3	HeV	NiV	GHV			H-N-G
3	GHV	HeV	CedV			G-H-C
3	GHV	HeV	NiV			G-H-N
3	GHV	HeV	NiV			G-H-N
3	GHV	NiV	HeV			G-N-H
4	HeV	GHV	NiV	CedV		H-G-N-C
4	HeV	GHV	NiV	CedV		H-G-N-C
3	GHV	NiV	HeV			G-N-H
2	GHV	MoJV				G-M
4	GHV	MoJV	HeV	CedV		G-M-H-C
2	MoJV	GHV				M-G
2	GHV	MoJV				G

MFI range for positive reaction

	4776	12117
Yellow	4	HeV, NiV, GHV
	5000	20100
Yellow	2	NiV, MoJV
Light ora	2	HeV. CedV
Dark ora	1	GHV
	5042	5042
	4834	19905
Yellow	21	HeV. NiV. CedV. GHV
Light ora	4	HeV, GHV
	6149	
	7249	
	8162	
	9125	
	11118	
	19805	
	4477	
	4141	
	4035	
	5731	
	11295	
	10369	
	7795	
	5644	
	8225	
	4814	
	13133	
	13062	
	10408	
	10302	
	10879	
	15434	
	9416	
	2752	
	17170	
	496	
	11225	
	526	
	24050	
	6949	
	13414	

	3 sigma	LCA
Filo	4182 14	4347 46
Henipa	4641 82	4859 74
Marbi	4450 25	4616 65

Total rea 52
Filo 45
Henipa 36

*RESTV bats to ecats?

EBOV related	4	EBOV	4
BDBV related	16	BDBV	14
SUDV related	1	BOMV	2
RESTVp related	2	SUDV	15
Total	23	RESTVm	2
		RESTVa	15
		MARV	1
		RAVV	5

Yellow	10		
Dark ora	2		
Total	12		

GHV is closer to ancestral strains of HeV and NiV
MoJV is antigenically distinct from henipas. So the 2 MoJV n
There's 2 unknown viruses - 1 closely related to GHV & Meh
The MoJVs are only found in cattle & doe from same farm -

GHV related	9	HeV 21
MoJV	2	NiV 19
Total	11	CedV 9
	30.6	MoJV 5
		GHV 23

Yellow	30	
Light ora	9	
Dark ora	5	
Total	48	

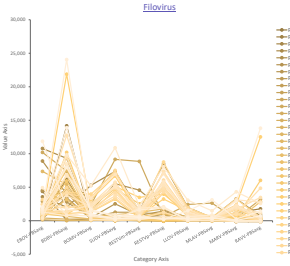
High reactivity for Filovirus

	-							
for		Sera	Antigen	Sera	Antigen	Sera	antigen	mean
Bat	4382	10784	2	EBOV, BDBv	0	-	0	-
Cattle	4246	10205	1	EBOV	0	-	0	-
Dog	5429	7561	0		0	-	0	-
Goat	4183	21882	3	BDBV, RAVv	0	-	1	BDBV
Sheep	4317	10890	1	SUDV	0	-	0	-
Human	4928	24050	3	EBOV, BDBv	0	-	1	BDBV
Total			10		0		2	

	Mean
	4382-107
	4246-102
	5429-756
	4183-218
	4317-108
	4928-240
	8095

High reactivity for henipavirus

	MFI range for positive reaction s	No. of Sera with 10000-14999 MFI	Type of Antigen reaction	No. of Sera with 15000-19999 MFI	Type of Antigen reaction	No. of Sera with >20000 MFI	Type of antigen reaction	MFI mean (range)	Mean
Bat	4776	12117	4		0		0	-	4776-12117	8383
Cattle	5000	20100	3	-	2	HeV, CedV	1	GHV	5000-20100	11907
Doe	5042	-	0	-	0	-	0	-	5042	5042
Goat	4834	19905	21	HeV, NiV, CedV,	4	HeV, GHV	0	-	4834-19905	9473
Sheep	9894	17401	0	-	3	HeV, NiV, GHV	0	-	9894-17401	13137
Human	7170	24089	2	GHV	0	-	4	HeV. CedV	7170-24089	14922
Total		30			9		5			10777



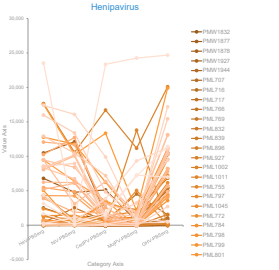


Plate	Well	Type	label	Region	Farm	Description	Description	HeV	NiV	CedV	MojV	GhV
FARM PIC	A2	X9	NA1	Northern	NA	PML99901	PML99901	2539	1295	1368	2230	1399
FARM PIC	B2	X10	NA2	Northern	NA	PML99902	PML99902	4823	2773	3331	5661	3580
FARM PIC	C2	X11	NA3	Northern	NA	PML99903	PML99903	3953	2353	3594	5821	3019
FARM PIC	D2	X12	NA4	Northern	NA	PML99904	PML99904	3462	2216	2422	4824	7299
FARM PIC	E2	X13	NA5	Northern	NA	PML99905	PML99905	5072	3628	3537	4961	3739
FARM PIC	F2	X14	NA6	Northern	NA	PML99906	PML99906	8653	5822	6682	9634	7071
FARM PIC	A3	X17	NA9	Northern	NA	PML99909	PML99909	7872	4375	5581	7738	5604
FARM PIC	C3	X19	NB1	Northern	NB	PML99911	PML99911	4023	2104	3085	5728	3507
FARM PIC	D3	X20	NB2	Northern	NB	PML99912	PML99912	9408	4359	7924	13020	9948
FARM PIC	E3	X21	NB3	Northern	NB	PML99913	PML99913	7975	3157	6762	11928	9078
FARM PIC	F3	X22	NB4	Northern	NB	PML99914	PML99914	5762	3624	4489	6503	4250
FARM PIC	G3	X23	NB5	Northern	NB	PML99915	PML99915	11471	5902	10230	16709	11716
FARM PIC	H3	X24	NB6	Northern	NB	PML99916		12784	6902	10610	19586	11183
FARM PIC	B4	X26	NB8	Northern	NB	PML99918	PML99918	20017	9851	20366	22626	20573
FARM PIC	C4	X27	NB9	Northern	NB	PML99919	PML99919	4387	2346	3439	6763	4553
FARM PIC	D4	X28	NB10	Northern	NB	PML99920	PML99920	21592	11235	21715	24142	23812
FARM PIC	E4	X29	NC1	Northern	NC	PML99921	PML99921	3803	2424	2645	4933	3569
FARM PIC	A5	X33	NC5	Northern	NC	PML99925	PML99925	5541	2755	3908	5983	4623
FARM PIC	B5	X34	NC6	Northern	NC	PML99926	PML99926	13798	5745	12334	18180	15501
FARM PIC	C5	X35	NC7	Northern	NC	PML99927	PML99927	10449	5194	9438	14382	11961
FARM PIC	D5	X36	NC8	Northern	NC	PML99928	PML99928	15441	6865	13952	19400	17081
FARM PIC	E5	X37	NC9	Northern	NC	PML99929	PML99929	22509	11295	21347	24650	23921
FARM PIC	F5	X38	NC10	Northern	NC	PML99930	PML99930	12142	6144	8652	14890	10805
FARM PIC	G5	X39	MA1	Middle	MA	PML99931	PML99931	4635	3829	3438	4788	4874
FARM PIC	H5	X40	MA2	Middle	MA	PML99932	PML99932	11165	7222	7743	16168	13205
FARM PIC	C6	X43	MA5	Middle	MA	PML99935	PML99935	7537	3424	6563	10902	9397
FARM PIC	E6	X45	MA7	Middle	MA	PML99937	PML99937	4132	2330	2833	5027	3248
FARM PIC	F6	X46	MA8	Middle	MA	PML99938	PML99938	4925	3092	4243	5265	5274
FARM PIC	G6	X47	MA9	Middle	MA	PML99939	PML99939	4721	2664	3940	5862	5243
FARM PIC	H6	X48	MA10	Middle	MA	PML99940	PML99940	4275	3195	3559	4221	4648
FARM PIC	B7	X50	MB2	Middle	MB	PML99942	PML99942	24245	15780	22813	25581	25028
FARM PIC	D7	X52	MB4	Middle	MB	PML99944	PML99944	4397	3151	4669	6098	4788
FARM PIC	E7	X53	MB5	Middle	MB	PML99945	PML99945	11836	6200	10643	16182	14432
FARM PIC	F7	X54	MB6	Middle	MB	PML99946	PML99946	8937	4407	9154	13126	8627
FARM PIC	G7	X55	MB7	Middle	MB	PML99947	PML99947	10181	4515	6821	11655	8275
FARM PIC	H7	X56	MB8	Middle	MB	PML99948	PML99948	8502	4379	7847	10511	8958
FARM PIC	A8	X57	MB9	Middle	MB	PML99949	PML99949	3750	1929	3041	5112	4073
FARM PIC	B8	X58	MB10	Middle	MB	PML99950	PML99950	21665	10327	20946	23807	23018
FARM PIC	C8	X59	MC1	Middle	MC	PML99951	PML99951	4501	2474	3914	6085	4491
FARM PIC	H8	X64	MC6	Middle	MC	PML99956	PML99956	7175	3372	6199	9854	7955
FARM PIC	A9	X65	MC7	Middle	MC	PML99957	PML99957	4979	2842	3534	5371	4370
FARM PIC	C9	X67	MC9	Middle	MC	PML99959	PML99959	7407	5306	6305	9718	7631
FARM PIC	B10	X74	SA6	Southern	SA	PML99966	PML99966	3935	2189	2810	4942	3833
FARM PIC	D10	X76	SA8	Southern	SA	PML99968	PML99968	14910	8740	10417	14106	12227
FARM PIC	E10	X77	SA9	Southern	SA	PML99969	PML99969	3696	2141	2956	4403	3567
FARM PIC	G10	X79	SB1	Southern	SB	PML99971	PML99971	12537	6387	10845	18233	15673
FARM PIC	C11	X83	SB5	Southern	SB	PML99975	PML99975	7828	5230	6150	9339	7485
FARM PIC	D11	X84	SB6	Southern	SB	PML99976	PML99976	4331	2588	3246	6317	4480
FARM PIC	F11	X86	SB8	Southern	SB	PML99978	PML99978	7789	2901	5493	8858	7419
FARM PIC	G11	X87	SB9	Southern	SB	PML99979	PML99979	10609	4020	8118	11511	8162
FARM PIC	H11	X88	SB10	Southern	SB	PML99980	PML99980	14197	6103	13013	19284	17543
FARM PIC	E2	X11	SC3	Southern	SC	PML99983	PML99983	3987	1950	2892	5592	3159
FARM PIC	A3	X13	SC5	Southern	SC	PML99985	PML99985	4151	3044	3546	6828	4612
FARM PIC	C3	X14	SC6	Southern	SC	PML99986	PML99986	3630	2017	2569	4827	3295
FARM PIC	G3	X16	SC8	Southern	SC	PML99988	PML99988	5962	4009	4430	6195	4916
FARM PIC	A4	X17	SC9	Southern	SC	PML99989	PML99989	11254	5421	8965	16174	13117

Henipa	No. of reactio	Rx1	Rx2	Rx3	Rx4	Rx5
	2	MojV	HeV			
	1	MojV related				
	2	GhV	MojV			
	2	GhV	MojV			
	5	MojV	HeV	GhV	CedV	NiV
	4	HeV	MojV	GhV	CedV	
	1	MojV related				
	4	MojV	GhV	HeV	CedV	
	4	MojV	GhV	HeV	CedV	
	2	MojV	HeV			
	5	MojV	GhV	HeV	CedV	NiV
	5	MojV	HeV	GhV	CedV	NiV
	5	MojV	GhV	CedV	HeV	NiV
	1	MojV related				
	5	MojV	GhV	CedV	HeV	NiV
	1	MojV related				
	2	MojV	HeV			
	5	MojV	GhV	HeV	CedV	NiV
	5	MojV	GhV	HeV	CedV	NiV
	5	MojV	GhV	HeV	CedV	NiV
	5	MojV	GhV	HeV	CedV	NiV
	5	MojV	HeV	GhV	CedV	NiV
	2	GhV	MojV			
	5	MojV	GhV	HeV	CedV	NiV
	5	MojV	GhV	HeV	CedV	NiV
	1	MojV related				
	3	GhV	MojV	HeV		
	3	MojV	GhV	HeV		
	1	MojV related				
	5	MojV	GhV	HeV	CedV	NiV
	3	MojV	GhV	CedV		
	5	MojV	GhV	HeV	CedV	NiV
	4	MojV	CedV	HeV	GhV	
	4	MojV	HeV	GhV	CedV	
	4	MojV	GhV	HeV	CedV	
	1	MojV related				
	5	MojV	GhV	HeV	CedV	NiV
	1	MojV related				
	4	MojV	GhV	HeV	CedV	
	2	MojV	HeV			
	5	MojV	GhV	HeV	CedV	NiV
	1	MojV related				
	5	HeV	MojV	GhV	CedV	NiV
	5	MojV	GhV	HeV	CedV	NiV
	5	MojV	HeV	GhV	CedV	NiV
	1	MojV related				
	4	MojV	HeV	GhV	CedV	
	4	MojV	HeV	GhV	CedV	
	5	MojV	GhV	HeV	CedV	NiV
	1	MojV related				
	1	MojV related				
	1	MojV related				
	3	MojV	HeV	GhV		
	5	MojV	GhV	HeV	CedV	NiV

Indicators

Red bo	Positive samples	Positive samp	38	20	31	53	37	179
Yellow	Above 10-15k M	Above 10-15k	13	3	8	10	8	42
Orange	Above 15-20k M	Above 15-20k	1	1	0	9	4	15
Dark or	Above 20k MFI	Above 20k MI	5	0	5	5	5	20

41

MojV related	13	HeV	37
		NiV	21
		CedV	31
		MojV	41
		GhV	37

MojV GhV HeV CedV NiV 13

*This sheet only contains ID with either Filo or/and Heni positives = 56 pigs

Total pigs	90	90	90	90	90
Prev (3 sigma)	38	20	31	53	37
Prev (3 sigma)	42.2%	22.2%	34.4%	58.9%	41.1%
Prev (LCA)	37	20	29	52	2

Filo
Henipa
MenV

High reactivity for henipavirus

	MFI range for positi	10000-14999 M	Antigen	00-19999	Antigen	!0000 M	Antigen
Pig	4648	25581	42	HeV, Ced	15	HeV, NiV, MojV,	20	HeV, CedV, MojV, GhV

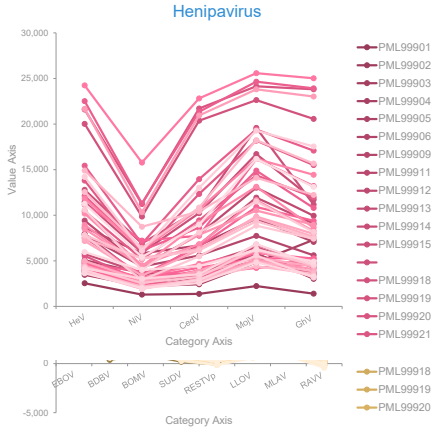
HeV, CedV, MojV, GhV

High reactivity for henipavirus

	MFI range for positives reactions	No. of Sera with 10000-14999 MFI	Type of Antigen reaction	No. of Sera with 15000-19999 MFI	Type of Antigen reaction	No. of Sera with >20000 MFI	Type pf antigen reaction	MFI mean (range)	Mean MFI
Pig	4648	25581	42	HeV, Ced	15	HeV, NiV, MojV,	20	HeV, CedV, M	4648-25581	10452

Total pigs
Positives
%

Positive
Negative
Positive
%



Region	Mean MFI
North	11572
Middle	10059
South	9725
	10452

Chapter 3

FILOVIRUSES AND HENIPAVIRUSES AT THE HUMAN-LIVESTOCK-WILDLIFE INTERFACE IN FOREST FRINGE CATTLE FARMS IN SELECTED STATES OF PENINSULAR MALAYSIA

3.1 Introduction

Filoviruses and henipaviruses are high consequence emerging zoonotic pathogens that have caused severe outbreaks in many parts of the world leading to high mortality and morbidity and grave economic losses in both human and animal health sectors (Knust et al., 2017; Looi & Chua, 2007). The economic losses due to Ebola has been estimated to be USD2.8 billion in the 2014 Ebola outbreak and USD446 million in 1998 Nipah outbreak (FAO and APHCA, 2002; World Bank, 2016).

The viruses within the family Filoviridae; Ebola (EBOV) and Marburg virus (MARV) cause haemorrhagic fever outbreaks in Africa, Germany and isolated cases in other parts of the world like Spain, United States of America, United Kingdom, Philippines, Italy, and Russia (CDC, 2018; C. E. Smith et al., 1967; WHO, 2014). The mortality from Ebola disease in Africa was 50-90% in humans and almost 100% mortality in non-human primates (Bowen et al., 1978; Jaax et al., 1996; Jahrling et al., 1996; K. M. Johnson, 1978; Simpson, 1978). While *Henipavirus*; Hendra virus (HeV) and Nipah virus (NiV) have cause respiratory and neurological signs in humans, pigs and horses in

Malaysia, Australia, Bangladesh and India (Chadha et al., 2006; Chua et al., 1999; Hsu et al., 2004; Nor, Gan, Ong, et al., 2000; O'Sullivan et al., 1997). The mortality from Nipah virus infection was 40-70% in humans in Malaysia and Bangladesh; and of 1-5% in adult pigs and 40% in suckling pigs in Malaysia (Chua et al., 2000; Goh et al., 2000; Hsu et al., 2004; Luby et al., 2009; Nor, Gan, & Ong, 2000).

With an exception of Nipah virus which emerged in 1998 (Fikri et al., 2011; Laing et al., 2019; Rahman et al., 2013; Sohayati et al., 2011), limited information is available on both filoviruses and henipaviruses in Malaysia. The investigative work on Nipah has led to numerous works on bat ecology and bat-borne microbial agents. It is now understood that bats have co-evolved with multiple viruses that may be of public health significance such a Nipah (Hayman, 2016). Filoviruses have been detected in bats in Singapore, Bangladesh, China, and the Philippines (Laing et al., 2018; Olival et al., 2013; Taniguchi et al., 2011; Yuan et al., 2012). While henipaviruses widely circulate in bats in Malaysia, India, Vietnam, Cambodia, Indonesia and Australia (Breed et al., 2013; Epstein et al., 2008; Hasebe et al., 2012; Rahman et al., 2013; Reynes et al., 2005; Sendow et al., 2006). Besides bats, ffiloviruses' and henipaviruses can infect a broad range of mammals such as human primates, rodents, shrews, guinea pigs, bats, dogs, pigs, cattle, donkey, chicken; and rodents, horse, cats, pigs, dogs, goats respectively (Chua et al., 2000; Edson et al., 2015; Gonzalez et al., 2005; Leroy et al., 2004; Morvan et al., 1999; Wu et al., 2014).

Despite the apparently wide distribution and reported prevalence of these pathogens amongst the reservoir hosts in Southeast Asia, outbreak has been rare in both humans and domestic animals. Following the Nipah outbreak in 1998-1999 in Malaysia, no further cases have been reported in both humans and animals to date (Fikri et al., 2011; Muniandy et al., 2001; Naama et al., 2013; Sohayati et al., 2016). There has also been no human outbreaks in Cambodia despite serological and molecular evidence widely found in Cambodian bats (Olson et al., 2002; Reynes et al., 2005). However, the SEA region is considered a hotspot for emerging pathogens because several localized emerging zoonosis have occurred in the past two decades and because multiple factors that drives emerging infectious diseases are highly prevalent. For example, wide circulation and abundance of reservoir hosts, topographical or landscape changes and intensified systems of agriculture and animal production may increase contact opportunities for viral transmission at the wildlife-livestock-human interfaces (Breed et al., 2013; Horby et al., 2013; Jung & Threlfall, 2016; I. Smith & Wang, 2013). In additon,the global climatological changes may impact distribution and concentration of disease vectors which subsequently facilitates spatiotemporal changes of filovirus and henipavirus viral spillover events (Martin et al., 2018; McFarlane et al., 2011; Schmidt et al., 2017).

The one health approach of managing complex emerging diseases requires the understanding of all actors that take part at the environment-animal-human interface. For example, food animals may become infected with pathogens from wildlife that can

then be transmitted to human such as Nipah, Highly Pathogenic Avian Influenza H5N1, Pandemic Influenza H1N1 (2009) amongst others. Many livestock farms in this region are located in the rural or peri-urban areas bordering primary or secondary forests. In Malaysia, livestock especially cattle are commonly managed extensively or semi-intensively at forest fringes thus may have higher probability of contact with infectious materials from infected wildlife or domestic animals. Beef cattle are most commonly raised using the aforementioned systems where animals are let out to graze in a field, plantations or in a village located at forest fringes where wildlife and peri-domestic animals are plentiful. Such setting improves the chance and frequency for pathogen spillover events to livestock or humans (Hassell et al., 2017). The spillover occurrences varies given available drivers and rarely result in an epidemic, however frequent spillover events will increase the chance of an outbreak (Clayton et al., 2013; Hayman et al., 2011; Parrish et al., 2008; Plowright et al., 2017).

In this chapter, we targeted cattle farms located at forest fringe areas with high contact risk and probability of pathogen spillover events. We hypothesise that cattle, other domestic animals, wild animals and farmers are seropositive for filoviruses and henipaviruses. The objectives of this study are: 1) To determine the serological evidence of filoviruses and/or henipaviruses in cattle, farmers, and other domestic animals and wildlife in and at close proximity to the targeted cattle farm; 2) To determine risk factors of virus infection in those species.

3.2 Materials and Methods

3.2.1 Ethics statement

All human and animal samples from cattle farms were collected in accordance to an established USAID Emerging Pandemic Threats PREDICT Operating Procedures with permission from the local institutional and ministerial Institutional Animal Care and Use Committee from UPM (AUP-R064/2018), UC Davis (#19300) and Tufts (G2021-131) and the Institutional Review Board from the Medical Research and Ethics committee (MERC) from National Medical Research Register (NMRR), Malaysia (NMRR-17-3356-37135, IIR) and UC Davis (#804522) in collaboration with Ministry of Health (MOH), Malaysia, Department of Veterinary Services (DVS), Malaysia, and the Department of Wildlife and National Parks (PERHILITAN). The work performed for this project is part of a larger project in collaboration with the EcoHealth Alliance.

3.2.2 Study design and study sites

A cross-sectional survey was performed in 3 semi-intensive, 2 integrated and 1 intensive cattle farm. These farms were selected based on a defined selection criteria which include, 1) located in districts with existing NMRR and IRB approval for human study, 2) located 5km from periphery of forests as approved by the Department of Veterinary Services, Putrajaya, 3) a minimum of 30 cattle in farm, and 4) has other domestic animals and wildlife in or around farm. The approved districts were Kuala Kangsar in the state of Perak, Gua Musang in Kelantan and Kuala Lipis in Pahang. In

these districts, the district health officers had worked with EcoHealth Alliance on previous projects and have agreed to assist the human sampling aspects in this project.

The identification of the cattle farms consisted of several steps as in Figure 3.1. The details of the steps in identifying location and farms are further described in Appendix X. All animals sampled in this study were within 5km radius of a primary forest.

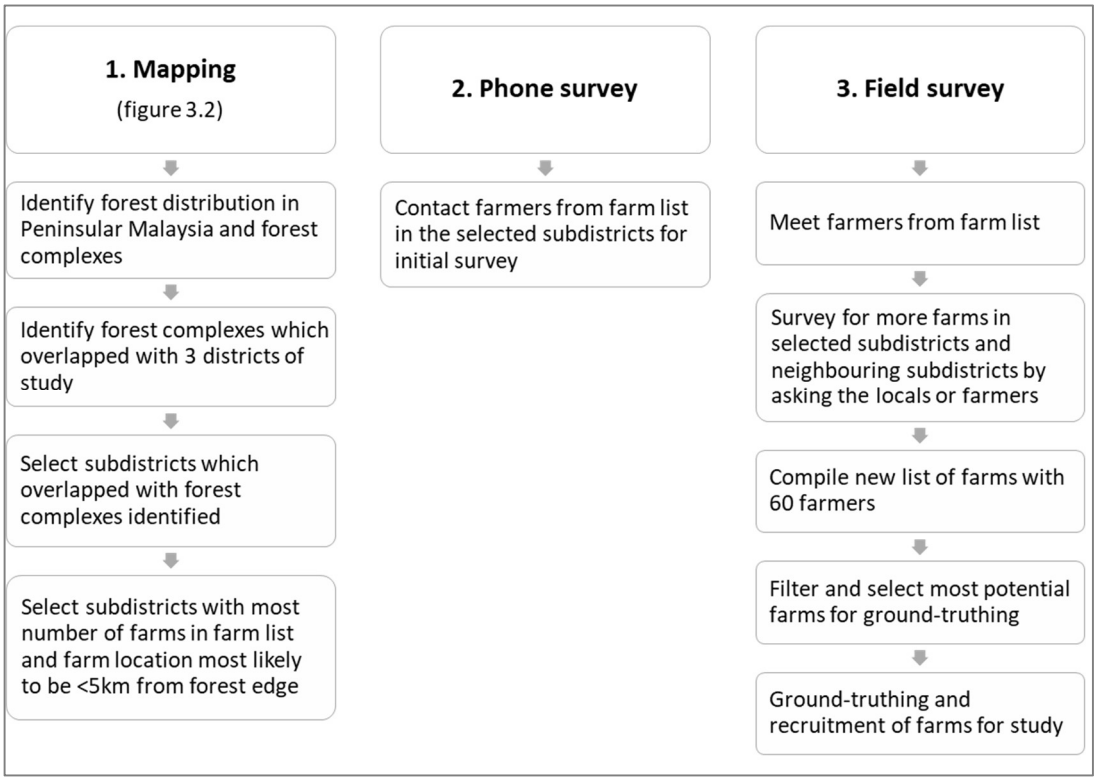


Figure 3. 1 Identification of forest-fringed cattle farm for the study on filovirus and henipavirus at the wildlife-livestock-human interface

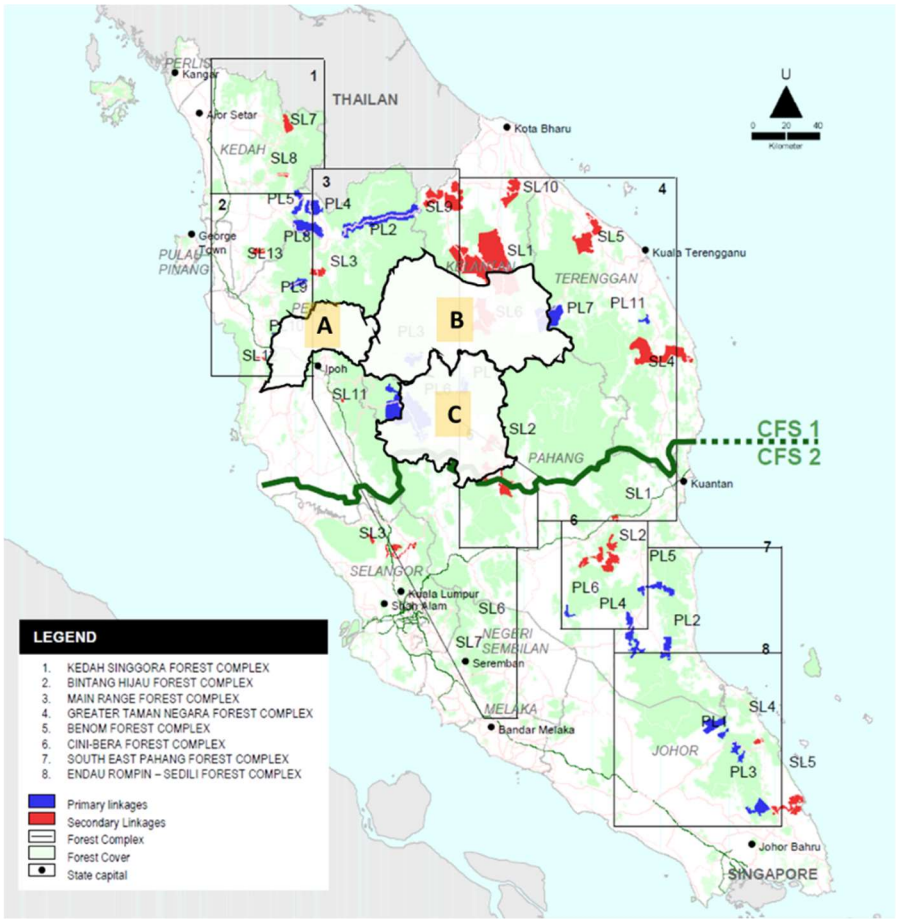


Figure 3. 2 Map of forest complexes in Peninsular Malaysia and the overlapping targeted districts in our study marked by A: Kuala Kangsar, B: Gua Musang, C: Lipis. (Source: Regional Planning Division, Department of Town and Country Planning, Peninsular Malaysia, 2009)

3.2.3 Sample size

Four cattle farms from Kuala Kangsar district, Perak and two cattle farms from Gua Musang district, Kelantan within 5km radius of forests were sampled beginning 2019.

Targeted sample size for each animal species per farm were 30 cattle from participating target farms, 30 other domestic animals (eg, goat, sheep, dog, etc) and 60 wildlife (eg, bats and rodents). The sample size (n) for this study (Table 3.1) was determined using

the Open-Epi Version 3 calculator. The software program applies the formula of n = [DEFF*Np (1-p)]/[(d2/Z21-□/2 *(N-1)+p*(1-p)], where N = population size, p = prevalence, d = confidence limits = 10%, and DEFF = design effect for cluster surveys = 1. The estimated prevalence used in the current study was 5% based on prevalence studies of filoviruses and henipaviruses in the region which reported seroprevalence of 0-6.5% in domestic animals and 1-20% in bats (Table). Other domestic animals and wildlife were opportunistically captured and sampled with priority given to animals in and closest to the participating target farms.

Table 3. 1 Sample size calculation for the sero-detection of filovirus and henipavirus in cattle, other domestic animals and wildlife in participating cattle farms located at the forest fringe in Perak and Kelantan, Malaysia

	Estimated Population Size, N per farm	Estimated Prevalence, p (% +/-5)[a]	Sample size, n per Farm	No. of farms	Total
Cattle : (Semi-intensive (n=3), Integrated oil palm (n=2), Intensive farm(n=1)	50	5	30	6	180
Other domestic animals : Goat, sheep, dog, etc.	50	5	30	6	180
Wildlife : Bat, rodent, other small mammal	1249 per roost for bats	5	70 (max of 60)[c]	6	360

[a]Prevalence of 5% was decided based on prevalence studies of filoviruses and henipaviruses in the region (see Table ???).

[b]Estimated population size for bats is based on the average roost size of bats. Total number of bats calculated is an overestimation as it will be highly dependent on the bats captured in nets and number of days the nets are placed in a location.

[c]Total number of wildlife are set to the maximum of 60 due to logistical and financial limitations.

3.2.4 Sampling of cattle and other domestic animals

Cattle and other domestic animals such as goat, sheep, dogs and rabbit were conducted with the team from the EcoHealth Alliance in accordance to USAID Emerging Pandemic Threats PREDICT Operating Procedures (PREDICT One Health Consortium, 2016b, 2016e).

All domestic animals were manually restraint for the sampling procedures (Appendix B). Blood samples were obtained from the appropriate venepuncture sites according to species. In cattle, goat, and sheep, the jugular vein or coccygeal vein was used (Figure 3.3). In dogs, blood was collected from the cephalic vein, jugular vein, femoral vein, lateral or medial saphenous vein. In rabbits, blood was collected from the lateral saphenous vein. Three to five ml of blood were taken from these animals and distributed equally in a BD Vacutainer® serum tube and EDTA tube.

A basic physical examination was performed by veterinarian to assess the health status and identify the age and sex of the animal. Photographs of sampled animal was taken from the sides and front, including dentition, or presence of any lesions, abnormalities or identifying marks or characteristics for future reference.

All blood tubes were labelled appropriately and placed in cooler boxes and transported back to sampling processing base. Blood in BD Vacutainer® serum tubes were left to

clot for serum collection within 1 hour. After clot is form, serum tubes were centrifuged at 1300 r.p.m for 15 minutes and 1 ml of serum was transferred into 0.5ml cryovial tubes to produce at least two aliquots. Cryovial tubes were labelled appropriately. Samples were then placed in MVE Doble 34 liquid nitrogen tanks in the field and later stored at -80° freezer in the lab according to proper cold chain methods.

Figure 3. 3 Locating the jugular vein in the jugular groove for blood sampling

3.2.5 Sampling of wildlife

Permission was obtained from the local authorities or landowners before bat and rodent trapping and sampling begun. Selected sites were first surveyed with priorities given to potential trapping locations in the farm and nearest to farm within 5 km radius. All personnel handling trapping and sampling of wildlife had to be immunized

against rabies, Japanese Encephalitis, Hepatitis B, tetanus and negative sputum tests for Tuberculosis.

3.2.5.1 Sampling of bat

Bats were captured using mist nets suitable for capturing bats in open spaces as described by FAO Manual for Investigating the Role of Bats in Emerging Zoonoses (Food and Agriculture Organisation of the United Nations, 2011). Further description on the sampling procedure is in Appendix X. Briefly, bats were captured using mist nets placed at anticipated bat flyways in the farm, village and in nearby plantations such as across streams or trails. The nets were also positioned near roosting or feeding sites such as near flowering banana trees and flowering durian trees (Figure 3.4).

Figure 3. 4 A bat trapping site with banana and durian plantation in a village near a participating cattle farm.

The mist nets were raised at 7.30pm at sunset and left opened until 12am for trapping to occur (Figure 3.5) and closed neatly after trapping period (Figure 3.6). Bat nets were repositioned to new locations when recapture rates were high, or captures were absent. The GPS coordinates for each mist nets were recorded.

Figure 3. 5 Extended mist nets for capture of bats at sunset.

Figure 3. 6 Retracted mist nets that is secured after trapping period

Mist nets were checked for bats every 30 mins to an hour. Captured bats were extracted from the nets carefully using at least one leather gloves on one hand and with the use of forceps or small stick to move the netting or bat mandibles then placed into a porous cotton bag with a draw-string mouth and taken to a sampling processing site where sampling was performed immediately (Figure 3.7). Approximately 20-30 bats were sampled in a night. No bats should be kept for more than 6 hours.

Figure 3. 7 Bags containing bats hung in draw-string cloth bags on a raffia string between two pillars

Bats were sampled and identified according to the USAID Emerging Pandemic Threats PREDICT Operating Procedures with some modifications (Hoffmann et al., 2010; PREDICT One Health Consortium, 2016a; C. Smith et al., 2010). Bat was removed from the cloth bag and manually restrained for sampling using leather gloves. In bats that were >100g, a non-heparinised syringe was used to collect blood from the cephalic vein, brachial vein or saphenous vein. Blood was then placed in a serum vacutainer tube containing serum-clotting factor and centrifuge. Extracted serum was placed into 2 cryovials of a minimum of 60µl if volume. If bats were <100g, the tip of a sterile 25G needle was used to puncture the brachial or propatagial vein (C. Smith et al., 2010).

A 1:10 serum dilution (10µl blood into 90µl PBS) was centrifuged, and the supernatant was transferred into a new cryovial tube. Cryovial tubes was labelled appropriately and placed in MVE Doble 34 liquid nitrogen tanks in the field and later stored at -80° freezer in the lab according to proper cold chain methods.

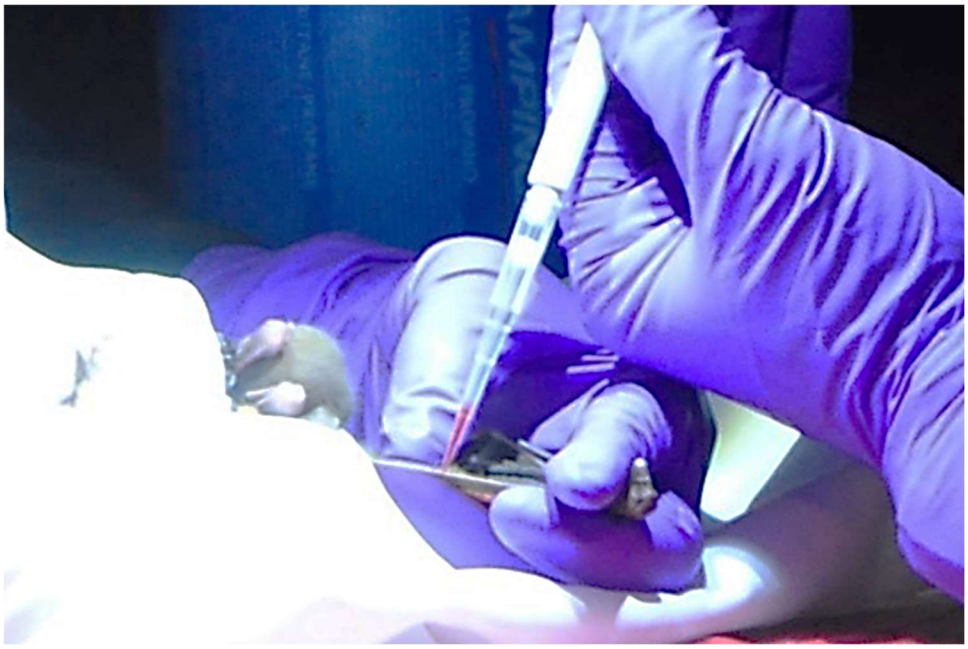


Figure 3. 8 Blood sampling from the cephalic vein of a bat by aspirating the drop of blood using a pipette.

Morphometric measurements and photographs of each bat were taken during the sampling procedure for the purpose of species identification. Identification of bat species was performed by an experienced rangers and field veterinarian based on the morphology and biometric measurements. Guide books were used for identification of bats on the field (Francis, 2008; Kingston et al., 2006; Payne et al., 1985; Phillipps & Phillipps, 2016).

Age of bats were classified according to adult, subadult and juvenile based on sexual characteristics and size. Males with descended testis and relative body size were considered adults while those with unfused epiphyses and undescended testis were considered subadults. Female bats with prominent nipples, were pregnant or lactating, relative body size and complete fusion of the phalangeal symphysis are classified as adult while those that were fully grown with unfused epiphyses were considered subadults. Pregnancy status was identified by gently palpating the abdomen and lactating status were identified by attempting to express milk from the teats. Bats that were small, clinging to dam and suckling were considered juveniles.

Typically, sampling per bat took less than 15 minutes with one person handling and performing the morphometric measurements, while the other person focused on the sampling. Bats nails were painted with a red nail polish as identification in case of recapture. Frugivorous or nectivorous bats were fed with approximately 1ml of 100% fruit juice or honey water prior to release. Bats were released after sampling.

3.2.5.2 Sampling of rodent and other small mammal

Rodents and other small mammals were trapped and sampled according to the USAID Emerging Pandemic Threats PREDICT Operating Procedures with some modifications (Aplin et al., 2003; Hoffmann et al., 2010; Mills et al., 1995; PREDICT One Health Consortium, 2016d, 2016e). Further description on the sampling technique is in

Appendix X. A total of 100 traps were used to trap rodents which encompasses 45 Sherman traps, 45 small Tomahawk for smaller mammals such as mice, rats, tree shrews and 10 large Tomahawk traps for larger mammals, such as civet (Figure 3.9).

Figure 3. 9 Sorting of 100 rodent traps according to transect lines before deployment

Sites for traps were first surveyed and divided between inside farm or nearby plantation or forest. Transect lines were used for placement of traps as opposed to grid to reduce overlapping and increase sampling area coverage and increase chance of capturing more species through coverage of small mammal home ranges and microhabitats (Pearson et al., 2003).

Traps containing appropriate baits were placed at strategic pathways for rodents (Figure 3.10). Oil palm fruit was the preferred option as it does not attract insects. Traps were placed at the same sites for a minimum of 3 consecutive nights from 6pm to 8am the next day. If adverse weather such as heavy rain is expected, traps will not be opened for that night.

Figure 3. 10 Placement of rodent trap by the fences in a farm

Figure 3. 11 Roasting of oil palm fruits that were used as baits for rodents

Trapped small mammal were gently transferred into a draw-string cloth bag by covering the bag over the opening of the trap and slightly tilting the trap downwards. Animals that were too stressed will be released, while animals found to be injured or weak will be brought back to sampling base for veterinary treatment.

Smaller rodents were removed from the bag gently and placed in a modified container for anaesthetic induction. Whereas, in small mammal species like civets, a gas mask was used for induction without completely removing animal out from the bag to reduce stress (Figure 3.12). Once animal is motionless and unresponsive, it is taken out and placed on the table with an appropriate fitting face mask for continue induction or

maintenance of anaesthesia. Monitoring of animal was performed by a veterinarian throughout during procedure along with a basic physical examination to access health status.

Figure 3. 12 Induction of general anesthesia using isoflurane in a small Indian civet (*Viverricula indica*)

Blood samples were collected from the lateral tail vein, ventral tail artery or lateral saphenous vein or lateral saphenous vein in rodents and other small mammals (Hem et al., 1998; Lee & Goosens, 2015; Mills et al., 1995; Office of the University Veterinarian, 2017a; PREDICT One Health Consortium, 2016d). In smaller rodents, approximately 1ml of blood was collected, and in larger small mammals (~200g), 3-5ml of blood was collected. Blood collected was placed into 1:10 PBS and spin to collect serum. Subsequent steps in processing and storage are as mentioned in 3.2.5.1.

After blood collection, fluid volume replacement was given subcutaneously using Lactated Ringer's or 0.9% NaCL solution to avoid hypovolemic shock. Marking on sampled rodent or small mammal is done by clipping 3-5mm of hair at the right ventral thigh. Morphometric measurements and photographs of each animal were taken during the sampling procedure for the purpose of species identification.

Age was classified into adult, subadult and juvenile based on secondary sexual characteristics and size. Adults are fully grown with adult pelage and is sexually mature. Subadults are not fully grown with developing pelage and may or may not be sexually mature. Juveniles are smaller than subadults and not sexually mature with grey and soft pelage (Hoffmann et al., 2010). Animals were monitored in a small cage until fully recovered from anaesthesia before release at place of capture.

3.2.5.3 Sampling of Wild boar

Wild boar were sampled according to established protocols (Office of the University Veterinarian, 2017b, 2017c; PREDICT One Health Consortium, 2016b). Further description on the sampling technique is in Appendix X. Wild boar was trapped according to trapping protocol described for rodent and other small mammals. It is transported to sampling base in the cage for sampling (Figure 3.13).

Figure 3. 13 A subadult wild boar in a Tomahawk trap from an oil palm plantation

Wild boar is sedated with by creating an anaesthetic chamber for induction and maintained with a gas mask. Monitoring of anaesthesia was performed by a veterinarian throughout during procedure. Blood samples were collected from the anterior vena cava and is distributed equally in a BD Vacutainer® serum tube and an EDTA tube. Processing of blood for serum extraction and storage were similar to previously mentioned in 3.2.4. After blood collection, fluid volume replacement was given subcutaneously using Lactated Ringer's or 0.9% NaCL solution. A basic physical examination was performed by veterinarian to access health status. Morphometric measurements and photographs of each animal were taken during the sampling procedure for the purpose of species identification.

Wild boar was classified into adult, subadult and juvenile based on secondary sexual characteristics and size. Adults are fully grown with adult pelage and is sexually mature. Subadults are not fully grown with developing pelage and may or may not be sexually mature. Juveniles are smaller than subadults and not sexually mature with grey and soft pelage (Hoffmann et al., 2010).

3.2.5.4 Sampling of Non-human primate

Non-human primates (NHP) were kept as pets hence trapping was not required. NHP were anaesthetized and sampled upon the agreement of its owner according to established protocols (Hoffmann et al., 2010; PREDICT One Health Consortium, 2016c). Further description on the sampling technique is in Appendix X. Briefly, aesthesia drug used to sedate animals were the combination of: 1) 4mg/kg Ketamine and 0.15mg/kg Xylazine with Yohimbine 1:1 as reversal agent for Xylazine, or 2) Tiletamine-Zolazepam, Zoletil® 3-5mg/kg in more aggressive primates. A dart syringe and blowpipe were used to deliver anaesthetic drug intra-muscularly by an experienced and trained ranger. Anaesthetic monitoring was performed by a veterinarian. After immobilization, basic physical examination was performed. A sterile lubricant is placed into animal's eyes to prevent corneal drying or scratches during sedation.

NHP is placed in a supine position. Blood is withdrawn from femoral vein located in the inguinal region lateral and parallel to the femoral artery (Figure 3.14). Three to five ml

of blood were taken from these animals and distributed equally in a BD Vacutainer® serum tube and EDTA tube. Processing of blood for serum extraction and storage are similar to previously mentioned in 3.2.4.

Figure 3. 14 Sampling of an anaesthetized pig-tailed macaque (*Macaca nemestrina*)

Photographs of the anterior and ventral view of the full body, anterior and lateral face, upper and lower dentition and genitalia were taken for future reference. Age was classified to 7 age class according to the PREDICT Operating Procedures for Non-Human Primates. Neonates are animals that show signs of being born within few days; infants are young that were still suckling and clinging on to mother; juveniles are mostly independent from mother, sexually immature and have not developed into adult size; immature primates are individual that have not sexually mature, subadults are fully independent, appears sexually mature but has not fully grown into adult size; adults are fully grown size with sexually mature sexual characteristics; old adults are adults with

signs of age degeneration. Monitoring is done from a safe distance until NHP is fully awake and ambulatory.

3.2.6 Sampling of Human

Human samples were collected from farmers in participating cattle farms. Sample size was limited to 10 farmers per farm due to logistical and budget constraints. Individuals were recruited based on their willingness to participate, were above 18 years old and have worked or assisted with handling of animals in participating farms. Individuals were recruited based on recommendations from the farm owner.

Human sampling was conducted by district health officers from Ministry of Health, Malaysia and staffs of EcoHealth Alliance in accordance to USAID Emerging Pandemic Threats PREDICT Operating Procedures (Huppenthal, 2009; C. K. Johnson & Saylors, 2016). A written informed consent from the participant was obtained for biological sampling collection and questionnaire. Participants were explained about the study objectives and that the biological testing is exploratory and not diagnostic for their health, the types of question asked, duration of questionnaire and potential risks of their participation. They were also informed that the study was voluntary and anonymous in nature.

Biological sampling and questionnaire were conducted in a convenient, comfortable and private environment. Further description on the sampling is in Appendix X. Briefly, blood was taken from the median cubital vein on the upper limb or metacarpal plexus or the dorsal venous arch on the dorsal surface of the hand. Three ml of blood were taken from these animals and distributed equally in a BD Vacutainer® serum tube and EDTA tube. Processing of blood for serum extraction and storage are similar to previously mentioned in 3.2.4.

3.2.7 Data collection

Following biological sampling, interviews with farmers were conducted using questionnaire designed to gauge exposure or behavioural risks to zoonotic spillover events. The questionnaire has been approved for used by the Institutional Review Board for studies on human research subjects and has been verified in previous studies (Kamau et al., 2021; Li et al., 2019; The PREDICT Consortium, 2021). The questionnaire was designed into several parts to gather information on participants' demographics, exposure to different species of domestic animal and wildlife, forms of exposure to domestic animal and wildlife, reported unusual illnesses within the past year. The questionnaire took less than 30 minutes to be completed and were administered by trained EcoHealth Alliance staffs in Malay language.

Data for livestock, other domestic animals and wildlife were recorded accordingly during the sampling process as described above. Table below shows the data collected for each species (Table 3.2).

Table 3. 2 Types of data collected during sampling of animals and human from participating cattle farms located at the forest fringe in Perak and Kelantan, Malaysia

Species	Data collected
Livestock/Dogs	Date of sampling, weather (clear, cloudy, rain), moon (full, half, new moon), weight, animal classification (domestic, feral domestic, captive wild, wild), species scientific name, species' English name, breed, farm location GPS coordinates, husbandry, ecological setting, other animals at site, tag number, sex, pregnancy status, lactating status, descended testes, age class, age, age in captivity, health condition during sampling, presence of ectoparasite, notes (special characteristics/health status/injury/body condition), specimen type, specimen collected, GPS coordinates of sampling base
Rodent/Small mammal/Non-human primates/Wild boar	Date of capture, time of trap check, trap ID and type, weather (clear, cloudy, rain), moon (full, half, new moon), weight, animal taxa, species scientific name, species' English name, sex, pregnancy status, lactating status, descended testes, age class, health condition during sampling, presence of ectoparasite, morphometric measurements (body length, right ear height, right hind foot, tail length), recapture marking, notes (special characteristics/health status/injury/body condition), specimen type, specimen collected, GPS coordinates of traps, GPS coordinates of sampling base
Bats	Date of capture, time of capture, GPS coordinate of net, weather (clear, cloudy, rain), moon (full, half, new moon), weight, animal taxa, species scientific name, species' English name, sex, pregnancy status, lactating status, descended testes, age class, health condition during sampling, presence of ectoparasite, morphometric measurements (forearm or radius length, body length, ear length, hind foot length, tail length), recapture marking, notes (special characteristics/health status/injury/body condition), specimen type, specimen collected, volume of serum dilution, of traps, GPS coordinates of sampling base
Human	Participant ID, site location, GPS coordinates, date of sampling, body temperature, blood pressure, pulse, weight, height, obvious signs of illness, age, sex, ethnicity, education level, years of residence in village, frequency of entering forest, exposure to different species of domestic animal and wildlife (primate, bat, rodent, small mammal, wild boar, wild bird, swine, cattle, dog, cat, goat, chicken, others), forms of exposure to domestic animal and wildlife (1. General exposure: lived with pet, handled live animals, raised animals, share water source with animals, seen faeces in or near food, animal entered house, eaten food that were touched, disturbed or damaged by animal, scratched or bitten by animal; and 2. Exposure to wildlife: cook or handle recently slaughtered meat, offal or blood of wildlife, eaten raw or undercooked meat, offal or blood of wildlife, eaten wildlife of unknown health status, eaten or shared dead wildlife, collect and sell dead wildlife, slaughter or butcher wildlife), reported unusual illnesses within the past year (fever with headaches and lethargy @ encephalitis, haemorrhagic fever and rash, severe

	acute respiratory infection (SARI), Influenza-like illnesses (ILI), fever with diarrhoea and vomiting, fever with skin rash), interviewer's name

3.2.8 Luminex Serology

Serum samples from all species of animals and from humans were analysed using a Luminex bead-based multiplex microsphere assay in a Bio-Rad Bio-Plex 200 (Bossart et al., 2007). This is an assay derived from the Luminex xMAP (xMAP = multianalyte profile) technology based on flow-cytometry principles which allows high-throughput, multiplex and simultaneous detection of up to 500 analytes in a 96-well plate. Since its invention in the late 1990s, this technology has been extensively used for detection of viral, bacterial, parasitical and fungal agents (Reslova et al., 2017). It has also been used increasingly for seroprevalence study of filoviruses and henipaviruses globally (Chowdhury et al., 2014; Dovih et al., 2019; Hayman et al., 2008; Laing et al., 2018; Peel et al., 2013b).

Filoviruses viral envelope glycoproteins (GPs) and henipaviruses receptor-binding proteins (G) were coupled to 5.6 µm diameter polystyrene magnetic beads for antigen-antibody binding. The list of filoviruses and henipaviruses tested are as shown in Table 3.3.

Table 3. 3 Filoviruses and Henipaviruses conjugated Bio-Plex beads used in multiplex assay for antibody detection.

Filoviruses (GPs)	Henipaviruses (Gs)
EBOV, Ebola virus	HeV, Hendra virus
BDBV, Bundibugyo virus	NiV, Nipah virus

BOMV, Bombali virus	CedV, Cedar virus
SUDV, Sudan virus	MojV, Mojiang virus
MLAV, Mengla virus	GhV, Ghana virus.
RESTVm, Reston virus monkey isolate	
RESTVp, Reston virus pig isolate	
LLOV, Lloviu virus	
MARV, Marburg virus	
RAVV, Ravn virus	

Prior to testing, individual samples were diluted 1:100 in phosphate-buffered saline (PBS) in 1.5ml Eppendorf tube and vortexed briefly to mix followed by heat inactivation at 56°C for 30 mins. A duplicate of a 100 µl diluted sera were added into 96-wells plate containing 100µl of bead master mix containing specific sGP- and sG-coated beads. Mixture is covered with plastic shield and aluminium foil and incubated on a shaker for 1200 r.p.m for 1 minute followed by 900 r.p.m for 45 minutes. Samples were washed to remove unbound antibodies three times and incubated on shaker with 100µl mastermix of 1:1000 biotinylated-Protein A and biotinylated Protein G (Thermo Fisher Scientific, Waltham, MA, USA) in PBS-Tween 20 (0.05%) (Bio-Rad). Mixture was washed three times and lastly incubated on a shaker with 100µl 1:1000 streptavidin-phycoerythrin (PE) in into each well and shook for 10 minutes. Results are read on Bio-Rad Bio-Plex 200 with at least 50 per spectral region and reported as Median fluorescence intensities (MFI). Cut-off values in the first round of testing at dilutions 1:100 were determined by 3*Sigma (3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations) (Dovih et al., 2019).

Positive samples in the first round were rerun at the optimum dilutions of 1:400 and 1:500 with added positive and negative controls. Sera from EBOV infected bats at dilutions of 1:6400 were used as positive controls for EBOV which resulted in an average MFI value of 20359. Negative rabbit polysera and PBS were used as negative controls with a maximum MFI value of 1629 and 1851 respectively. Naïve livestock sera (N=46) comprising of cattle, goat, sheep and pigs tested with filovirus and henipavirus antigens by collaborators in Uniformed Services University of the Health Sciences, USA yield a maximum MFI value of less than 900. Antigen-antibody binding were measured and read using a Bio-Plex 200 system. Data were transformed to adjusted MFI values by taking the mean MFI values duplicates of each sample and subtracting average PBS MFI per plate.

Cut-off were determined by using 3*Sigma and Latent cluster analysis (LCA). LCA was used in absence of gold standard assays and lack of positive and negative reference samples for the viral and animal species tested (Peel et al., 2013a). To improve cut-off determination, MFI values from local samples collected as part of the research programme were combined with this study dataset. Serum samples above the cut-off values were considered seropositive. The 3*Sigma cut-off values for filoviruses and henipaviruses were determined to be 4182 MFI and 4641 MFI respectively. LCA for filoviruses and henipaviruses were attempted but were not possible due to insufficient samples reactive samples.

3.2.9 Data Analysis

Serodetection of filoviruses and henipavirus for different species were calculated based on the number of seropositive animals over the total number of sample and reported as percentage (%). The association between the serological evidence and determinants from animals (such as, farm, sex, age, age class, species, reproductive status and health status) and humans (such as, farm, husbandry, sex, age range, ethnicity, education level, years of residence in village, frequency of entering forest, exposure to different species of domestic animal and wildlife, forms of exposure to domestic animal and wildlife, reported unusual illnesses within the past year) were analysed using Pearson Chi-Square test, X^2 or Fisher's exact test (two-tailed), when X^2 is not appropriate (one of expected cell value is less than 5) at significance level $\alpha = 0.05$. Post-hoc test Bonferroni corrections and Bonferroni adjusted p-value were used for multiple comparison. Odds ratio (OR) and OR 95% Confidence intervals (CI) with Haldane-Anscombe correction were applied when contingency table has values of 0. The difference between seropositivity and hypothesized risk factors were tested using Kruskal-Wallis, KW (more than two groups) test at $\alpha=0.05$ and post-hoc Dunn's test was applied if applicable. Variable was excluded from further analysis if data is insufficient or too small ($n<10$). All analysis were conducted using the Statistical Package for Social Sciences (SPSS 27) and Microsoft Excel.

3.3 Results

3.3.1 Population characteristics

3.3.1.1 Domestic animals

Six forest-fringed cattle farms were enrolled in the study in 2018 from Kuala Kangsar district, Perak and Gua Musang district, Kelantan. Serum samples were collected from farmers (n=55), cattle (n=156), goat (n=158), sheep (n=11), dogs (n=23), bats (n=265), rodents (n=36), civet (n=1), wild boar (n=1), rabbits (n=3) and non-human primates (n=2). In total, 709 serum samples were collected.

Cattle were mostly sampled from integrated cattle farms (Table 3.13). Other domestic animals such as goats and sheep were mainly sampled from semi-intensive goat and sheep farms (Table 3.14 and 3.15). Dogs sampled were either tethered or free-roaming in the village or plantation (Table 3.16). Besides these animals, a pet rabbit was also sampled. Majority of the farm animals sampled were adults.

3.3.1.2 Wildlife

A total of 265 bats that were sampled during the study with an average of 44 (25-56) bats sampled per farm. Out of 9 species captured, 6 species were frugivores or nectarivores and 3 species were insectivores. Species that were most sampled were mostly adult male *C. brachyotis* (n=108), *E. spelaea* (n=70) and *C. horsfieldii* (n=52) (Table 3.17).

Table 3. 4 Bats based on sex, age class and species sampled from the surrounding targeted cattle farm at forest fringe

Bat	
	n
Sex	
Female	139
Male	126
	265
Age class	
Adult	242
Subadult	23
Juvenile	0
	265
Species	
Balionycteris maculata	3
Cynopterus brachyotis	108
Cynopterus horsfieldii	52
Eonycteris spelaea	70
Hipposideros galeritus	1
Macroglossus minimus	6
Macroglossus sobrinus	5
Nycteris tragata	1
Tylonycteris pachypus	19
	265

Overall, 36 rodents were sampled with an average of 6 (3-5) rodents per farm (Table 3.5). Twelve species of rodents mostly adult *Rattus exulans* (n=8) and *Rattus tiomanicus* (n=7) were captured.

Table 3. 5 Rodents based on sex, age class and species sampled from the surrounding targeted cattle farm at forest fringe.

Rodent	
	n
Sex	
Female	12
Male	24
	36
Age class	
Adult	29
Subadult	7
Juvenile	0

		36
Species		
	Chiropodomys gliroides	5
	Lariscus insignis	1
	Leopoldamys sabanus	5
	Maxomys rajah	1
	Maxomys surifer	3
	Niviventer cremoriventer	1
	Rattus argentiventer	2
	Rattus exulans	8
	Rattus rattus	1
	Rattus tiomanicus	7
	Sundasciurus lowii	1
	Tupaia glis	1
		36

Other wildlife sampled were pet macaques (n=2), a wild boar (n=1) and a civet, *Viverricula indica* (n=1).

3.3.1.3 Human

A total of 56 farmers from participating cattle farms were enrolled but only 55 samples were obtained. An average of 9 farmers were sampled from each farm. Majority sampled were male, between 20-29 years old, with mean age of 42 (18-68) of the Malay race. Most have completed secondary school or above and have resided in the village for more than 10 years (51/55, 92.7%) (Table 3.19).

The most common domestic animals that farmers were exposed to within the past one year of sampling were cattle, cat and poultry. Whereas the most common wildlife exposed to were rodents and bats (Table 3.120). Farmers had general exposure to

domesticated animals and wildlife in the past one year primarily through handling of live animals, animal entering living quarters and living with pet. Farmers were exposed to wildlife primarily through slaughter, butchering, cooking and handling of recently slaughtered meat, offal or blood of wildlife (Table 3.21).

Out of the 55 farmers, many farmers reported Influenza-like illnesses (ILI), followed by fever with diarrhoea and vomiting, Severe acute respiratory infection (SARI), fever with headaches and lethargy (3/55, 5.5%), and fever with skin rash (1/55, 1.8%). No farmers reported haemorrhagic fever (Table 3.22).

3.3.2 Serological result

3.1.1.1 Filovirus

A total of 45 sera were reactive to filovirus (%??). The serological detection for filovirus among species in descending order were goat (15.2%, 95% CI 10-22), sheep (9.1%, 95% CI 0.2-41.3), human (9.1%, 95% CI 3-20), dog (8.7%, 95% CI 1.1-28), cattle (5.1%, 95% CI 2.2-9.9) and bat (1.9%, 95% CI 0.6-4.3) (Table 3.6, Figure 16-22). Bats species that were seropositive for filovirus were *C. brachyotis*, *E. spelaea* and *C. horsfieldii* (Table 3.7).

Table 3. 6 Serological evidence of filovirus based on farm and species sampled using Luminex bead-based multiplex microsphere assay

	Filovirus						
Participating cattle farms	**1**	**2**	**3**	**4**	**5**	**6**	**Total**

Category	Husbandry	SI	SI	SI	It	It	In	
Human								
	Individual sampled	10	9	10	10	10	6	55
	Seropositive, n (%)	1 (10)	0	1 (10)	2 (20)	0	1 (16.7)	5 (9.1)
Cattle								
	Individual sampled	22	15	24	29	36	30	156
	Seropositive, n (%)	2 (9.1)	2 (13.3)	2 (8.3)	1 (3.4)	1 (2.8)	0	8 (5.1)
Goat								
	Individual sampled	20	44	31	22	22	19	158
	Seropositive, n (%)	0	7 (15.9)	8 (25.8)	5 (22.7)	0	4 (21.7)	24 (15.2)
Sheep								
	Individual sampled	0	0	0	9	0	2	11
	Seropositive, n (%)	0	0	0	0	0	1 (50)	1 (9.1)
Dog								
	Individual sampled	10	1	2	1	0	9	23
	Seropositive, n (%)	1 (10)	1 (100)	0	0	0	0	2 (8.7)
Bat								
	Individual sampled	45	49	47	43	25	56	265
	Seropositive, n (%)	0	3 (6.1)	1 (2.1)	0	1 (4)	0	5 (1.9)
Rodent								
	Individual sampled	10	5	8	3	7	3	36
	Seropositive, n (%)	0	0	0	0	0	0	0
Civet								
	Individual sampled	0	1	0	0	0	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Wild boar								
	Individual sampled	0	0	0	0	1	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Rabbit								
	Individual sampled	0	0	3	0	0	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Primate								
	Individual sampled	0	1	1	0	0	0	2
	Seropositive, n (%)	0	0	0	0	0	0	0
Total		4	13	12	8	2	6	45

Production system: SI, semi-intensive system; It, integrated system; In, intensive system

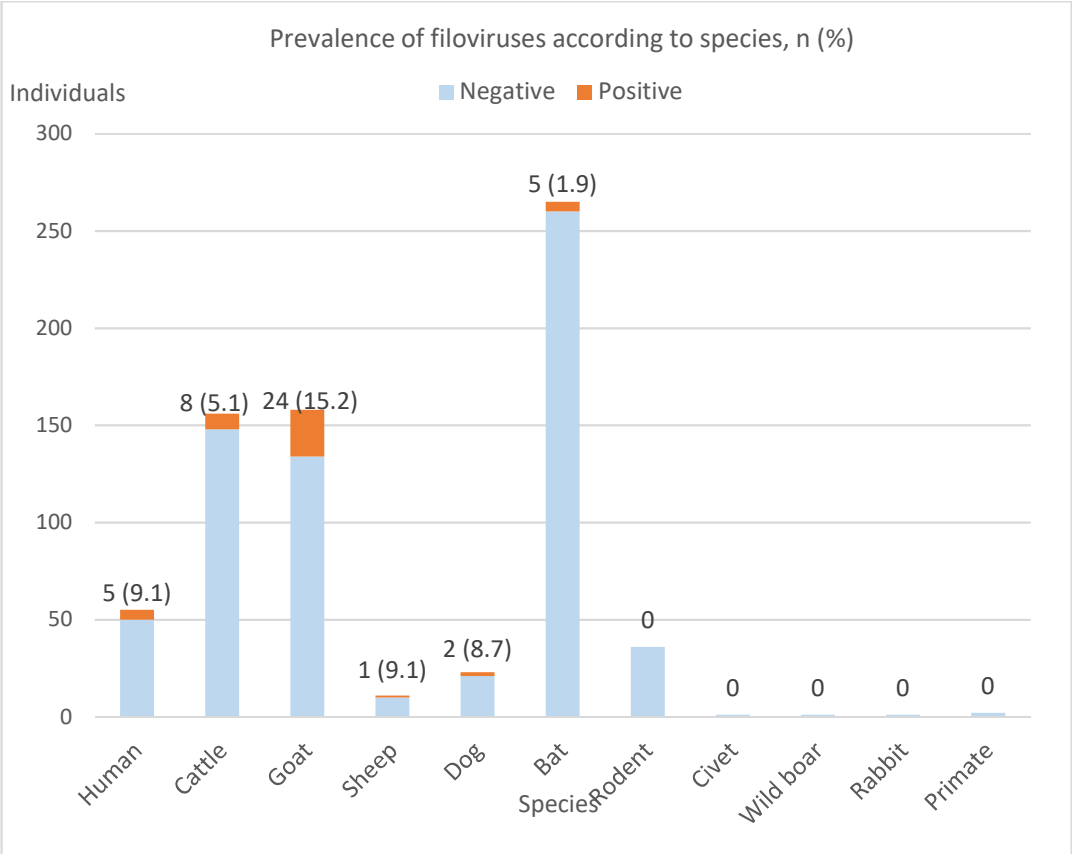


Figure 3. 15 Serological evidence of filovirus based on species sampled using Luminex bead-based multiplex microsphere assay

Table 3. 7 Serological evidence of filovirus in bats using Luminex bead-based multiplex microsphere assay

No	Species	Individuals sampled (n)	Filovirus positive (%)
1	*Balionycteris maculata*	3	0
2	*Cynopterus brachyotis*	108	2 (1)
3	*Cynopterus horsfieldii*	52	1 (2)
4	*Eonycteris spelaea*	70	2 (2)
5	*Hipposideros galeritus*	1	0
6	*Macroglossus minimus*	6	0
7	*Macroglossus sobrinus*	5	0
8	*Nycteris tragata*	1	0
9	*Tylonycteris pachypus*	19	0
	Total	265	5

Most filovirus reactive samples were reactive to BDBV (29/45) followed by SUDV (16/45) and RESTV (16/45) (Table 3.8). Twenty-three (23/45, 51%) samples were reactive to only one antigen, with the most being BDBV (16/23), followed by EBOV (4/23), RESTVp (2/23) and SUDV (1/23). Whereas twenty-two (22/45, 48.9%) samples were reactive to two to five filovirus antigens which involved BDBV, SUDV, RESTVp the most. Cross-reactivity were observed between EBOV, BDBV, BOMV, SUDV, RESTVm, RESTVp, MARV, and RAVV (Appendix).

Table 3. 8 Reactivity with filovirus across species using Luminex bead-based multiplex microsphere assay

			Reactivity to filovirus, n (%)									
*Category	Total	Total reactive	EBOV	BDBV	BOMV	SUDV	RESTVm	RESTVp	LLOV	MLAV	MARV	RAVV
Human	55	5	2 (3.6)	3 (5.5)	0	0	0	0	0	0	0	1 (1.7)
Cattle	156	8	3 (1.9)	6 (3.8)	0	2 (1.3)	2 (1.3)	0	0	0	0	0
Goat	158	24	0	14 (8.9)	0	12 (7.6)	0	14 (8.9)	0	0	0	0
Sheep	11	1	0	1 (9.1)	1 (9.1)	1 (9.1)	0	1 (9.1)	0	0	1 (9.1)	0
Dog	23	2	1 (4.3)	2 (8.7)	0	0	0	0	0	0	0	0
Bat	265	5	2 (0.8)	3 (1.1)	1 (0.4)	1 (0.4)	0	1 (0.4)	0	0	0	0
Total		**45**	**8 (17.8)**	**29 (64.4)**	**2 (4.4)**	**16 (35.6)**	**2 (4.4)**	**16 (35.6)**	**0**	**0**	**1 (2.2)**	**4 (8.9)**

EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

*Non-reactive category not included

The range of MFI for filovirus positive sera was between 4183 and 24059, with the highest being 24059 MFI for BDBV in a human. There were 10 reactions which produced an MFI reading of 10000-14999 MFI (EBOV, BDBV, SUDV and RAVV) and 2 reactions with >20000 MFI (BDBV) (Table 3.9).

Table 3. 9 Filovirus range of MFI of positive sera, number of high reactivity and its antigen among different species using Luminex bead-based multiplex microsphere assay

	High reactivity for filovirus						
	No. of Sera with 10000-14999 MFI	**Type of Antigen reaction**	**No. of Sera with 15000-19999 MFI**	**Type of Antigen reaction**	**No. of Sera with >20000 MFI**	**Type pf antigen reaction**	**MFI range**
Bat	2	EBOV, BDBV	0	-	0	-	4382-10784
Cattle	1	EBOV	0	-	0	-	4246-10205
Dog	0	-	0	-	0	-	5429-7561
Goat	3	BDBV, RAVV	0	-	1	BDBV	4183-21882
Sheep	1	SUDV	0	-	0	-	4317-10890
Human	3	EBOV, BDBV, RAVV	0	-	1	BDBV**	4928-24059
Total	10		0		2		

**Among all reactivity to filoviruses, a human reported highest MFI for BDBV
EBOV, Ebola virus; BDBV, Bundibugyo virus; SUDV, Sudan virus; RAVV, Ravn virus

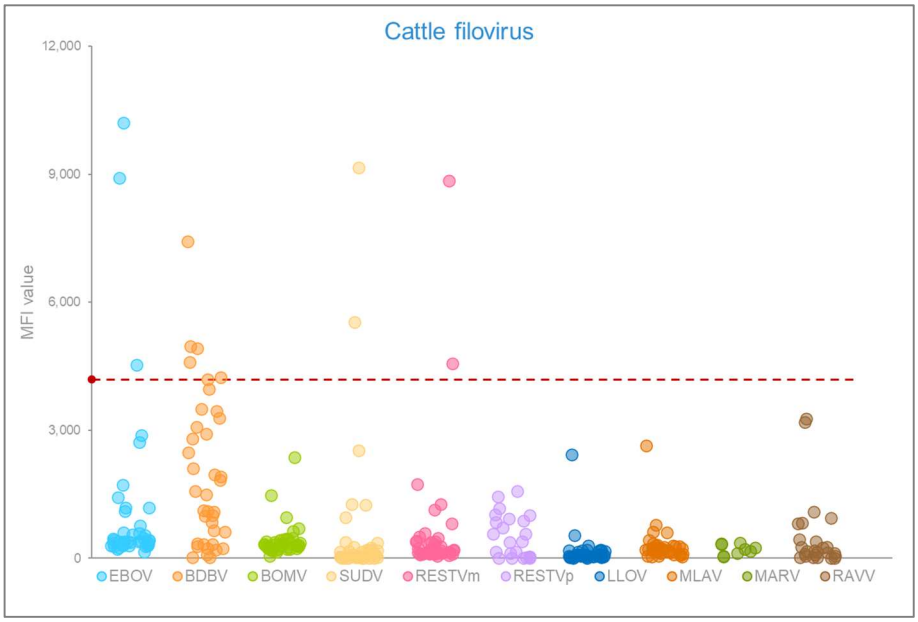


Figure 3. 16 Filovirus range of MFI of positive cattle sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

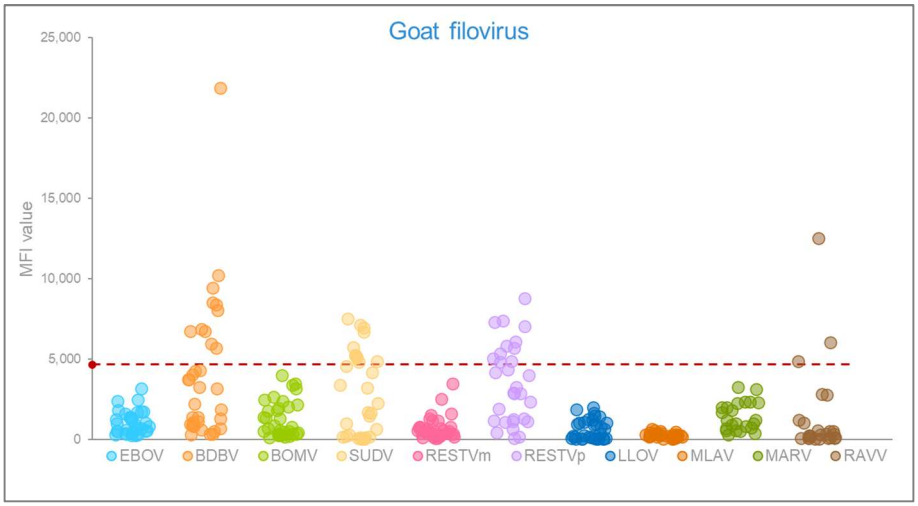


Figure 3. 17 Filovirus range of MFI of positive goat sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

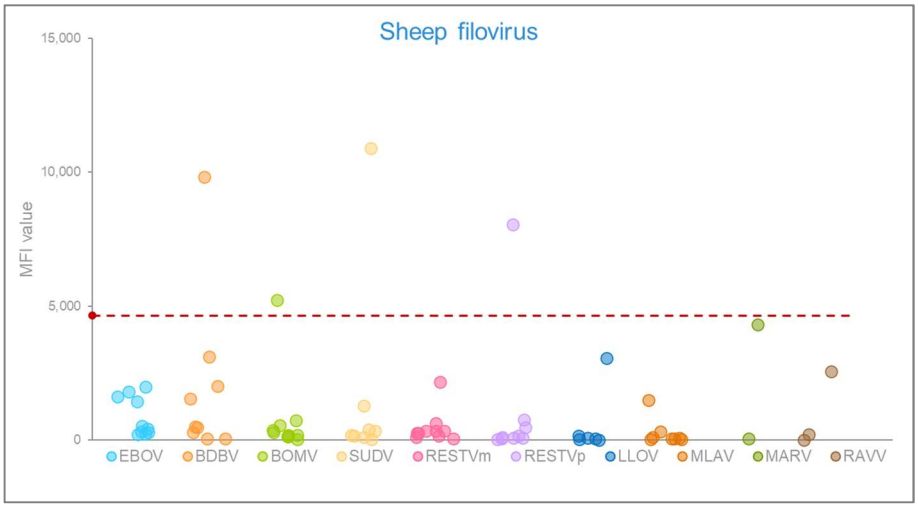


Figure 3. 18 Filovirus range of MFI of positive sheep sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

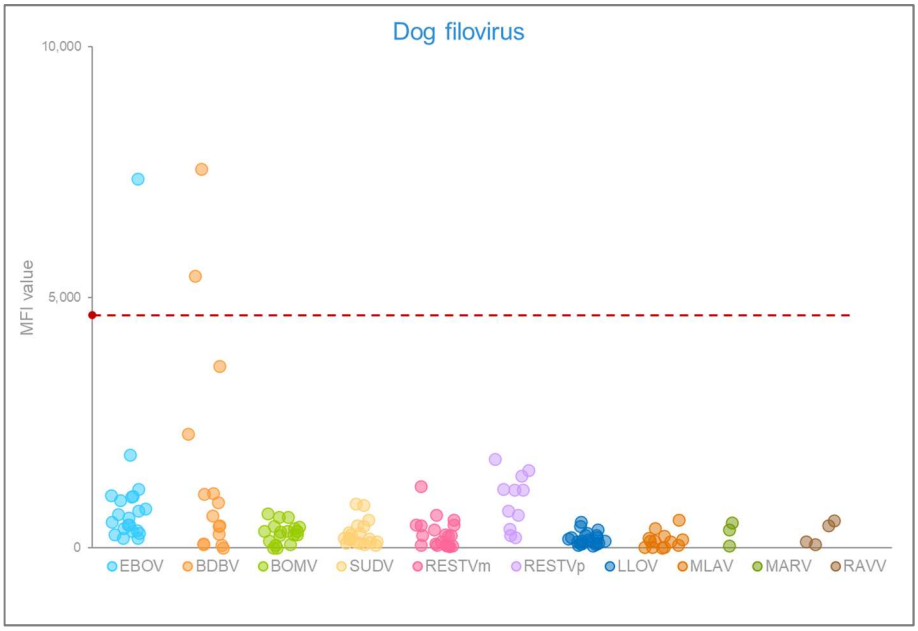


Figure 3. 19 Filovirus range of MFI of positive dog sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

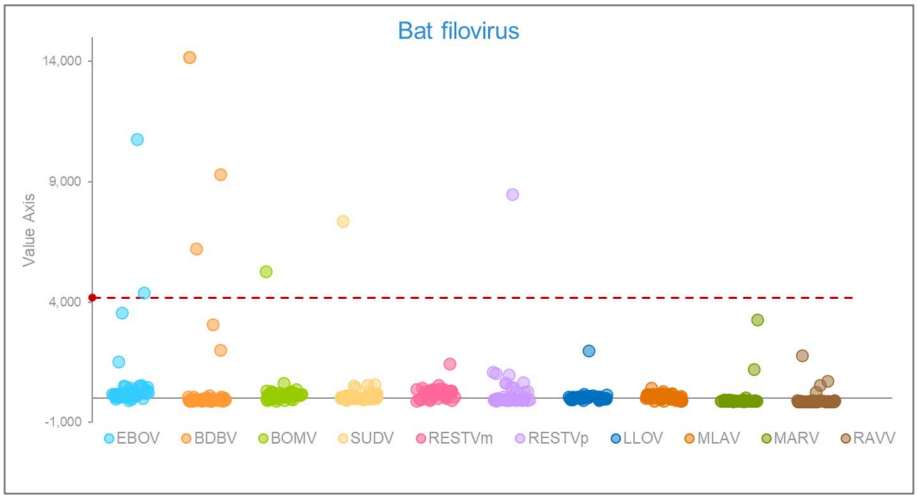


Figure 3. 20 Filovirus range of MFI of positive bat sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

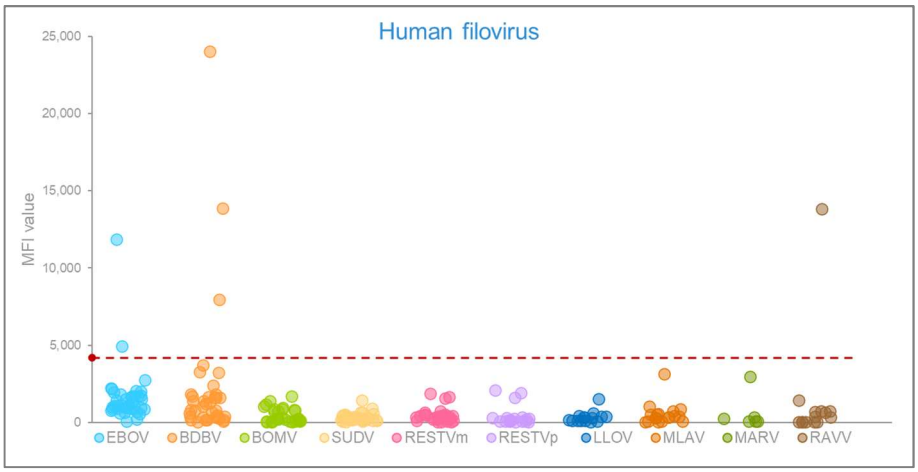


Figure 3. 21 Filovirus range of MFI of positive human sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

3.1.1.2 Henipavirus

A total of 36 sera were reactive to henipavirus. The reactive sera were in goat (13.9%, 95% CI 8.9-20.3), sheep (9.1%, 95% CI 0.2-41.3), human (7.3%, 95% CI 2-17.6), dog (4.3%, 95% CI 0.1-21.9), cattle (3.2%, 95% CI 1.0-7.3) and bat (1.1%, 95% CI 0.2-3.3) (Table 3.10 and Figure 3.23-29). Bats seropositive for henipavirus were *C. brachyotis* and *C. horsfieldii* (Table 3.11).

Table 3. 10 Serological evidence of Henipavirus based on farm and species using Luminex bead-based multiplex microsphere assay

		Henipavirus						
	Participating cattle farms	**1**	**2**	**3**	**4**	**5**	**6**	**Total**
	Husbandry	**SI**	**SI**	**SI**	**It**	**It**	**In**	
Human	Individual sampled	10	9	10	10	10	6	55
	Seropositive, n (%)	0	1 (10)	0	2 (20)	0	1 (16.7)	4 (7.3)
Cattle	Individual sampled	22	15	24	29	36	30	156
	Seropositive, n (%)	1 (4.5)	0	1 (4.2)	1 (3.4)	0	2 (6.7)	5 (3.2)
Goat	Individual sampled	20	44	31	22	22	19	158
	Seropositive, n (%)	0	7 (15.9)	7 (22.6)	5 (22.7)	0	3 (15.8)	22 (13.9)
Sheep	Individual sampled	0	0	0	9	0	2	11
	Seropositive, n (%)	0	0	0	0	0	1 (9.1)	1 (9.1)
Dog	Individual sampled	10	1	2	1	0	9	23
	Seropositive, n (%)	0	0	0	0	0	1 (11.1)	1 (4.3)
Bat	Individual sampled	45	49	47	43	25	56	265
	Seropositive, n (%)	0	2 (4.1)	1 (2.1)	0	0	0	3 (1.1)
Rodent	Individual sampled	10	5	8	3	7	3	36
	Seropositive, n (%)	0	0	0	0	0	0	0
Civet	Individual sampled	0	1	0	0	0	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Wild boar	Individual sampled	0	0	0	0	1	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Rabbit	Individual sampled	0	0	3	0	0	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Primate	Individual sampled	0	1	1	0	0	0	2
	Seropositive, n (%)	0	0	0	0	0	0	0
Total		1	10	9	8	0	8	

Production system: SI, semi-intensive system; It, integrated system; In, intensive system

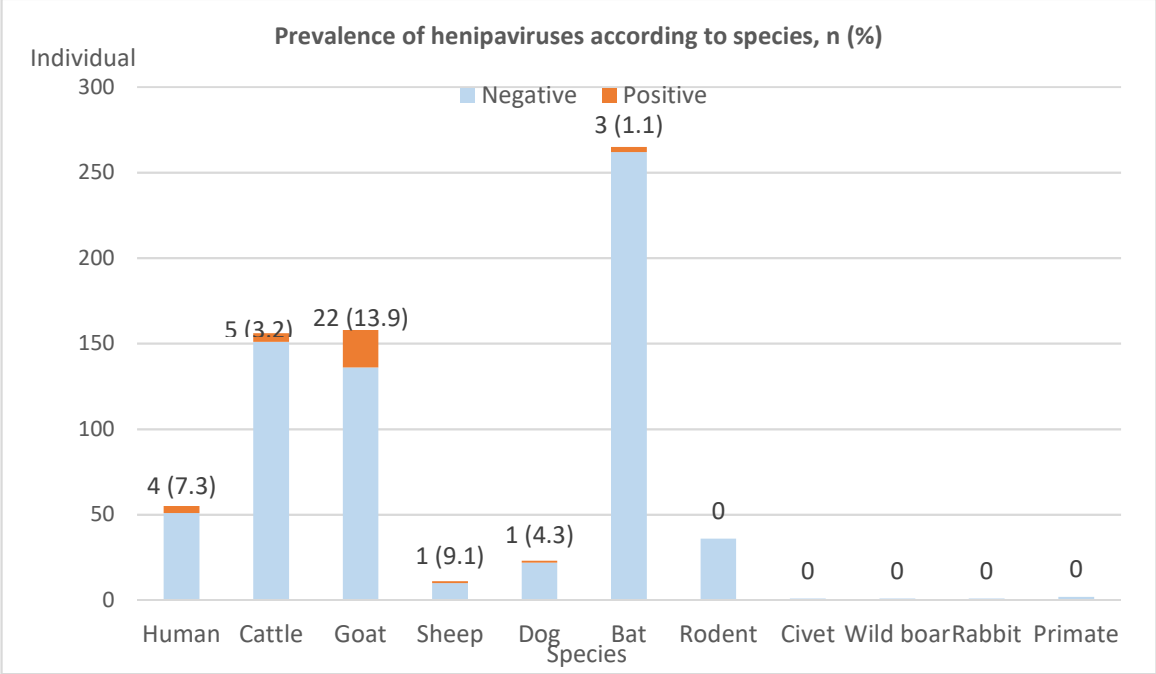


Figure 3. 22 Serological evidence of henipaviruses based on species sampled using Luminex bead-based multiplex microsphere assay

Table 3. 11 Serological evidence of henipavirus in bats using Luminex bead-based multiplex microsphere assay

No	Species	Individuals sampled (n)	Henipavirus positive (%)
1	*Balionycteris maculata*	3	0
2	*Cynopterus brachyotis*	108	2 (1)
3	*Cynopterus horsfieldii*	52	1 (2)
4	*Eonycteris spelaea*	70	0
5	*Hipposideros galeritus*	1	0
6	*Macroglossus minimus*	6	0
7	*Macroglossus sobrinus*	5	0
8	*Nycteris tragata*	1	0
9	*Tylonycteris pachypus*	19	0
	Total	265	3

Most samples were reactive to GhV (32/36), HeV (22/36), NiV (19/36), CedV (9/36) and MojV (7/36) (Table 3.11). Eleven (11/36, 31%) samples were reactive to only one antigen, with the most being GhV (9/11), followed by MpjV (2/11). Twenty-two (25/36, 69.4%) samples were reactive to two to five henipavirus antigens which involved GhV,

HeV and NiV the most. Cross-reactivity were observed between all five antigens tested (Appendix).

Table 3. 12 Reactivity with henipavirus across species using Luminex bead-based multiplex microsphere assay

			Reactivity with henipavirus, n (%)				
	Total sampled	Total reactive	HeV	NiV	CedV	MojV	GhV
Human	55	4	1 (1.7)	0	1 (1.7)	4 (6.7)	4 (6.7)
Cattle	156	5	1 (0.6)	0	1 (0.6)	2 (1.3)	4 (2.6)
Goat	158	22	17 (10.8)	15 (9.5)	5 (3.2)	0	20 (12.7)
Sheep	11	1	1 (9)	1 (9.1)	1 (9.1)	0	1 (9)
Dog	23	1	0	0	0	1 (4.3)	0
Bat	265	3	2 (0.8)	2 (0.8)	1 (0.4)	0	3 (1.1)
Total		36	22 (61.1)	19 (52.8)	9 (25)	7 (19.4)	32 (88.9)

HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

The range of MFI for henipavirus positive sera was between 4182 and 24689 with the highest being 24689 MFI against GhV in a farmer. There were 39 reactions with an MFI reading of 10000-19999 MFI (HeV, NiV, CedV, and GhV), and 5 reactions of >20000 MFI (HeV, CedV, MojV and GhV). Goat sera displayed many high MFI readings of >10000. In general, almost all henipavirus reactive sera had reactivity to GhV with many being >10000 MFI. (Table 3.13 and Appendix).

Table 3. 13 Henipavirus range of MFI of positive sera, number of high reactivity and its antigen among different species using Luminex bead-based multiplex microsphere assay

	High reactivity for henipavirus						
	No. of Sera with 10000-14999 MFI	Type of Antigen reaction	No. of Sera with 15000-19999 MFI	Type of Antigen reaction	No. of Sera with >20000 MFI	Type pf antigen reaction	MFI range
Bat	4	-	0	-	0	-	4776-12117
Cattle	3	-	2	HeV, CedV	1	GhV	5000-20100
Dog	0	-	0	-	0	-	5042[a]
Goat	21	HeV, NiV, CedV, GhV	4	HeV, GhV	0	-	4834-19905
Sheep	0	-	3	HeV, NiV, GhV	0	-	9894-17401
Human	2	GhV	0	-	4	HeV, CedV, MojV, GhV	7170-24689
Total	30		9		5		

[a]no upper MFI range as there was only 1 reactivity in 1 dog serum
HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

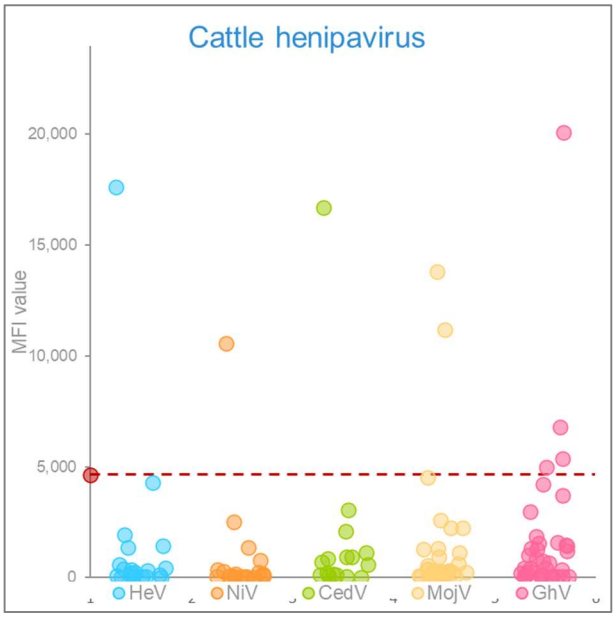


Figure 3. 23 Henipavirus range of MFI of positive cattle sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

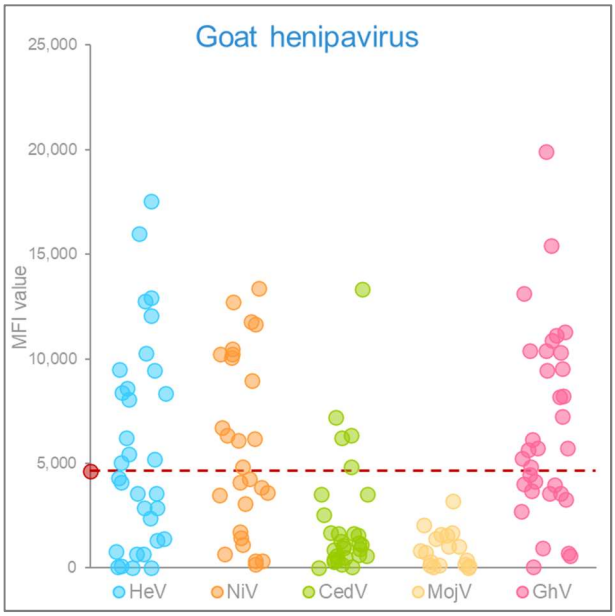


Figure 3. 24 Henipavirus range of MFI of positive goat sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

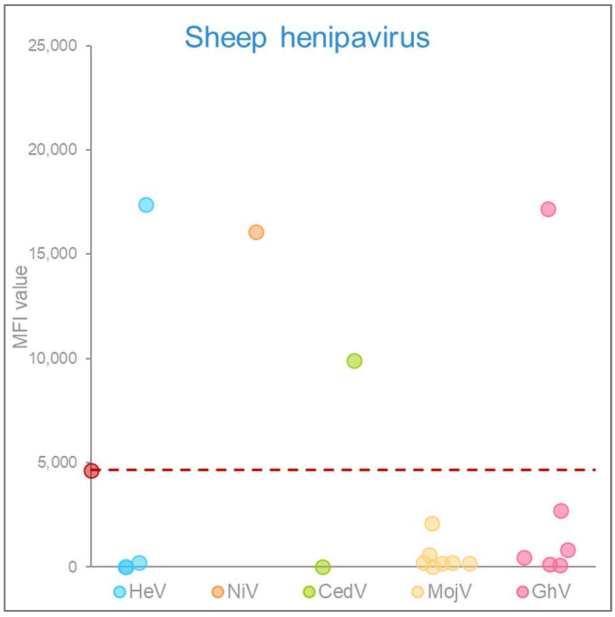
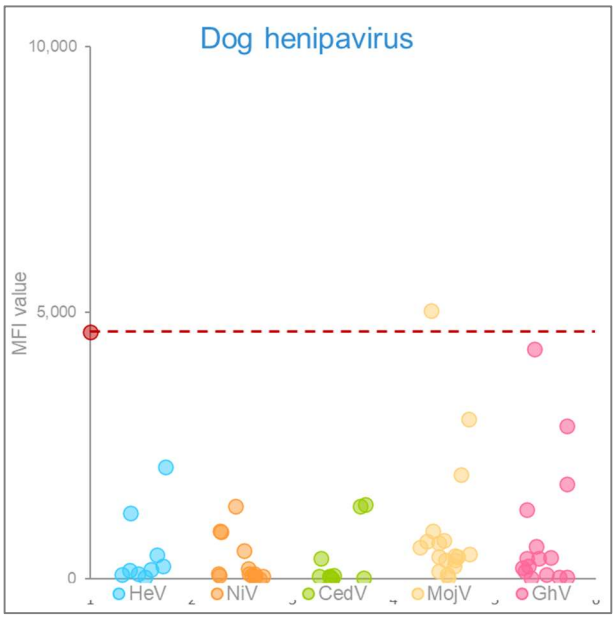


Figure 3. 25 Henipavirus range of MFI of positive sheep sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

Figure 3. 26 Henipavirus range of MFI of positive dog sera using Luminex bead-based multiplex microsphere assay



Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

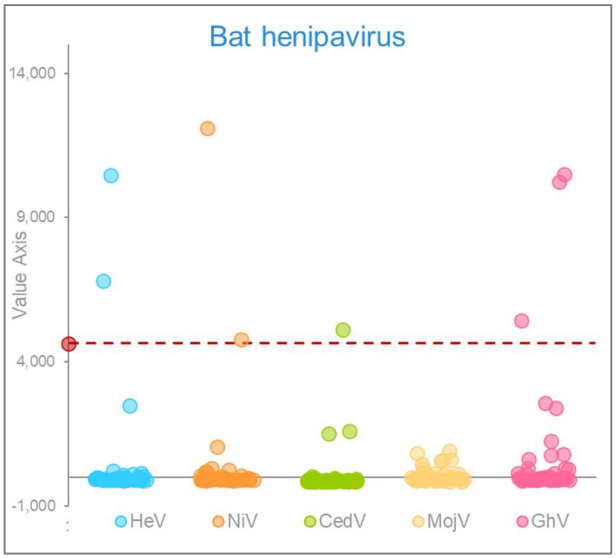


Figure 3. 27 Henipavirus range of MFI of positive bat sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

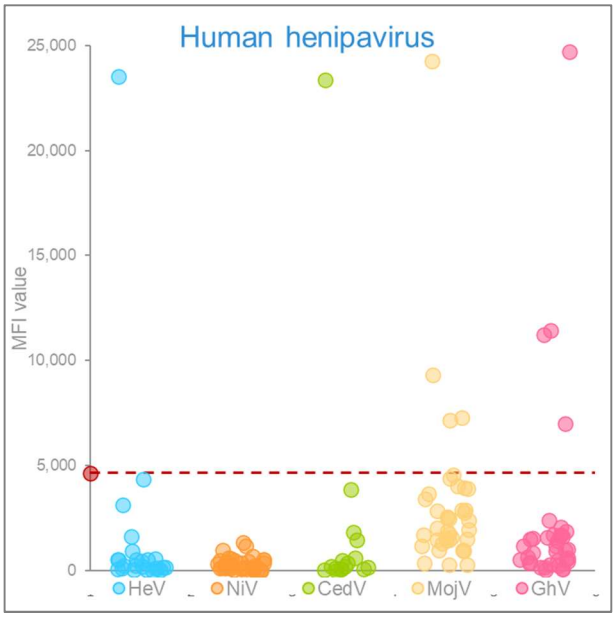


Figure 3. 28 Henipavirus range of MFI of positive human sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

3.3.3 Serological evidence and demographic distribution

3.3.3.1 Cattle

The serological evidence of filovirus and henipavirus in cattle were 5.1%, 8/156 (95% CI 2.2-9.9) and 3.2%, 5/156 (95% CI 1.0-7.3) respectively. Among the filovirus positive (8/156) and henipavirus positive (5/156) cattle sera, most were female, adults and apparently healthy and had no observable ectoparasite at the time of sampling. No significant difference was observed between serological evidence of filovirus or henipavirus with husbandry, sex, age class and health status (Table 3.14).

Table 3. 14 Serological evidence of filovirus and henipavirus in cattle based on farm, sex, age class, reproduction status, health status and presence of ectoparasite

	Cattle				
	n	Filovirus positive	P-value	Henipavirus positive	P-value
Farm			a		a
1	22	2		1	
2	15	2		0	
3	24	2		1	
4	29	1		1	
5	36	1		0	
6	30	0		2	
	156	8		5	
Husbandry			0.108		0.343
Intensive	30	0		2	
Semi-intensive	61	6		2	
Integrated	65	2		1	
	156	8		5	
Sex			1.000		a
Female	101	5		5	
Male	55	3		0	
	156	8		5	
Age class			1.000		1.000
Adult (>2 yr)	121	7		4	
Subadult (6m-2yr)	28	1		1	
Juvenile (<6m)	7	0		0	
	156	8		5	
Reproduction status					
Pregnant	18	0	a	0	a
Lactating	33	1	a	2	0.285
		1		2	
Health status			0.192		0.123
Apparently healthy	153	7		4	
Sick	3	1		1	
	156	8		5	
Ectoparasite			a		a
No	147	8		5	
Yes	9	0		0	
	156	8		5	

[a]Variable is excluded from further analysis due to insufficient or too small data

3.3.3.2 Goat

The serological evidence of filovirus and henipavirus in goat were 15.2%, 24/158 (95% CI 10-22) and 13.9%, 22/158 (95% CI 8.9-20.3) respectively. Among the filovirus positive (24/158) and henipavirus positive (22/158) goat sera, most were semi-intensively reared, female, adults and apparently healthy. Subadult goats were 6.3 times less likely to be seropositive for filoviruses as compared to adults (P= 0.028, OR=6.3, 95% CI=1.149, 34.383). No significant difference was observed between serological evidence of henipavirus and the various factors (Table 3.15).

Table 3. 15 Serological evidence of filovirus and henipavirus in goat based on farm, sex, age class, reproduction status, health status and presence of ectoparasite sampled from goat farms surrounding participating targeted cattle farms

	Goat				
	n	**Filovirus positive**	**P-value**	**Henipavirus positive**	**P-value**
Targeted Cattle Farm			a		a
1	20	0		0	
2	44	7		7	
3	31	8		7	
4	22	5		5	
5	22	0		0	
6	19	4		3	
	158	24		22	
Husbandry of goat farm			0.009[b]		0.004[b]
Intensive	52	2		1	
Semi-intensive	101	21		20	
Extensive	5	1		1	
	158	24		22	
Sex			0.243		0.629
Female	104	13		13	
Male	54	11		9	
	158	24		22	
Age class			0.028		0.114
Adult (>1 yr)	106	21		19	
Subadult (6m-1yr)	38	1		2	
Juvenile (<6m)	14	2		1	
	158	24		22	
Reproduction status					
Pregnant	28	5	0.771	5	0.548
Lactating	28	4	1.000	4	1.000

Health status			0.148		0.403
Apparently healthy	122	21		18	
Sick	36	3		4	
	158	24		22	
Ectoparasite			0.604		0.788
No	120	17		16	
Yes	38	7		6	
	158	24		22	

[a]Variable is excluded from further analysis due to insufficient or too small data
[b]Not significant after Bonferroni correction

3.3.4 Sheep

The serological evidence of filovirus and henipavirus in sheep were 9.1%, 1/11 (95% CI 0.2-41.3) for both. Out of 11 sheep sampled, only one sheep was seropositive for filoviruses and henipaviruses. The sheep was a female pregnant and lactating adult that was raised extensively, and apparently healthy (Table 3.16). Further analysis with the variables were not performed due to insufficient or too small data.

Table 3. 16 Serological evidence of filovirus and henipavirus in sheep based on farm, sex, age class, reproduction status, health status and presence of ectoparasite sampled from sheep farms surrounding participating targeted cattle farm

	Sheep		
	n	Filovirus positive	Henipavirus positive
Targeted Cattle Farm			
1	0	0	0
2	0	0	0
3	0	0	0
4	9	0	0
5	0	0	0
6	2	1	1
	11	1	1
Husbandry of sheep farm			
Semi-intensive	9	0	0
Extensive	2	1	1
	11	1	1
Sex			
Female	6	1	1
Male	5	0	0
	11	1	1
Age class			

Adult (>1 yr)	7	1	1
Subadult (6m-1yr)	2	0	0
Juvenile (<6m)	2	0	0
	11	1	1
Reproduction status			
Pregnant	2	1	1
Lactating	2	1	1
Health status			
Apparently healthy	11	1	1
Sick	0	0	0
	11	1	1
Ectoparasite			
No	11	1	1
Yes	0	0	0
	11	1	1

3.3.5 Dog

The serological evidence of filovirus and henipavirus in dog were 8.7%, 2/23 (95% CI 1.1-28) and 4.3%, 1/23 (95% CI 0.1-21.9) respectively. Filovirus positive dog (2/23) were adult and subadult tethered males. Whereas henipavirus positive dog was an adult male raised extensively. All filovirus and henipavirus positive dogs were apparently healthy with no observable ectoparasite at time of sampling. Further analysis with the variables were not performed due to insufficient or too small data (Table 3.17).

Table 3. 17 Serological evidence of filovirus and henipavirus in dog based on farm, sex, age class, reproduction status, health status and presence of ectoparasite sampled surrounding participating targeted cattle farm

		Dog	
	n	**Filovirus positive**	**Henipavirus positive**
Targeted Cattle Farm			
1	10	1	0
2	1	1	0
3	2	0	0
4	1	0	0
5	0	0	0
6	9	0	1
	23	2	1
Husbandry of dog			

Extensive	18	0	1
Tethered	5	2	0
	23	2	1
Sex			
Female	14	0	0
Male	9	2	1
	23	2	1
Age class			
Adult (>1y)	12	1	1
Subadult (<1y)	6	1	0
Juvenile (<1m)	5	0	0
	23	2	1
Reproduction status			
Pregnant	0	0	0
Lactating	1	0	0
Health status			
Apparently healthy	23	2	1
Sick	0	0	0
Ectoparasite			
No	10	2	0
Yes	13	0	1
	23	2	1

3.3.6 Bat

The serological evidence of filovirus and henipavirus in bat were 1.9%, 5/265 (95% CI 0.6-4.3) and 1.1%, 3/265 (95% CI 0.2-3.3) respectively. Majority of the filovirus positive (5/265) and henipavirus positive (3/265), bat sera were adult females that were apparently healthy (Table 3.18). Seropositivity was detected in 3 bat species (*C. brachyotis*, *E. spelaea* and *C. horsfieldii).* Further analysis with the variables were not performed due to insufficient or too small data.

Table 3. 18 Serological evidence of filovirus and henipavirus in bat based on sex, age class, reproduction status, health status and presence of ectoparasite surrounding participating targeted cattle farms

	Bat		
	n	**Filovirus positive**	**Henipavirus positive**
Sex			
Female	139	3	2
Male	126	2	1
	265	5	3
Age class			
Adult	242	5	3
Subadult	23	0	0
Juvenile	0	0	0
	265	5	3
Species			
Balionycteris maculata	3	0	0
Cynopterus brachyotis	108	1	1
Cynopterus horsfieldii	52	2	2
Eonycteris spelaea	70	2	0
Hipposideros galeritus	1	0	0
Macroglossus minimus	6	0	0
Macroglossus sobrinus	5	0	0
Nycteris tragata	1	0	0
Tylonycteris pachypus	19	0	0
	265	5	3
Reproduction status			
Pregnant	14	0	0
Lactating	20	0	0
Health status			
Apparently healthy	263	5	3
Sick	2	0	0
	265	5	3
Ectoparasite			
No	209	4	3
Yes	56	1	0
	265	5	3

3.1.1.1 Human

The serological evidence of filovirus and henipavirus in human were 9.1%, 5/55 (95% CI 3-20) and 7.3%, 4/55 (95% CI 2-17.6) respectively. Humans positive for filovirus (5/55) and henipavirus (4/55) were mostly males of Malay ethnicity, completed secondary level education and have resided in village for more than 10 years. No significant difference was observed between serological evidence of filovirus or henipavirus with the various factors (Table 3.19).

Table 3. 19 Serological evidence of filovirus and henipavirus in human based on demographics of participating farmers sampled from targeted cattle farms

	Human				
	n	**Filovirus positive**	**P-value**	**Henipavirus positive**	**P-value**
Targeted Cattle Farm			a		a
1	10	1		0	
2	9	0		1	
3	10	1		0	
4	10	2		2	
5	10	0		0	
6	6	1		1	
	55	5		4	
Husbandry of cattle farm			0.664		0.417
Intensive	6	1		1	
Semi-intensive	29	2		1	
Integrated	20	2		2	
	55	5		4	
Sex					
Male	45	3	0.220	3	0.563
Female	10	2		1	
	55	5		4	
Age range			0.069		0.291
10-29	16	1		2	
30-49	20	0		0	
50-69	19	4		2	
	55	5		4	
Years of residence in village			0.325		0.203
1 month - 1 year	1	0		0	
1-10 years	3	1		1	
>10 years	51	4		3	
	55			4	
Ethnic					

	Melayu	51	4	a	3	a
	Semai	1	0		0	
	Indian	1	0		0	
	Bugis	1	1		1	
	Indonesian	1	0		0	
		55	5		4	
Education level				0.433		0.406
	No education	3	0		0	
	Primary	16	0		0	
	Secondary	29	4		4	
	Tertiary	7	1		0	
		55	5		4	
Frequency of entering forest				0.205		0.900
	Never	8	2		1	
	Once a year	3	0		0	
	≥1 in a month	31	1		2	
	≥1 in a week	13	2		1	
		55	5		4	

[a]Variable is excluded from further analysis due to insufficient or too small data

Filovirus and henipavirus serologically positive humans reported exposure to cat, cattle, poultry, goat, rabbit, rodent, wild bird, bat and deer. One henipavirus positive human had exposure to non-human primates. No significant finding were found between serological evidence of filovirus and henipavirus and exposure to different animal species (Table 3.20).

Table 3. 20 Serological evidence of filovirus and henipavirus in human based on exposure to different species of domestic animals and wildlife in the past year

		Human, n=55					
No.	**Species**	**Exposure to animal species**	**%**	**Filovirus positive, n=5**	**P-value**	**Henipavirus positive, n=4**	**P-value**
					FE		**FE**
Domestic animal							
1	**Cattle**	50	90.9	4	0.391	3	0.325
2	**Cat**	49	89.1	5	a	4	a
3	**Poultry**	41	74.5	3	0.592	3	1.000
4	**Goat**	27	49.1	2	1.000	2	1.000
5	**Dog**	6	10.9	0	a	0	a
6	**Rabbit**	2	3.6	1	a	1	a
Wildlife							
1	**Rodent (rats/shrews/squirrel)**	34	61.8	5	a	4	a
2	**Wild bird**	19	34.5	2	1.000	2	0.602

3	**Non-human primates**	15	27.3	0	a	1	1.000
4	**Bat**	13	23.6	2	0.582	2	0.234
5	**Reptiles (snake, monitor lizard)**	9	16.4	0	a	0	a
6	**Deer**	7	12.7	1	0.508	1	0.429
7	**Porcupine**	6	10.9	0	a	0	a
8	**Mousedeer**	3	5.5	0	a	0	a
9	**Wild boar**	1	1.8	0	a	0	a
10	**Pangolin**	1	1.8	0	a	0	a
11	**Fox**	1	1.8	0	a	0	a
12	**Binturong**	1	1.8	0	a	0	a
13	**Bear**	1	1.8	0	a	0	a

[a]Variable is excluded from further analysis due to insufficient or too small data

A majority (26.7%, 4/15) filovirus seropositive humans have eaten food that were touched, disturbed or damaged by animals. These animals were 10 times more likely to be seropositive for filovirus compared to those who have not (P=0.017, OR=10.3, 95% CI=1.45, 73.24,). For henipavirus, there was no significant association between seropositivity to the different forms of exposure to various animal species. However, there is a meaningful increase of risk for consuming food that were touched, disturbed or damaged by animal (P=0.057) and eating raw or undercooked meat, offal or blood of wildlife (0.097) (Table 3.21).

Table 3. 21 Serological evidence of filovirus and henipavirus in human based on different forms of exposures to animals and wildlife in the past year

		Human, n=55					
	Forms of exposure	**Reported forms of exposure**	**%**	**Filovirus positive, n=5**	**Fischer Exact p-value**	**Henipavirus positive, n=4**	**Fischer Exact p-value**
General exposure to domesticated animals or wildlife							
1	**Handled live animals**	53	96.4	5	1.000	4	1.000
2	**Animal entered house**	49	89.1	5	1.000	4	1.000
3	**Lived with pet**	44	80	3	0.259	4	0.573

4	**Raised animals**	39	70.9	4	1.000	3	1.000
5	**Eaten food that were touched, disturbed or damaged by animal**	15	27.3	4	0.017*	3	0.057
6	**Share water source with animals**	14	25.5	0	a	0	a
7	**Scratched or bitten by animal**	13	23.6	1	a	2	0.234
8	**Seen feces in or near human food**	7	12.7	0	a	0	a
Specific exposure to wildlife							
1	**Cook or handle recently slaughtered meat, offal or blood of wildlife**	18	32.7	1	a	1	a
2	**Slaughter or butcher wildlife**	18	32.7	2	1.000	2	0.59
3	**Eaten raw or undercooked meat, offal or blood of wildlife**	8	14.5	1	a	2	0.097
4	**Hunt or trap wildlife**	6	10.9	0	a	0	a
5	**Eaten wildlife of unknown health status**	2	3.6	0	a	0	a
6	**Cut or injured oneself when butchering wildlife**	2	3.6	0	a	0	a
7	**Eaten or shared dead wildlife**	0	0	0	a	0	a
8	**Collect and sell dead wildlife**	0	0	0	a	0	a

[a]Variable is excluded from further analysis due to insufficient or too small data
*Humans that consumed food damaged by animal were more likely to be seropositive for filovirus (P=0.017, OR=10.3, 95% CI=1.45, 73.24).

Most farmers reported have had symptoms of Influenza-like illnesses (ILI) in the past one year. A few filoviruses and henipavirus seropositive farmers also reported other symptoms such as severe acute respiratory infection and fever with diarrhea and vomiting (Table 3.22). However, bivariate analysis between seropositivity and reported symptoms were not performed due to insufficient data for meaningful analysis.

Table 3. 22 Seroprevalence of filovirus and henipavirus in human based on symptoms experienced by farmers in participating cattle farms within the past year

		Human, n=55			
No.	**Symptoms**	**Individuals with reported symptoms**	**%**	**Filovirus positive, n=5**	**Henipavirus positive, n=4**
1	**Fever with headaches and lethargy**	3	5.5	0	0
2	**Haemorrhagic fever**	0	0	0	0
3	**Severe acute respiratory infection (SARI)**	3	5.5	1	0
4	**Influenza-like illnesses (ILI) symptoms**	10	18.2	0	1

5	**Fever with diarrhea and vomiting**	4	7.3	1[a]	1[a]
6	**Fever with skin rash**	1	1.8	0	0
	Total	21		2	2

*3/5 filovirus seropositive humans had no symptoms, 2/4 henipavirus seropositive humans had no symptoms. Two seropositive humans for both filovirus and henipavirus had no symptoms.
[a]The same individual

A comparison was made to determine if a particular exposure to animal species influenced reported ILI symptoms within the past year in farmers. ILI was most reported in farmers with exposure to cats and cattle, but no significant differences were observed (Table 3.23)

Table 3. 23 Influenza-like illnesses (ILI) symptoms and exposure to animals and wildlife species in human within the past year

		Human, n=55			
No.	**Species**	**Exposure to animal species**	**%**	**Influenza-like illnesses (ILI) symptoms, n=10**	**Fisher exact P-value**
Domestic animal					
1	**Cattle**	50	90.9	9	1.000
2	**Cat**	49	89.1	10	0.579
3	**Poultry**	41	74.5	6	0.255
4	**Goat**	27	49.1	6	0.340
5	**Dog**	6	10.9	1	1.000
Wildlife					
1	**Rodent (rats/shrews/squirrel)**	34	61.8	7	0.725
2	**Wild bird**	19	34.5	4	0.723
3	**Primate**	15	27.3	4	0.434
4	**Bat**	13	23.6	1	0.421
5	**Reptiles (snake, monitor lizard)**	9	16.4	3	0.340
6	**Deer**	7	12.7	2	0.599
7	**Porcupine**	6	10.9	1	0.702

*Only exposure to animal species with >2 reports were included in this comparison

ILI symptoms reported were also compared to forms of exposure to animals and wildlife within the past year of survey in farmers. All farmers that reported ILI symptoms have reported handling live animals and most of them lived with a pet animal. No significant findings were found between ILI and forms of exposure to animals and wildlife within the past year of survey (Table 3.24).

Table 3. 24 Influenza-like illnesses (ILI) symptoms in human based on forms of exposure to animals and wildlife within the past year

	Human				
	Forms of exposure	**n=55**	**%**	**Influenza-like illnesses (ILI) symptoms, n=10**	**Fisher exact P-value**
General exposure					
1	**Lived with pet**	44	80	8	1.000
2	**Handled live animals**	53	96.4	10	1.000
3	**Raised animals**	39	70.9	6	0.453
4	**Share water source with animals**	14	25.5	2	0.503
5	**Seen feces in or near food**	7	12.7	0	0.328
6	**Animal entered house**	49	89.1	9	1.000
7	**Eaten food that were touched, disturbed or damaged by animal**	15	27.3	2	0.710
Exposure to wildlife					
1	**Cook or handle recently slaughtered meat, offal or blood of wildlife**	18	32.7	3	1.000
2	**Eaten raw or undercooked meat, offal or blood of wildlife**	8	14.5	2	0.627
3	**Scratched or bitten by animal**	13	23.6	3	0.685
4	**Slaughter or butcher wildlife**	18	32.7	2	0.470
5	**Hunt or trap wildlife**	6	10.9	0	0.347

*Only exposure to animal species with >2 reports were included in this comparison

3.4 Discussion

In this study, domestic animals and wildlife in and around forest-fringe cattle farms were tested for exposure to the family filoviruses and the genus henipaviruses using Luminex bead-based multiplex microsphere assay. This technology has been developed and widely used for screening of viruses globally (Chowdhury et al., 2014; Dovih et al., 2019; Hayman et al., 2008, 2011; Laing et al., 2018; Peel et al., 2013b) but is not meant for diagnostic determinations. The findings can be used to target areas of surveillance for emerging pathogens but should not be used for policy decisions such as trade, quarantine or culling (MacNeil et al., 2011; Peel et al., 2013a). Serologically positive samples may indicate positivity to the tested virus or to antigenically related viruses. Antigenically related viruses share similar epitopes on its surface antigenic molecule that stimulates binding to antibodies or T cell receptor (Frank, 2020). Phylogenetically closely related viruses has higher epitope amino acid sequence similarity and binding to similar antibodies which result in cross-reactivity of antibodies (Brangel et al., 2018; Marsh et al., 2012; Schuh et al., 2019). Further testing and confirmation is necessary using virus neutralisation test (OIE, 2018).

Filovirus

Study found serological evidence for filoviruses is highest in goat (24/158, 15.2%), sheep (1/11, 9.1%), human (5/55, 9.1%), cattle (5/156, 5.1%) and lowest in bat (5/265, 1.9%). Seropositivity indicates evidence of past exposure to specific viruses

antigenically related to filoviruses. Studies on filoviruses in domestic animals has been scarce. Only dogs and pigs have been demonstrated to be infected with filoviruses (Allela et al., 2005; Chowdhury et al., 2014). In Gabon, 28% of dogs from Ebola epidemic areas were found to be IgG positive for Ebola virus (Allela et al., 2005). Meanwhile, Reston ebolavirus (RESTV) was identified in a spillover event between pigs and human in the Philippines and later in sick pigs in China (Barrette et al., 2009; Pan et al., 2014). To our knowledge, this study is the first to report evidence of exposure to filoviruses in cattle, goat and sheep. This is also the first evidence of exposure of filoviruses in human and dogs beyond Africa.

Highest seropositivity was reported among goats in this study particularly in semi-intensively reared goats which were 4 times more compared to cattle. The reasons for this may need further exploration however we suggest a few reasons for this findings: 1) Goat is known to browse extensively relative to other species (Hofmann, 1989; Rutagwenda et al., 1989) and have the habit of sampling wide ranges of items (edible and non-edible), therefore may increase their risk of exposure to multiple disease agents; 2) Many of the seropositive goats reported in this study were managed semi-intensively to browse freely around village and raised in close proximity where flowering and fruiting trees are abundant which attracts bats such as *C. brachyotis* (V. C. Lim et al., 2017; Tan et al., 1998). Goats are very likely to pick up partially eaten fruits consistent with their feeding behaviour therefore increases risk of contracting multiple disease agents.

Seropositivity of bats in this study is lower than previous reports of bats in this region. Local *P. hypomelanus* reported 10% and 5% reactivity to filoviruses closely related to EBOV and SUDV respectively (Laing et al., 2019). Study from neighbouring country, Singapore reported 9.1% (17/186) in *E. spelaea*, 8.5% (13/153) in *C. brachyotis* and 4.3% (3/70) in *P. lucasi*. Besides *E. spelaea* and *C. brachyotis*, *C. horsfieldii* was seropositive for filoviruses in our study. *Cynopterus sp*. and *E. spelaea* are adaptive species that occupy a range of habitats from orchards, plantations, primary and secondary forests and urban sites (Campbell et al., 2004; Fukuda et al., 2009; Heidenan & Heaney, 1989; Kitchener et al., 1990; V. Lim, 2018; Tan et al., 1998). Livestock farming in rural and sub-rural areas where fruit trees are plenty provides bat roosting and foraging areas (Fukuda et al., 2009). This overlapping spatial distribution of bats and livestock increase risk of viral spillover to livestock and agricultural workers (Mikail et al., 2017). Longitudinal spatial-temporal distribution studies will ascertain the level of such risk (Zhao et al., 2022).

Antibody reactivity to specific filovirus antigen in our study suggests that circulating filovirus in this region is predominantly more closely related to BDBV, SUDV, EBOV, RESTV and less so with BOMV, MARV, and RAVV. Reactivity to filoviruses closely related to EBOV, BDBV and SUDV corresponds to a similar study in Singapore bats (Laing et al., 2018). However, in contrast to that study, we report a considerably high number of reactivity to antigenically related but not identical to RESTV-pig in a *C. brachyotis* and many goats. RESTV is an zoonotic Asiatic filovirus responsible for sporadic cases in non-

human primates, pigs and human (Barrette et al., 2009; CDC, 1993; Demetria et al., 2018; Miranda et al., 1999). Bats may be natural reservoirs for RESTV in the Philippines, Bangladesh and China (Jayme et al., 2015; Olival et al., 2013; Taniguchi et al., 2011; Yang et al., 2017). Finding of our study suggests direct transmission of RESTV-related viruses from bats to goat. Detection of RESTV genome is necessary to confirm spillover.

Henipavirus

Seropositivity to henipaviruses was highest in goats (13.9%), sheep (9.1%), human (7.3%), dog (4.3%), cattle (3.2%) and bat (1.1%). Seropositivity indicates evidence of past exposure to viruses antigenically related to henipaviruses. Our result is supported by several studies. Surveillance of NiV in human outbreak areas near Pteropus sp. bat roosts in Bangladesh using the Luminex platform found that 6.5% (26/400) of cattle and 4.3% (17/400) of goats were positive for henipavirus (Chowdhury et al., 2014). During the Nipah virus outbreak in Malaysia, dogs sampled near infected pig farms had detectable antibodies for Nipah virus (Mills et al., 2009; Yob, Field, et al., 2001). Since 2001, NiV have not been reported in domestic animals and wildlife locally using ELISA (Fikri et al., 2011; Naama et al., 2013). This study is the first report that uses Luminex technology of henipavirus infection in dogs since 1999 and in local cattle, goat and sheep. Reported seropositivity of henipaviruses in goats was high at 13.9% (22/158) particularly in semi-intensively reared goats and was 4 times more as compared to cattle 3.2% (5/156) for hypothetical reasons of risk of exposure to infectious materials as explained before.

Previous survey of bats in Malaysia were mostly focused in the *P. vampyrus* and *P. hypomelanus* species as reservoirs for Hendra virus and Nipah virus with a reported seroprevalence of 1-33% (Rahman et al., 2013; Yob, Jamaluddin, et al., 2001). Those studies used indirect ELISA and serum neutralization test to detect the antibodies against Nipah virus in samples. A recent survey using multiplex serological assay in the local *P. hypomelanus* bats samples found 25% reactivity to NiV (Laing et al., 2019). The reported seroprevalence in our study of 1% (2/108) in *C. brachyotis* and *C. horsfieldii* 2% (1/52) is much less than that of Laing et al. (2109) *Pteropus sp*. surveys and are comparable to the seroprevalence of 4% (2/56) in *C. brachyotis* and 5% (2/38) in *E. spelaea* in a separate study in 1999 (Yob, Field, et al., 2001).

Antibody reactivity to specific henipavirus antigen in our study suggests that circulating henipavirus in this region is predominantly more closely related to GhV, HeV, NiV and less so to CedV and MojV. The MFI values for GhV is high and many cross-reacted with other henipaviruses. Presently, little is known about GhV which was first detected in African bats and is the only African henipavirus (Drexler et al., 2009, 2012). GhV is also antigenically closer to ancestral henipaviruses (Pernet et al., 2014). Thus, reaction to GhV in our study indicates prevalence of henipavirus more antigenically related to the ancestral GhV than the other Asiatic viruses.

Interestingly, our study found two reactions only to MojV with no cross-reactivity in a cattle and dog from the same intensive cattle farm. MojV is a rodent henipavirus discovered in anal specimens of *Rattus flavipectus* in Southern China. This novel virus was identified in the same cave 6 months later where three patients died of respiratory illness of unidentified aetiology. MojV pathogenicity remains to be determined due to the absence of isolate (Wu et al., 2014). Cattle and dog may have acquired MojV closely related virus through contact with rodent excrement. Rodents are probable reservoir hosts or amplifying host for MojV (Cheliout et al., 2021). Increasing the sample size of rodents to detect antibodies and genome of MojV or MojV-like virus will improve chances of detection and determine its circulation locally.

Risk Factors

We detected exposure against viruses antigenically related to filoviruses, EBOV, BDVD and RAVV; and henipaviruses HeV, CedV, MojV in humans. Our study found those that consumed food that were touched, disturbed or damaged by animal were more 10 times more likely to be seropositive for filovirus. This is supported by the study by Adjemian et al., amongst febrile tribesmen presented to healthcare facilities in Uganda. Increased risk of filovirus infection was observed in activities such as hunting (37.5 times), touching (5.6 times) or eating wildlife (10.7 times) (Adjemian et al., 2011). Likewise, bushmeat slaughtering or working closely with infected pigs or horse enhanced risk of henipavirus infection (Chua, 2010; Pernet et al., 2014; Rogers et al., 1996). The finding is expected because the both viruses are transmitted via direct

contact with body fluids (Amal et al., 2000; Bausch et al., 2007; O'Sullivan et al., 1997; Saéz et al., 2015; Williamson et al., 1998). Therefore, poor hygiene practices or improper personal protective equipment may expose farmers to infectious materials, especially when handling sick or infected animals, animal waste or carcasses.

Approximately half of the humans sampled (21/55) reported Influenza-like illnesses (ILI), fever with diarrhoea and vomiting, fever with headaches and lethargy and severe acute respiratory infection (SARI) symptoms within the past year of survey. Half of the seropositive human for filovirus and/or henipavirus in our study had reported one of the symptoms in the past year, while the other half did not. The symptoms can be due to several multitude of reasons, unfortunately causal relationship between viral infection and symptoms in our study cannot be ascertain without clinical and longitudinal data. Syndromic surveillance incorporating important pathogens at the family or genus level will improve early detection and appropriate intervention.

The catastrophic Nipah outbreak in 1998 is a turning point for cross-sectoral ministerial integration in managing zoonotic disease and recognition that the country is susceptible to spillover of pathogenic viruses (Chua, 2003). Unfortunately, at present, novel or emerging viral surveillance in animal population is sporadic and scanty; usually linked to specific research endeavour. The ecological concerns and agricultural intensification in Malaysia strongly indicate the need for regular monitoring and

surveillance of livestock and humans working in close proximity to animals especially those that are located in geographic areas with high potential for wildlife viral spillover events. A better understanding on the interaction and viral transmission between human, livestock, other domestic animals and wildlife will inform disease surveillance and control policy to reduce the risk of transmission and prevent undesirable animal and public health consequences.

Limitations

This study was performed on highly targeted populations to increase the chance of viral detection in high-risk populations at the wildlife-livestock-human interface. Therefore, the findings should be interpreted in light of the sampling bias, limited sample size, non-random sampling, cross-reactions between antigenically related viruses and the diversity of uncharacterized filoviruses and henipaviruses that is missed from the test panel. Further characterization of filoviruses and henipaviruses in the region, larger data set, improved diagnostic assays and species-specific positive controls will influence cut-off values and reported sero-detection.

Additionally, infection status in bats fluctuates seasonally and are influenced by reproduction phases, nutritional stress, migrations or contacts with infected animals (Amman et al., 2012; Baker et al., 2014; Hayman, 2015; Plowright et al., 2008; Sohayati et al., 2011; Wang et al., 2013). This infection pattern in bats as a reservoir host would

subsequently impact degree of spillover to other potential hosts like cattle, goats or humans in our case (Amman et al., 2012; Plowright et al., 2017). Antibody post-infection also wax and wanes over time (Boardman et al., 2020; Diallo et al., 2021). Thus, the sero-detection reported in this cross-sectional study only provides information at the time of sampling which may be an underestimation or overestimation and will differ depending on time of sampling.

In addition, maternal antibodies to NiV can last up to 14 months, hence without longitudinal data, some reactivity in our study may be misrepresented (Sohayati et al., 2011). As a result of lack of longitudinal data, serological findings only demonstrate past exposure to filoviruses and henipaviruses. Despite these limitations, our study generated valuable information of filoviruses and henipaviruses in the multiple animal host species to inform future studies. Besides that, research on viral diversity characterization in this region will improve the depth of interpreting serological results.

3.5 Conclusion

This study provides the first evidence of exposure to viruses antigenically related to filoviruses and henipaviruses in domestic animals and humans in Malaysia, as well as viruses antigenically related to henipaviruses in previously unreported bat species. Results from our study supports more extensive and longitudinal viral surveillance in these populations to enhance disease preparedness and improve our understanding on

the viral distribution, diversity and potential spillover at the human-livestock-wildlife interface.

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Chapter 3

FILOVIRUSES AND HENIPAVIRUSES AT THE HUMAN-LIVESTOCK-WILDLIFE INTERFACE IN FOREST FRINGE CATTLE FARMS IN SELECTED STATES OF PENINSULAR MALAYSIA

3.1 Introduction

Filoviruses and henipaviruses are high consequence emerging zoonotic pathogens that have caused severe outbreaks in many parts of the world leading to high mortality and morbidity and grave economic losses in both human and animal health sectors (Knust et al., 2017; Looi & Chua, 2007). The economic losses due to Ebola has been estimated to be USD2.8 billion in the 2014 Ebola outbreak and USD446 million in 1998 Nipah outbreak (FAO and APHCA, 2002; World Bank, 2016).

The viruses within the family Filoviridae; Ebola (EBOV) and Marburg virus (MARV) cause haemorrhagic fever outbreaks in Africa, Germany and isolated cases in other parts of the world like Spain, United States of America, United Kingdom, Philippines, Italy, and Russia (CDC, 2018; C. E. Smith et al., 1967; WHO, 2014). The mortality from Ebola disease in Africa was 50-90% in humans and almost 100% mortality in non-human primates (Bowen et al., 1978; Jaax et al., 1996; Jahrling et al., 1996; K. M. Johnson, 1978; Simpson, 1978). While *Henipavirus*); Hendra virus (HeV) and Nipah virus (NiV) have cause respiratory and neurological signs in humans, pigs and horses in

Malaysia, Australia, Bangladesh and India (Chadha et al., 2006; Chua et al., 1999; Hsu et al., 2004; Nor, Gan, Ong, et al., 2000; O'Sullivan et al., 1997). The mortality from Nipah virus infection was 40-70% in humans in Malaysia and Bangladesh; and of 1-5% in adult pigs and 40% in suckling pigs in Malaysia (Chua et al., 2000; Goh et al., 2000; Hsu et al., 2004; Luby et al., 2009; Nor, Gan, & Ong, 2000).

With an exception of Nipah virus which emerged in 1998 (Fikri et al., 2011; Laing et al., 2019; Rahman et al., 2013; Sohayati et al., 2011), limited information is available on both filoviruses and henipaviruses in Malaysia. The investigative work on Nipah has led to numerous works on bat ecology and bat-borne microbial agents. It is now understood that bats have co-evolved with multiple viruses that may be of public health significance such a Nipah (Hayman, 2016). Filoviruses have been detected in bats in Singapore, Bangladesh, China, and the Philippines (Laing et al., 2018; Olival et al., 2013; Taniguchi et al., 2011; Yuan et al., 2012). While henipaviruses widely circulate in bats in Malaysia, India, Vietnam, Cambodia, Indonesia and Australia (Breed et al., 2013; Epstein et al., 2008; Hasebe et al., 2012; Rahman et al., 2013; Reynes et al., 2005; Sendow et al., 2006). Besides bats, ffiloviruses' and henipaviruses can infect a broad range of mammals such as human primates, rodents, shrews, guinea pigs, bats, dogs, pigs, cattle, donkey, chicken; and rodents, horse, cats, pigs, dogs, goats respectively (Chua et al., 2000; Edson et al., 2015; Gonzalez et al., 2005; Leroy et al., 2004; Morvan et al., 1999; Wu et al., 2014).

Despite the apparently wide distribution and reported prevalence of these pathogens amongst the reservoir hosts in Southeast Asia, outbreak has been rare in both humans and domestic animals. Following the Nipah outbreak in 1998-1999 in Malaysia, no further cases have been reported in both humans and animals to date (Fikri et al., 2011; Muniandy et al., 2001; Naama et al., 2013; Sohayati et al., 2016). There has also been no human outbreaks in Cambodia despite serological and molecular evidence widely found in Cambodian bats (Olson et al., 2002; Reynes et al., 2005)s. However, the SEA region is considered a hotspot for emerging pathogens because several localized emerging zoonosis have occurred in the past two decades and because multiple factors that drives emerging infectious diseases are highly prevalent. For example, wide circulation and abundance of reservoir hosts, topographical or landscape changes and intensified systems of agriculture and animal production may increase contact opportunities for viral transmission at the wildlife-livestock-human interfaces (Breed et al., 2013; Horby et al., 2013; Jung & Threlfall, 2016; I. Smith & Wang, 2013). In additon,the global climatological changes may impact distribution and concentration of disease vectors which subsequently facilitates spatiotemporal changes of filovirus and henipavirus viral spillover events (Martin et al., 2018; McFarlane et al., 2011; Schmidt et al., 2017).

The one health approach of managing complex emerging diseases requires the understanding of all actors that take part at the environment-animal-human interface. For example, food animals may become infected with pathogens from wildlife that can

then be transmitted to human such as Nipah, Highly Pathogenic Avian Influenza H5N1, Pandemic Influenza H1N1 (2009) amongst others. Many livestock farms in this region are located in the rural or peri-urban areas bordering primary or secondary forests. In Malaysia, livestock especially cattle are commonly managed extensively or semi-intensively at forest fringes thus may have higher probability of contact with infectious materials from infected wildlife or domestic animals. Beef cattle are most commonly raised using the aforementioned systems where animals are let out to graze in a field, plantations or in a village located at forest fringes where wildlife and peri-domestic animals are plentiful. Such setting improves the chance and frequency for pathogen spillover events to livestock or humans (Hassell et al., 2017). The spillover occurrences varies given available drivers and rarely result in an epidemic, however frequent spillover events will increase the chance of an outbreak (Clayton et al., 2013; Hayman et al., 2011; Parrish et al., 2008; Plowright et al., 2017).

In this chapter, we targeted cattle farms located at forest fringe areas with high contact risk and probability of pathogen spillover events. We hypothesise that cattle, other domestic animals, wild animals and farmers are seropositive for filoviruses and henipaviruses. The objectives of this study are: 1) To determine the serological evidence of filoviruses and/or henipaviruses in cattle, farmers, and other domestic animals and wildlife in and at close proximity to the targeted cattle farm; 2) To determine risk factors of virus infection in those species.

3.2 Materials and Methods

3.2.1 Ethics statement

All human and animal samples from cattle farms were collected in accordance to an established USAID Emerging Pandemic Threats PREDICT Operating Procedures with permission from the local institutional and ministerial Institutional Animal Care and Use Committee from UPM (AUP-R064/2018), UC Davis (#19300) and Tufts (G2021-131) and the Institutional Review Board from the Medical Research and Ethics committee (MERC) from National Medical Research Register (NMRR), Malaysia (NMRR-17-3356-37135, IIR) and UC Davis (#804522) in collaboration with Ministry of Health (MOH), Malaysia, Department of Veterinary Services (DVS), Malaysia, and the Department of Wildlife and National Parks (PERHILITAN). The work performed for this project is part of a larger project in collaboration with the EcoHealth Alliance.

3.2.2 Study design and study sites

A cross-sectional survey was performed in 3 semi-intensive, 2 integrated and 1 intensive cattle farm. These farms were selected based on a defined selection criteria which include, 1) located in districts with existing NMRR and IRB approval for human study, 2) located 5km from periphery of forests as approved by the Department of Veterinary Services, Putrajaya, 3) a minimum of 30 cattle in farm, and 4) has other domestic animals and wildlife in or around farm. The approved districts were Kuala Kangsar in the state of Perak, Gua Musang in Kelantan and Kuala Lipis in Pahang. In

these districts, the district health officers had worked with EcoHealth Alliance on previous projects and have agreed to assist the human sampling aspects in this project.

The identification of the cattle farms consisted of several steps as in Figure 3.1. The details of the steps in identifying location and farms are further described in Appendix X. All animals sampled in this study were within 5km radius of a primary forest.

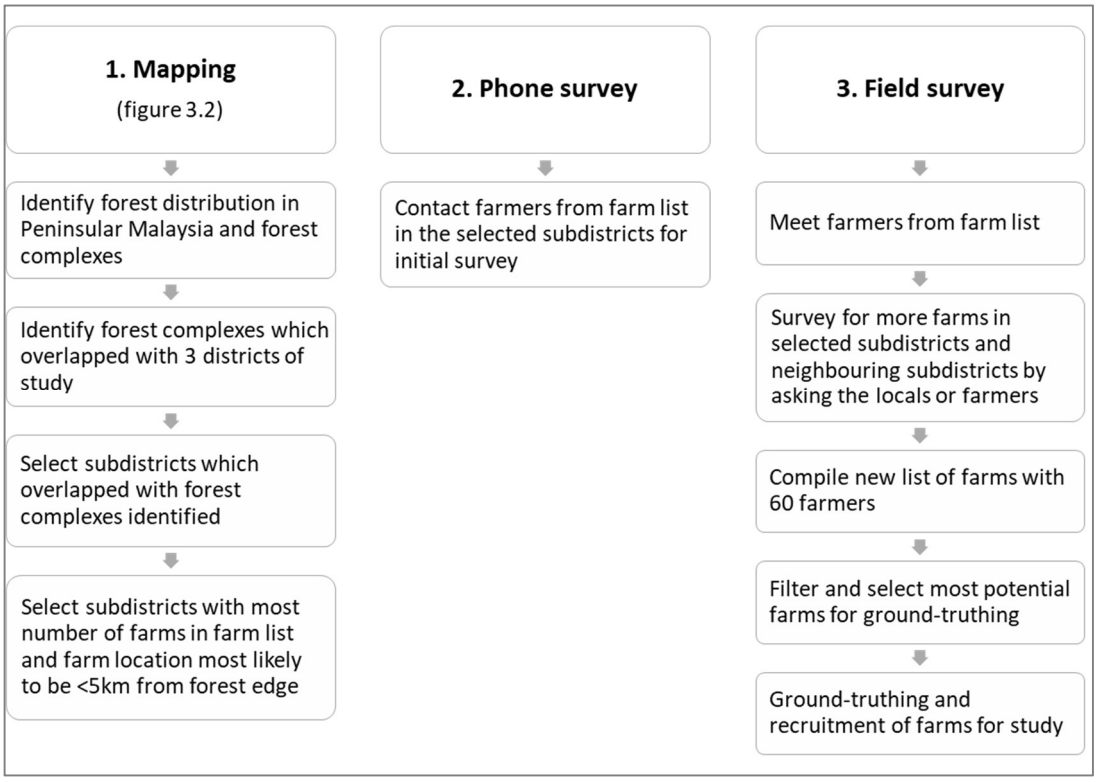


Figure 3. 1 Identification of forest-fringed cattle farm for the study on filovirus and henipavirus at the wildlife-livestock-human interface

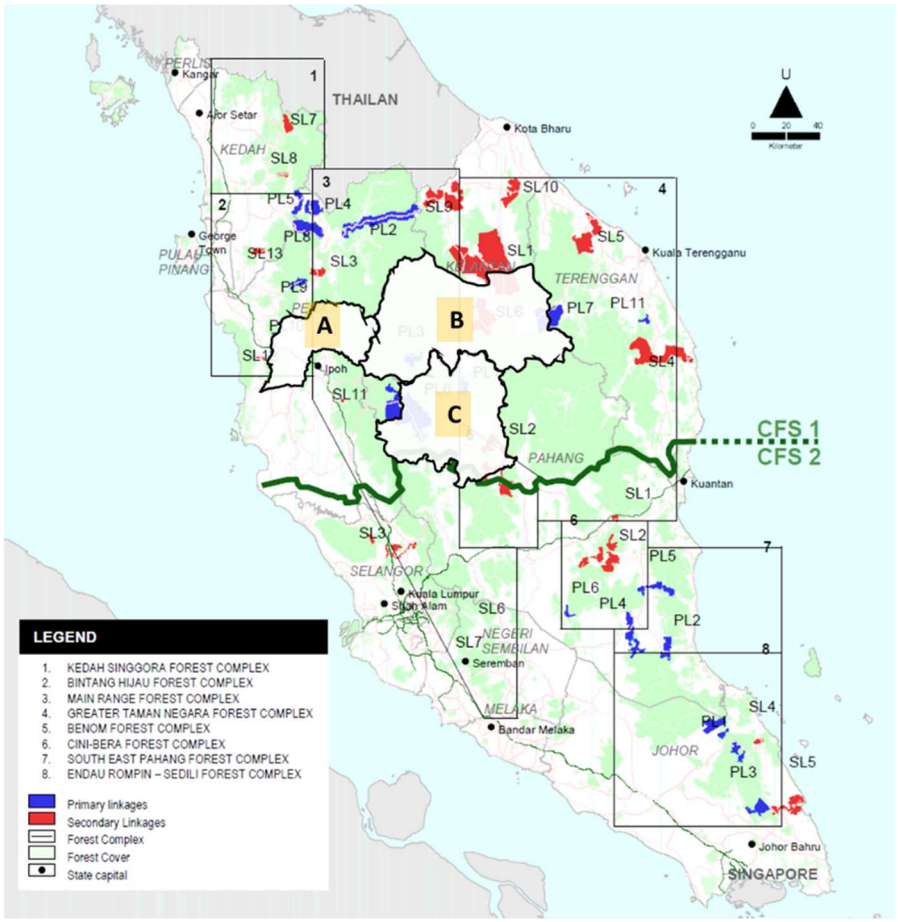


Figure 3. 2 Map of forest complexes in Peninsular Malaysia and the overlapping targeted districts in our study marked by A: Kuala Kangsar, B: Gua Musang, C: Lipis. (Source: Regional Planning Division, Department of Town and Country Planning, Peninsular Malaysia, 2009)

3.2.3 Sample size

Four cattle farms from Kuala Kangsar district, Perak and two cattle farms from Gua Musang district, Kelantan within 5km radius of forests were sampled beginning 2019.

Targeted sample size for each animal species per farm were 30 cattle from participating target farms, 30 other domestic animals (eg, goat, sheep, dog, etc) and 60 wildlife (eg, bats and rodents). The sample size (n) for this study (Table 3.1) was determined using

the Open-Epi Version 3 calculator. The software program applies the formula of n = [DEFF*Np (1-p)]/[(d2/Z21-□/2 *(N-1)+p*(1-p)], where N = population size, p = prevalence, d = confidence limits = 10%, and DEFF = design effect for cluster surveys = 1. The estimated prevalence used in the current study was 5% based on prevalence studies of filoviruses and henipaviruses in the region which reported seroprevalence of 0-6.5% in domestic animals and 1-20% in bats (Table). Other domestic animals and wildlife were opportunistically captured and sampled with priority given to animals in and closest to the participating target farms.

Table 3. 1 Sample size calculation for the sero-detection of filovirus and henipavirus in cattle, other domestic animals and wildlife in participating cattle farms located at the forest fringe in Perak and Kelantan, Malaysia

	Estimated Population Size, N per farm	Estimated Prevalence, p (% +/-5)[a]	Sample size, n per Farm	No. of farms	Total
Cattle : (Semi-intensive (n=3), Integrated oil palm (n=2), Intensive farm(n=1)	50	5	30	6	180
Other domestic animals : Goat, sheep, dog, etc.	50	5	30	6	180
Wildlife : Bat, rodent, other small mammal	1249 per roost for bats	5	70 (max of 60)[c]	6	360

[a]Prevalence of 5% was decided based on prevalence studies of filoviruses and henipaviruses in the region (see Table ???).

[b]Estimated population size for bats is based on the average roost size of bats. Total number of bats calculated is an overestimation as it will be highly dependent on the bats captured in nets and number of days the nets are placed in a location.

[c]Total number of wildlife are set to the maximum of 60 due to logistical and financial limitations.

3.2.4 Sampling of cattle and other domestic animals

Cattle and other domestic animals such as goat, sheep, dogs and rabbit were conducted with the team from the EcoHealth Alliance in accordance to USAID Emerging Pandemic Threats PREDICT Operating Procedures (PREDICT One Health Consortium, 2016b, 2016e).

All domestic animals were manually restraint for the sampling procedures (Appendix B). Blood samples were obtained from the appropriate venepuncture sites according to species. In cattle, goat, and sheep, the jugular vein or coccygeal vein was used (Figure 3.3). In dogs, blood was collected from the cephalic vein, jugular vein, femoral vein, lateral or medial saphenous vein. In rabbits, blood was collected from the lateral saphenous vein. Three to five ml of blood were taken from these animals and distributed equally in a BD Vacutainer® serum tube and EDTA tube.

A basic physical examination was performed by veterinarian to assess the health status and identify the age and sex of the animal. Photographs of sampled animal was taken from the sides and front, including dentition, or presence of any lesions, abnormalities or identifying marks or characteristics for future reference.

All blood tubes were labelled appropriately and placed in cooler boxes and transported back to sampling processing base. Blood in BD Vacutainer® serum tubes were left to

clot for serum collection within 1 hour. After clot is form, serum tubes were centrifuged at 1300 r.p.m for 15 minutes and 1 ml of serum was transferred into 0.5ml cryovial tubes to produce at least two aliquots. Cryovial tubes were labelled appropriately. Samples were then placed in MVE Doble 34 liquid nitrogen tanks in the field and later stored at -80° freezer in the lab according to proper cold chain methods.

Figure 3. 3 Locating the jugular vein in the jugular groove for blood sampling

3.2.5 Sampling of wildlife

Permission was obtained from the local authorities or landowners before bat and rodent trapping and sampling begun. Selected sites were first surveyed with priorities given to potential trapping locations in the farm and nearest to farm within 5 km radius. All personnel handling trapping and sampling of wildlife had to be immunized

against rabies, Japanese Encephalitis, Hepatitis B, tetanus and negative sputum tests for Tuberculosis.

3.2.5.1 Sampling of bat

Bats were captured using mist nets suitable for capturing bats in open spaces as described by FAO Manual for Investigating the Role of Bats in Emerging Zoonoses (Food and Agriculture Organisation of the United Nations, 2011). Further description on the sampling procedure is in Appendix X. Briefly, bats were captured using mist nets placed at anticipated bat flyways in the farm, village and in nearby plantations such as across streams or trails. The nets were also positioned near roosting or feeding sites such as near flowering banana trees and flowering durian trees (Figure 3.4).

Figure 3. 4 A bat trapping site with banana and durian plantation in a village near a participating cattle farm.

The mist nets were raised at 7.30pm at sunset and left opened until 12am for trapping to occur (Figure 3.5) and closed neatly after trapping period (Figure 3.6). Bat nets were repositioned to new locations when recapture rates were high, or captures were absent. The GPS coordinates for each mist nets were recorded.

Figure 3. 5 Extended mist nets for capture of bats at sunset.

Figure 3. 6 Retracted mist nets that is secured after trapping period

Mist nets were checked for bats every 30 mins to an hour. Captured bats were extracted from the nets carefully using at least one leather gloves on one hand and with the use of forceps or small stick to move the netting or bat mandibles then placed into a porous cotton bag with a draw-string mouth and taken to a sampling processing site where sampling was performed immediately (Figure 3.7). Approximately 20-30 bats were sampled in a night. No bats should be kept for more than 6 hours.

Figure 3. 7 Bags containing bats hung in draw-string cloth bags on a raffia string between two pillars

Bats were sampled and identified according to the USAID Emerging Pandemic Threats PREDICT Operating Procedures with some modifications (Hoffmann et al., 2010; PREDICT One Health Consortium, 2016a; C. Smith et al., 2010). Bat was removed from the cloth bag and manually restrained for sampling using leather gloves. In bats that were >100g, a non-heparinised syringe was used to collect blood from the cephalic vein, brachial vein or saphenous vein. Blood was then placed in a serum vacutainer tube containing serum-clotting factor and centrifuge. Extracted serum was placed into 2 cryovials of a minimum of 60µl if volume. If bats were <100g, the tip of a sterile 25G needle was used to puncture the brachial or propatagial vein (C. Smith et al., 2010).

A 1:10 serum dilution (10µl blood into 90µl PBS) was centrifuged, and the supernatant was transferred into a new cryovial tube. Cryovial tubes was labelled appropriately and placed in MVE Doble 34 liquid nitrogen tanks in the field and later stored at -80° freezer in the lab according to proper cold chain methods.

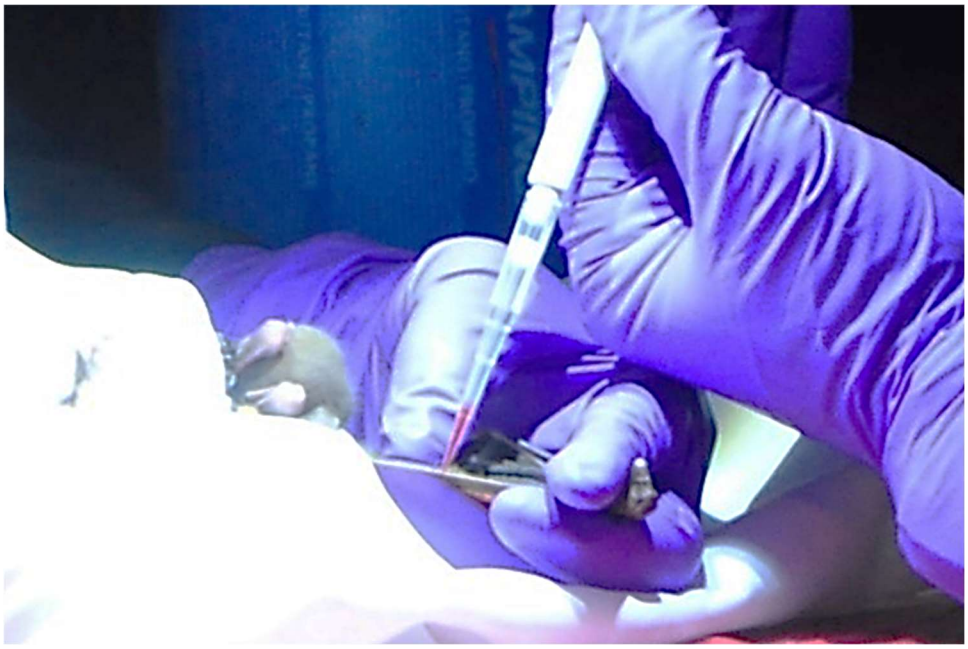


Figure 3. 8 Blood sampling from the cephalic vein of a bat by aspirating the drop of blood using a pipette.

Morphometric measurements and photographs of each bat were taken during the sampling procedure for the purpose of species identification. Identification of bat species was performed by an experienced rangers and field veterinarian based on the morphology and biometric measurements. Guide books were used for identification of bats on the field (Francis, 2008; Kingston et al., 2006; Payne et al., 1985; Phillipps & Phillipps, 2016).

Age of bats were classified according to adult, subadult and juvenile based on sexual characteristics and size. Males with descended testis and relative body size were considered adults while those with unfused epiphyses and undescended testis were considered subadults. Female bats with prominent nipples, were pregnant or lactating, relative body size and complete fusion of the phalangeal symphysis are classified as adult while those that were fully grown with unfused epiphyses were considered subadults. Pregnancy status was identified by gently palpating the abdomen and lactating status were identified by attempting to express milk from the teats. Bats that were small, clinging to dam and suckling were considered juveniles.

Typically, sampling per bat took less than 15 minutes with one person handling and performing the morphometric measurements, while the other person focused on the sampling. Bats nails were painted with a red nail polish as identification in case of recapture. Frugivorous or nectivorous bats were fed with approximately 1ml of 100% fruit juice or honey water prior to release. Bats were released after sampling.

3.2.5.2 Sampling of rodent and other small mammal

Rodents and other small mammals were trapped and sampled according to the USAID Emerging Pandemic Threats PREDICT Operating Procedures with some modifications (Aplin et al., 2003; Hoffmann et al., 2010; Mills et al., 1995; PREDICT One Health Consortium, 2016d, 2016e). Further description on the sampling technique is in

Appendix X. A total of 100 traps were used to trap rodents which encompasses 45 Sherman traps, 45 small Tomahawk for smaller mammals such as mice, rats, tree shrews and 10 large Tomahawk traps for larger mammals, such as civet (Figure 3.9).

Figure 3. 9 Sorting of 100 rodent traps according to transect lines before deployment

Sites for traps were first surveyed and divided between inside farm or nearby plantation or forest. Transect lines were used for placement of traps as opposed to grid to reduce overlapping and increase sampling area coverage and increase chance of capturing more species through coverage of small mammal home ranges and microhabitats (Pearson et al., 2003).

Traps containing appropriate baits were placed at strategic pathways for rodents (Figure 3.10). Oil palm fruit was the preferred option as it does not attract insects. Traps were placed at the same sites for a minimum of 3 consecutive nights from 6pm to 8am the next day. If adverse weather such as heavy rain is expected, traps will not be opened for that night.

Figure 3. 10 Placement of rodent trap by the fences in a farm

Figure 3. 11 Roasting of oil palm fruits that were used as baits for rodents

Trapped small mammal were gently transferred into a draw-string cloth bag by covering the bag over the opening of the trap and slightly tilting the trap downwards. Animals that were too stressed will be released, while animals found to be injured or weak will be brought back to sampling base for veterinary treatment.

Smaller rodents were removed from the bag gently and placed in a modified container for anaesthetic induction. Whereas, in small mammal species like civets, a gas mask was used for induction without completely removing animal out from the bag to reduce stress (Figure 3.12). Once animal is motionless and unresponsive, it is taken out and placed on the table with an appropriate fitting face mask for continue induction or

maintenance of anaesthesia. Monitoring of animal was performed by a veterinarian throughout during procedure along with a basic physical examination to access health status.

Figure 3. 12 Induction of general anesthesia using isoflurane in a small Indian civet (*Viverricula indica*)

Blood samples were collected from the lateral tail vein, ventral tail artery or lateral saphenous vein or lateral saphenous vein in rodents and other small mammals (Hem et al., 1998; Lee & Goosens, 2015; Mills et al., 1995; Office of the University Veterinarian, 2017a; PREDICT One Health Consortium, 2016d). In smaller rodents, approximately 1ml of blood was collected, and in larger small mammals (~200g), 3-5ml of blood was collected. Blood collected was placed into 1:10 PBS and spin to collect serum. Subsequent steps in processing and storage are as mentioned in 3.2.5.1.

After blood collection, fluid volume replacement was given subcutaneously using Lactated Ringer's or 0.9% NaCL solution to avoid hypovolemic shock. Marking on sampled rodent or small mammal is done by clipping 3-5mm of hair at the right ventral thigh. Morphometric measurements and photographs of each animal were taken during the sampling procedure for the purpose of species identification.

Age was classified into adult, subadult and juvenile based on secondary sexual characteristics and size. Adults are fully grown with adult pelage and is sexually mature. Subadults are not fully grown with developing pelage and may or may not be sexually mature. Juveniles are smaller than subadults and not sexually mature with grey and soft pelage (Hoffmann et al., 2010). Animals were monitored in a small cage until fully recovered from anaesthesia before release at place of capture.

3.2.5.3 Sampling of Wild boar

Wild boar were sampled according to established protocols (Office of the University Veterinarian, 2017b, 2017c; PREDICT One Health Consortium, 2016b). Further description on the sampling technique is in Appendix X. Wild boar was trapped according to trapping protocol described for rodent and other small mammals. It is transported to sampling base in the cage for sampling (Figure 3.13).

Figure 3. 13 A subadult wild boar in a Tomahawk trap from an oil palm plantation

Wild boar is sedated with by creating an anaesthetic chamber for induction and maintained with a gas mask. Monitoring of anaesthesia was performed by a veterinarian throughout during procedure. Blood samples were collected from the anterior vena cava and is distributed equally in a BD Vacutainer® serum tube and an EDTA tube. Processing of blood for serum extraction and storage were similar to previously mentioned in 3.2.4. After blood collection, fluid volume replacement was given subcutaneously using Lactated Ringer's or 0.9% NaCL solution. A basic physical examination was performed by veterinarian to access health status. Morphometric measurements and photographs of each animal were taken during the sampling procedure for the purpose of species identification.

Wild boar was classified into adult, subadult and juvenile based on secondary sexual characteristics and size. Adults are fully grown with adult pelage and is sexually mature. Subadults are not fully grown with developing pelage and may or may not be sexually mature. Juveniles are smaller than subadults and not sexually mature with grey and soft pelage (Hoffmann et al., 2010).

3.2.5.4 Sampling of Non-human primate

Non-human primates (NHP) were kept as pets hence trapping was not required. NHP were anaesthetized and sampled upon the agreement of its owner according to established protocols (Hoffmann et al., 2010; PREDICT One Health Consortium, 2016c). Further description on the sampling technique is in Appendix X. Briefly, aesthesia drug used to sedate animals were the combination of: 1) 4mg/kg Ketamine and 0.15mg/kg Xylazine with Yohimbine 1:1 as reversal agent for Xylazine, or 2) Tiletamine-Zolazepam, Zoletil® 3-5mg/kg in more aggressive primates. A dart syringe and blowpipe were used to deliver anaesthetic drug intra-muscularly by an experienced and trained ranger. Anaesthetic monitoring was performed by a veterinarian. After immobilization, basic physical examination was performed. A sterile lubricant is placed into animal's eyes to prevent corneal drying or scratches during sedation.

NHP is placed in a supine position. Blood is withdrawn from femoral vein located in the inguinal region lateral and parallel to the femoral artery (Figure 3.14). Three to five ml

of blood were taken from these animals and distributed equally in a BD Vacutainer® serum tube and EDTA tube. Processing of blood for serum extraction and storage are similar to previously mentioned in 3.2.4.

Figure 3. 14 Sampling of an anaesthetized pig-tailed macaque (*Macaca nemestrina*)

Photographs of the anterior and ventral view of the full body, anterior and lateral face, upper and lower dentition and genitalia were taken for future reference. Age was classified to 7 age class according to the PREDICT Operating Procedures for Non-Human Primates. Neonates are animals that show signs of being born within few days; infants are young that were still suckling and clinging on to mother; juveniles are mostly independent from mother, sexually immature and have not developed into adult size; immature primates are individual that have not sexually mature, subadults are fully independent, appears sexually mature but has not fully grown into adult size; adults are fully grown size with sexually mature sexual characteristics; old adults are adults with

signs of age degeneration. Monitoring is done from a safe distance until NHP is fully awake and ambulatory.

3.2.6 Sampling of Human

Human samples were collected from farmers in participating cattle farms. Sample size was limited to 10 farmers per farm due to logistical and budget constraints. Individuals were recruited based on their willingness to participate, were above 18 years old and have worked or assisted with handling of animals in participating farms. Individuals were recruited based on recommendations from the farm owner.

Human sampling was conducted by district health officers from Ministry of Health, Malaysia and staffs of EcoHealth Alliance in accordance to USAID Emerging Pandemic Threats PREDICT Operating Procedures (Huppenthal, 2009; C. K. Johnson & Saylors, 2016). A written informed consent from the participant was obtained for biological sampling collection and questionnaire. Participants were explained about the study objectives and that the biological testing is exploratory and not diagnostic for their health, the types of question asked, duration of questionnaire and potential risks of their participation. They were also informed that the study was voluntary and anonymous in nature.

Biological sampling and questionnaire were conducted in a convenient, comfortable and private environment. Further description on the sampling is in Appendix X. Briefly, blood was taken from the median cubital vein on the upper limb or metacarpal plexus or the dorsal venous arch on the dorsal surface of the hand. Three ml of blood were taken from these animals and distributed equally in a BD Vacutainer® serum tube and EDTA tube. Processing of blood for serum extraction and storage are similar to previously mentioned in 3.2.4.

3.2.7 Data collection

Following biological sampling, interviews with farmers were conducted using questionnaire designed to gauge exposure or behavioural risks to zoonotic spillover events. The questionnaire has been approved for used by the Institutional Review Board for studies on human research subjects and has been verified in previous studies (Kamau et al., 2021; Li et al., 2019; The PREDICT Consortium, 2021). The questionnaire was designed into several parts to gather information on participants' demographics, exposure to different species of domestic animal and wildlife, forms of exposure to domestic animal and wildlife, reported unusual illnesses within the past year. The questionnaire took less than 30 minutes to be completed and were administered by trained EcoHealth Alliance staffs in Malay language.

Data for livestock, other domestic animals and wildlife were recorded accordingly during the sampling process as described above. Table below shows the data collected for each species (Table 3.2).

Table 3. 2 Types of data collected during sampling of animals and human from participating cattle farms located at the forest fringe in Perak and Kelantan, Malaysia

Species	Data collected
Livestock/Dogs	Date of sampling, weather (clear, cloudy, rain), moon (full, half, new moon), weight, animal classification (domestic, feral domestic, captive wild, wild), species scientific name, species' English name, breed, farm location GPS coordinates, husbandry, ecological setting, other animals at site, tag number, sex, pregnancy status, lactating status, descended testes, age class, age, age in captivity, health condition during sampling, presence of ectoparasite, notes (special characteristics/health status/injury/body condition), specimen type, specimen collected, GPS coordinates of sampling base
Rodent/Small mammal/Non-human primates/Wild boar	Date of capture, time of trap check, trap ID and type, weather (clear, cloudy, rain), moon (full, half, new moon), weight, animal taxa, species scientific name, species' English name, sex, pregnancy status, lactating status, descended testes, age class, health condition during sampling, presence of ectoparasite, morphometric measurements (body length, right ear height, right hind foot, tail length), recapture marking, notes (special characteristics/health status/injury/body condition), specimen type, specimen collected, GPS coordinates of traps, GPS coordinates of sampling base
Bats	Date of capture, time of capture, GPS coordinate of net, weather (clear, cloudy, rain), moon (full, half, new moon), weight, animal taxa, species scientific name, species' English name, sex, pregnancy status, lactating status, descended testes, age class, health condition during sampling, presence of ectoparasite, morphometric measurements (forearm or radius length, body length, ear length, hind foot length, tail length), recapture marking, notes (special characteristics/health status/injury/body condition), specimen type, specimen collected, volume of serum dilution, of traps, GPS coordinates of sampling base
Human	Participant ID, site location, GPS coordinates, date of sampling, body temperature, blood pressure, pulse, weight, height, obvious signs of illness, age, sex, ethnicity, education level, years of residence in village, frequency of entering forest, exposure to different species of domestic animal and wildlife (primate, bat, rodent, small mammal, wild boar, wild bird, swine, cattle, dog, cat, goat, chicken, others), forms of exposure to domestic animal and wildlife (1. General exposure: lived with pet, handled live animals, raised animals, share water source with animals, seen faeces in or near food, animal entered house, eaten food damaged by animal, scratched or bitten by animal; and 2. Exposure to wildlife: cook or handle recently slaughtered meat, offal or blood of wildlife, eaten raw or undercooked meat, offal or blood of wildlife, eaten wildlife of unknown health status, eaten or shared dead wildlife, collect and sell dead wildlife, slaughter or butcher wildlife), reported unusual illnesses within the past year (fever with headaches and lethargy @ encephalitis, haemorrhagic fever and rash, severe acute respiratory infection (SARI),

	Influenza-like illnesses (ILI), fever with diarrhoea and vomiting, fever with skin rash), interviewer's name

3.2.8 Luminex Serology

Serum samples from all species of animals and from humans were analysed using a Luminex bead-based multiplex microsphere assay in a Bio-Rad Bio-Plex 200 (Bossart et al., 2007). This is an assay derived from the Luminex xMAP (xMAP = multianalyte profile) technology based on flow-cytometry principles which allows high-throughput, multiplex and simultaneous detection of up to 500 analytes in a 96-well plate. Since its invention in the late 1990s, this technology has been extensively used for detection of viral, bacterial, parasitical and fungal agents (Reslova et al., 2017). It has also been used increasingly for seroprevalence study of filoviruses and henipaviruses globally (Chowdhury et al., 2014; Dovih et al., 2019; Hayman et al., 2008; Laing et al., 2018; Peel et al., 2013b).

Filoviruses viral envelope glycoproteins (GPs) and henipaviruses receptor-binding proteins (G) were coupled to 5.6 µm diameter polystyrene magnetic beads for antigen-antibody binding. The list of filoviruses and henipaviruses tested are as shown in Table 3.3.

Table 3. 3 Filoviruses and Henipaviruses conjugated Bio-Plex beads used in multiplex assay for antibody detection.

Filoviruses (GPs)	Henipaviruses (Gs)
EBOV, Ebola virus	HeV, Hendra virus
BDBV, Bundibugyo virus	NiV, Nipah virus

BOMV, Bombali virus	CedV, Cedar virus
SUDV, Sudan virus	MojV, Mojiang virus
MLAV, Mengla virus	GhV, Ghana virus.
RESTVm, Reston virus monkey isolate	
RESTVp, Reston virus pig isolate	
LLOV, Lloviu virus	
MARV, Marburg virus	
RAVV, Ravn virus	

Prior to testing, individual samples were diluted 1:100 in phosphate-buffered saline (PBS) in 1.5ml Eppendorf tube and vortexed briefly to mix followed by heat inactivation at 56°C for 30 mins. A duplicate of a 100 µl diluted sera were added into 96-wells plate containing 100µl of bead master mix containing specific sGP- and sG-coated beads. Mixture is covered with plastic shield and aluminium foil and incubated on a shaker for 1200 r.p.m for 1 minute followed by 900 r.p.m for 45 minutes. Samples were washed to remove unbound antibodies three times and incubated on shaker with 100µl mastermix of 1:1000 biotinylated-Protein A and biotinylated Protein G (Thermo Fisher Scientific, Waltham, MA, USA) in PBS-Tween 20 (0.05%) (Bio-Rad). Mixture was washed three times and lastly incubated on a shaker with 100µl 1:1000 streptavidin-phycoerythrin (PE) in into each well and shook for 10 minutes. Results are read on Bio-Rad Bio-Plex 200 with at least 50 per spectral region and reported as Median fluorescence intensities (MFI). Cut-off values in the first round of testing at dilutions 1:100 were determined by 3*Sigma (3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations) (Dovih et al., 2019).

Positive samples in the first round were rerun at the optimum dilutions of 1:400 and 1:500 with added positive and negative controls. Sera from EBOV infected bats were used as positive controls for EBOV. Negative rabbit polysera and PBS were used as negative controls. Antigen-antibody binding were measured and read using a Bio-Plex 200 system. Data were transformed to adjusted MFI values by taking the mean MFI values duplicates of each sample and subtracting average PBS MFI per plate.

Cut-off were determined by using 3*Sigma and Latent cluster analysis (LCA). LCA was used in absence of gold standard assays and lack of positive and negative reference samples for the viral and animal species tested (Peel et al., 2013a). To improve cut-off determination, MFI values from local samples collected as part of the research programme were combined with this study dataset. Serum samples above the cut-off values were considered seropositive. The 3*Sigma cut-off values for filoviruses and henipaviruses were determined to be 4182 MFI and 4641 MFI respectively. LCA for filoviruses and henipaviruses were attempted but were not possible due to insufficient samples reactive samples.

3.2.9 Data Analysis

Serodetection of filoviruses and henipavirus for different species were calculated based on the number of seropositive animals over the total number of sample and reported as percentage (%). The association between the serological evidence and determinants

from animals (such as, farm, sex, age, age class, species, reproductive status and health status) and humans (such as, farm, husbandry, sex, age range, ethnicity, education level, years of residence in village, frequency of entering forest, exposure to different species of domestic animal and wildlife, forms of exposure to domestic animal and wildlife, reported unusual illnesses within the past year) were analysed using Pearson Chi-Square test, X^2 or Fisher's exact test (two-tailed), when X^2 is not appropriate (one of expected cell value is less than 5) at significance level $\alpha = 0.05$. Post-hoc test Bonferroni corrections and Bonferroni adjusted p-value were used for multiple comparison. Odds ratio (OR) and OR 95% Confidence intervals (CI) with Haldane-Anscombe correction were applied when contingency table has values of 0. The difference between seropositivity and hypothesized risk factors were tested using Kruskal-Wallis, KW (more than two groups) test at $\alpha=0.05$ and post-hoc Dunn's test was applied if applicable. Variable was excluded from further analysis if data is insufficient or too small ($n<10$). All analysis were conducted using the Statistical Package for Social Sciences (SPSS 27) and Microsoft Excel.

3.3 Results

3.3.1 Population characteristics

3.3.1.1 Domestic animals

Six forest-fringed cattle farms were enrolled in the study in 2018 from Kuala Kangsar district, Perak and Gua Musang district, Kelantan. Serum samples were collected from farmers (n=55), cattle (n=156), goat (n=158), sheep (n=11), dogs (n=23), bats (n=265),

rodents (n=36), civet (n=1), wild boar (n=1), rabbits (n=3) and non-human primates (n=2). In total, 709 serum samples were collected.

Cattle were mostly sampled from integrated cattle farms (Table 3.13). Other domestic animals such as goats and sheep were mainly sampled from semi-intensive goat and sheep farms (Table 3.14 and 3.15). Dogs sampled were either tethered or free-roaming in the village or plantation (Table 3.16). Besides these animals, a pet rabbit was also sampled. Majority of the farm animals sampled were adults.

3.3.1.2 Wildlife

A total of 265 bats that were sampled during the study with an average of 44 (25-56) bats sampled per farm. Out of 9 species captured, 6 species were frugivores or nectarivores and 3 species were insectivores. Species that were most sampled were mostly adult male *C. brachyotis* (n=108), *E. spelaea* (n=70) and *C. horsfieldii* (n=52) (Table 3.17).

Table 3. 4 Bats based on sex, age class and species sampled from the surrounding targeted cattle farm at forest fringe

	Bat
	n
Sex	
Female	139
Male	126
	265
Age class	
Adult	242
Subadult	23
Juvenile	0
	265

Species	
Balionycteris maculata	3
Cynopterus brachyotis	108
Cynopterus horsfieldii	52
Eonycteris spelaea	70
Hipposideros galeritus	1
Macroglossus minimus	6
Macroglossus sobrinus	5
Nycteris tragata	1
Tylonycteris pachypus	19
	265

Overall, 36 rodents were sampled with an average of 6 (3-5) rodents per farm (Table 3.5). Twelve species of rodents mostly adult *Rattus exulans* (n=8) and *Rattus tiomanicus* (n=7) were captured.

Table 3. 5 Rodents based on sex, age class and species sampled from the surrounding targeted cattle farm at forest fringe.

Rodent		
		n
Sex		
	Female	12
	Male	24
		36
Age class		
	Adult	29
	Subadult	7
	Juvenile	0
		36
Species		
	Chiropodomys gliroides	5
	Lariscus insignis	1
	Leopoldamys sabanus	5
	Maxomys rajah	1
	Maxomys surifer	3
	Niviventer cremoriventer	1
	Rattus argentiventer	2
	Rattus exulans	8
	Rattus rattus	1
	Rattus tiomanicus	7
	Sundasciurus lowii	1
	Tupaia glis	1

Other wildlife sampled were pet macaques (n=2), a wild boar (n=1) and a civet, *Viverricula indica* (n=1).

3.3.1.3 Human

A total of 56 farmers from participating cattle farms were enrolled but only 55 samples were obtained. An average of 9 farmers were sampled from each farm. Majority sampled were male, between 20-29 years old, with mean age of 42 (18-68) of the Malay race. Most have completed secondary school or above and have resided in the village for more than 10 years (51/55, 92.7%) (Table 3.19).

The most common domestic animals that farmers were exposed to within the past one year of sampling were cattle, cat and poultry. Whereas the most common wildlife exposed to were rodents and bats (Table 3.120). Farmers had general exposure to domesticated animals and wildlife in the past one year primarily through handling of live animals, animal entering living quarters and living with pet. Farmers were exposed to wildlife primarily through slaughter, butchering, cooking and handling of recently slaughtered meat, offal or blood of wildlife (Table 3.21).

Out of the 55 farmers, many farmers reported Influenza-like illnesses (ILI), followed by fever with diarrhoea and vomiting, Severe acute respiratory infection (SARI), fever with headaches and lethargy (3/55, 5.5%), and fever with skin rash (1/55, 1.8%). No farmers reported haemorrhagic fever (Table 3.22).

3.3.2 Serological result

3.1.1.1 Filovirus

A total of 45 sera were reactive to filovirus (%??). The serological detection for filovirus among species in descending order were goat (15.2%, 95% CI 10-22), sheep (9.1%, 95% CI 0.2-41.3), human (9.1%, 95% CI 3-20), dog (8.7%, 95% CI 1.1-28), cattle (5.1%, 95% CI 2.2-9.9) and bat (1.9%, 95% CI 0.6-4.3) (Table 3.6, Figure 16-22). Bats species that were seropositive for filovirus were *C. brachyotis*, *E. spelaea* and *C. horsfieldii* (Table 3.7).

Table 3. 6 Serological evidence of filovirus based on farm and species sampled using Luminex bead-based multiplex microsphere assay

		Filovirus						
	Participating cattle farms	**1**	**2**	**3**	**4**	**5**	**6**	**Total**
Category	**Husbandry**	**SI**	**SI**	**SI**	**It**	**It**	**In**	
Human								
	Individual sampled	10	9	10	10	10	6	55
	Seropositive, n (%)	1 (10)	0	1 (10)	2 (20)	0	1 (16.7)	5 (9.1)
Cattle								
	Individual sampled	22	15	24	29	36	30	156
	Seropositive, n (%)	2 (9.1)	2 (13.3)	2 (8.3)	1 (3.4)	1 (2.8)	0	8 (5.1)
Goat								
	Individual sampled	20	44	31	22	22	19	158
	Seropositive, n (%)	0	7 (15.9)	8 (25.8)	5 (22.7)	0	4 (21.7)	24 (15.2)
Sheep								

	Individual sampled	0	0	0	9	0	2	11
	Seropositive, n (%)	0	0	0	0	0	1 (50)	1 (9.1)
Dog								
	Individual sampled	10	1	2	1	0	9	23
	Seropositive, n (%)	1 (10)	1 (100)	0	0	0	0	2 (8.7)
Bat								
	Individual sampled	45	49	47	43	25	56	265
	Seropositive, n (%)	0	3 (6.1)	1 (2.1)	0	1 (4)	0	5 (1.9)
Rodent								
	Individual sampled	10	5	8	3	7	3	36
	Seropositive, n (%)	0	0	0	0	0	0	0
Civet								
	Individual sampled	0	1	0	0	0	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Wild boar								
	Individual sampled	0	0	0	0	1	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Rabbit								
	Individual sampled	0	0	3	0	0	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Primate								
	Individual sampled	0	1	1	0	0	0	2
	Seropositive, n (%)	0	0	0	0	0	0	0
Total		4	13	12	8	2	6	45

Production system: SI, semi-intensive system; It, integrated system; In, intensive system

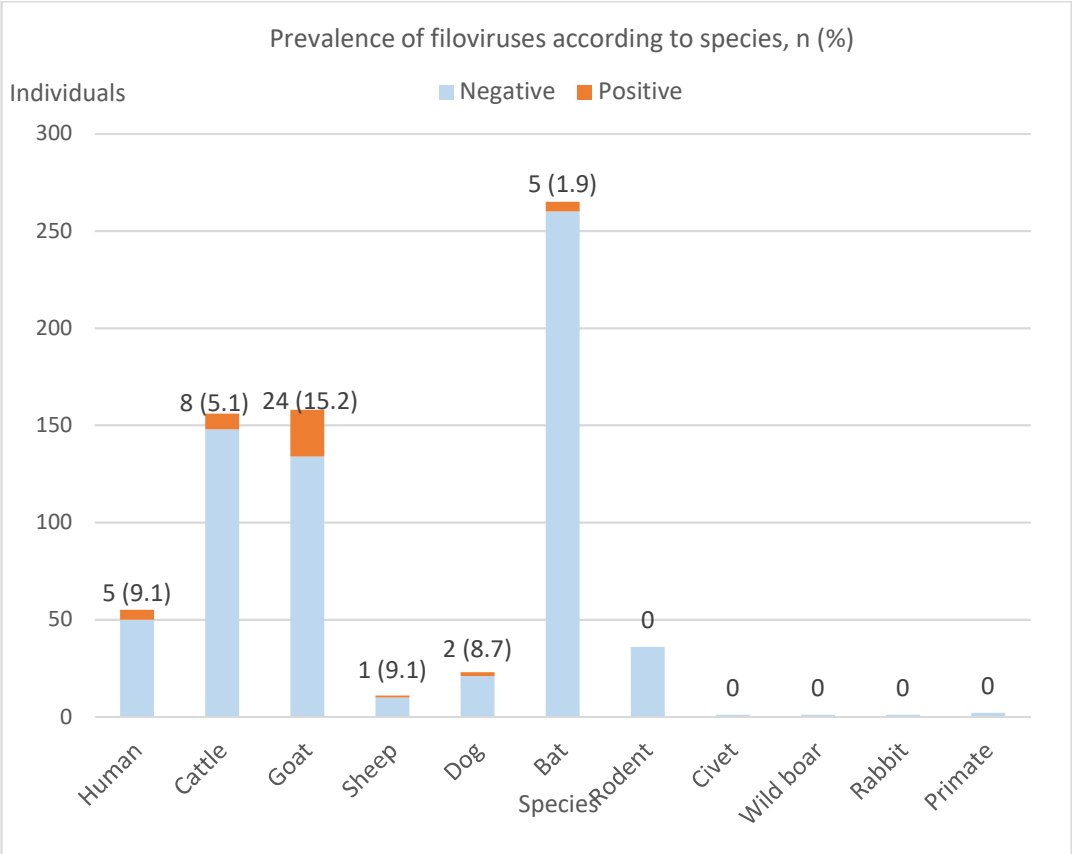


Figure 3. 15 Serological evidence of filovirus based on species sampled using Luminex bead-based multiplex microsphere assay

Table 3. 7 Serological evidence of filovirus in bats using Luminex bead-based multiplex microsphere assay

No	Species	Individuals sampled (n)	Filovirus positive (%)
1	*Balionycteris maculata*	3	0
2	*Cynopterus brachyotis*	108	2 (1)
3	*Cynopterus horsfieldii*	52	1 (2)
4	*Eonycteris spelaea*	70	2 (2)
5	*Hipposideros galeritus*	1	0
6	*Macroglossus minimus*	6	0
7	*Macroglossus sobrinus*	5	0
8	*Nycteris tragata*	1	0
9	*Tylonycteris pachypus*	19	0
	Total	265	5

Most filovirus reactive samples were reactive to BDBV (29/45) followed by SUDV (16/45) and RESTV (16/45) (Table 3.8). Twenty-three (23/45, 51%) samples were reactive to only one antigen, with the most being BDBV (16/23), followed by EBOV (4/23), RESTVp (2/23) and SUDV (1/23). Whereas twenty-two (22/45, 48.9%) samples were reactive to two to five filovirus antigens which involved BDBV, SUDV, RESTVp the most. Cross-reactivity were observed between EBOV, BDBV, BOMV, SUDV, RESTVm, RESTVp, MARV, and RAVV (Appendix).

Table 3. 8 Reactivity with filovirus across species using Luminex bead-based multiplex microsphere assay

			Reactivity to filovirus, n (%)									
*Category	Total	Total reactive	EBOV	BDBV	BOMV	SUDV	RESTVm	RESTVp	LLOV	MLAV	MARV	RAVV
Human	55	5	2 (3.6)	3 (5.5)	0	0	0	0	0	0	0	1 (1.7)
Cattle	156	8	3 (1.9)	6 (3.8)	0	2 (1.3)	2 (1.3)	0	0	0	0	0
Goat	158	24	0	14 (8.9)	0	12 (7.6)	0	14 (8.9)	0	0	0	0
Sheep	11	1	0	1 (9.1)	1 (9.1)	1 (9.1)	0	1 (9.1)	0	0	1 (9.1)	0
Dog	23	2	1 (4.3)	2 (8.7)	0	0	0	0	0	0	0	0
Bat	265	5	2 (0.8)	3 (1.1)	1 (0.4)	1 (0.4)	0	1 (0.4)	0	0	0	0
Total		**45**	**8 (17.8)**	**29 (64.4)**	**2 (4.4)**	**16 (35.6)**	**2 (4.4)**	**16 (35.6)**	**0**	**0**	**1 (2.2)**	**4 (8.9)**

EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

*Non-reactive category not included

The range of MFI for filovirus positive sera was between 4183 and 24059, with the highest being 24059 MFI for BDBV in a human. There were 10 reactions which produced an MFI reading of 10000-14999 MFI (EBOV, BDBV, SUDV and RAVV) and 2 reactions with >20000 MFI (BDBV) (Table 3.9).

Table 3. 9 Filovirus range of MFI of positive sera, number of high reactivity and its antigen among different species using Luminex bead-based multiplex microsphere assay

	High reactivity for filovirus						
	No. of Sera with 10000-14999 MFI	**Type of Antigen reaction**	**No. of Sera with 15000-19999 MFI**	**Type of Antigen reaction**	**No. of Sera with >20000 MFI**	**Type pf antigen reaction**	**MFI range**
Bat	2	EBOV, BDBV	0	-	0	-	4382-10784
Cattle	1	EBOV	0	-	0	-	4246-10205
Dog	0	-	0	-	0	-	5429-7561
Goat	3	BDBV, RAVV	0	-	1	BDBV	4183-21882
Sheep	1	SUDV	0	-	0	-	4317-10890
Human	3	EBOV, BDBV, RAVV	0	-	1	BDBV**	4928-24059
Total	10		0		2		

**Among all reactivity to filoviruses, a human reported highest MFI for BDBV
EBOV, Ebola virus; BDBV, Bundibugyo virus; SUDV, Sudan virus; RAVV, Ravn virus

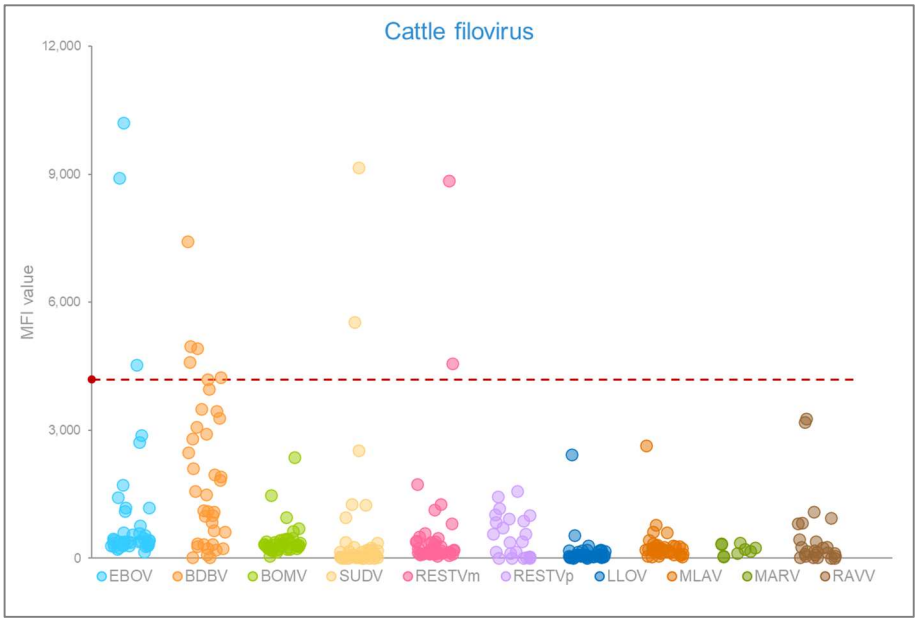


Figure 3. 16 Filovirus range of MFI of positive cattle sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

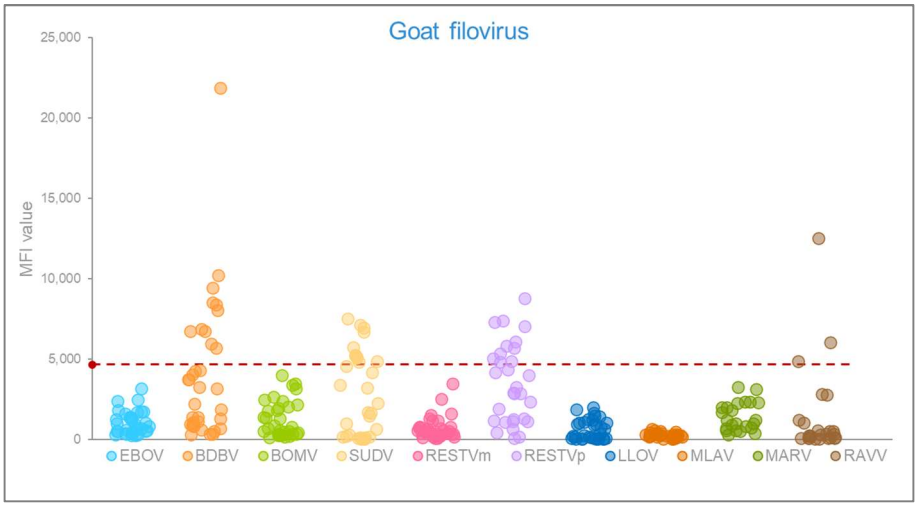


Figure 3. 17 Filovirus range of MFI of positive goat sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

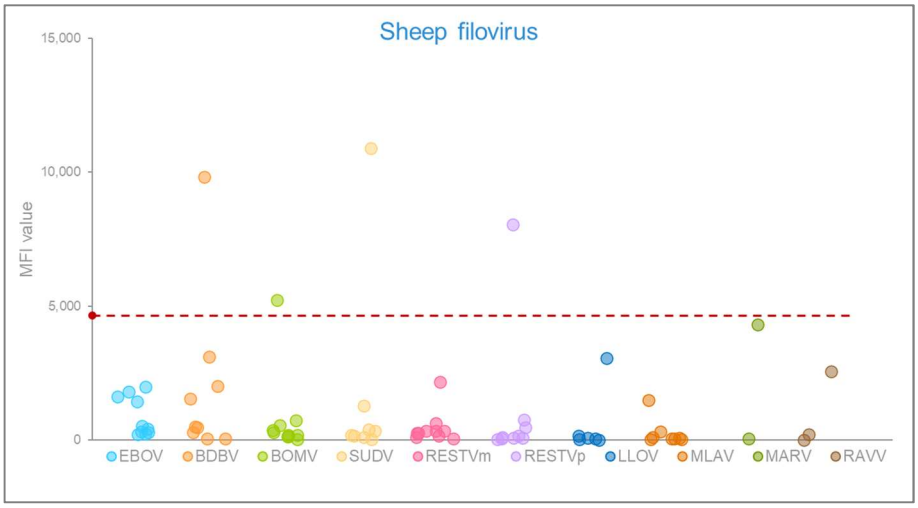


Figure 3. 18 Filovirus range of MFI of positive sheep sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

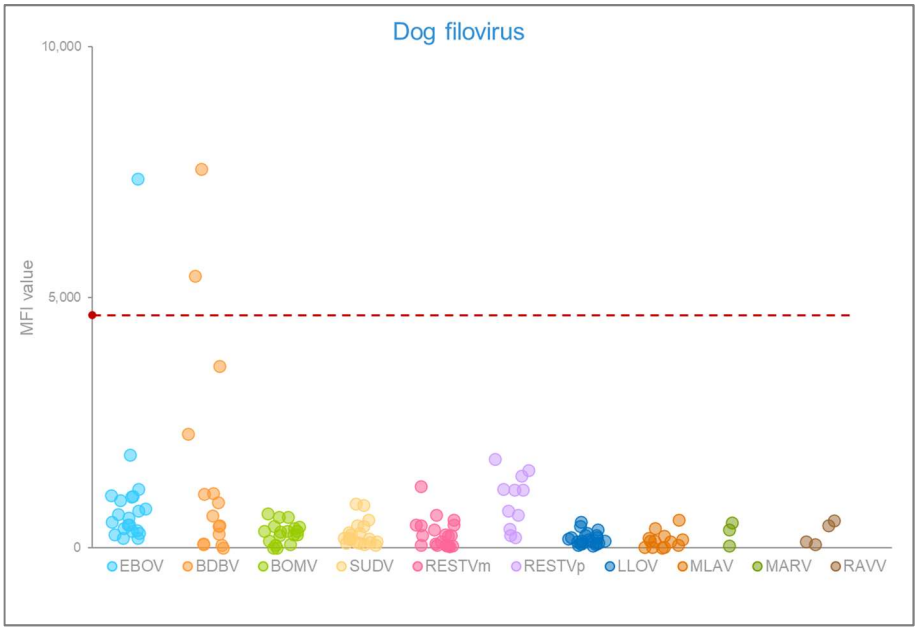


Figure 3. 19 Filovirus range of MFI of positive dog sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

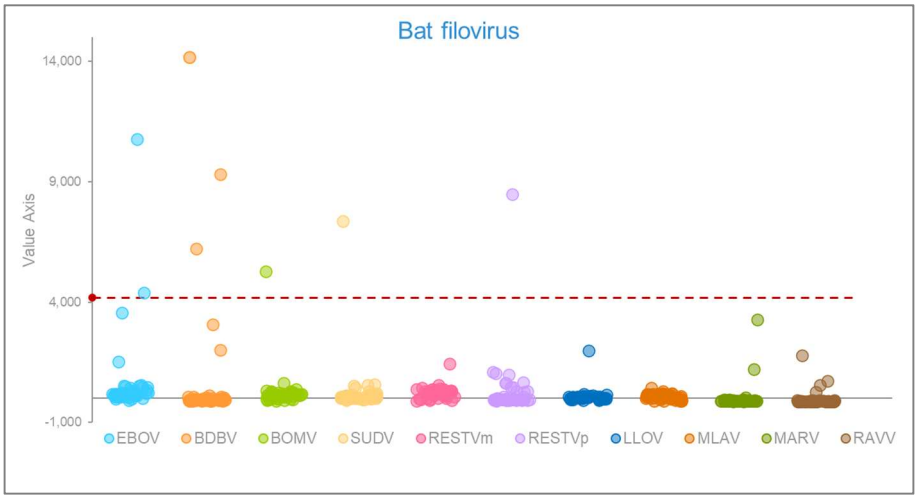


Figure 3. 20 Filovirus range of MFI of positive bat sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

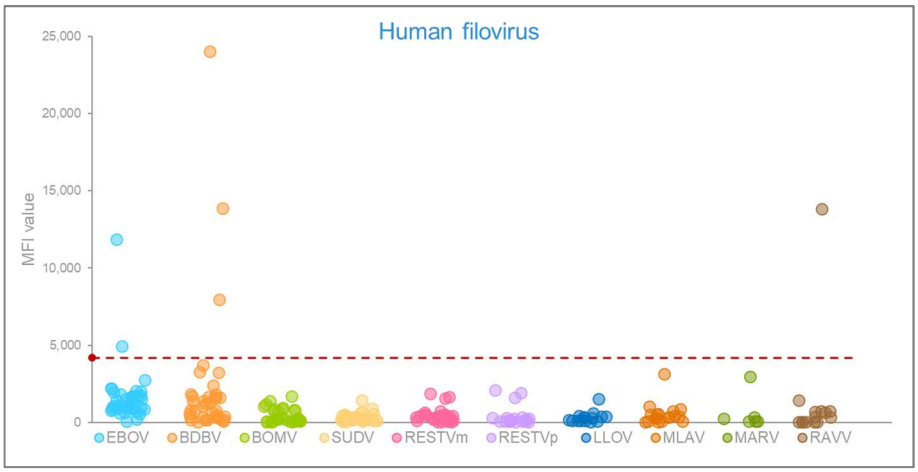


Figure 3. 21 Filovirus range of MFI of positive human sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4182 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

3.1.1.2 Henipavirus

A total of 36 sera were reactive to henipavirus. The reactive sera were in goat (13.9%, 95% CI 8.9-20.3), sheep (9.1%, 95% CI 0.2-41.3), human (7.3%, 95% CI 2-17.6), dog (4.3%, 95% CI 0.1-21.9), cattle (3.2%, 95% CI 1.0-7.3) and bat (1.1%, 95% CI 0.2-3.3) (Table 3.10 and Figure 3.23-29). Bats seropositive for henipavirus were *C. brachyotis* and *C. horsfieldii* (Table 3.11).

Table 3. 10 Serological evidence of Henipavirus based on farm and species using Luminex bead-based multiplex microsphere assay

		Henipavirus						
	Participating cattle farms	**1**	**2**	**3**	**4**	**5**	**6**	**Total**
	Husbandry	**SI**	**SI**	**SI**	**It**	**It**	**In**	
Human	Individual sampled	10	9	10	10	10	6	55
	Seropositive, n (%)	0	1 (10)	0	2 (20)	0	1 (16.7)	4 (7.3)
Cattle	Individual sampled	22	15	24	29	36	30	156
	Seropositive, n (%)	1 (4.5)	0	1 (4.2)	1 (3.4)	0	2 (6.7)	5 (3.2)
Goat	Individual sampled	20	44	31	22	22	19	158
	Seropositive, n (%)	0	7 (15.9)	7 (22.6)	5 (22.7)	0	3 (15.8)	22 (13.9)
Sheep	Individual sampled	0	0	0	9	0	2	11
	Seropositive, n (%)	0	0	0	0	0	1 (9.1)	1 (9.1)
Dog	Individual sampled	10	1	2	1	0	9	23
	Seropositive, n (%)	0	0	0	0	0	1 (11.1)	1 (4.3)
Bat	Individual sampled	45	49	47	43	25	56	265
	Seropositive, n (%)	0	2 (4.1)	1 (2.1)	0	0	0	3 (1.1)
Rodent	Individual sampled	10	5	8	3	7	3	36
	Seropositive, n (%)	0	0	0	0	0	0	0
Civet	Individual sampled	0	1	0	0	0	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Wild boar	Individual sampled	0	0	0	0	1	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Rabbit	Individual sampled	0	0	3	0	0	0	1
	Seropositive, n (%)	0	0	0	0	0	0	0
Primate	Individual sampled	0	1	1	0	0	0	2
	Seropositive, n (%)	0	0	0	0	0	0	0
Total		1	10	9	8	0	8	

Production system: SI, semi-intensive system; It, integrated system; In, intensive system

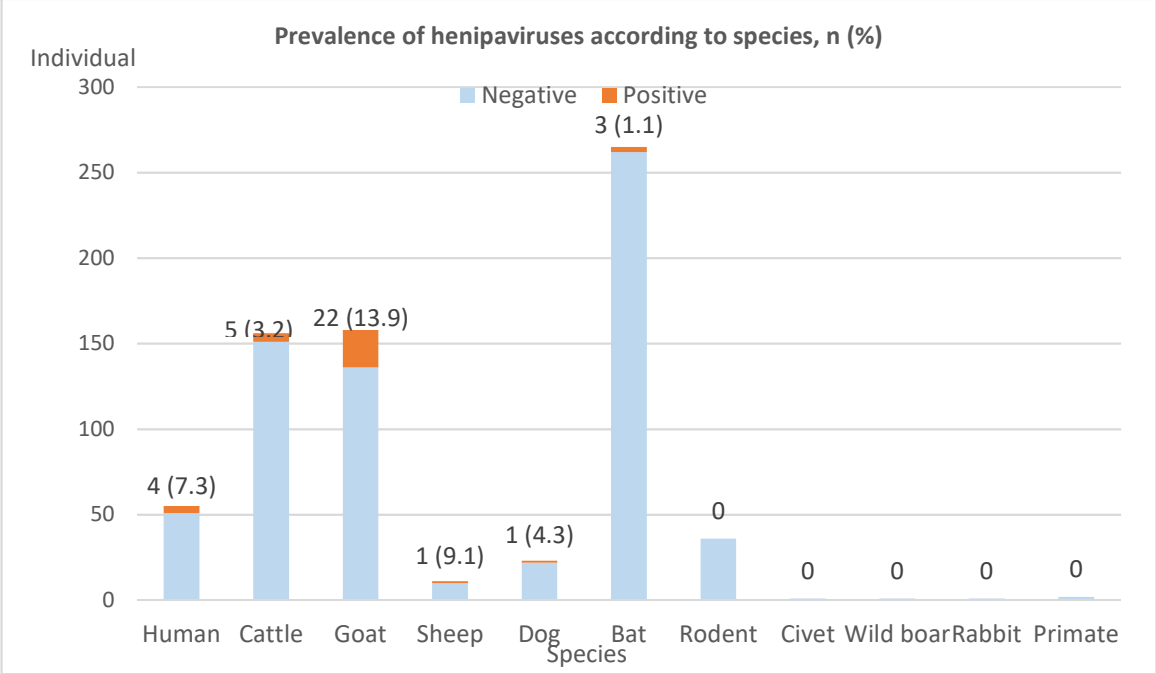


Figure 3. 22 Serological evidence of henipaviruses based on species sampled using Luminex bead-based multiplex microsphere assay

Table 3. 11 Serological evidence of henipavirus in bats using Luminex bead-based multiplex microsphere assay

No	Species	Individuals sampled (n)	Henipavirus positive (%)
1	*Balionycteris maculata*	3	0
2	*Cynopterus brachyotis*	108	2 (1)
3	*Cynopterus horsfieldii*	52	1 (2)
4	*Eonycteris spelaea*	70	0
5	*Hipposideros galeritus*	1	0
6	*Macroglossus minimus*	6	0
7	*Macroglossus sobrinus*	5	0
8	*Nycteris tragata*	1	0
9	*Tylonycteris pachypus*	19	0
	Total	265	3

Most samples were reactive to GhV (32/36), HeV (22/36), NiV (19/36), CedV (9/36) and MojV (7/36) (Table 3.11). Eleven (11/36, 31%) samples were reactive to only one antigen, with the most being GhV (9/11), followed by MpjV (2/11). Twenty-two (25/36, 69.4%) samples were reactive to two to five henipavirus antigens which involved GhV,

HeV and NiV the most. Cross-reactivity were observed between all five antigens tested (Appendix).

Table 3. 12 Reactivity with henipavirus across species using Luminex bead-based multiplex microsphere assay

			Reactivity with henipavirus, n (%)				
	Total sampled	Total reactive	HeV	NiV	CedV	MojV	GhV
Human	55	4	1 (1.7)	0	1 (1.7)	4 (6.7)	4 (6.7)
Cattle	156	5	1 (0.6)	0	1 (0.6)	2 (1.3)	4 (2.6)
Goat	158	22	17 (10.8)	15 (9.5)	5 (3.2)	0	20 (12.7)
Sheep	11	1	1 (9)	1 (9.1)	1 (9.1)	0	1 (9)
Dog	23	1	0	0	0	1 (4.3)	0
Bat	265	3	2 (0.8)	2 (0.8)	1 (0.4)	0	3 (1.1)
Total		36	22 (61.1)	19 (52.8)	9 (25)	7 (19.4)	32 (88.9)

HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

The range of MFI for henipavirus positive sera was between 4182 and 24689 with the highest being 24689 MFI against GhV in a farmer. There were 39 reactions with an MFI reading of 10000-19999 MFI (HeV, NiV, CedV, and GhV), and 5 reactions of >20000 MFI (HeV, CedV, MojV and GhV). Goat sera displayed many high MFI readings of >10000. In general, almost all henipavirus reactive sera had reactivity to GhV with many being >10000 MFI. (Table 3.13 and Appendix).

Table 3. 13 Henipavirus range of MFI of positive sera, number of high reactivity and its antigen among different species using Luminex bead-based multiplex microsphere assay

	High reactivity for henipavirus						
	No. of Sera with 10000-14999 MFI	Type of Antigen reaction	No. of Sera with 15000-19999 MFI	Type of Antigen reaction	No. of Sera with >20000 MFI	Type pf antigen reaction	MFI range
Bat	4	-	0	-	0	-	4776-12117
Cattle	3	-	2	HeV, CedV	1	GhV	5000-20100
Dog	0	-	0	-	0	-	5042[a]
Goat	21	HeV, NiV, CedV, GhV	4	HeV, GhV	0	-	4834-19905
Sheep	0	-	3	HeV, NiV, GhV	0	-	9894-17401
Human	2	GhV	0	-	4	HeV, CedV, MojV, GhV	7170-24689
Total	30		9		5		

[a]no upper MFI range as there was only 1 reactivity in 1 dog serum
HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

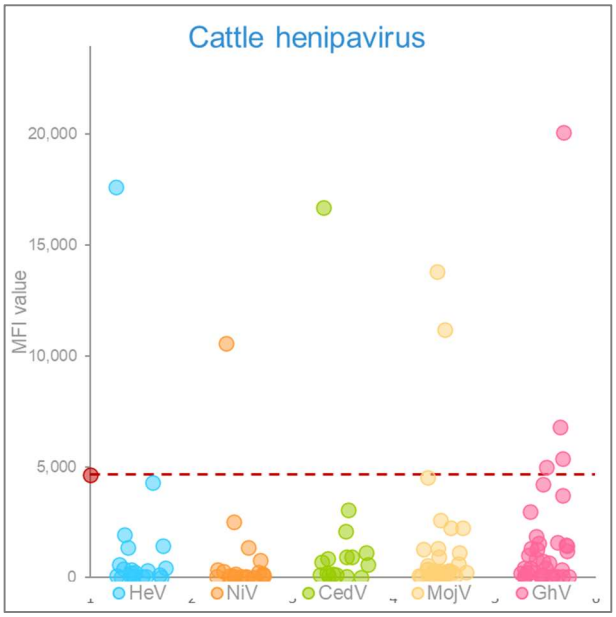


Figure 3. 23 Henipavirus range of MFI of positive cattle sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

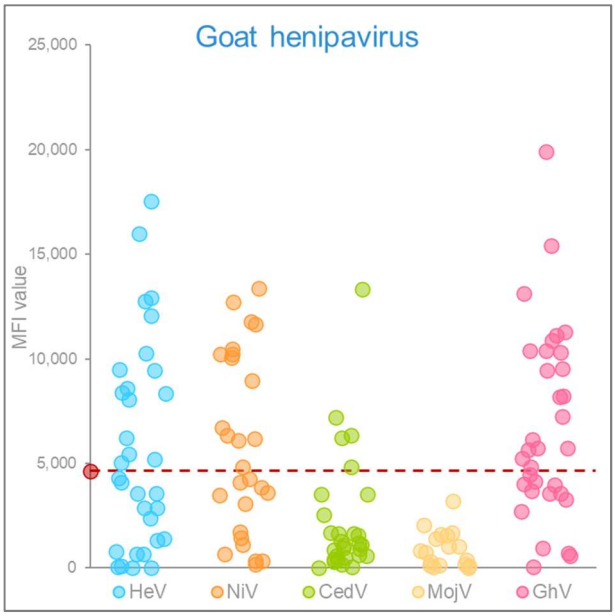


Figure 3. 24 Henipavirus range of MFI of positive goat sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

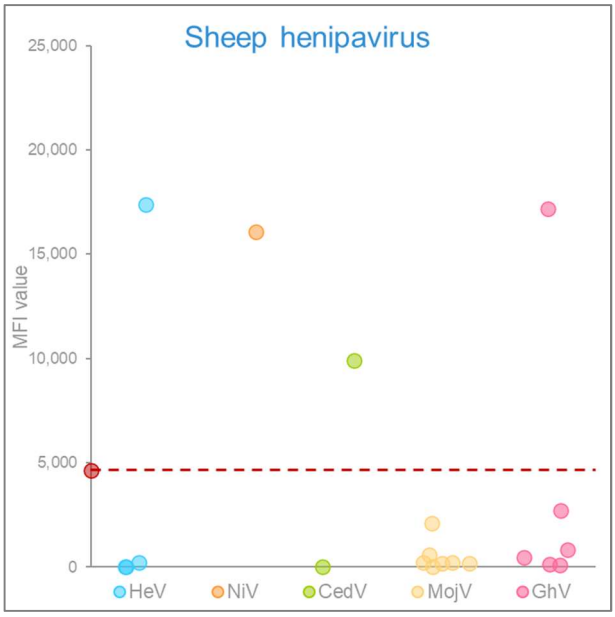
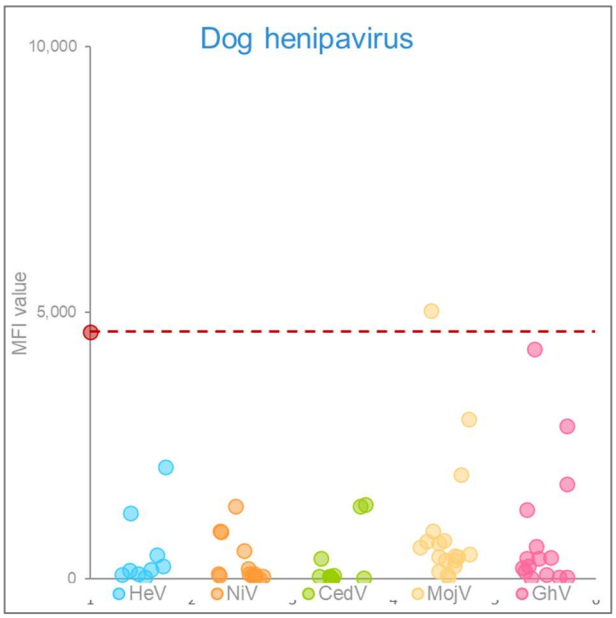


Figure 3. 25 Henipavirus range of MFI of positive sheep sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

Figure 3. 26 Henipavirus range of MFI of positive dog sera using Luminex bead-based multiplex microsphere assay



Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

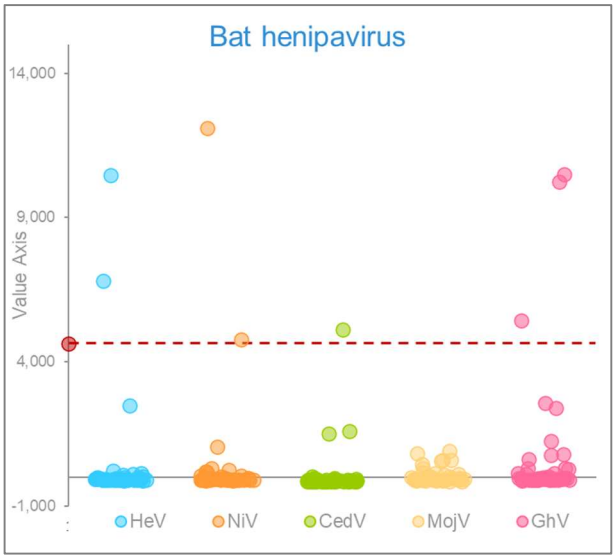


Figure 3. 27 Henipavirus range of MFI of positive bat sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

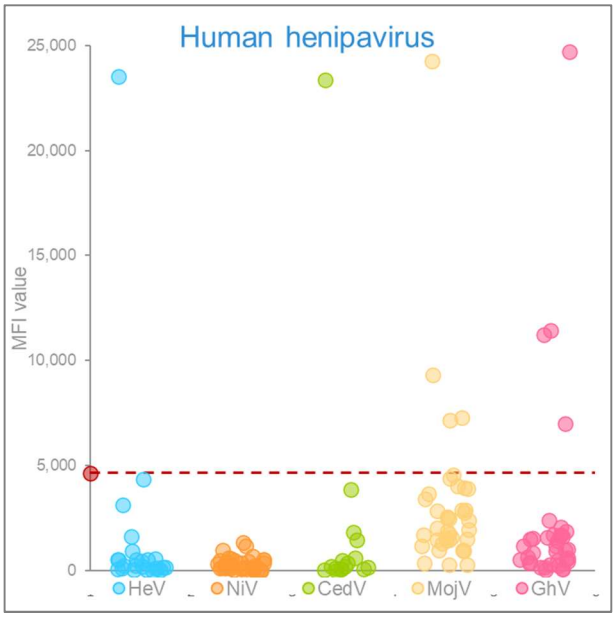


Figure 3. 28 Henipavirus range of MFI of positive human sera using Luminex bead-based multiplex microsphere assay

Coloured dots indicate specific sera samples. Red lines indicate cut-off value of 4641 MFI that was determined by 3-fold change of the mean of adjusted MFI values or above the mean plus 3 standard deviations. HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

3.3.3 Serological evidence and demographic distribution

3.3.3.1 Cattle

The serological evidence of filovirus and henipavirus in cattle were 5.1%, 8/156 (95% CI 2.2-9.9) and 3.2%, 5/156 (95% CI 1.0-7.3) respectively. Among the filovirus positive (8/156) and henipavirus positive (5/156) cattle sera, most were female, adults and apparently healthy and had no observable ectoparasite at the time of sampling. No significant difference was observed between serological evidence of filovirus or henipavirus with husbandry, sex, age class and health status (Table 3.14).

Table 3. 14 Serological evidence of filovirus and henipavirus in cattle based on farm, sex, age class, reproduction status, health status and presence of ectoparasite

	Cattle				
	n	Filovirus positive	P-value	Henipavirus positive	P-value
Farm			a		a
1	22	2		1	
2	15	2		0	
3	24	2		1	
4	29	1		1	
5	36	1		0	
6	30	0		2	
	156	8		5	
Husbandry			0.108		0.343
Intensive	30	0		2	
Semi-intensive	61	6		2	
Integrated	65	2		1	
	156	8		5	
Sex			1.000		a
Female	101	5		5	
Male	55	3		0	
	156	8		5	
Age class			1.000		1.000
Adult (>2 yr)	121	7		4	
Subadult (6m-2yr)	28	1		1	
Juvenile (<6m)	7	0		0	
	156	8		5	
Reproduction status					
Pregnant	18	0	a	0	a
Lactating	33	1	a	2	0.285
		1		2	
Health status			0.192		0.123
Apparently healthy	153	7		4	
Sick	3	1		1	
	156	8		5	
Ectoparasite			a		a
No	147	8		5	
Yes	9	0		0	
	156	8		5	

[a]Variable is excluded from further analysis due to insufficient or too small data

3.3.3.2 Goat

The serological evidence of filovirus and henipavirus in goat were 15.2%, 24/158 (95% CI 10-22) and 13.9%, 22/158 (95% CI 8.9-20.3) respectively. Among the filovirus positive (24/158) and henipavirus positive (22/158) goat sera, most were semi-intensively reared, female, adults and apparently healthy. Subadult goats were 6.3 times less likely to be seropositive for filoviruses as compared to adults (P= 0.028, OR=6.3, 95% CI=1.149, 34.383). No significant difference was observed between serological evidence of henipavirus and the various factors (Table 3.15).

Table 3. 15 Serological evidence of filovirus and henipavirus in goat based on farm, sex, age class, reproduction status, health status and presence of ectoparasite sampled from goat farms surrounding participating targeted cattle farms

	Goat				
	n	**Filovirus positive**	**P-value**	**Henipavirus positive**	**P-value**
Targeted Cattle Farm			a		a
1	20	0		0	
2	44	7		7	
3	31	8		7	
4	22	5		5	
5	22	0		0	
6	19	4		3	
	158	24		22	
Husbandry of goat farm			0.009[b]		0.004[b]
Intensive	52	2		1	
Semi-intensive	101	21		20	
Extensive	5	1		1	
	158	24		22	
Sex			0.243		0.629
Female	104	13		13	
Male	54	11		9	
	158	24		22	
Age class			0.028		0.114
Adult (>1 yr)	106	21		19	
Subadult (6m-1yr)	38	1		2	
Juvenile (<6m)	14	2		1	
	158	24		22	
Reproduction status					
Pregnant	28	5	0.771	5	0.548
Lactating	28	4	1.000	4	1.000

Health status			0.148		0.403
Apparently healthy	122	21		18	
Sick	36	3		4	
	158	24		22	
Ectoparasite			0.604		0.788
No	120	17		16	
Yes	38	7		6	
	158	24		22	

[a]Variable is excluded from further analysis due to insufficient or too small data
[b]Not significant after Bonferroni correction

3.3.4 Sheep

The serological evidence of filovirus and henipavirus in sheep were 9.1%, 1/11 (95% CI 0.2-41.3) for both. Out of 11 sheep sampled, only one sheep was seropositive for filoviruses and henipaviruses. The sheep was a female pregnant and lactating adult that was raised extensively, and apparently healthy (Table 3.16). Further analysis with the variables were not performed due to insufficient or too small data.

Table 3. 16 Serological evidence of filovirus and henipavirus in sheep based on farm, sex, age class, reproduction status, health status and presence of ectoparasite sampled from sheep farms surrounding participating targeted cattle farm

	Sheep		
	n	Filovirus positive	Henipavirus positive
Targeted Cattle Farm			
1	0	0	0
2	0	0	0
3	0	0	0
4	9	0	0
5	0	0	0
6	2	1	1
	11	1	1
Husbandry of sheep farm			
Semi-intensive	9	0	0
Extensive	2	1	1
	11	1	1
Sex			
Female	6	1	1
Male	5	0	0
	11	1	1
Age class			

Adult (>1 yr)	7	1	1
Subadult (6m-1yr)	2	0	0
Juvenile (<6m)	2	0	0
	11	1	1
Reproduction status			
Pregnant	2	1	1
Lactating	2	1	1
Health status			
Apparently healthy	11	1	1
Sick	0	0	0
	11	1	1
Ectoparasite			
No	11	1	1
Yes	0	0	0
	11	1	1

3.3.5 Dog

The serological evidence of filovirus and henipavirus in dog were 8.7%, 2/23 (95% CI 1.1-28) and 4.3%, 1/23 (95% CI 0.1-21.9) respectively. Filovirus positive dog (2/23) were adult and subadult tethered males. Whereas henipavirus positive dog was an adult male raised extensively. All filovirus and henipavirus positive dogs were apparently healthy with no observable ectoparasite at time of sampling. Further analysis with the variables were not performed due to insufficient or too small data (Table 3.17).

Table 3. 17 Serological evidence of filovirus and henipavirus in dog based on farm, sex, age class, reproduction status, health status and presence of ectoparasite sampled surrounding participating targeted cattle farm

		Dog	
	n	**Filovirus positive**	**Henipavirus positive**
Targeted Cattle Farm			
1	10	1	0
2	1	1	0
3	2	0	0
4	1	0	0
5	0	0	0
6	9	0	1
	23	2	1
Husbandry of dog			

Extensive	18	0	1
Tethered	5	2	0
	23	2	1
Sex			
Female	14	0	0
Male	9	2	1
	23	2	1
Age class			
Adult (>1y)	12	1	1
Subadult (<1y)	6	1	0
Juvenile (<1m)	5	0	0
	23	2	1
Reproduction status			
Pregnant	0	0	0
Lactating	1	0	0
Health status			
Apparently healthy	23	2	1
Sick	0	0	0
Ectoparasite			
No	10	2	0
Yes	13	0	1
	23	2	1

3.3.6 Bat

The serological evidence of filovirus and henipavirus in bat were 1.9%, 5/265 (95% CI 0.6-4.3) and 1.1%, 3/265 (95% CI 0.2-3.3) respectively. Majority of the filovirus positive (5/265) and henipavirus positive (3/265), bat sera were adult females that were apparently healthy (Table 3.18). Seropositivity was detected in 3 bat species (*C. brachyotis*, *E. spelaea* and *C. horsfieldii).* Further analysis with the variables were not performed due to insufficient or too small data.

Table 3. 18 Serological evidence of filovirus and henipavirus in bat based on sex, age class, reproduction status, health status and presence of ectoparasite surrounding participating targeted cattle farms

	Bat		
	n	**Filovirus positive**	**Henipavirus positive**
Sex			
Female	139	3	2
Male	126	2	1
	265	5	3
Age class			
Adult	242	5	3
Subadult	23	0	0
Juvenile	0	0	0
	265	5	3
Species			
Balionycteris maculata	3	0	0
Cynopterus brachyotis	108	1	1
Cynopterus horsfieldii	52	2	2
Eonycteris spelaea	70	2	0
Hipposideros galeritus	1	0	0
Macroglossus minimus	6	0	0
Macroglossus sobrinus	5	0	0
Nycteris tragata	1	0	0
Tylonycteris pachypus	19	0	0
	265	5	3
Reproduction status			
Pregnant	14	0	0
Lactating	20	0	0
Health status			
Apparently healthy	263	5	3
Sick	2	0	0
	265	5	3
Ectoparasite			
No	209	4	3
Yes	56	1	0
	265	5	3

3.1.1.1 Human

The serological evidence of filovirus and henipavirus in human were 9.1%, 5/55 (95% CI 3-20) and 7.3%, 4/55 (95% CI 2-17.6) respectively. Humans positive for filovirus (5/55) and henipavirus (4/55) were mostly males of Malay ethnicity, completed secondary level education and have resided in village for more than 10 years. No significant difference was observed between serological evidence of filovirus or henipavirus with the various factors (Table 3.19).

Table 3. 19 Serological evidence of filovirus and henipavirus in human based on demographics of participating farmers sampled from targeted cattle farms

	Human				
	n	**Filovirus positive**	**P-value**	**Henipavirus positive**	**P-value**
Targeted Cattle Farm			a		a
1	10	1		0	
2	9	0		1	
3	10	1		0	
4	10	2		2	
5	10	0		0	
6	6	1		1	
	55	5		4	
Husbandry of cattle farm			0.664		0.417
Intensive	6	1		1	
Semi-intensive	29	2		1	
Integrated	20	2		2	
	55	5		4	
Sex					
Male	45	3	0.220	3	0.563
Female	10	2		1	
	55	5		4	
Age range			0.069		0.291
10-29	16	1		2	
30-49	20	0		0	
50-69	19	4		2	
	55	5		4	
Years of residence in village			0.325		0.203
1 month - 1 year	1	0		0	
1-10 years	3	1		1	
>10 years	51	4		3	
	55			4	
Ethnic					

	Melayu	51	4	a	3	a
	Semai	1	0		0	
	Indian	1	0		0	
	Bugis	1	1		1	
	Indonesian	1	0		0	
		55	5		4	
Education level				0.433		0.406
	No education	3	0		0	
	Primary	16	0		0	
	Secondary	29	4		4	
	Tertiary	7	1		0	
		55	5		4	
Frequency of entering forest				0.205		0.900
	Never	8	2		1	
	Once a year	3	0		0	
	≥1 in a month	31	1		2	
	≥1 in a week	13	2		1	
		55	5		4	

[a]Variable is excluded from further analysis due to insufficient or too small data

Filovirus and henipavirus serologically positive humans reported exposure to cat, cattle, poultry, goat, rabbit, rodent, wild bird, bat and deer. One henipavirus positive human had exposure to non-human primates. No significant finding were found between serological evidence of filovirus and henipavirus and exposure to different animal species (Table 3.20).

Table 3. 20 Serological evidence of filovirus and henipavirus in human based on exposure to different species of domestic animals and wildlife in the past year

		Human, n=55					
No.	**Species**	**Exposure to animal species**	**%**	**Filovirus positive, n=5**	**P-value**	**Henipavirus positive, n=4**	**P-value**
					FE		**FE**
Domestic animal							
1	**Cattle**	50	90.9	4	0.391	3	0.325
2	**Cat**	49	89.1	5	a	4	a
3	**Poultry**	41	74.5	3	0.592	3	1.000
4	**Goat**	27	49.1	2	1.000	2	1.000
5	**Dog**	6	10.9	0	a	0	a
6	**Rabbit**	2	3.6	1	a	1	a
Wildlife							
1	**Rodent (rats/shrews/squirrel)**	34	61.8	5	a	4	a
2	**Wild bird**	19	34.5	2	1.000	2	0.602

3	**Non-human primates**	15	27.3	0	a	1	1.000
4	**Bat**	13	23.6	2	0.582	2	0.234
5	**Reptiles (snake, monitor lizard)**	9	16.4	0	a	0	a
6	**Deer**	7	12.7	1	0.508	1	0.429
7	**Porcupine**	6	10.9	0	a	0	a
8	**Mousedeer**	3	5.5	0	a	0	a
9	**Wild boar**	1	1.8	0	a	0	a
10	**Pangolin**	1	1.8	0	a	0	a
11	**Fox**	1	1.8	0	a	0	a
12	**Binturong**	1	1.8	0	a	0	a
13	**Bear**	1	1.8	0	a	0	a

[a]Variable is excluded from further analysis due to insufficient or too small data

A majority (26.7%, 4/15) filovirus seropositive humans have eaten food damaged by animals. Those that consumed food damaged by animal were 10 times more likely to be seropositive for filovirus compared to those who have not (P=0.017, OR=10.3, 95% CI=1.45, 73.24,). For henipavirus, there was no significant association between seropositivity to the different forms of exposure to various animal species. However, there is a meaningful increase of risk for consuming food damaged by animal (P=0.057) and eating raw or undercooked meat, offal or blood of wildlife (0.097) (Table 3.21).

Table 3. 21 Serological evidence of filovirus and henipavirus in human based on different forms of exposures to animals and wildlife in the past year

		Human, n=55					
	Forms of exposure	**Reported forms of exposure**	**%**	**Filovirus positive, n=5**	**Fischer Exact p-value**	**Henipavirus positive, n=4**	**Fischer Exact p-value**
General exposure to domesticated animals or wildlife							
1	**Handled live animals**	53	96.4	5	1.000	4	1.000
2	**Animal entered house**	49	89.1	5	1.000	4	1.000
3	**Lived with pet**	44	80	3	0.259	4	0.573
4	**Raised animals**	39	70.9	4	1.000	3	1.000
5	**Eaten food damaged by animal**	15	27.3	4	0.017*	3	0.057

6	**Share water source with animals**	14	25.5	0	a	0	a
7	**Scratched or bitten by animal**	13	23.6	1	a	2	0.234
8	**Seen feces in or near human food**	7	12.7	0	a	0	a
Specific exposure to wildlife							
1	**Cook or handle recently slaughtered meat, offal or blood of wildlife**	18	32.7	1	a	1	a
2	**Slaughter or butcher wildlife**	18	32.7	2	1.000	2	0.59
3	**Eaten raw or undercooked meat, offal or blood of wildlife**	8	14.5	1	a	2	0.097
4	**Hunt or trap wildlife**	6	10.9	0	a	0	a
5	**Eaten wildlife of unknown health status**	2	3.6	0	a	0	a
6	**Cut or injured oneself when butchering wildlife**	2	3.6	0	a	0	a
7	**Eaten or shared dead wildlife**	0	0	0	a	0	a
8	**Collect and sell dead wildlife**	0	0	0	a	0	a

[a]Variable is excluded from further analysis due to insufficient or too small data
*Humans that consumed food damaged by animal were more likely to be seropositive for filovirus (P=0.017, OR=10.3, 95% CI=1.45, 73.24).

Most farmers reported have had symptoms of Influenza-like illnesses (ILI) in the past one year. A few filoviruses and henipavirus seropositive farmers also reported other symptoms such as severe acute respiratory infection and fever with diarrhea and vomiting (Table 3.22). However, bivariate analysis between seropositivity and reported symptoms were not performed due to insufficient data for meaningful analysis.

Table 3. 22 Seroprevalence of filovirus and henipavirus in human based on symptoms experienced by farmers in participating cattle farms within the past year

		Human, n=55			
No.	**Symptoms**	**Individuals with reported symptoms**	**%**	**Filovirus positive, n=5**	**Henipavirus positive, n=4**
1	**Fever with headaches and lethargy**	3	5.5	0	0
2	**Haemorrhagic fever**	0	0	0	0
3	**Severe acute respiratory infection (SARI)**	3	5.5	1	0
4	**Influenza-like illnesses (ILI) symptoms**	10	18.2	0	1
5	**Fever with diarrhea and vomiting**	4	7.3	1[a]	1[a]
6	**Fever with skin rash**	1	1.8	0	0

Total	21	2	2

*3/5 filovirus seropositive humans had no symptoms, 2/4 henipavirus seropositive humans had no symptoms. Two seropositive humans for both filovirus and henipavirus had no symptoms.
[a]The same individual

A comparison was made to determine if a particular exposure to animal species influenced reported ILI symptoms within the past year in farmers. ILI was most reported in farmers with exposure to cats and cattle, but no significant differences were observed (Table 3.23)

Table 3. 23 Influenza-like illnesses (ILI) symptoms and exposure to animals and wildlife species in human within the past year

		Human, n=55			
No.	**Species**	**Exposure to animal species**	**%**	**Influenza-like illnesses (ILI) symptoms, n=10**	**Fisher exact P-value**
Domestic animal					
1	**Cattle**	50	90.9	9	1.000
2	**Cat**	49	89.1	10	0.579
3	**Poultry**	41	74.5	6	0.255
4	**Goat**	27	49.1	6	0.340
5	**Dog**	6	10.9	1	1.000
Wildlife					
1	**Rodent (rats/shrews/squirrel)**	34	61.8	7	0.725
2	**Wild bird**	19	34.5	4	0.723
3	**Primate**	15	27.3	4	0.434
4	**Bat**	13	23.6	1	0.421
5	**Reptiles (snake, monitor lizard)**	9	16.4	3	0.340
6	**Deer**	7	12.7	2	0.599
7	**Porcupine**	6	10.9	1	0.702

*Only exposure to animal species with >2 reports were included in this comparison

ILI symptoms reported were also compared to forms of exposure to animals and wildlife within the past year of survey in farmers. All farmers that reported ILI symptoms have reported handling live animals and most of them lived with a pet animal. No significant findings were found between ILI and forms of exposure to animals and wildlife within the past year of survey (Table 3.24).

Table 3. 24 Influenza-like illnesses (ILI) symptoms in human based on forms of exposure to animals and wildlife within the past year

	Human				
	Forms of exposure	**n=55**	**%**	**Influenza-like illnesses (ILI) symptoms, n=10**	**Fisher exact P-value**
General exposure					
1	**Lived with pet**	44	80	8	1.000
2	**Handled live animals**	53	96.4	10	1.000
3	**Raised animals**	39	70.9	6	0.453
4	**Share water source with animals**	14	25.5	2	0.503
5	**Seen feces in or near food**	7	12.7	0	0.328
6	**Animal entered house**	49	89.1	9	1.000
7	**Eaten food damaged by animal**	15	27.3	2	0.710
Exposure to wildlife					
1	**Cook or handle recently slaughtered meat, offal or blood of wildlife**	18	32.7	3	1.000
2	**Eaten raw or undercooked meat, offal or blood of wildlife**	8	14.5	2	0.627
3	**Scratched or bitten by animal**	13	23.6	3	0.685
4	**Slaughter or butcher wildlife**	18	32.7	2	0.470
5	**Hunt or trap wildlife**	6	10.9	0	0.347

*Only exposure to animal species with >2 reports were included in this comparison

3.4 Discussion

In this study, domestic animals and wildlife in and around forest-fringe cattle farms were tested for exposure to the family filoviruses and the genus henipaviruses using Luminex bead-based multiplex microsphere assay. This technology has been developed and widely used for screening of viruses globally (Chowdhury et al., 2014; Dovih et al., 2019; Hayman et al., 2008, 2011; Laing et al., 2018; Peel et al., 2013b) but is not meant for diagnostic determinations. The findings can be used to target areas of surveillance for emerging pathogens but should not be used for policy decisions such as trade, quarantine or culling (MacNeil et al., 2011; Peel et al., 2013a). Serologically positive samples may indicate positivity to the tested virus or to antigenically related viruses. Antigenically related viruses share similar epitopes on its surface antigenic molecule that stimulates binding to antibodies or T cell receptor (Frank, 2020). Phylogenetically closely related viruses has higher epitope amino acid sequence similarity and binding to similar antibodies which result in cross-reactivity of antibodies (Brangel et al., 2018; Marsh et al., 2012; Schuh et al., 2019). Further testing and confirmation is necessary using virus neutralisation test (OIE, 2018).

Filovirus

Study found serological evidence for filoviruses is highest in goat (24/158, 15.2%), sheep (1/11, 9.1%), human (5/55, 9.1%), cattle (5/156, 5.1%) and lowest in bat (5/265, 1.9%). Seropositivity indicates evidence of past exposure to specific viruses

antigenically related to filoviruses. Studies on filoviruses in domestic animals has been scarce. Only dogs and pigs have been demonstrated to be infected with filoviruses (Allela et al., 2005; Chowdhury et al., 2014). In Gabon, 28% of dogs from Ebola epidemic areas were found to be IgG positive for Ebola virus (Allela et al., 2005). Meanwhile, Reston ebolavirus (RESTV) was identified in a spillover event between pigs and human in the Philippines and later in sick pigs in China (Barrette et al., 2009; Pan et al., 2014). To our knowledge, this study is the first to report evidence of exposure to filoviruses in cattle, goat and sheep. This is also the first evidence of exposure of filoviruses in human and dogs beyond Africa.

Highest seropositivity was reported among goats in this study particularly in semi-intensively reared goats which were 4 times more compared to cattle. The reasons for this may need further exploration however we suggest a few reasons for this findings: 1) Goat is known to browse extensively relative to other species (Hofmann, 1989; Rutagwenda et al., 1989) and have the habit of sampling wide ranges of items (edible and non-edible), therefore may increase their risk of exposure to multiple disease agents; 2) Many of the seropositive goats reported in this study were managed semi-intensively to browse freely around village and raised in close proximity where flowering and fruiting trees are abundant which attracts bats such as *C. brachyotis* (V. C. Lim et al., 2017; Tan et al., 1998). Goats are very likely to pick up partially eaten fruits consistent with their feeding behaviour therefore increases risk of contracting multiple disease agents.

Seropositivity of bats in this study is lower than previous reports of bats in this region. Local *P. hypomelanus* reported 10% and 5% reactivity to filoviruses closely related to EBOV and SUDV respectively (Laing et al., 2019). Study from neighbouring country, Singapore reported 9.1% (17/186) in *E. spelaea*, 8.5% (13/153) in *C. brachyotis* and 4.3% (3/70) in *P. lucasi*. Besides *E. spelaea* and *C. brachyotis*, *C. horsfieldii* was seropositive for filoviruses in our study. *Cynopterus sp*. and *E. spelaea* are adaptive species that occupy a range of habitats from orchards, plantations, primary and secondary forests and urban sites (Campbell et al., 2004; Fukuda et al., 2009; Heidenan & Heaney, 1989; Kitchener et al., 1990; V. Lim, 2018; Tan et al., 1998). Livestock farming in rural and sub-rural areas where fruit trees are plenty provides bat roosting and foraging areas (Fukuda et al., 2009). This overlapping spatial distribution of bats and livestock increase risk of viral spillover to livestock and agricultural workers (Mikail et al., 2017). Longitudinal spatial-temporal distribution studies will ascertain the level of such risk (Zhao et al., 2022).

Antibody reactivity to specific filovirus antigen in our study suggests that circulating filovirus in this region is predominantly more closely related to BDBV, SUDV, EBOV, RESTV and less so with BOMV, MARV, and RAVV. Reactivity to filoviruses closely related to EBOV, BDBV and SUDV corresponds to a similar study in Singapore bats (Laing et al., 2018). However, in contrast to that study, we report a considerably high number of reactivity to antigenically related but not identical to RESTV-pig in a *C. brachyotis* and many goats. RESTV is an zoonotic Asiatic filovirus responsible for sporadic cases in non-

human primates, pigs and human (Barrette et al., 2009; CDC, 1993; Demetria et al., 2018; Miranda et al., 1999). Bats may be natural reservoirs for RESTV in the Philippines, Bangladesh and China (Jayme et al., 2015; Olival et al., 2013; Taniguchi et al., 2011; Yang et al., 2017). Finding of our study suggests direct transmission of RESTV-related viruses from bats to goat. Detection of RESTV genome is necessary to confirm spillover.

Henipavirus

Seropositivity to henipaviruses was highest in goats (13.9%), sheep (9.1%), human (7.3%), dog (4.3%), cattle (3.2%) and bat (1.1%). Seropositivity indicates evidence of past exposure to viruses antigenically related to henipaviruses. Our result is supported by several studies. Surveillance of NiV in human outbreak areas near Pteropus sp. bat roosts in Bangladesh using the Luminex platform found that 6.5% (26/400) of cattle and 4.3% (17/400) of goats were positive for henipavirus (Chowdhury et al., 2014). During the Nipah virus outbreak in Malaysia, dogs sampled near infected pig farms had detectable antibodies for Nipah virus (Mills et al., 2009; Yob, Field, et al., 2001). Since 2001, NiV have not been reported in domestic animals and wildlife locally using ELISA (Fikri et al., 2011; Naama et al., 2013). This study is the first report that uses Luminex technology of henipavirus infection in dogs since 1999 and in local cattle, goat and sheep. Reported seropositivity of henipaviruses in goats was high at 13.9% (22/158) particularly in semi-intensively reared goats and was 4 times more as compared to cattle 3.2% (5/156) for hypothetical reasons of risk of exposure to infectious materials as explained before.

Previous survey of bats in Malaysia were mostly focused in the *P. vampyrus* and *P. hypomelanus* species as reservoirs for Hendra virus and Nipah virus with a reported seroprevalence of 1-33% (Rahman et al., 2013; Yob, Jamaluddin, et al., 2001). Those studies used indirect ELISA and serum neutralization test to detect the antibodies against Nipah virus in samples. A recent survey using multiplex serological assay in the local *P. hypomelanus* bats samples found 25% reactivity to NiV (Laing et al., 2019). The reported seroprevalence in our study of 1% (2/108) in *C. brachyotis* and *C. horsfieldii* 2% (1/52) is much less than that of Laing et al. (2109) *Pteropus sp*. surveys and are comparable to the seroprevalence of 4% (2/56) in *C. brachyotis* and 5% (2/38) in *E. spelaea* in a separate study in 1999 (Yob, Field, et al., 2001).

Antibody reactivity to specific henipavirus antigen in our study suggests that circulating henipavirus in this region is predominantly more closely related to GhV, HeV, NiV and less so to CedV and MojV. The MFI values for GhV is high and many cross-reacted with other henipaviruses. Presently, little is known about GhV which was first detected in African bats and is the only African henipavirus (Drexler et al., 2009, 2012). GhV is also antigenically closer to ancestral henipaviruses (Pernet et al., 2014). Thus, reaction to GhV in our study indicates prevalence of henipavirus more antigenically related to the ancestral GhV than the other Asiatic viruses.

Interestingly, our study found two reactions only to MojV with no cross-reactivity in a cattle and dog from the same intensive cattle farm. MojV is a rodent henipavirus discovered in anal specimens of *Rattus flavipectus* in Southern China. This novel virus was identified in the same cave 6 months later where three patients died of respiratory illness of unidentified aetiology. MojV pathogenicity remains to be determined due to the absence of isolate (Wu et al., 2014). Cattle and dog may have acquired MojV closely related virus through contact with rodent excrement. Rodents are probable reservoir hosts or amplifying host for MojV (Cheliout et al., 2021). Increasing the sample size of rodents to detect antibodies and genome of MojV or MojV-like virus will improve chances of detection and determine its circulation locally.

Risk Factors

We detected exposure against viruses antigenically related to filoviruses, EBOV, BDVD and RAVV; and henipaviruses HeV, CedV, MojV in humans. Our study found those that consumed food damaged by animal were more 10 times more likely to be seropositive for filovirus. This is supported by the study by Adjemian et al., amongst febrile tribesmen presented to healthcare facilities in Uganda. Increased risk of filovirus infection was observed in activities such as hunting (37.5 times), touching (5.6 times) or eating wildlife (10.7 times) (Adjemian et al., 2011). Likewise, bushmeat slaughtering or working closely with infected pigs or horse enhanced risk of henipavirus infection (Chua, 2010; Pernet et al., 2014; Rogers et al., 1996). The finding is expected because the both viruses are transmitted via direct contact with body fluids (Amal et al., 2000;

Bausch et al., 2007; O'Sullivan et al., 1997; Saéz et al., 2015; Williamson et al., 1998). Therefore, poor hygiene practices or improper personal protective equipment may expose farmers to infectious materials, especially when handling sick or infected animals, animal waste or carcasses.

Approximately half of the humans sampled (21/55) reported Influenza-like illnesses (ILI), fever with diarrhoea and vomiting, fever with headaches and lethargy and severe acute respiratory infection (SARI) symptoms within the past year of survey. Half of the seropositive human for filovirus and/or henipavirus in our study had reported one of the symptoms in the past year, while the other half did not. The symptoms can be due to several multitude of reasons, unfortunately causal relationship between viral infection and symptoms in our study cannot be ascertain without clinical and longitudinal data. Syndromic surveillance incorporating important pathogens at the family or genus level will improve early detection and appropriate intervention.

The catastrophic Nipah outbreak in 1998 is a turning point for cross-sectoral ministerial integration in managing zoonotic disease and recognition that the country is susceptible to spillover of pathogenic viruses (Chua, 2003). Unfortunately, at present, novel or emerging viral surveillance in animal population is sporadic and scanty; usually linked to specific research endeavour. The ecological concerns and agricultural intensification in Malaysia strongly indicate the need for regular monitoring and

surveillance of livestock and humans working in close proximity to animals especially those that are located in geographic areas with high potential for wildlife viral spillover events. A better understanding on the interaction and viral transmission between human, livestock, other domestic animals and wildlife will inform disease surveillance and control policy to reduce the risk of transmission and prevent undesirable animal and public health consequences.

Limitations

This study was performed on highly targeted populations to increase the chance of viral detection in high-risk populations at the wildlife-livestock-human interface. Therefore, the findings should be interpreted in light of the sampling bias, limited sample size, non-random sampling, cross-reactions between antigenically related viruses and the diversity of uncharacterized filoviruses and henipaviruses that is missed from the test panel. Further characterization of filoviruses and henipaviruses in the region, larger data set, improved diagnostic assays and species-specific positive controls will influence cut-off values and reported sero-detection.

Additionally, infection status in bats fluctuates seasonally and are influenced by reproduction phases, nutritional stress, migrations or contacts with infected animals (Amman et al., 2012; Baker et al., 2014; Hayman, 2015; Plowright et al., 2008; Sohayati et al., 2011; Wang et al., 2013). This infection pattern in bats as a reservoir host would

subsequently impact degree of spillover to other potential hosts like cattle, goats or humans in our case (Amman et al., 2012; Plowright et al., 2017). Antibody post-infection also wax and wanes over time (Boardman et al., 2020; Diallo et al., 2021). Thus, the sero-detection reported in this cross-sectional study only provides information at the time of sampling which may be an underestimation or overestimation and will differ depending on time of sampling.

In addition, maternal antibodies to NiV can last up to 14 months, hence without longitudinal data, some reactivity in our study may be misrepresented (Sohayati et al., 2011). As a result of lack of longitudinal data, serological findings only demonstrate past exposure to filoviruses and henipaviruses. Despite these limitations, our study generated valuable information of filoviruses and henipaviruses in the multiple animal host species to inform future studies. Besides that, research on viral diversity characterization in this region will improve the depth of interpreting serological results.

3.5 Conclusion

This study provides the first evidence of exposure to viruses antigenically related to filoviruses and henipaviruses in domestic animals and humans in Malaysia, as well as viruses antigenically related to henipaviruses in previously unreported bat species. Results from our study supports more extensive and longitudinal viral surveillance in these populations to enhance disease preparedness and improve our understanding on

the viral distribution, diversity and potential spillover at the human-livestock-wildlife interface.

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Appendix A

Study design

First step involved mapping to identify the sub districts for phone and ground survey. In the mapping process, forest distribution in Peninsular Malaysia and forest complexes which overlapped the 3 targeted districts in our study were first identified using Google Earth (version 7.1.2.2600). Out of 8 forest complexes in Peninsular Malaysia, the forest complexes which overlapped with our targeted districts were the Bintang Hijau Forest Complex (FC2) which overlapped with Kuala Kangsar, the Main Range Forest Complex (FC3) which overlapped with Kuala Kangsar, Gua Musang and Lipis, and the Greater Taman Negara Forest Complex (FC4) which overlapped with Gua Musang and Lipis (Figure 3.2).

Secondly, the subdistricts which overlapped the 3 forest complexes were selected for the next step. Since FC2 overlaps with Kuala Kangsar, the subdistricts from this region of Kuala Kangsar was selected. They were Chegar Galah, Kampung Buaya, Kota Lama Kiri, Kota Lama Kanan, Lubuk Merbau, Senggang, Sungai Siput and Sayong. The east side of Gua Musang which overlapped with FC4 were selected because there were more subdistricts on the overlapping forest region. The subdistricts were Relai, Ketil, Limau Kasturi, Kuala Sungai and partial of Renok, Pulai and Bandar Gua Musang. While the west side of Lipis which overlapped FC3 were selected. The subdistricts were Ulu Jelai and partial of Batu Yon, Telang and Penjom.

Next, the list of sub-districts selected were compared to a list of cattle farm in Peninsular Malaysia. The farm list registered 339 farms from the three districts of interest. The subdistrict/s with the most number of farms and with farm location likely to be less than 5km from the fringe of forests were identified. This was done by using forest mapping data from state governments and marking positions of potential farms in Google Earth (version 7.1.2.2600) and measuring the farm distance to the nearest forest. The subdistrict/s identified were Chegar Galah in Kuala Kangsar, Relai and Renok in Gua Musang and Ulu Jelai in Lipis.

A phone survey was then conducted to contact the farmers in the selected sub-districts to obtain basic information about their production system, grazing practices, number of livestock and type of production. Only 50% of the farmers were contactable out of which only 19 farms had more than 30 cattle. Subsequently, a field survey was conducted to meet farmers in the selected sub districts from the three states. New farmers were enrolled using the snowball method where researchers were introduced to the new farmers by the locals or other interviewed farmers. At the initial discussion information of the farm such as production system, number of livestock, grazing and feeding practices, herd health, presence of wildlife in and around farm, estimated distance to nearest forest, and the willingness to participate in the research were noted. In cases where farmers were absent, information were obtained from their family members and consent was obtained to contact farmers through call at a later date. These conversations mainly occurred in their house, coffee shops or in the farm. Due to insufficient farms that meet the criteria of study in selected sub districts, other neighbouring sub districts in the same selected forest complex were also surveyed.

At the end of the field survey, a new list of farms was compiled with more than 60 potential farmers. A total of 11 farms were selected from the 60 as suitable for further ground-truthing. Ground-truthing and recruitment of farms were performed with a field manager and ranger from EcoHealth Alliance. Appointments with farmers were made in advance to meet and visit the farms. Ground-truthing is a process where ground information on the farm was obtained, formal farmers' participation was sought and sampling logistic requirements were noted. Information gathered comprised of: 1) the GPS location of farm and GPS location of the nearest forest to calculate the distance between farm and forest to confirm that farm is within 5km to forest, 2) observation of farm setting, size and periphery of farm and availability of facilities for handling and sampling of animals, processing of samples and storage space, 3) farm production system and type, number of animals, sex and age of animal, feeding and grazing schedule, 4) number of farm personnel, 5) presence of other domestic animals and wildlife, 6) pictures of farms, and 7) interest and willingness to participate in research.

At the end of these process, six farms were recruited from Kuala Kangsar, Perak and Gua Musang, Kelantan for the study. Only 2 districts were sampled due to the unavailability of collaborating district health officers from Lipis, Pahang. Sample size of farms is small and purposely selected to only include those with the set criteria listed for logistical ease and enhance viral detection probability. Due to the highly targeted nature of the design and sampling, the findings from this study may not be inferred to other farms, animals or humans in Malaysia.

Appendix B

Sampling of cattle and other domestic animals

Species	Restraining method	Blood collection
Cattle, goat, sheep	Cattle were first herded into an enclosure or chute system. For farms that did not have any chutes, chutes were built by field sampling team to ensure ease and safety of both animals and handlers during sampling. Fixing and modifying existing fences or chutes were also done to facilitate with herding and restraining of the cattle.	An 18G vacutainer needle attached to a vacutainer holder was used with needle inserted at a 45° angle for blood withdrawal from the jugular vein, whereas a needle was inserted perpendicularly to skin surface midway along the coccygeal vertebra. An approximate 3-5ml of blood was withdrawn and pressure was placed on venepuncture site until bleeding stops. Blood collected was distributed equally in a BD Vacutainer® serum tube (red top) and an EDTA tube (purple top).
Dog	Dogs were manually restrained with assistance from the owner. A gauze tie around the muzzle and a towel were used to cover eyes if necessary.	Blood collection was performed from the cephalic vein, jugular vein, femoral vein, lateral or medial saphenous vein using a 21G needle and 3ml syringe. BD Vacutainer® serum tubes (red top) and EDTA tube (purple top) were used to collect 3-5ml of blood. Blood volume is limited to a maximum of 1% body weight. Blood collected was distributed equally in a BD Vacutainer® serum tube (red top) and an EDTA tube (purple top).
Rabbit	Rabbit was manually restrained holding rabbit on its side. Towel was used to cover the eyes if necessary.	Rear leg was extended while putting pressure on the stifle area. Venepuncture site was disinfected with alcohol before withdrawal. Blood was taken withdrawal was performed from the lateral saphenous vein using a 23G needle and 3ml syringe to collect 3-5ml of blood. Blood volume is limited to a maximum of 1% body weight. Blood collected was distributed equally in a BD Vacutainer® serum tube (red top) and an EDTA tube (purple top).

Appendix C

Sampling of wildlife

Species	Trapping	Restraining method	Blood collection	Species identification
Bat	Bats were trapped using mist nets. Mist nets used in our study were 6m x 3m in dimension, size no. 4, black, nylon material mist nets with 3-pouch construction. Mist nets were looped at the top end of an extendable aluminium pole which enables raising the net for capture and lowering the net for extraction of captured bats for sampling. Poles for the mist nets were typically secured by pressing it into the ground. The first pole was placed first followed by looping of mist net to the top of the pole and careful unravelling of the mist nets without touching the ground to prevent entangling of debris or leaves while walking to the other end where the second pole is located. Then, both poles were raised carefully by 2 persons on each pole by ensuring proper tension and pouches are formed for the bats to fall into. A total of 15 mist nets were employed to suitable sites with priority given to the participating cattle farm and its surrounding locations. The mist nets were raised at 7.30pm at sunset and left opened until 12am for trapping to occur (Figure 3.5). Bat nets were repositioned to new locations when recapture rates were high or captures were absent. The GPS coordinates for each mist nets were recorded.	Prior to taking bat out from the cloth bag, the bag is weighed with a Pesola digital hanging scale. After bat is taken out, the weight of the bag re-measured and subtracted from previous total to obtain the weight of the bat. The weight is important for calculating the maximum of blood (1% of bat body weight) that can be withdrawn. Bat is removed from the cloth bag and manually restrained for sampling using leather gloves. This was done while having a handler manually restraining a bat with one hand with the wing gently extended with another hand until a 90° angle.	Venipuncture site was swab with 70% alcohol swab prior to puncture. The droplet of blood to form on the surface of skin was then collected with a pipette tip and pipette (Figure 3.8). A cotton ball was used to apply pressure on venepuncture site until bleeding ceases (approximately 1 minute). The droplet of blood was expelled into a cryovial tube containing phosphate buffered saline (PBS). In bats that are >100g, a non-heparinized syringe was used to collect blood from the cephalic vein, brachial vein or saphenous vein. Blood is then placed in a serum vacutainer tube containing serum-clotting factor and centrifuge. A sterile pipette tip and pipette was used to extract serum and placed into 2 cryovials of a minimum of 60µl if volume. In bats that are <100g, the tip of a sterile 25G needle was used to puncture the brachial or propatagial vein (C. Smith et al., 2010). A dilution of 1:10 PBS is prepared by placing 10µl blood (1 drop) into 90µl PBS. The serum dilution is then centrifuged and the clear supernatant containing the serum dilution is transferred into a new cryovial tube.	Morphometric measurements such as, forearm or radius length (from elbow to wrist), ear length (from distal tip of ear to middle of base of ear), body length (from tip of nose to base of tail when bat is placed on a ventral recumbency), hind foot length (from ankle to toe) and tail length (from base of body to tip of tail) were measured in millimeters using a ruler or a caliper. Photographs of the full body, anterior face, lateral face and view of parted pelage on ventrum and dorsum were taken for the purpose of identification.

	Mist nets were checked for bats every 30 mins to an hour. Captured bats were extracted from the nets carefully using at least one leather gloves on one hand and with the use of forceps or small stick to move the netting or bat mandibles. In cases where bats were found badly entangled in the net, a small scissor was used to cut the net to release the bats. Bats were then placed into a porous cotton bag with a draw-string mouth and taken to a sampling processing site where sampling was performed immediately or hung on a rope until further handling. Net is erected after bats were released from the mist nets. Approximately 20-30 bats were sampled in a night. Sampling procedure starts from when the first bat is captured until sampling is done. No bats should be kept for more than 6 hours.		Cryovial tubes is labelled appropriately and placed in MVE Doble 34 liquid nitrogen tanks in the field and later stored at -80° freezer in the lab according to proper cold chain methods.	
Rodent and other small mammal	A total of 100 traps were used to trap rodents which encompasses 45 Sherman traps (12L x 3W x 3.75H), 45 small Tomahawk (19L x 6W x 6H) and 10 large Tomahawk traps (32L x 10W x 12H). Sherman trap is made of aluminum with a galvanized door and treadle for small mammals. The Tomahawk trap is a collapsible trap made from gauge galvanized wire mesh. Two sizes of Tomahawk trap were used to capture smaller mammals such as mice, rats, tree shrews and larger mammals, such as civet. A hundred traps were divided into 10 transect lines, each line consisted of 10 traps. Odd number transect lines had 1 big Tomahawk trap, 4 small Tomahawk traps	Prior to taking animal out from the cloth bag, the bag is weighed with a Pesola digital hanging scale. After animal is removed from the bag, the weight of the bag was re-measured and subtracted from previous total to obtain the weight of animal. The weight is used for calculating the maximum of blood (1% of bat body weight) that can be withdrawn. Gas anaesthesia was used for induction (5% isoflurane and 0.5% O2) and maintained (2% isoflurane and 0.2% O2) and adjusted according to depth of anaesthesia and vital signs. Small rodents like	Blood samples were collected from the lateral tail vein, ventral tail artery or lateral saphenous vein or lateral saphenous vein in rodents and other small mammals. Puncture site was swab with 70% alcohol swab prior to puncture. In the lateral tail venepuncture, animal is placed on ventral recumbency. The tail is immobilized with a non-dominant hand and rotated ¼ to access the lateral tail vein. Veins are located on either side of the tail which is quite superficial. Pressure is then placed proximal to the venepuncture site occlude the veins for venepuncture. Needle for blood collection is inserted	Morphometric measurements such as body length, length of ear and length of hind foot and tail length were taken for purpose of identification. Identification of animal species was performed by an experienced rangers and field veterinarian. Guide books were also used as reference for identification

	and 5 Sherman traps, whereas even number transect lines had 1 big Tomahawk trap, 5 small Tomahawk traps and 5 Sherman traps. The Tomahawk and Sherman traps were placed alternately in each line. Traps in the farms were placed along fences for the paddocks or periphery near the forests in a transect line and/or in strategic positions in and around the farmhouse or store room. A red and white ribbon was used to mark the beginning of each trap or at every trap site in dense bushy areas. GPS coordinates for the front and ends of the first and last transect line were taken. Forest areas were selected based on its proximity to the participating cattle farm, topography and canopy cover. Site selected should have no flooding risk and have sufficient canopy to avoid direct sunshine and heat stress on the trapped animals. Materials such as leaves and branches are placed above and around the trap to provide shelter and camouflage. The traps may be placed on the ground, or secured on trees or fences with raffia string, away from human activity disturbance and livestock damage (Figure 3.10). Areas with stream or water source were also chosen to improve capture rate. Roasted oil palm fruits were used as baits and occasionally roasted coconuts, jackfruit, cempedak fruit (*Artocapus integer*), bananas or grapes when capture rates were low (Figure 3.11). Oil palm fruit was the preferred option as it does not attract insects. Traps were placed at the same sites for a minimum of 3 consecutive nights to improve capture rates which may be influenced by	rats and tree shrews were secured by scuffing dorsal to neck region with thumb and forefinger carefully without restricting airway and cause cyanosis to be transferred into modified container with an inlet for anaesthetic gas for induction. Once animal is motionless and unresponsive, it is taken out and placed on the table. A modified tube for appropriate fitting of the face was used for maintenance of anaesthesia. In small mammal species like civets, a gas mask was used for induction without completely removing animal out from the bag to reduce stress. Upon successful induction, it is removed from bag and placed on table with the gas mask attached. Monitoring of animal was performed by a veterinarian throughout during handling to observe breathing rate, colour of ears, nose and oral cavity and withdrawal reflex. Isoflurane is turned off if an animal becomes cyanotic. A sterile lubricant is placed into animal's eyes to prevent corneal drying or scratches during sedation.	one third down the length of tail. In ventral tail artery blood collection, animal is placed on dorsal recumbency and vein is located on the centrally in the tail and pressure is place proximal to the venepuncture site occlude the artery for venepuncture. Needle for blood collection is inserted one third down the length of tail. In lateral saphenous venepuncture, animal is placed on lateral recumbency while an assistant stretch out the rear legs to its natural position and place pressure on the upper leg to occlude blood flow for visualization of vein and venepuncture site. A 25G or 27G needle and 1ml syringe is used to withdraw blood from lateral tail vein, ventral tail artery or lateral saphenous vein. Attempts to withdraw blood from animal is minimized to no more than three needle sticks. In cases where no blood is aspirated but a drop of blood is formed on the surface of the skin, a pipette tip and pipette is used to collect it. A cotton ball is used to apply pressure on venepuncture site until bleeding ceases. Blood collected is placed into 1:10 PBS and spin to collect serum. The serum dilution is then centrifuged and the clear supernatant containing the serum dilution is transferred into a new cryovial tube. Cryovial tubes is labelled appropriately and placed in MVE Doble 34 liquid nitrogen tanks in the field and later stored at -80° freezer in the lab according to proper cold chain	while in the field (Francis, 2008; Payne et al., 1985; Phillipps & Phillipps, 2016). Photographs of the full body dorsally and laterally, anterior face and lateral face or any special marking or characteristics were taken for future reference.

	weather conditions and rodents' neophobic behaviour (Aplin et al., 2003). Rodent traps were set and opened at 6pm, left overnight and checked at 8am the next day. If adverse weather such as heavy rain is expected, traps will not be opened for that night. Trapped small mammal were gently transferred into a draw-string cloth bag by covering the bag over the opening of the trap and slightly tilting the trap downwards. Animals that were too stressed will be released, while animals found to be injured or weak will be brought back to sampling base for veterinary treatment.		methods. After blood collection, fluid volume replacement is given subcutaneously using Lactated Ringer's or 0.9% NaCL solution to avoid hypovolemic shock. A 25G or 27G needle is used to administer 1ml in small rodents (~25g) and 3-5ml in larger rodents (~200g) fluid replacement under the loose skin on the dorsal neck or flank. Gentle pressure is placed to ensure no leaking is seen from injection site. Marking on sampled rodent or small mammal is done by clipping 3-5mm of hair at the right ventral thigh.	
Wild boar	Wild boar was trapped according to trapping protocol described for rodent and other small mammals. It is transported to sampling base in the cage for sampling. *Wild boar trapping was incidental in our study.	Prior to taking animal out from the cage, the cage is weighed with a Pesola digital hanging scale. After animal is removed from the cage, the weight of the cage was re-measured and subtracted from previous total to obtain the weight of animal. A portable anaesthetic chamber was made for the induction procedure by placing a plastic large enough to cover entire cage with an anaesthetic gas tubing input to the opening of the plastic. Once animal is motionless and unresponsive, it is quickly taken out of the cage and placed on the table for sampling with gas mask fitted on the muzzle. Wild boar was induced with 5% isoflurane and 0.5% O2 and maintained with 2% isoflurane and 0.2% O2 and adjusted according to depth of anaesthesia and vital signs. A sterile	Blood sample is collected from the anterior vena cava using a 21G needle and 3ml syringe while animal is placed on dorsal recumbency. Venepuncture site was swab with 70% alcohol swab prior to puncture. Blood volume is limited to a maximum of 1% body weight. A cotton ball is used to apply pressure on venepuncture site until bleeding ceases. Blood collected is distributed equally in a BD Vacutainer® serum tube (red top) and an EDTA tube (purple top). Blood in BD Vacutainer® serum tubes were left to clot for serum collection within 1 hour. After clotting is form, serum tubes were centrifuged for 15 minutes and 1 ml of serum was transferred into 2 0.5ml cryovial tubes. Cryovial tubes were labelled appropriately. Samples were then placed in MVE Doble 34 liquid nitrogen tanks in the field and later stored at -80° freezer in the lab according to	Morphometric measurements such as body length, length of ear and length of hind foot and tail length were taken for purpose of identification. Identification of animal species was performed by an experienced rangers and field veterinarian. Guide books were also used as reference for identification while in the field (Francis, 2008; Payne et al., 1985; Phillipps & Phillipps, 2016). Photographs of the

		lubricant is placed into animal's eyes to prevent corneal drying or scratches during sedation. Monitoring of animal was performed by a veterinarian throughout during handling to observe heart rate, breathing rate, mucous membrane colour and withdrawal reflex. Isoflurane is turned off immediately if animal becomes cyanotic. A basic physical examination was performed by veterinarian to access health status.	proper cold chain methods. After blood collection, fluid replacement was given subcutaneously using Lactated Ringer's or 0.9% NaCL solution. In young pigs, <2ml were given at the axillary area caudal to elbow or in the inguinal region and in adults, <3ml were given under the loose skin behind the ear (Office of the University Veterinarian, 2017c). Animal were monitored in a small cage until fully recovered from anaesthesia before release back at place of capture.	full body dorsally and laterally, anterior face and lateral face or any special marking or characteristics were taken for future reference.
Non-human primate	Non-human primates (NHP) were in our study were kept as pets hence trapping was not required.	NHP were anaesthetized and sampled upon the agreement of its owner according to established protocols (Hoffmann et al., 2010; PREDICT One Health Consortium, 2016c). Anaesthesia drug were the combination of: 1) 4mg/kg Ketamine and 0.15mg/kg Xylazine with Yohimbine 1:1 as reversal agent for Xylazine, or 2) Tiletamine-Zolazepam, Zoletil® 3-5mg/kg in more aggressive primates. A dart syringe and blowpipe was used to deliver anaesthetic drug intra-muscularly by an experienced and trained ranger. The dart is targeted to muscles of the thigh, shoulder or upper arm. Immobilization is recognized by droopy eyelids, reduced movement, leaning against wall or lying on floor and unreactive to stimuli. Anaesthetic monitoring was performed by a veterinarian. Respiratory rate, heart rate, pulse	NHP is placed in a supine position. Blood is withdrawn from femoral vein located in the inguinal region lateral and parallel to the femoral artery. Venepuncture site is swabbed with 70% alcohol swab prior to puncture with a 21G needle and 3ml syringe. Blood volume is limited to a maximum of 1% estimated body weight. Firm pressure with a cotton ball is placed on venepuncture site for 1 minute to prevent hematoma formation. Blood collected was distributed equally in a BD Vacutainer® serum tube (red top) and an EDTA tube (purple top). All blood tubes were labelled appropriately and placed in cooler boxes and transported back to base station. Blood in BD Vacutainer® serum tubes were left to clot for serum collection within 1 hour. After clotting is form, serum tubes were centrifuged for 15 minutes and 1 ml of serum was transferred into 2 0.5ml cryovial tubes.	Photographs of the anterior and ventral view of the full body, anterior and lateral face, upper and lower dentition and genitalia were taken for future reference.

		quality, mucous membrane colour and muscle tone were taken and recorded. After immobilization, basic physical examination was performed. A sterile lubricant is placed into animal's eyes to prevent corneal drying or scratches during sedation.	Cryovial tubes were labelled appropriately. Samples were then placed in MVE Doble 34 liquid nitrogen tanks in the field and later stored at -80° freezer in the lab according to proper cold chain methods.	

Appendix D

Sampling of human

Species	Blood collection
Human	Participants were asked to sit comfortably on a chair with their arm for venepuncture on the table. A quick release tourniquet is placed approximately 4 inches above intended venepuncture site. Blood is withdrawn from the median cubital vein on the upper limb or metacarpal plexus or the dorsal venous arch on the dorsal surface of the hand. Venepuncture site is swabbed with 70% alcohol swab prior to puncture with a 21G needle and 3ml syringe. Tourniquet is released upon filling of the syringe. A sterile gauze pad is placed over venepuncture site and needle is removed in a quick careful motion. Venepuncture site should be observed to ensure bleeding has stopped, followed by a bandage over site. Blood collected is transferred equally in a BD Vacutainer® serum tube (red top) and an EDTA tube (purple top). All blood tubes were labelled appropriately and placed in cooler boxes and transported back to base station. Blood in BD Vacutainer® serum tubes were left to clot for serum collection within 1 hour. After clot is form, serum tubes were centrifuged for 15 minutes at 1300 r.p.m and serum was transferred 0.5ml cryovial tubes to produce at least a minimum of two aliquots. Cryovial tubes were labelled appropriately. Samples were then placed in MVE Doble 34 liquid nitrogen tanks in the field and later stored at -80° freezer in the lab according to proper cold chain methods.

Appendix E

Table.. Median fluorescence intensity values of positive filovirus reactive samples across species using Luminex bead-based multiplex microsphere assay

ID	Farm	EBOV	BDBV	BOMV	SUDV	RESTVm	RESTVp	MARV	RAVV
Human									
PMH1533	1	**4928**	828	707	166	1876	2081	-144	-189
PMH1553	2	**11867***	1838	1025	288	1635	1906	-43	-38
PMH1563	4	1649	**24059****	861	335	374	304	2946	**13819***
PMH1568	4	2053	**7949**	1686	570	625	218	261	1440
PMH1583	6	1948	**13891***	938	287	485	247	58	725
Bat									
PMW1832									
C. brachyotis	2	518	**6212**	239	139	291	473	49	269
PMW1927									
C. brachyotis	3	3567	2027	**5284**	**7361**	1448	**8475**	3280	719
PMW1877									
C. horsfieldii	2	458	**14168***	282	64	312	995	1219	1774
PMW1878									
E. spelaea	2	**4382**	63	287	65	384	1085	-132	-145
PMW1944									
E. spelaea	5	**10784***	**9312**	629	551	327	626	-106	-130
Cattle									
PML707	1	**8916**	3971	1475	**5527**	**4564**	1573	-97	-57
PML716	1	**4535**	2810	439	2526	819	1441	-95	-117
PML766	2	2890	**4596**	475	1266	1263	882	-63	127
PML769	2	364	**4976**	329	19	185	11	-53	-65
PML832	3	766	**4187**	422	258	242	159	-17	-5
PML839	3	**10205***	**7417**	2369	**9159**	**8851**	571	176	247
PML896	4	301	**4927**	236	20	122	-22	365	3190
PML927	5	536	**4246**	437	190	400	1019	-76	-78
Goat									
PML784	2	659	**8374**	344	167	524	1160	-47	86
PML798	2	328	**6738**	281	46	174	167	-5	2825
PML799	2	511	**5932**	431	206	500	1269	562	312
PML801	2	1181	1396	2042	**4794**	2508	**4861**	1673	270
PML803	2	589	**10199***	266	8	179	-7	353	**6030**
PML811	2	983	**21882****	1815	**5116**	3484	3975	2336	**12518***
PML812	2	1318	941	3150	**6679**	775	3233	568	-15
PML845	3	931	839	1301	3214	390	**4183**	889	8
PML853	3	1596	1065	1320	**4544**	761	**4796**	1209	82
PML856	3	287	**8040**	101	79	270	402	-10	182
PML861	3	3150	**4250**	3971	**7143**	1170	**8761**	3248	502
PML863	3	2460	4011	3456	**7519**	754	**7310**	2342	519
PML865	3	315	**4938**	243	86	271	542	-5	35
PML872	3	1385	2223	1364	3390	668	**5054**	1219	98
PML873	3	2378	1872	2656	**4850**	632	**6064**	1830	259
PML942	4	1737	**4293**	1756	4161	727	**4320**	1000	-2
PML945	4	456	**9440**	532	646	320	1250	979	**4855**
PML960	4	1594	**12756***	1591	3703	949	**4875**	1722	3466
PML963	4	1802	1362	2447	**5746**	556	**7375**	2003	206
PML965	4	1254	1310	2361	**5226**	1292	**5669**	2302	547
PML1032	6	1378	3742	2163	**5271**	1145	**5353**	2000	1199
PML1034	6	1735	**6712**	3373	**6929**	1597	**7037**	3120	2773
PML1051	6	1042	**6853**	1942	**5067**	1510	**5819**	2245	338
PML1053	6	411	**8499**	169	117	155	656	-84	-37
Sheep									

PML1030	6	1977	**9834**	**5226**	**10890***	2178	**8059**	**4317**	2572
Dog									
PML755	1	**7364**	**5429**	347	311	457	1163	-83	-85
PML797	2	1861	**7561**	618	887	1238	1776	372	453

Boldface indicates seropositive reactivity.

*indicates MFI of >10000; **indicates MFI of >20000.

EBOV, Ebola virus; BDBV, Bundibugyo virus; BOMV, Bombali virus; SUDV, Sudan virus; MLAV, Mengla virus; RESTVm, Reston virus monkey isolate; RESTVp, Reston virus pig isolate; LLOV, Lloviu virus; MARV, Marburg virus; RAVV, Ravn virus

Appendix F

Table Median fluorescence intensity values of positive henipavirus reactive samples across species using Luminex bead-based multiplex microsphere assay

ID	Farm	HeV	NiV	CedPV	MojPV	GHV
Human						
PMH1546	2	4366	530	3845	**9334**	**11221***
PMH1563	4	**23519****	967	**23354****	**24257****	**24689****
PMH1568	4	3133	468	1826	**7264**	**6980**
PMH1583	6	1643	134	1477	**7170**	**11434***
Bat						
PMW1832 *C. brachyotis*	2	2497	1058	1515	103	**5429**
PMW1927 *C. brachyotis*	3	**10478***	**12117***	1593	128	**10509***
PMW1877 *C. horsfieldii*	2	**6797**	**4776**	**5113**	600	**10246***
Cattle						
PML717	1	1420	115	1144	1111	**5000**
PML832	3	580	173	178	78	**5354**
PML896	4	4294	782	3072	2262	**6813**
PML1002	6	**17641***	**10580***	**16712***	**11175***	**20100****
PML1011	6	-33	-54	-65	**13791***	40
Goat						
PML772	2	792	329	284	72	**6149**
PML784	2	1401	1437	580	758	**7249**
PML798	2	2372	1106	1080	1033	**8182**
PML801	2	**12778***	**11762***	**6234**	838	**9525**
PML803	2	**5054**	-30	3521	1668	**11123***
PML811	2	**17528***	**10245***	**13342***	2063	**19905***
PML812	2	**9483**	**6354**	3539	-89	4477
PML853	3	**5459**	**4828**	946	-9	4035
PML856	3	679	-22	408	-53	**5731**
PML861	3	**10255***	**12723***	1671	-95	**11291***
PML863	3	**12061***	**11660***	2537	-37	**10369***
PML865	3	3865	1880	1781	242	**7795**
PML872	3	**5202**	**6086**	910	-56	**5644**
PML873	3	**8066**	**10054**	855	-70	**8225**
PML942	4	**6240**	**6168**	1279	-101	**4851**
PML945	4	**9464**	3086	**6339**	1552	**13133***
PML960	4	**9464**	**8401**	3091	1869	**13062***
PML963	4	**8356**	**6726**	1630	-124	**10400***
PML965	4	**8376**	**10213***	1557	1387	**10302***
PML1032	6	**12918***	**10462***	**7191**	292	**10879***
PML1034	6	**15985***	**13379***	**4834**	1034	**15434***
PML1051	6	**8595**	**8953**	1104	3206	**9458**
Sheep						
PML1030	6	**17401***	**16075***	**9894**	575	**17178***
Dog						
PML1045	6	70	89	-38	**5042**	24

Boldface indicates seropositive reactivity.

*indicates MFI of >10000; **indicates MFI of >20000.

HeV, Hendra virus; NiV, Nipah virus; CedV, Cedar virus; MojV, Mojiang virus; GhV, Ghana virus.

From: C Yong on behalf of C Yong <gem_cysb@hotmail.com>
To: Laing, Eric; Christopher Broder
Cc: Jon Epstein; latiffah hassan; Broder, Christopher
Subject: Re: Thesis review - cattle farm chapter 3
Date: Thursday, March 2, 2023 4:51:47 PM
Attachments: Chap 3 Serology_draft8.docx
Table on prev of neighbouring countries.docx
Ag reactions for DTRA livestock and archived pigs.xlsx

Hi Eric,

Thank you for going through the chapter early. I appreciate the many great suggestions and helpful feedback.

Kindly see attached for the updated draft and attachment for the table of seroprevalence of filovirus and henipavirus in neighbouring countries mentioned in 3.2.3 Sample Size.

I'm also reattaching the breakdown of antigen reaction which I sent to you and Jon last time for Dr Broder. @Christopher Broder, happy to hear from you.

Thank you for guiding me,
Chyna

From: Laing, Eric <eric.laing@usuhs.edu>
Sent: Tuesday, February 28, 2023 9:05 AM
To: Broder, Christopher <christopher.broder@usuhs.edu>; C Yong <gem_cysb@hotmail.com>
Cc: Jon Epstein <epstein@ecohealthalliance.org>; latiffah hassan <tiffah_hassan@yahoo.com>
Subject: Re: Thesis review - cattle farm chapter 3

Hi Chyna,

I'm looping in Dr. Broder for additional comments.

Eric

On Mon, Feb 27, 2023 at 11:38 AM Laing, Eric <eric.laing@usuhs.edu> wrote:

> Hi Chyna,
>
> I enjoyed reading the chapter and seeing the totality of your thesis work. Excellent work! I've made some edits, suggestions, and comments. Feel free to send back updates or questions if you need more clarification.
>
> - 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 Sun, Feb 26, 2023 at 6:46 PM C Yong <gem_cysb@hotmail.com> wrote:

> Hi Eric,
>
> Thank you so much.
>
> Chyna
>
> ---
>
> **From:** Laing, Eric <eric.laing@usuhs.edu>
> **Sent:** Monday, February 27, 2023 6:54:30 AM
> **To:** C Yong <gem_cysb@hotmail.com>
> **Cc:** Jon Epstein <epstein@ecohealthalliance.org>; latiffah hassan <tiffah_hassan@yahoo.com>
> **Subject:** Re: Thesis review - cattle farm chapter 3
>
> Hi Chyna,
>
> I'll finish by today.
>
> Eric
>
> On Mon, Feb 27, 2023 at 2:40 AM C Yong <gem_cysb@hotmail.com> wrote:
>
> > Hi Eric,
> >
> > The due date is 13 March. I think after 4th March is still doable. But if you have time to scheme through the results section and point out any problems before the 4th would be awesome too.
> >
> > Safe travels!
> >
> > Thanks,
> > Chyna
> >
> > ---
> >
> > **From:** Laing, Eric <eric.laing@usuhs.edu>
> > **Sent:** Thursday, February 23, 2023 1:28 PM
> > **To:** C Yong <gem_cysb@hotmail.com>

Cc: Jon Epstein <epstein@ecohealthalliance.org>; latiffah hassan <tiffah_hassan@yahoo.com>
Subject: Re: Thesis review - cattle farm chapter 3

Hi Chyna,

Email received. Can I have a few days to review this, what's a due date? I'm traveling now and time is limited thru March 4. But if this is critical before then I can do my best.

- 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 Wed, Feb 22, 2023 at 6:01 PM C Yong <gem_cysb@hotmail.com> wrote:

> Dear Jon and Eric,
>
> This version is a newer version with more details on the positive & negative controls + results of USU's naive livestock sera.
>
> If you have not reviewed the previous version, please review this one.
>
> Looking forward to hearing from you.
>
> Thanks!
> Chyna
>
> ---
>
> **From:** C Yong
> **Sent:** Monday, February 20, 2023 7:07 AM
> **To:** Jon Epstein <epstein@ecohealthalliance.org>; Eric Laing <eric.laing@usuhs.edu>
> **Cc:** latiffah hassan <tiffah_hassan@yahoo.com>
> **Subject:** Thesis review - cattle farm chapter 3
>
> Hi Jon and Eric,
>
> Hope the both of you are well.

> > > Attached is a chapter from my thesis that covers the cattle farm project + its Appendix for your review.
> > > Please ignore the green highlights as those are for me to double-check when I compile the thesis.
> > >
> > > @Eric, should I add the MFI baseline for the livestock samples that you've conducted in USU?
> > >
> > > Appreciate if you could review it by end of this month if possible. Will send the archived pig chapter as soon as I finalize it with Prof Latiffah.
> > >
> > > Thank you.
> > >
> > > Kind regards,
> > > Chyna

--
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
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--
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office: (301) 295-3419
lab: (301) 295-9618

eric.laing@usuhs.edu

RESEARCH & RELATED BUDGET - Budget Period 1

OMB Number: 4040-0001
Expiration Date: 12/31/2022

UEI: UYLKBRENAPG5 **Enter name of Organization:** The Henry M. Jackson Fdn. for the Adv'mnt of Mil. Med., Inc.

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 1** **Start Date:** 10/01/2023 **End Date:** 09/30/2024

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Eric		Laing	Ph.D.		0.80			0.00	0.00	0.00

Project Role: Co-Investigator

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	Scientist I	3.00			18,500.00	5,443.00	23,943.00
1	Research Assistant	4.00			13,733.00	4,040.00	17,773.00

2 **Total Number Other Personnel**

Total Other Personnel 41,716.00

Total Salary, Wages and Fringe Benefits (A+B) 41,716.00

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	
Total Travel Cost	

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	30,631.00
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.		
9.		
10.		
11.		
12.		
13.		
14.		
15.		
16.		
17.		
	Total Other Direct Costs	30,631.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	72,347.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
FY23 Onsite Overhead	36.74	72,347.00	26,580.00
FY23 G&A Companywide	16.70	98,927.00	16,521.00
Contract Fee	5.00	115,448.00	5,772.00

Total Indirect Costs 48,873.00

Cognizant Federal Agency
(Agency Name, POC Name, and POC Phone Number)

USAMRAA, Jennifer C. Jackson, 301-619-2054

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	121,220.00

J. Fee

	Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	121,220.00

L. Budget Justification

(Only attach one file.)

Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Budget Period 2

OMB Number: 4040-0001
Expiration Date: 12/31/2022

UEI: UYLKBRENAPG5 **Enter name of Organization:** The Henry M. Jackson Fdn. for the Adv'mnt of Mil. Med., Inc.

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 2** **Start Date:** 10/01/2024 **End Date:** 09/30/2025

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Eric		Laing	Ph.D.		0.80			0.00	0.00	0.00

Project Role: Co-Investigator

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	Scientist I	2.00			12,333.00	3,628.00	15,961.00
1	Research Associate/Scientific Project Coordinator	2.00			13,000.00	3,825.00	16,825.00

2 **Total Number Other Personnel**

Total Other Personnel 32,786.00

Total Salary, Wages and Fringe Benefits (A+B) 32,786.00

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	23,920.00
Total Travel Cost	23,920.00

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	5,000.00
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.		
9.		
10.		
11.		
12.		
13.		
14.		
15.		
16.		
17.		
	Total Other Direct Costs	5,000.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	61,706.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
FY23 Onsite Overhead	36.74	61,706.00	22,671.00
FY23 G&A Companywide	16.70	84,377.00	14,091.00
Contract Fee	5.00	98,468.00	4,923.00

Total Indirect Costs 41,685.00

Cognizant Federal Agency
(Agency Name, POC Name, and POC Phone Number)

USAMRAA, Jennifer C. Jackson, 301-619-2054

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	103,391.00

J. Fee

	Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	103,391.00

L. Budget Justification

(Only attach one file.) Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Budget Period 3

OMB Number: 4040-0001
Expiration Date: 12/31/2022

UEI: UYLKBRENAPG5 **Enter name of Organization:** The Henry M. Jackson Fdn. for the Adv'mnt of Mil. Med., Inc.

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 3** **Start Date:** 10/01/2025 **End Date:** 09/30/2026

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Eric		Laing	Ph.D.		0.80			0.00	0.00	0.00

Project Role: Co-Investigator

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	Scientist I	2.00			12,333.00	3,629.00	15,962.00
1	Research Associate/Scientific Project Coordinator	2.00			13,000.00	3,825.00	16,825.00

2 **Total Number Other Personnel**

Total Other Personnel 32,787.00

Total Salary, Wages and Fringe Benefits (A+B) 32,787.00

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	7,920.00
Total Travel Cost	7,920.00

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	5,000.00
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.		
9.		
10.		
11.		
12.		
13.		
14.		
15.		
16.		
17.		
	Total Other Direct Costs	5,000.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	45,707.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
FY23 Onsite Overhead	36.74	45,707.00	16,792.00
FY23 G&A Companywide	16.70	62,499.00	10,437.00
Contract Fee	5.00	72,936.00	3,646.00

Total Indirect Costs 30,875.00

Cognizant Federal Agency
(Agency Name, POC Name, and POC Phone Number)

USAMRAA, Jennifer C. Jackson, 301-619-2054

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	76,582.00

J. Fee

	Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	76,582.00

L. Budget Justification

(Only attach one file.)

Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Cumulative Budget

		Totals ($)
Section A, Senior/Key Person		0.00
Section B, Other Personnel		107,289.00
Total Number Other Personnel	6	
Total Salary, Wages and Fringe Benefits (A+B)		107,289.00
Section C, Equipment		
Section D, Travel		31,840.00
1. Domestic		
2. Foreign	31,840.00	
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		40,631.00
1. Materials and Supplies	40,631.00	
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		
9. Other 2		
10. Other 3		
11. Other 4		
12. Other 5		
13. Other 6		
14. Other 7		
15. Other 8		
16. Other 9		
17. Other 10		

Section G, Direct Costs (A thru F)	179,760.00
Section H, Indirect Costs	121,433.00
Section I, Total Direct and Indirect Costs (G + H)	301,193.00
Section J, Fee	
Section K, Total Costs and Fee (I + J)	301,193.00

Y1	Y2	Y3	OY1	OY2	Base Salary
0.8	0.8	0.8	0	0	

3	2	2	0	0	$ 74,000.00
4	0	0	0	0	$ 41,200.00
0	2	2	0	0	$ 78,000.00
				Fringe Rate-->	29.42%

IDC Rate-->	36.74%
IDC Rate-->	16.70%

USU	Y1	Y2	Y3
A. Senior/Key Personnel			
Eric Laing	$ -	$ -	$ -
Fringe	$ -	$ -	$ -
Total Senior/Key Personnel	$ -	$ -	$ -
B. Other Personnel			
Scientist (Dr. Si'Ana Coggins)	$ 18,500.00	$ 12,333.33	$ 12,333.33
Fringe	$ 5,442.70	$ 3,628.47	$ 3,628.47
Research Assistant (TBD)	$ 13,733.33	$ -	$ -
Fringe	$ 4,040.35	$ -	$ -
Research Associate/Scientific Project Coorindator (Mr. Spencer Sterling)	$ -	$ 13,000.00	$ 13,000.00
Fringe	$ -	$ 3,824.60	$ 3,824.60
Total Other Personnel	$ 41,716.38	$ 32,786.40	$ 32,786.40
C. Equipment			
Total Equipment	$ -	$ -	$ -
D. Travel			
1. Domestic Travel	$ -	$ -	$ -
2. Foreign Travel		$ 23,920.00	$ 7,920.00
Total Travel	$ -	$ 23,920.00	$ 7,920.00
E. Participant/Trainee Support Costs			
1. Tuition/Fees/Health Insurance	$ -	$ -	$ -
2. Stipends	$ -	$ -	$ -
3. Travel	$ -	$ -	$ -
4. Subsistence	$ -	$ -	$ -
5. Other	$ -	$ -	$ -
Total Participant/Trainee Support Costs	$ -	$ -	$ -
F. Other Direct Costs			
1. Materials and Supplies	$30,630.60	$ 5,000.00	$ 5,000.00
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. Other	$ -	$ -	$ -
Total Other Direct Costs	$ 30,630.60	$ 5,000.00	$ 5,000.00
G. Direct Costs and Modified Direct Costs			
Direct Costs	$ 72,346.98	$ 61,706.40	$ 45,706.40
Modified Direct Costs	$ 72,346.98	$ 61,706.40	$ 45,706.40
H. Indirect Costs			
1. IDC Rate (Onsite OH Expense)	$ 26,580.28	$ 22,670.93	$ 16,792.53
2. IDC Rate (G&A Expense)	$ 16,520.85	$ 14,091.01	$ 10,437.32
I. Total Direct and Indirect Costs			
Direct + Indirect	$ 115,448.11	$ 98,468.35	$ 72,936.25
J. Fee			
Fee	$ 5,772.00	$ 4,923.00	$ 3,646.00
K. Total Costs and Fee			
Total Costs	**$ 121,220.11**	**$ 103,391.35**	**$ 76,582.25**

Serology Training	**Y1**	**Y2**	**Y3**
Georgia			
Home <-> Airport Taxi	-	$120.00	$120.00
International Flight	-	$2,000.00	$2,000.00
Airport <-> Hotel Taxi	-	$100.00	$100.00
Hotel <-> Lab Taxi		$160.00	$200.00
Hotel Per Diem	-	$230.00	$230.00
Meals and Incidental Expenses Per Diem	-	$60.00	$60.00
5 nights and 6.5 days (2 travel days, 5 training days)	-	$1,540.00	$1,540.00
Trip Total	-	$3,920.00	$3,960.00
Oman			
Home <-> Airport Taxi	-	$120.00	
International Flight	-	$2,000.00	

Airport <-> Hotel Taxi	-	$100.00	
Hotel <-> Lab Taxi		$160.00	
Hotel Per Diem	-	$250.00	
Meals and Incidental Expenses Per Diem	-	$60.00	
5 nights and 6.5 days (2 travel days, 5 training days)	-	$1,640.00	
Trip Total	-	$4,020.00	
Turkey			
Home <-> Airport Taxi	-	$120.00	
International Flight	-	$2,000.00	
Airport <-> Hotel Taxi	-	$100.00	
Hotel <-> Lab Taxi		$160.00	
Hotel Per Diem	-	$250.00	
Meals and Incidental Expenses Per Diem	-	$60.00	
5 nights and 6.5 days (2 travel days, 5 training days)	-	$1,640.00	
Trip Total	-	$4,020.00	
No. Trips/Year		1	1
No. of EHA staff/trip		2	2
Per Person Total	$0.00	$11,960.00	$3,960.00
TOTAL	$0.00	**$23,920.00**	**$7,920.00**

	Y1
SUPPLIES (Itemize by category)	
Protein antigen production (34 proteins, 1.0 mg)	
Buffers, DMEM	$15.00
Serum-free Medium	$57.00
Shaker Flasks	$90.00
Filter unit	$120.00
Protein tag purification resin	$166.20
SDS protein gels	$225.00
Antibody substrate	$1.20

ECL	$27.00	
Tag cleavage kits	$199.50	
subtotal	**$30,630.60**	
Multiplex serologies		To be procured by in-country labs
Bioplex beads (34 regions x $725, 1 mL per region)	$24,650.00	
Bio-Plex Amine Coupling Kit	$716.00	
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC)	$150.00	
Sulfo-N-hydroxysulfosuccinimide (S-NHS)	$266.00	
Streptavidin-PE	$1,662.00	
Protein A-biotinylated, lyophilized	$395.00	
Protein G-biotinylated, lyophilized	$557.00	
Anti-human IgG, biotinylated	$209.00	
TWEEN® 20	$67.00	
PBS (without Ca2+ or Mg2+)*	$768.00	
Bio-Plex Pro™ Flat Bottom Plates	$969.00	
xMap Sheath Concentrate PLUS, RUO	$355.00	
Reagent reservoirs 25ml	$386.00	
ELISA Plate Sealers	$190.00	
MAGPIX Calibration Kit*	$1,704.00	
MAGPIX Verification Kit*	$1,704.00	
1.5 mL eppendorf tubes	$726.00	
2.0 mL amber screw top tubes	$50.00	
50ml conical tubes	$314.00	
subtotal	**$35,838.00**	
TOTAL	**$66,468.60**	

OY1	OY2	Total	
$ -	$ -	$ -	In-kind contribution
$ -	$ -	$ -	
$ -	$ -	$ -	
		$ -	
$ -	$ -	$ 43,166.67	
$ -	$ -	$ 12,699.63	
$ -	$ -	$ 13,733.33	
$ -	$ -	$ 4,040.35	
$ -	$ -	$ 26,000.00	
$ -	$ -	$ 7,649.20	
$ -	$ -	$ 107,289.18	
$ -	$ -	$ -	
$ -	$ -	$ -	
$ -	$ -		Serology trainings in-region
$ -	$ -	$ 31,840.00	
$ -	$ -	$ -	
$ -	$ -	$ -	
$ -	$ -	$ -	
$ -	$ -	$ -	
$ -	$ -	$ -	
$ -	$ -	$ -	
$ -	$ -	$ 40,630.60	Supplies for protein antigen production
$ -	$ -	$ -	
$ -	$ -	$ -	
$ -	$ -	$ -	
$ -	$ -	$ -	

$ -	$ -	$ -
$ -	$ -	$ -
$ -	$ -	$ -
$ -	$ -	$ 40,630.60
$ -	$ -	$ 179,759.78
$ -	$ -	$ 179,759.78
$ -	$ -	$ 66,043.74
$ -	$ -	
$ -	$ -	$ 286,852.71
$ -	$ -	$ 14,341.00
$ -	**$ -**	**$ 301,193.71**

OY1	OY2	TOTAL	
-	-		
-	-		
-	-		
-	-		
-	-		Hotel per diem ($236)
-	-		M&I ($102)
-	-		
-	-		
-	-		
-	-		

-	-		
-	-		
-	-		Hotel per diem ($261)
-	-		M&I ($155)
-	-		
-	-		
-	-		
-	-		
-	-		
-	-		
-	-		Hotel per diem ($324)
-	-		M&I ($150)
-	-		
-	-		
		-	1 trip/year/country in Y1
		-	Eric and research assistant
$0.00	$0.00	-	
$0.00	**$0.00**	**$31,840.00**	

BUDGET JUSTIFICATION

The Henry M. Jackson Foundation for the Advancement of Military Medicine Inc. (HJF) in partnership with the Uniformed Services University of the Health Sciences (USUHS) will manage this proposal, if awarded.

Personnel

Key Personnel:

Eric Laing, Ph.D., Co-Investigator (0.8 calendar month). Dr. Laing will be responsible for the coordination of the project activities with the principal investigator and other co-investigators. Dr. Laing will oversee the lab activities pertaining to the production of recombinant virus protein antigens and the establishment of the multiplex serology test. Dr. Laing will provide mentorship to the postdoctoral student and assist with data analysis. Dr. Laing will oversee serological training activities at the partner lab in Bong, Liberia and assist with in-country testing and data interpretation. He will provide the necessary concepts and practice of viral glycoprotein antigen preparation for serological application to co-investigators and collaborators. Dr. Laing will also provide the virological and immunological expertise necessary to understand the serological profiles of humans and wildlife antisera. Dr. Laing will be a government employee and no salary support is requested.

Other Personnel:

Dr. Si'Ana A. Coggins, PhD, Scientist I (3 calendar months, YR1; 2 calendar months, YR2-3). Dr. Coggins will assist with the supervision of all lab activities at USU and interface with the project management teams. As a trained biochemist Dr. Coggins will oversee all protein expression and maintain the quality and rigor of the research. Dr. Coggins will work closely with the research assistant, in year 1 to prepare protein antigens, verification of material quality, coordinate the transfer for materials to the partner institute in the regional partner labs and assist in the project activities. Dr. Coggins will participate in and provide serological assay training at the regional partner labs in years 2-3. Dr. Coggins is an employee of the Henry M. Jackson Foundation and salary support is requested for years 2-3.

TBD, Res Asst I (4 calendar months, YR1). A research assistant (RA) will assist Dr. Coggins in the production of soluble glycoproteins and be responsible for microsphere coupling in YR1. The RA will participate in serological testing and quality verification of the material, and will also prepare shipments of materials to the regional partner lab. The RA will be an employee of the Henry M. Jackson Foundation and salary support is requested for year 1.

Mr. Spencer L. Sterling, MpH, Research Project Coordinator (2 calendar month, YR2-3). Mr. Sterling will participate in the transfer of serology assay methods and data analysis. Mr. Sterling will work with the regional partner teams and EcoHealth Alliance scientists to assist in the analysis and development of serological results. Mr. Sterling will work closely with scientists at the prime institute to generate data for yearly reports. Mr. Sterling is an employee of the Henry M. Jackson Foundation and salary support is requested for year 2-3.

International Travel:

Based on FY23 Department of State per diem rates and historical travel to the Republic of Georgia, Oman, and Turkey, we estimate $31,840 should cover the travel costs of two lab members to travel to initiate and maintain project activities in the partner labs. In year 3, only travel to the Republic of Georgia is required.

Supplies:

Based on current prices, the following equipment and supplies will be needed to support this project in year 1: Recombinant production of approximately 34 protein antigens, magnetic beads, plastic consumables, cell culture materials, affinity matrices and control antibody materials.

Year 1 - $30,631
Recombinant protein production

Year 2 and 3 - $5,000
Continuation of disposable and general lab supplies to support the research project.

Indirect Costs:

HJF indirect cost (IDC) is calculated based on the value-added cost base overhead rates. The fringe rate used is 29.42 % for Tier 1 employees and 7.29% for Tier 2 employees (all employees in this proposal are Tier 1).

The HJF indirect cost is calculated based on the value-added cost base overhead rates. The IDC rate is 36.74% USU Onsite Overhead. Additionally, 16.70% Companywide G&A and applied on the total direct cost less subaward plus the USU Onsite Overhead. For proposals including subawards, an additional 1.93% is applied on total external subaward costs.

The above fringe benefits and indirect cost rates for FY2023 were approved by the U.S. Army Medical Research Acquisition Activity on September 1, 2022.

Fixed Fee:

HJF has proposed a Fixed Fee of 5% for all the cost items in this proposal. The proposed fixed fee is deemed reasonable for the contract type proposed and necessary to compensate HJF for contractual risks, to meet financial obligations not covered under the contract, to provide working operating capital and stability, and as a stimulant for efficient and effective contractor performance. This rate will only apply if awarded as a contract.

Current and Pending Support

Investigator: Eric D. Laing, PhD

Support: ☐ Current ☒ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title:

Strengthening capacity for bat-borne coronavirus, paramyxovirus, and filovirus threat reduction in Western Asia (current proposal)

Objective: Detect and characterize known and novel CoVs, PMVs, and FVs at the human-wildlife interface in newly collected and archived bat samples using a combination of molecular screening, partial- and full-genome sequencing, and multiplex serological assays

Source of Support: DTRA | Address: 8725 John J Kingman Rd #6201, Fort Belvoir, VA 22060

Total Award Amount: $301,988 Total Award Period Covered: 1 October 2023 – 30 September 2028

Location of Project: US, Georgia, Jordan, Oman, Turkey

Person-Months Per Year Committed to Project: Cal: 0.8 Acad: Sumr:

Support: ☒ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: Efficacy testing of a novel human monoclonal antibody therapy for late-stage rabies/lyssavirus

Objective: The major goals of this project is to test the efficacy of mAb therapy at times beyond day seven post-infection, to more completely define the degree to which mAb therapy can confer protection from death post-onset of clinically evident severe neurological disease.

Source of Support: USUHS/CGHE | Address: 4301 Jones Bridge Road, Bethesda, MD 20814

Total Award Amount: $518,000 Total Award Period Covered: 09/30/2019 – 09/29/2023

Location of Project: US, Georgia, Kenya

Person-Months Per Year Committed to Project: Cal: 0.6 Acad: Sumr:

Support: ☒ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: Epidemiology, Immunology, and Clinical Characteristics of Emerging Infectious Diseases with Pandemic Potential (EpICC-EID)

Objective: The major goals of this project are to utilize SARS-CoV-2 serology to explore hospital and community infection, correlates of COVID-19, and vaccinology.

Source of Support: Defense Health Program | Address: 7700 Arlington Blvd. Suite 5101. Falls Church, VA 22042-5101

Total Award Amount: $1,178,876 Total Award Period Covered: 04/01/2020 – 09/30/2023

Location of Project: US

Person-Months Per Year Committed to Project: Cal: 1.0 Acad: Sumr:

Support: ☒ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: Prospective Assessment of SARS-CoV-Seroconversion

Objective: The major goals of this project are to monitor SARS-CoV-2 infection, the roleof human coronavirus antibodies in COVID-19 outcomes, and COVID-19 vaccinology

Source of Support: Defense Health Program/CARES Act, NIAID | Address: 7700 Arlington Blvd. Suite 5101. Falls Church, VA 22042-5101

Total Award Amount: $235,388 Total Award Period Covered: 04/01/2020-09/30/2024

Location of Project: US

Person-Months Per Year Committed to Project: Cal: 1.0 Acad: Sumr:

Support: ☒ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia

Objective: The major goals of this Centers for Research in Emerging Infectious Diseases (CREID) project are to develop multidisciplinary teams of investigators in the program will conduct pathogen/host surveillance, study pathogen transmission, pathogenesis and immunologic responses in the host, and will develop reagents and diagnostic assays for improved detection for important emerging pathogens and their vectors.

Source of Support: NIH/NIAID/CREID 07-049-7012-52338 | Address: 6701 Rockledge Drive, Room 1040 - MSC 7710 Bethesda, MD 20892-7710

Total Award Amount: $539,119 Total Award Period Covered: 06/17/2020-05/31/2025

Location of Project: US, Malaysia, Thailand, Singapore

Person-Months Per Year Committed to Project: Cal: 1.0 Acad: Sumr:

USE ADDITIONAL SHEETS AS NECESSARY

Support: ☒ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa

Objective: This project will focus on building capacity and enhancing surveillance, including early detection programs for henipa-, filo- and zoonotic coronaviruses in Southern Africa.

Source of Support: DTRA | Address: 8725 John J Kingman Rd #6201, Fort Belvoir, VA 22060

Total Award Amount: $1,120,965 Total Award Period Covered: 07/31/2020-07/30/2025

Location of Project: US, South Africa

Person-Months Per Year Committed to Project: Cal: 1.0 Acad: Sumr:

Support: ☒ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: Biosurveillance for Spillover of *Henipaviruses* and *Filoviruses* in Rural Communities in India

Objective: The major goals of this project are to conduct biosurveillance for evidence of henipavirus and filovirus infection in bats and cryptic infections in at-risk human communities

Source of Support: DTRA | Address: 8725 John J Kingman Rd #6201, Fort Belvoir, VA 22060

Total Award Amount: $1,195,275 Total Award Period Covered: 10/01/2020 – 09/30/2023

Location of Project: US, India

Person-Months Per Year Committed to Project: Cal: 0.5 Acad: Sumr:

Support: ☒ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: Informing Biosurveillance: Contribution of pteropodid fruit bats to virus spillover in the Philippines

Objective: The major goals of this project are to investigate Nipah virus and Reston virus circulation in bat populations in the Philippines, build research capacity for serological biosurveillance, and identify at-risk interfaces of virus zoonosis.

Source of Support: DTRA | Address: 8725 John J Kingman Rd #6201, Fort Belvoir, VA 22060

Total Award Amount: $1,213,746 Total Award Period Covered: 07/2022 – 06/2025

Location of Project: US, Philippines, Singapore

Person-Months Per Year Committed to Project: Cal: 2.0 Acad: Sumr:

Support: ☒ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: Solving Opportunities for Spillover (SOS): Frequency and Mechanisms of Cross-species Transmission of Henipaviruses in Bangladesh

Objective: We aim to better understand and prevent spillovers of bat-borne viruses into intermediate hosts and humans. The knowledge gained from this study will be immediately applicable to human and animal health programs because if we know which henipaviruses infect humans and domesticated animals, and how they are infected, we can advise public health surveillance programs on how to optimize detection and prevention of infections.

Source of Support: NIH/NIAID | Address: 6701 Rockledge Drive, Room 1040 - MSC 7710 Bethesda, MD
Total Award Amount: $3,356,172 Total Award Period Covered: 01/03/2022- 12/31/2027
Location of Project: US, Bangladesh
Person-Months Per Year Committed to Project: Cal: 1.0 Acad: Sumr:

Support: ☐ Current ☒ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support
Project/Proposal Title: Establishment of a Bat Resource for Infectious Disease Research

Objective: The major goals of this proposal is to contribute significant insight into the viral determinants that confer henipaviral pathogenesis. The construction and generation of recombinant henipavirus chimeric viruses will be the primary means for determining how virus receptor usage and the expression of virulence factors act individually or in conjunction to cause limited or severe disease. The recombinant constructs generated from the proposed studies will also be used to determine host determinants of immunity in Jamaican fruit bats and animal models.

Source of Support: NIH / NIAID | Address: 6701 Rockledge Drive, Room 1040 - MSC 7710 Bethesda, MD
Total Award Amount: $695,937 Total Award Period Covered: 01/01/2023-12/30/2027
Location of Project: US
Person-Months Per Year Committed to Project: Cal: 1.0 Acad: Sumr:

USE ADDITIONAL SHEETS AS NECESSARY

Eric D. Laing
Uniformed Services University, 4301 Jones Bridge Road, Bethesda, MD 20814
Email: eric.laing@usuhs.edu

Professional Preparation

University of Maryland	Biology	BS (hons)	2008
Uniformed Services University	Emerg Infect Dis	PhD	2016
Uniformed Services University	Virology	Post-doc.	2019

Appointments

Assistant Professor, Dept. of Micro. and Immun., Uniformed Services University	2021 - pres.
Joint appointment, EID Graduate Program, Uniformed Services University	2021 - pres.
Research Assistant Professor, Uniformed Services University	2019 - 2021

Publications

Paskey AC, Ng JHJ, Rice GK, Chia WN, Philipson CW, Foo RJH, Cer RZ, Long KA, Lueder MR, Lim XF, Frey KG, Hamilton T, Anderson DE, **Laing ED**, Mendenhall IH, Smith GJ, Wang LF, Bishop-Lilly KA. Detection of Recombinant Rousettus Bat Coronavirus GCCDC1 in Lesser Dawn Bats (Eonycteris spelaea) in Singapore. Viruses. 2020 May 14;12(5):539. doi: 10.3390/v12050539. PMID: 32422932; PMCID: PMC7291116.

Seifert SN, Letko MC, Bushmaker T, **Laing ED**, Saturday G, Meade-White K, van Doremalen N, Broder CC, Munster VJ. Rousettus aegyptiacus Bats Do Not Support Productive Nipah Virus Replication. J Infect Dis. 2020 May 11;221(Suppl 4):S407-S413. doi: 10.1093/infdis/jiz429. PMID: 31682727; PMCID: PMC7199784.

Schulz JE, Seifert SN, Thompson JT, Avanzato V, Sterling SL, Yan L, Letko MC, Matson MJ, Fischer RJ, Tremeau-Bravard A, Seetahal JFR, Ramkissoon V, Foster J, Goldstein T, Anthony SJ, Epstein JH, **Laing ED**, Broder CC, Carrington CVF, Schountz T, Munster VJ. Serological Evidence for Henipa-like and Filo-like Viruses in Trinidad Bats. J Infect Dis. 2020 May 11;221(Suppl 4):S375-S382. doi: 10.1093/infdis/jiz648. PMID: 32034942; PMCID: PMC7213578.

Laing ED, Navaratnarajah CK, Cheliout Da Silva S, Petzing SR, Xu Y, Sterling SL, Marsh GA, Wang LF, Amaya M, Nikolov DB, Cattaneo R, Broder CC, Xu K. Structural and functional analyses reveal promiscuous and species specific use of ephrin receptors by Cedar virus. Proc Natl Acad Sci U S A. 2019 Oct 8;116(41):20707-20715. doi: 10.1073/pnas.1911773116. Epub 2019 Sep 23. PMID: 31548390; PMCID: PMC6789926.

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UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799
www.usuhs.edu

February 15, 2023

To: DTRA C-WMD Thrust Area 6 Program
From: Eric D. Laing, PhD

I am writing to express my commitment to collaborate with EcoHealth Alliance on the project proposal, *Strengthening capacity for bat-borne coronavirus, paramyxovirus, and filovirus threat reduction in Western Asia*. I agree to undertake the tasks assigned to me as described in the project narrative of the proposal. I strongly support this project, which is designed to increase the capacity of Western Asian scientists to conduct and sustain bat-borne disease surveillance in the region. This will be accomplished through active surveillance of bat populations, improved capacity to conduct molecular and serological screening of viral families that pose a high risk to humans, and increased multi-sectoral engagement of governmental and non-governmental partners in the region to improve the timeliness of data sharing, zoonotic risk assessments, and mitigation of emerging diseases. This project is of mutual benefit in that it will enhance the existing capacity and build future sustainability for a proactive early warning system for bat-borne zoonoses throughout Western Asia. Moreover, this project will leverage the professional networks of the Western Asia Bat Research Network (WAB-Net), improve our scientific understanding of bat- borne viral diversity throughout Western Asia, and elucidate the risk factors associated with bat-borne virus shedding and spillover into human populations.

As you know one of my lab's focus in on the utility of serosurveillance to identify emergent zoonotic viruses such as coronaviruses, henipaviruses, and filovirurses. I have been developing tools for and analyzing serosurveillance data collected from wildlife (bats) and humans since 2015. I worked as a postdoctoral fellow with EcoHealth Alliance on BTRP funded project dating back to 2017, "Serological Biosurveillance for Spillover of Henipaviruses and Filoviruses at Agricultural and Hunting Human-Animal Interfaces in Peninsular Malaysia (HDTRA11710037)." In addition, my lab supported follow-up serological analysis of bat sera collected during the Ebola Host Project that was performed in collaboration with EcoHealth Alliance. Presently, I am a co-investigator within the EID-Southeast Asia Research Collaborative Hub (EID-SEARCH), one of the NIH Centers for Research in Emerging Infectious Diseases, of which you are a co-principal investigator. Within EID-SEARCH my lab develops, implements, and analyzes data generated from serological profiles of bats and human study participants. This proposed project will provide invaluable evidence to strengthen our understanding of and capacity to detect filovirus, coronaviruses, henipavirus (paramyxoviruses) circulation in bats in west Asia.

Sincerely,

(b) (6)

Eric D. Laing
Assistant Professor
Department of Microbiology and Immunology
Uniformed Services University

(b) (6)

Christopher Broder, Ph.D.
Chair and Professor
Department of Microbiology and Immunology - USU

Facilities and Other Resources

Uniformed Services University of the Health Sciences
Dr. Eric Laing (Co-Investigator)

Overall Scientific Environment: Uniformed Services University (USU) is the medical school at which approximately half of the physicians in the Armed Services receive their graduate training. Research at USU is supported primarily by extramural grants, as in other medical schools. Dr. Laing is an Assistant Professor in the Department of Microbiology and Immunology, which includes 12 full-time Faculty members. The overall focus of the Department is 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. Dr. Laing is currently involved in active collaborations within the University, in areas of viral immunology and vaccine and antiviral therapeutics and animal model development. USU is also physically located directly across from the main NIH campus in Bethesda. The overall broad scientific environment at both USU and the NIH is highly conducive to productive collaborations. The PI often uses these resources to his advantage, both for his research objectives and interests and in assisting in adjunct faculty appointments within the Emerging Infectious Diseases graduate program for both on and off-campus scientists interested in participating in graduate education and graduate student training. Overall, the available technical resources (and University support for continually improving technical resources) is exceptional. In addition, multi-lab Immunology journal club, Immunology data club, Virology journal club, and Virology data club exist. Approximately 10 groups regularly participate in the immunology journal/data club and 5 groups regularly participate in the virology journal/data club.

Dr. Laing has been well-supported by the Department of Microbiology and Immunology and the USU Office of Research. Although USU is a modest-sized medical school, the technical resources are outstanding, rivaling many very large institutions (see below). The modest size of the school is an advantage, as key pieces of equipment (e.g., Bruker In Vivo Xtreme II; Siemens Inveon SPECT/PET/CT Scanner) are not overbooked and therefore readily available to the laboratory personnel. In addition to University funds, we have successfully used the NIH S10 mechanism to purchase advanced and very expensive equipment, including the Zeiss 710NLO system and the FACSAria 15-parameter cell sorter. Overall, the available technical resources (and University support for continually improving technical resources) is exceptional, as is illustrated by the most recent USU purchase of cutting-edge equipment: the Zeiss Elyra PS.1 super-resolution microscope.

Laboratory: Dr. Laing's laboratory is 453 sq. ft. and is currently supported by two graduate students, two research assistants, two research associates, and one scientist. Dr. Laing also has access to 3 rooms within the department totaling 1,440 sq. ft. where BSL-2 virus rescues are performed and blood specimen samples are handled. Dr. Laing has access to or possesses routine equipment for microbiology, molecular biology, and biochemistry including incubators, centrifuges, inverted microscopes, gel electrophoresis equipment for protein and DNA gels, thermal cyclers, balances, pH meters, refrigerators, freezers, platform orbital shakers, sonicator, UV transilluminator, shakers, heating blocks, waterbaths, free-standing biosafety cabinets, and CO_2-tissue culture incubators. Additionally, Dr. Laing has access to three BioPlex 200 HTF multiplexing systems, one MAGPIX multiplexing system, an epifluorescence microscopy system (a Zeiss AxioObserver inverted fluorescence microscope, upgraded in 2015), and two complete GE-ATKA low pressure chromatography systems, with integrated UV detectors, fraction collectors, and pump systems, and gradient fractionator apparatus.

Animal: Animals are maintained in University facilities under the supervision of a full-time veterinarian. The USUHS maintains a modern AAALC Accredited, Central Animal Facility of about 50,000 square feet. It, and the University's animal care and use program, is managed by the Center for Laboratory Animal Medicine, which is directed by a veterinarian who is an ACLAM Diplomate and staffed with three other veterinarians, a graduate animal husbandryman, and about 30 technicians. The University is able to provide appropriate care for a wide variety of laboratory animal species, from invertebrates to lower vertebrates, to higher vertebrates including non-human primates and domestic livestock, as well as the more commonly used species such as rodents and rabbits. The facility also includes a number of properly equipped ABSL-2 rooms.

Computer: Windows-based computers (Intel i5/i7 or similar processors and ≥2GB of RAM) are available for routine use by postdoctoral fellows, graduate students, and technicians located in the laboratories of both investigators. The University maintains site licenses for other image and data analysis software, including Zeiss Axiovision, VisioPharm, and OriginPro.

Office: Dr. Laing has an office separate from but proximal to the laboratories. Trainees and technicians have desks in the laboratory or in a separate office area proximal to the laboratory. The department employees one senior program manager, administrative officer, administrative specialist and a microbiologist.

Clinical: N/A

Other: The Biostatistics Consulting Center (BCC), a service of the Department of Preventive Medicine and Biometrics, provides statistical consulting to USUHS scientific investigators. We routinely consult with Cara Olsen, Research Assistant Professor (the full-time Biostatistics Consultant of the BCC), regarding proper design of experiments for statistical testing and for statistical analysis of the resulting data.
The USU Translational Imaging Facility (TIF) houses state-of-the art equipment for live animal imaging, including a Siemens Inveon SPECT/PET/CT Scanner, a Bruker Biospec 70/20 USR Magnetic Resonance Imaging system, and a Bruker In-Vivo Xtreme II bioluminescence and X-ray imaging system.

The USU Biomedical Instrumentation Center (BIC) houses core equipment for use by investigators throughout the University. Instrumentation is available either free or on a fee-for-service basis, depending on which instruments have annual service contracts (which are paid largely through per-hour use fees). The BIC Flow Cytometry Core includes two Becton-Dickinson (10- and 13-parameter) LSRII FACS analyzers, one 15-parameter FACSAria FACS sorter, and one Amnis Image Stream X Mark II imaging flow cytometer, as well as off-line analysis workstations.

The BIC Imaging Core houses three confocal microscopes, including a Zeiss 700 inverted system with 405/458/488/514/561/633 laser excitation; a Zess 710NLO inverted system with 405/458/488/514/561/633 conventional lasers and a Coherent Ultra2 Ti-Sapphire laser for multiphoton excitation, continuously tunable over the range of 690 to 1080 nm; and a Zeiss AxioExaminer-Z1 upright microscope equipped with a direct-coupled Coherent Chameleon tunable infrared laser for ex vivo and in vivo mutliphoton imaging projects. A Becker-Hickl two-detector FLIM system (for FRET analyses) is connected to the inverted Zeiss 710NLO system. Recently, the BIC has also acquired a Zeiss Elyra PS.1 super-resolution microscope, which is capable of 4-parameter SR-SIM (super-resolution structured illumination) imaging, 3-parameter PALM (Photoactivation localization microscopy) and dSTORM (direct stochastic optical reconstruction microscopy), as well as 3D-PALM/dSTORM. The BIC also houses a Leica AF6000 system, consisting of an inverted microscope equipped with a fully motorized 3-axis stage plus atmosphere and temperature control, allowing extended term (days) live cell analyses. Additionally, there is a stereology system consisting of a Zeiss AxioImager.M2 upright microscope connected to MicroBrightField's Stereo Investigator software package. The facility also includes several additional wide-field fluorescence microscopes, and three offline data analysis stations with software packages including: Zeiss Zen software and full Physiology package; Media Cybernetics' 3D Constructor, Image Pro Analyzer, Autodeblur, and Autovisualize; Metamorph Basic. The Imaging Core also includes a transmission electron microscope (Philips CM100 transmission EM) and an ultramicrotome (Leica EM UC6 with EM FC6 cryo attachment).

The BIC Genomics core includes an ABI 3900 DNA synthesizer, an ABI3500xl Genetic Analyzer (for sequencing), a RochLightCycler 480 for real-time PCR, and Systec Mediaprep and Plate Pourer instrument. There is also an integrated Fuji FLA-5000/LAS-3000 imaging system for many applications that involve fluorescence and chemiluminescence imaging of gels and blots. The BIC Proteomics Core includes two Agilent 1100 HPLCs, an AB SCIEX Voyager DSTR MALDI-TOF mass spectrometer, and an AB SCIEX Q-TOF tandem mass spectrometer.

The BIC Structural Biology Core includes a Rigaku HighFlux HomeLab X-ray diffraction system, with a MicroMax-007 HF microfocus rotating anode generator, an R-AXIS Imaging Plate detector, and an X-stream 2000 cryogenic system. Other available BIC instruments and services include histopathology and PET/CT instrumentation for small animal research.

The University also has an equipment repair service, central duplicating service, audiovisual service, and microcomputer support service. The University Learning Resource Center is a high quality medical and scientific library with additional microcomputers and support. A wide variety of scientific journals are available in print and via remote computer access. A central autoclave/glassware washroom serves the Department of Microbiology and Immunology and is maintained through extramural grant support.

Investigator Initiatives-Business Development
Department Line: (240) 694-4016
Email: proposals@hjf.org

LETTER OF INTENT TO COLLABORATE

February 17, 2023

Luke Hamel
Program Coordinator
EcoHealth Alliance
520 8th Avenue
New York, New York 10018

Dear Mr. Hamel,

This letter confirms The Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc.'s (HJF) intent to collaborate with EcoHealth Alliance in response to HDTRA1-14-24-FRCWMD-BAA and is offered on the condition that EcoHealth Alliance agrees that if it receives an award for the subject project, it will issue a subaward to HJF for the work hereby proposed.

Confidentiality Notice: *This letter of Intent, including all proposal documents attached hereto, contains HJF confidential and proprietary information and is provided for use solely in connection with HJF's and EcoHealth Alliance's joint pursuit of the project listed below. Any unauthorized use, disclosure or distribution is prohibited.*

Prime Institution:	EcoHealth Alliance	
Prime Principal Investigator:	Kevin Olival, MA, PhD	
Project Title:	Strengthening capacity for bat-borne coronavirus, paramyxovirus, and filovirus threat reduction in Western Asia	
Proposed Principal Investigator:	Eric Laing, PhD	
Proposed Project Period:	October 1, 2023 – September 30, 2026	
Proposed Budget Amount:	$ 179,760	Direct Costs
	$ 107,092	Indirect Costs
	$ 14,341	Fee
	$ 301,193	Total Cost

HJF confirms that it has in place a written and enforced financial conflict of interest policy that applies to all HJF investigators and any other HJF employees responsible for the design, conduct or reporting of the budgeted activities. U.S. Government Principal Investigator(s) (if any) are subject to federal regulations governing conflicts of interest.

Your submission of a prime proposal that includes the subaward activities hereby proposed by HJF will constitute your acknowledgment of, and agreement to, the terms set forth in this letter.

Sincerely (b) (6)

Ms. Freda Denis-Cooper, CRA
Senior Proposal Manager-Team Lead
Investigator Initiatives-Business Development
Strategic Initiatives

cc: Eric Laing, PhD

Performance Site:

Organization Name:	Uniformed Services University of the Health Sciences
UEI Number:	Z83LJK5NWBC1
Street Address:	4301 Jones Bridge Road
City	Bethesda
County	Montgomery
State	MD
Zip+4	20814-4799
Project/ Performance Site Congressional District:	MD-008

Complete for each key person on the project. Copy and add additional tables as needed.
All key personnel providing a biosketch should have an era commons account included in their biosketch.

PI Key Personnel Info:

Name: (L, F. MI)	Laing, Eric
Position/Title	Assistant Professor
Department	MIC
Division:	
Street Address:	4301 Jones Bridge Road
City	Bethesda
County	Montgomery
State	MD
Zip+4	20814-4799
Phone:	301-295-3419
E-Mail	Eric.laing@USUHS.edu
eRA Login ID	Eric.laing

Key Personnel Info:

Name: (L, F. MI)	
Position/Title	
Department	
Division:	
Street Address:	
City	
County	
State	
Zip+4	
Phone:	
E-Mail	
eRA Login ID	

Key Personnel Info:

Name: (L, F. MI)	
Position/Title	
Department	
Division:	
Street Address:	
City	
County	
State	
Zip+4	
Phone:	
E-Mail	
eRA Login ID	

From:	Laing, Eric on behalf of Laing, Eric <eric.laing@usuhs.edu>
To:	Luke Hamel; Peter Okagaki; Kevin Olival, PhD; Kim Williamson
Subject:	Fwd: Subaward Proposal Dr. Laing
Date:	Saturday, February 18, 2023 9:30:01 AM
Attachments:	RR_Budget_Laing_DTRAv3.pdf USU subaward budget (WAB-Net 2) v4.xlsx DTRA_WABNet2_Laing_Budget Justificationv4.pdf Current and Pending Support Template (WAB-Net 2)_LaingEDv2.pdf BioSketch_Laing_DTRA.pdf DTRA_WABNet2_Laing_LOSv2-ccb.pdf Facilities_DTRA_Laing.pdf HJF LETTER OF INTENT E Laing_FDC021723.pdf Key Personnel and Performance Site_Laing_DTRA (5).pdf image001.png

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Hey Luke - your email isn't addressed correctly.

Please confirm receipt of this email.

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

---------- Forwarded message ---------
From: **Peter Okagaki** <pokagaki@hjf.org>
Date: Fri, Feb 17, 2023 at 4:28 PM
Subject: Subaward Proposal Dr. Laing
To: hamel@ecohelathalliance.org <hamel@ecohelathalliance.org>, olival@ecohealthalliance.org <olival@ecohealthalliance.org>
Cc: Kimberly Boxley <kimberly.boxley.ctr@usuhs.edu>, Eric Laing <eric.laing@usuhs.edu>

Dear Mr. Hamel,

On behalf of Dr. Laing of USUHS, I attach the following documents for your review:

- Budget—RR and Excel,
- Budget Justification,
- Current and Pending Support-Dr. Laing,

Biosketch-Dr. Laing,
- Letter of Support—Dr. Laing and Dr. Broder,
- Facilities/Equipment,
- Key Person/Performance site information,
- Letter of Intent.

If you have any questions or concerns, please let me know. Thank you for this opportunity to collaborate with Dr. Olival and EcoHealth Alliance.

Best,

Peter Okagaki, MS, MBA

Proposal Manager—International Portfolio

Investigator Initiatives – Business Development

Strategic Initiatives

tel. 240-694-2223

email: pokagaki@hjf.org

Henry M. Jackson Foundation for the

Advancement of Military Medicine

6720A Rockledge Drive, Suite 100

Bethesda, MD 20817

Eric D. Laing
Uniformed Services University, 4301 Jones Bridge Road, Bethesda, MD 20814
Email: eric.laing@usuhs.edu

Professional Preparation

University of Maryland	Biology	BS (hons)	2008
Uniformed Services University	Emerg Infect Dis	PhD	2016
Uniformed Services University	Virology	Post-doc.	2019

Appointments

Assistant Professor, Dept. of Micro. and Immun., Uniformed Services University	2021 - pres.
Joint appointment, EID Graduate Program, Uniformed Services University	2021 - pres.
Research Assistant Professor, Uniformed Services University	2019 - 2021

Publications

Paskey AC, Ng JHJ, Rice GK, Chia WN, Philipson CW, Foo RJH, Cer RZ, Long KA, Lueder MR, Lim XF, Frey KG, Hamilton T, Anderson DE, **Laing ED**, Mendenhall IH, Smith GJ, Wang LF, Bishop-Lilly KA. Detection of Recombinant Rousettus Bat Coronavirus GCCDC1 in Lesser Dawn Bats (Eonycteris spelaea) in Singapore. Viruses. 2020 May 14;12(5):539. doi: 10.3390/v12050539. PMID: 32422932; PMCID: PMC7291116.

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Current and Pending Support

Investigator: Eric D. Laing, PhD

Support: ☐ Current ☒ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support
Project/Proposal Title:
Strengthening capacity for bat-borne coronavirus, paramyxovirus, and filovirus threat reduction in Western Asia (current proposal)
Objective:
Source of Support: DTRA | Address: 8725 John J Kingman Rd #6201, Fort Belvoir, VA 22060
Total Award Amount: | Total Award Period Covered: 1 October 2023 – 30 September 2028
Location of Project: US, Georgia, Jordan, Oman, Turkey
Person-Months Per Year Committed to Project: Cal: 0.8 Acad: Sumr:

Support: ☒ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support
Project/Proposal Title: Efficacy testing of a novel human monoclonal antibody therapy for late-stage rabies/lyssavirus

Objective: The major goals of this project is to test the efficacy of mAb therapy at times beyond day seven post-infection, to more completely define the degree to which mAb therapy can confer protection from death post-onset of clinically evident severe neurological disease.

Source of Support: USUHS/CGHE | Address: 4301 Jones Bridge Road, Bethesda, MD 20814
Total Award Amount: $518,000 | Total Award Period Covered: 09/30/2019 – 09/29/2023
Location of Project: US, Georgia, Kenya
Person-Months Per Year Committed to Project: Cal: 0.6 Acad: Sumr:

Support: ☒ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support
Project/Proposal Title: Epidemiology, Immunology, and Clinical Characteristics of Emerging Infectious Diseases with Pandemic Potential (EpICC-EID)

Objective: The major goals of this project are to utilize SARS-CoV-2 serology to explore hospital and community infection, correlates of COVID-19, and vaccinology.

Source of Support: Defense Health Program | Address: 7700 Arlington Blvd. Suite 5101. Falls Church, VA 22042-5101
Total Award Amount: $1,178,876 | Total Award Period Covered: 04/01/2020 – 09/30/2023
Location of Project: US
Person-Months Per Year Committed to Project: Cal: 1.0 Acad: Sumr:

Support: ☒ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support
Project/Proposal Title: Prospective Assessment of SARS-CoV-Seroconversion

Objective: The major goals of this project are to monitor SARS-CoV-2 infection, the roleof human coronavirus antibodies in COVID-19 outcomes, and COVID-19 vaccinology
Source of Support: Defense Health Program/CARES Act, NIAID | Address: 7700 Arlington Blvd. Suite 5101. Falls Church, VA 22042-5101
Total Award Amount: $235,388 | Total Award Period Covered: 04/01/2020-09/30/2024
Location of Project: US
Person-Months Per Year Committed to Project: Cal: 1.0 Acad: Sumr:

Support: ☒ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support
Project/Proposal Title: Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia

Objective: The major goals of this Centers for Research in Emerging Infectious Diseases (CREID) project are to develop multidisciplinary teams of investigators in the program will conduct pathogen/host surveillance, study pathogen transmission, pathogenesis and immunologic responses in the host, and will develop reagents and diagnostic assays for improved detection for important emerging pathogens and their vectors.

Source of Support: NIH/NIAID/CREID 07-049-7012-52338 | Address: 6701 Rockledge Drive, Room 1040 - MSC 7710 Bethesda, MD 20892-7710

Total Award Amount: $539,119 Total Award Period Covered: 06/17/2020-05/31/2025

Location of Project: US, Malaysia, Thailand, Singapore

Person-Months Per Year Committed to Project: Cal: 1.0 Acad: Sumr:

USE ADDITIONAL SHEETS AS NECESSARY

Support: ☒ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa

Objective: This project will focus on building capacity and enhancing surveillance, including early detection programs for henipa-, filo- and zoonotic coronaviruses in Southern Africa.

Source of Support: DTRA | Address: 8725 John J Kingman Rd #6201, Fort Belvoir, VA 22060

Total Award Amount: $1,120,965 Total Award Period Covered: 07/31/2020-07/30/2025

Location of Project: US, South Africa

Person-Months Per Year Committed to Project: Cal: 1.0 Acad: Sumr:

Support: ☒ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: Biosurveillance for Spillover of *Henipaviruses* and *Filoviruses* in Rural Communities in India

Objective: The major goals of this project are to conduct biosurveillance for evidence of henipavirus and filovirus infection in bats and cryptic infections in at-risk human communities

Source of Support: DTRA | Address: 8725 John J Kingman Rd #6201, Fort Belvoir, VA 22060

Total Award Amount: $1,195,275 Total Award Period Covered: 10/01/2020 – 09/30/2023

Location of Project: US, India

Person-Months Per Year Committed to Project: Cal: 0.5 Acad: Sumr:

Support: ☒ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: Informing Biosurveillance: Contribution of pteropodid fruit bats to virus spillover in the Philippines

Objective: The major goals of this project are to investigate Nipah virus and Reston virus circulation in bat populations in the Philippines, build research capacity for serological biosurveillance, and identify at-risk interfaces of virus zoonosis.

Source of Support: DTRA | Address: 8725 John J Kingman Rd #6201, Fort Belvoir, VA 22060

Total Award Amount: $1,213,746 Total Award Period Covered: 07/2022 – 06/2025

Location of Project: US, Philippines, Singapore

Person-Months Per Year Committed to Project: Cal: 2.0 Acad: Sumr:

Support: ☒ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: Solving Opportunities for Spillover (SOS): Frequency and Mechanisms of Cross-species Transmission of Henipaviruses in Bangladesh

Objective: We aim to better understand and prevent spillovers of bat-borne viruses into intermediate hosts and humans. The knowledge gained from this study will be immediately applicable to human and animal health programs because if we know which henipaviruses infect humans and domesticated animals, and how they are infected, we can advise public health surveillance programs on how to optimize detection and prevention of infections.

Source of Support: NIH/NIAID | Address: 6701 Rockledge Drive, Room 1040 - MSC 7710 Bethesda, MD
Total Award Amount: $3,356,172 Total Award Period Covered: 01/03/2022- 12/31/2027
Location of Project: US, Bangladesh
Person-Months Per Year Committed to Project: Cal: 1.0 Acad: Sumr:

Support: ☐ Current ☒ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support
Project/Proposal Title: Establishment of a Bat Resource for Infectious Disease Research

Objective: The major goals of this proposal is to contribute significant insight into the viral determinants that confer henipaviral pathogenesis. The construction and generation of recombinant henipavirus chimeric viruses will be the primary means for determining how virus receptor usage and the expression of virulence factors act individually or in conjunction to cause limited or severe disease. The recombinant constructs generated from the proposed studies will also be used to determine host determinants of immunity in Jamaican fruit bats and animal models.

Source of Support: NIH / NIAID | Address: 6701 Rockledge Drive, Room 1040 - MSC 7710 Bethesda, MD
Total Award Amount: $695,937 Total Award Period Covered: 01/01/2023-12/30/2027
Location of Project: US
Person-Months Per Year Committed to Project: Cal: 1.0 Acad: Sumr:

USE ADDITIONAL SHEETS AS NECESSARY

Support: ☐ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support
Project/Proposal Title:

Objective:
Source of Support: DTRA | Address:
Total Award Amount: Total Award Period Covered:
Location of Project: Philippines
Person-Months Per Year Committed to Project: Cal: Acad: Sumr:

Support: ☐ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support
Project/Proposal Title:

Objective:
Source of Support: | Address:
Total Award Amount: Total Award Period Covered:
Location of Project:
Person-Months Per Year Committed to Project: Cal: Acad: Sumr:

Support: ☐ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support
Project/Proposal Title:

Objective:
Source of Support: | Address:
Total Award Amount: Total Award Period Covered:
Location of Project:
Person-Months Per Year Committed to Project: Cal: Acad: Sumr:

Support: ☐ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support
Project/Proposal Title:

Objective:
Source of Support: | Address:
Total Award Amount: Total Award Period Covered:
Location of Project:
Person-Months Per Year Committed to Project: Cal: Acad: Sumr:

Support: ☐ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support
Project/Proposal Title:

Objective:
Source of Support: | Address:
Total Award Amount: Total Award Period Covered:
Location of Project:
Person-Months Per Year Committed to Project: Cal: Acad: Sumr:

USE ADDITIONAL SHEETS AS NECESSARY

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799
www.usuhs.edu

February 15, 2023

To: DTRA C-WMD Thrust Area 6 Program
From: Eric D. Laing, PhD

I am writing to express my commitment to collaborate with EcoHealth Alliance on the project proposal, *Strengthening capacity for bat-borne coronavirus, paramyxovirus, and filovirus threat reduction in Western Asia*. I agree to undertake the tasks assigned to me as described in the project narrative of the proposal. I strongly support this project, which is designed to increase the capacity of Western Asian scientists to conduct and sustain bat-borne disease surveillance in the region. This will be accomplished through active surveillance of bat populations, improved capacity to conduct molecular and serological screening of viral families that pose a high risk to humans, and increased multi-sectoral engagement of governmental and non-governmental partners in the region to improve the timeliness of data sharing, zoonotic risk assessments, and mitigation of emerging diseases. This project is of mutual benefit in that it will enhance the existing capacity and build future sustainability for a proactive early warning system for bat-borne zoonoses throughout Western Asia. Moreover, this project will leverage the professional networks of the Western Asia Bat Research Network (WAB-Net), improve our scientific understanding of bat- borne viral diversity throughout Western Asia, and elucidate the risk factors associated with bat-borne virus shedding and spillover into human populations.

As you know one of my lab's focus in on the utility of serosurveillance to identify emergent zoonotic viruses such as coronaviruses, henipaviruses, and filovirurses. I have been developing tools for and analyzing serosurveillance data collected from wildlife (bats) and humans since 2015. I worked as a postdoctoral fellow with EcoHealth Alliance on BTRP funded project dating back to 2017, "Serological Biosurveillance for Spillover of Henipaviruses and Filoviruses at Agricultural and Hunting Human-Animal Interfaces in Peninsular Malaysia (HDTRA11710037)." In addition, my lab supported follow-up serological analysis of bat sera collected during the Ebola Host Project that was performed in collaboration with EcoHealth Alliance. Presently, I am a co-investigator within the EID-Southeast Asia Research Collaborative Hub (EID-SEARCH), one of the NIH Centers for Research in Emerging Infectious Diseases, of which you are a co-principal investigator. Within EID-SEARCH my lab develops, implements, and analyzes data generated from serological profiles of bats and human study participants. This proposed project will provide invaluable evidence to strengthen our understanding of and capacity to detect filovirus, coronaviruses, henipavirus (paramyxoviruses) circulation in bats in west Asia.

Sincerely,

(b) (6)

Eric D. Laing
Assistant Professor
Department of Microbiology and Immunology
Uniformed Services University

Christopher Broder, Ph.D.
Chair and Professor
Department of Microbiology and Immunology - USU

Facilities and Other Resources

Uniformed Services University of the Health Sciences
Dr. Eric Laing (Co-Investigator)

Overall Scientific Environment: Uniformed Services University (USU) is the medical school at which approximately half of the physicians in the Armed Services receive their graduate training. Research at USU is supported primarily by extramural grants, as in other medical schools. Dr. Laing is an Assistant Professor in the Department of Microbiology and Immunology, which includes 12 full-time Faculty members. The overall focus of the Department is 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. Dr. Laing is currently involved in active collaborations within the University, in areas of viral immunology and vaccine and antiviral therapeutics and animal model development. USU is also physically located directly across from the main NIH campus in Bethesda. The overall broad scientific environment at both USU and the NIH is highly conducive to productive collaborations. The PI often uses these resources to his advantage, both for his research objectives and interests and in assisting in adjunct faculty appointments within the Emerging Infectious Diseases graduate program for both on and off-campus scientists interested in participating in graduate education and graduate student training. Overall, the available technical resources (and University support for continually improving technical resources) is exceptional. In addition, multi-lab Immunology journal club, Immunology data club, Virology journal club, and Virology data club exist. Approximately 10 groups regularly participate in the immunology journal/data club and 5 groups regularly participate in the virology journal/data club.

Dr. Laing has been well-supported by the Department of Microbiology and Immunology and the USU Office of Research. Although USU is a modest-sized medical school, the technical resources are outstanding, rivaling many very large institutions (see below). The modest size of the school is an advantage, as key pieces of equipment (e.g., Bruker In Vivo Xtreme II; Siemens Inveon SPECT/PET/CT Scanner) are not overbooked and therefore readily available to the laboratory personnel. In addition to University funds, we have successfully used the NIH S10 mechanism to purchase advanced and very expensive equipment, including the Zeiss 710NLO system and the FACSAria 15-parameter cell sorter. Overall, the available technical resources (and University support for continually improving technical resources) is exceptional, as is illustrated by the most recent USU purchase of cutting-edge equipment: the Zeiss Elyra PS.1 super-resolution microscope.

Laboratory: Dr. Laing's laboratory is 453 sq. ft. and is currently supported by two graduate students, two research assistants, two research associates, and one scientist. Dr. Laing also has access to 3 rooms within the department totaling 1,440 sq. ft. where BSL-2 virus rescues are performed and blood specimen samples are handled. Dr. Laing has access to or possesses routine equipment for microbiology, molecular biology, and biochemistry including incubators, centrifuges, inverted microscopes, gel electrophoresis equipment for protein and DNA gels, thermal cyclers, balances, pH meters, refrigerators, freezers, platform orbital shakers, sonicator, UV transilluminator, shakers, heating blocks, waterbaths, free-standing biosafety cabinets, and CO_2-tissue culture incubators. Additionally, Dr. Laing has access to three BioPlex 200 HTF multiplexing systems, one MAGPIX multiplexing system, an epifluorescence microscopy system (a Zeiss AxioObserver inverted fluorescence microscope, upgraded in 2015), and two complete GE-ATKA low pressure chromatography systems, with integrated UV detectors, fraction collectors, and pump systems, and gradient fractionator apparatus.

Animal: Animals are maintained in University facilities under the supervision of a full-time veterinarian. The USUHS maintains a modern AAALC Accredited, Central Animal Facility of about 50,000 square feet. It, and the University's animal care and use program, is managed by the Center for Laboratory Animal Medicine, which is directed by a veterinarian who is an ACLAM Diplomate and staffed with three other veterinarians, a graduate animal husbandryman, and about 30 technicians. The University is able to provide appropriate care for a wide variety of laboratory animal species, from invertebrates to lower vertebrates, to higher vertebrates including non-human primates and domestic livestock, as well as the more commonly used species such as rodents and rabbits. The facility also includes a number of properly equipped ABSL-2 rooms.

Computer: Windows-based computers (Intel i5/i7 or similar processors and ≥2GB of RAM) are available for routine use by postdoctoral fellows, graduate students, and technicians located in the laboratories of both investigators. The University maintains site licenses for other image and data analysis software, including Zeiss Axiovision, VisioPharm, and OriginPro.

Office: Dr. Laing has an office separate from but proximal to the laboratories. Trainees and technicians have desks in the laboratory or in a separate office area proximal to the laboratory. The department employees one senior program manager, administrative officer, administrative specialist and a microbiologist.

Clinical: N/A

Other: The Biostatistics Consulting Center (BCC), a service of the Department of Preventive Medicine and Biometrics, provides statistical consulting to USUHS scientific investigators. We routinely consult with Cara Olsen, Research Assistant Professor (the full-time Biostatistics Consultant of the BCC), regarding proper design of experiments for statistical testing and for statistical analysis of the resulting data.
The USU Translational Imaging Facility (TIF) houses state-of-the art equipment for live animal imaging, including a Siemens Inveon SPECT/PET/CT Scanner, a Bruker Biospec 70/20 USR Magnetic Resonance Imaging system, and a Bruker In-Vivo Xtreme II bioluminescence and X-ray imaging system.

The USU Biomedical Instrumentation Center (BIC) houses core equipment for use by investigators throughout the University. Instrumentation is available either free or on a fee-for-service basis, depending on which instruments have annual service contracts (which are paid largely through per-hour use fees). The BIC Flow Cytometry Core includes two Becton-Dickinson (10- and 13-parameter) LSRII FACS analyzers, one 15-parameter FACSAria FACS sorter, and one Amnis Image Stream X Mark II imaging flow cytometer, as well as off-line analysis workstations.

The BIC Imaging Core houses three confocal microscopes, including a Zeiss 700 inverted system with 405/458/488/514/561/633 laser excitation; a Zess 710NLO inverted system with 405/458/488/514/561/633 conventional lasers and a Coherent Ultra2 Ti-Sapphire laser for multiphoton excitation, continuously tunable over the range of 690 to 1080 nm; and a Zeiss AxioExaminer-Z1 upright microscope equipped with a direct-coupled Coherent Chameleon tunable infrared laser for ex vivo and in vivo mutliphoton imaging projects. A Becker-Hickl two-detector FLIM system (for FRET analyses) is connected to the inverted Zeiss 710NLO system. Recently, the BIC has also acquired a Zeiss Elyra PS.1 super-resolution microscope, which is capable of 4-parameter SR-SIM (super-resolution structured illumination) imaging, 3-parameter PALM (Photoactivation localization microscopy) and dSTORM (direct stochastic optical reconstruction microscopy), as well as 3D-PALM/dSTORM. The BIC also houses a Leica AF6000 system, consisting of an inverted microscope equipped with a fully motorized 3-axis stage plus atmosphere and temperature control, allowing extended term (days) live cell analyses. Additionally, there is a stereology system consisting of a Zeiss AxioImager.M2 upright microscope connected to MicroBrightField's Stereo Investigator software package. The facility also includes several additional wide-field fluorescence microscopes, and three offline data analysis stations with software packages including: Zeiss Zen software and full Physiology package; Media Cybernetics' 3D Constructor, Image Pro Analyzer, Autodeblur, and Autovisualize; Metamorph Basic. The Imaging Core also includes a transmission electron microscope (Philips CM100 transmission EM) and an ultramicrotome (Leica EM UC6 with EM FC6 cryo attachment).

The BIC Genomics core includes an ABI 3900 DNA synthesizer, an ABI3500xl Genetic Analyzer (for sequencing), a RochLightCycler 480 for real-time PCR, and Systec Mediaprep and Plate Pourer instrument. There is also an integrated Fuji FLA-5000/LAS-3000 imaging system for many applications that involve fluorescence and chemiluminescence imaging of gels and blots. The BIC Proteomics Core includes two Agilent 1100 HPLCs, an AB SCIEX Voyager DSTR MALDI-TOF mass spectrometer, and an AB SCIEX Q-TOF tandem mass spectrometer.

The BIC Structural Biology Core includes a Rigaku HighFlux HomeLab X-ray diffraction system, with a MicroMax-007 HF microfocus rotating anode generator, an R-AXIS Imaging Plate detector, and an X-stream 2000 cryogenic system. Other available BIC instruments and services include histopathology and PET/CT instrumentation for small animal research.

The University also has an equipment repair service, central duplicating service, audiovisual service, and microcomputer support service. The University Learning Resource Center is a high quality medical and scientific library with additional microcomputers and support. A wide variety of scientific journals are available in print and via remote computer access. A central autoclave/glassware washroom serves the Department of Microbiology and Immunology and is maintained through extramural grant support.

BUDGET JUSTIFICATION

The Henry M. Jackson Foundation for the Advancement of Military Medicine Inc. (HJF) in partnership with the Uniformed Services University of the Health Sciences (USUHS) will manage this proposal, if awarded.

Personnel

Key Personnel:

Eric Laing, Ph.D., Co-Investigator (0.8 calendar month). Dr. Laing will be responsible for the coordination of the project activities with the principal investigator and other co-investigators. Dr. Laing will oversee the lab activities pertaining to the production of recombinant virus protein antigens and the establishment of the multiplex serology test. Dr. Laing will provide mentorship to the postdoctoral student and assist with data analysis. Dr. Laing will oversee serological training activities at the partner lab in Bong, Liberia and assist with in-country testing and data interpretation. He will provide the necessary concepts and practice of viral glycoprotein antigen preparation for serological application to co-investigators and collaborators. Dr. Laing will also provide the virological and immunological expertise necessary to understand the serological profiles of humans and wildlife antisera. Dr. Laing will be a government employee and no salary support is requested.

Other Personnel:

Dr. Si'Ana A. Coggins, PhD, Associate Scientist (3 calendar months, YR1; 2 calendar months, YR2-3). Dr. Coggins will assist with the supervision of all lab activities at USU and interface with the project management teams. As a trained biochemist Dr. Coggins will oversee all protein expression and maintain the quality and rigor of the research. Dr. Coggins will work closely with the research assistant, in year 1 to prepare protein antigens, verification of material quality, coordinate the transfer for materials to the partner institute in the regional partner labs and assist in the project activities. Dr. Coggins will participate in and provide serological assay training at the regional partner labs in years 2-3. Dr. Coggins is an employee of the Henry M. Jackson Foundation and salary support is requested for years 2-3.

TBD, Res Asst I (4 calendar months, YR1). A research assistant (RA) will assist Dr. Coggins in the production of soluble glycoproteins and be responsible for microsphere coupling in YR1. The RA will participate in serological testing and quality verification of the material, and will also prepare shipments of materials to the regional partner lab. The RA will be an employee of the Henry M. Jackson Foundation and salary support is requested for year 1.

Mr. Spencer L. Sterling, MpH, Research Project Coordinator (2 calendar month, YR2-3). Mr. Sterling will participate in the transfer of serology assay methods and data analysis. Mr. Sterling will work with the regional partner teams and EcoHealth Alliance scientists to assist in the analysis and development of serological results. Mr. Sterling will work closely with scientists at the prime institute to generate data for yearly reports. Mr. Sterling is an employee of the Henry M. Jackson Foundation and salary support is requested for year 2-3.

International Travel:

Based on FY23 Department of State per diem rates and historical travel to the Republic of Georgia, Oman, and Turkey, we estimate $32,300 should cover the travel costs of two lab members to travel to initiate and maintain project activities in the partner labs. In year 3, only travel to the Republic of Georgia is required.

Supplies:

Based on current prices, the following equipment and supplies will be needed to support this project in year 1: Recombinant production of approximately 34 protein antigens, magnetic beads, plastic consumables, cell culture materials, affinity matrices and control antibody materials.

Year 1 - $30,630.60
Recombinant protein production

Indirect Costs:

HJF indirect cost (IDC) is calculated based on the value-added cost base overhead rates. The fringe rate used is 29.42 % for Tier 1 employees and 7.29% for Tier 2 employees (all employees in this proposal are Tier 1).

The HJF indirect cost is calculated based on the value-added cost base overhead rates. The IDC rate is 36.74% USU Onsite Overhead. Additionally, 16.70% Companywide G&A and applied on the total direct cost less subaward plus the USU Onsite Overhead. For proposals including subawards, an additional 1.93% is applied on total external subaward costs.

The above fringe benefits and indirect cost rates for FY2023 were approved by the U.S. Army Medical Research Acquisition Activity on September 1, 2022.

FEE LANGUAGE HERE.

From:	Boxley, Kimberly on behalf of Boxley, Kimberly <kimberly.boxley.ctr@usuhs.edu>
To:	Luke Hamel
Cc:	Eric Laing
Subject:	Re: Submission
Date:	Thursday, February 16, 2023 7:41:08 AM
Attachments:	BioSketch_Laing_DTRA.pdf Current and Pending Support Template (WAB-Net 2)_LaingED.pdf DTRA_WABNet2_Laing_LOSv2.pdf Facilities_DTRA_Laing.pdf DTRA_WABNet2_Laing_Budget Justificationv2.pdf

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Hello Luke,

I don't anticipate any major changes .... But here is what we have. Note - there is a fee added to the project total costs. It is very minimal and we will include the language in the justification. Also, getting a countersignature from the chair, we should have that this morning also.

Thanks much .... Kim

On Wed, Feb 15, 2023 at 4:28 PM Luke Hamel <hamel@ecohealthalliance.org> wrote:

> Hi Kim,
>
> Could you please send me the biosketch, C&P, facilities, letter of support, and budget justification for Eric by tomorrow? Please let me know if that's not feasible. Thank you!
>
> Best,
>
> **Luke Hamel**
> *Program Coordinator and Research Assistant*
>
> EcoHealth Alliance
> 520 Eighth Avenue, Ste. 1200
> New York, NY 10018
>
> 1.212.380.4476 (direct)
> www.ecohealthalliance.org
>
> ***EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation***
>
> On Mon, Feb 13, 2023 at 3:52 PM Luke Hamel <hamel@ecohealthalliance.org> wrote:
>
> > Hi Kim,
> >
> > Those are the correct documents, and I have not received any of them yet.
> >
> > **Luke Hamel**
> > *Program Coordinator and Research Assistant*
> >
> > EcoHealth Alliance
> > 520 Eighth Avenue, Ste. 1200
> > New York, NY 10018

1.212.380.4476 (direct)
www.ecohealthalliance.org

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

On Mon, Feb 13, 2023 at 12:46 PM Boxley, Kimberly <kimberly.boxley.ctr@usuhs.edu> wrote:

> Hello Luke,
>
> Please confirm the following is necessary:
>
> *Budget
> *Budget Justification
> *Current & pending support
> *Biosketch
> *Letter of support
> *Facilities / Equipment
>
> Have you received any of the documents yet?
>
> Thanks much ..... Kim
>
> --
> **Kim N. Boxley l Program Manager 3 l Henry M Jackson Foundation**
> Uniformed Services University of the Health Sciences l Microbiology and Immunology
> 4301 Jones Bridge Road, Room B4122 l Bethesda, Maryland 20814
> Phone: **301-295-1941** l Fax: **301-295-1545** l Cell: **301-793-1883**
> Email: **KIMBERLY.BOXLEY.CTR@USUHS.EDU**
>
>

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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
4301 Jones Bridge Road, Room B4122 l Bethesda, Maryland 20814
Phone: **301-295-1941** l Fax: **301-295-1545** l Cell: **301-793-1883**
Email: **KIMBERLY.BOXLEY.CTR@USUHS.EDU**

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Title: Strengthening capacity for bat-borne coronavirus, paramyxovirus, and filovirus threat reduction in Western Asia

Abstract: Bats are reservoir hosts for several viral families that pose significant threats to human and livestock health. The diversity of bat viruses and their potential for emergence remain largely unknown across Western Asia – a region with a mixture of bat species from northern Africa, Europe, and Asia, which may facilitate viral sharing and diversification. Over the last five years, our team established a field-to-laboratory biosurveillance network including wildlife biologists, virologists, and public health experts from seven Western Asian countries. Here we propose to build on that regional network with an in-depth zoonotic disease surveillance project targeting bats and multiple pathogens. Specifically, we will: 1) detect and characterize known and novel viral threats (coronaviruses, paramyxoviruses, and filoviruses) from newly collected and archived bat samples from Western Asian countries using virus-family specific and next-generation sequencing approaches and multiplex serological assays; 2) identify risk factors for virus shedding and transmission at the individual-, population-, and metapopulation-levels using repeated, longitudinal sampling of key bat species that are known reservoirs for zoonotic viruses (i.e. *Rousettus aegyptiacus*, *Rhinolophus ferrumequinum*); 3) strengthen the professional capacity of regional partners and local students to conduct and sustain One Health disease surveillance; and 4) expand multi-sectoral engagement of governmental and non-governmental partners in the Western Asia Bat Research Network to improve the timeliness of data sharing, zoonotic risk assessments, and mitigation. Our project will leverage prior DTRA investments to further enhance the capacity of regional stakeholders to rapidly detect, diagnose, report, and develop policies to mitigate zoonotic disease threats.

Background and Justification: Bats are important reservoirs for zoonotic viruses, including coronaviruses (CoVs, e.g. SARS, MERS), paramyxoviruses (PMVs, e.g. Hendra and Nipah), and filoviruses (FVs, e.g. Ebola and Marburg) that cause fatal diseases in human populations, livestock, and other wildlife species [1]. Yet there remains a lack of knowledge about bat-borne viral threats in Western Asia that may lead to a heightened risk of destabilizing zoonotic disease outbreaks [2]. Under the BTRP-funded project 'Understanding the Risk of Bat-Borne Zoonotic Disease Emergence in Western Asia' (HDTRA1170064) from 2017-22, we conducted a robust, regional surveillance effort to characterize CoV prevalence and diversity in 37 bat species distributed in 7 countries and established the Western Asia Bat Research Network (WAB-Net) [2]. Overall CoV prevalence across all species in Western Asia was relatively high (15%) as compared to previous surveillance findings from other regions, e.g. SE Asia (1.7 - 8.6%) [3].

Our previous BTRP project led to several key discoveries warranting further investigation. Using partial-gene sequencing, we characterized a wide diversity of newly-discovered CoVs circulating in the region, including a large cluster of alpha- and beta-CoVs with potential to cause human and/or livestock disease outbreaks. While our cross-sectional surveillance across many bat species and sites led to critical insights into the vast diversity of bat CoVs, there remains a critical need for *longitudinal studies* of bat species with high potential for transmitting these viruses across broad geographic distributions. Two bat species distributed in Western Asia are ideal candidates for such studies: *Rhinolophus ferrumequinum* and *Rousettus aegyptiacus.* Specifically, *R. ferrumequinum*, a species with the largest distribution of any bat species in Western Asia, had the highest CoV prevalence (33%) of all 37 species we tested, and is a known host for other CoVs and PMVs [4]. Whereas *R. aegyptiacus* has a patchy distribution across Western Asia and Africa, and is host to FVs with high mortality rates (i.e. Marburg virus

[5]), CoVs, and PMVs [4]. Longitudinal studies of these target bat species would allow for a more complete understanding of viral emergence risk, including what factors promote viral shedding and when (seasonally) spillover is most likely. Conducting in-depth longitudinal studies from sites across a wide geographic area will inform bat-borne viral dynamics at multiple levels, from individual to population and metapopulation, and across spatio-temporal scales [6].

Scientific & Threat Reduction Impact: Our project will fill in critical knowledge gaps related to the diversity of viruses in high consequence pathogen groups circulating in Western Asia, and identify drivers of viral dynamics and transmission risk at the individual-, population-, and metapopulation-levels in target bat reservoir species. **Our project will additionally serve multiple objectives of the Cooperative Biological Engagement Program (Thrust Area 6)** by 1) engaging country partners in hypothesis-driven, One Health research; 2) advancing our scientific knowledge of how, when, and why bats shed viruses that can lead to outbreaks; 3) supporting enhanced biosurveillance capacity of five partner laboratories to rapidly detect, diagnose, and report diverse zoonotic viruses using molecular and serological approaches; 4) employing best practices for biosafety management during hands-on field and lab trainings; 5) promoting sustainability via multidisciplinary, cross-border student training initiatives from field-to-lab-to-analysis; and 6) promoting multi-sectoral government engagement in regional disease surveillance and monitoring to improve data sharing and reporting.

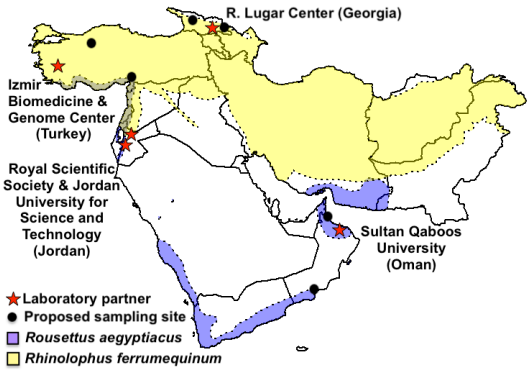
Objectives:

1) Detect and characterize known and novel CoVs, PMVs, and FVs at the human-wildlife interface in newly collected and archived bat samples using a combination of molecular screening, partial- and full-genome sequencing, and multiplex serological assays;
2) Identify factors that influence virus shedding and transmission at the individual, population, and metapopulation-level via targeted, longitudinal sampling of key bat species in order to better forecast disease spillover risk;
3) Strengthen field and laboratory capacity of in-country partners, including the next-generation of One Health students, to conduct zoonotic disease surveillance and research;
4) Increase engagement of community and multisectoral government stakeholders to improve data sharing, zoonotic risk assessment and mitigation activities.

Our proposed project will test the following initial hypotheses to understand the risk of bat-borne viral emergence, to be refined during the project in collaboration with in-country partners: **H1)** Molecular and serological screening of diagnostic samples will lead to the detection of currently unrecognized virus threats circulating in bat populations of Western Asia (e.g. known or novel FVs); **H2)** Individual-level host traits (i.e. demographic and physiological) influence infection dynamics and transmission (e.g. transmission may increase during the reproductive period when immune suppression is greatest); **H3)** Population-level interactions (i.e. bat density, species diversity) play a role in infection dynamics within a site (e.g. greater species diversity leads to increased opportunities for transmission within and among species); **H4)** Viral diversity and infection dynamics will vary across spatiotemporal scales at the metapopulation-level, such that spillover risk will not be uniform across the geographic distribution in the region; and **H5)** Sites with increased bat-human interactions (e.g. cave tourism, rapid habitat modification) will be at greatest risk for a spillover event.

Methodology: *Site selection* (Y1): We will identify 6 sites that serve as roosts for our target bat species: *Rousettus aegyptiacus* (3) and *Rhinolophus ferrumequinum* (3) for prospective,

longitudinal sampling. Sites will be located in 3 countries: Turkey, Georgia, and Oman (*see map*).



Site characterization (Y1-OY1): Using a multiscale approach, we will characterize environmental context at sampling sites for potential bat-human interfaces that may promote transmission risk. We will assess ecological and human disturbance factors during each sampling visit to monitor changes in bat-human interactions and spillover risk factors over time at each site, and assess broader land-cover and habitat changes using satellite images.

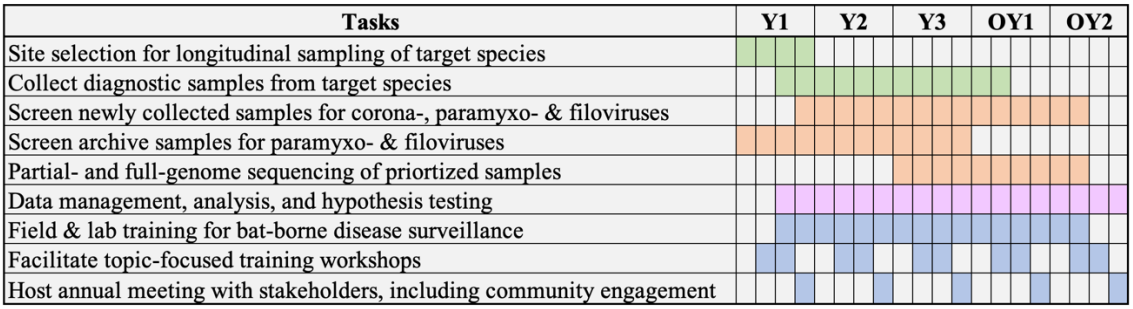
Bat sampling (Y1-OY1): We will conduct quarterly sampling (4 events/year) for 3 years at sites identified as roosts of target bat species. Specifically, we will collect non-lethal and field-inactivated (DNA/RNA Shield) diagnostic samples to screen for CoVs, PMVs, and FVs by: 1) capturing individual bats to safely collect non-lethal samples, specifically oral swabs, feces/rectal swabs, and blood samples, to assess the role of individual traits (e.g. sex, age, reproductive status) on infection dynamics; and 2) placing sterile plastic sheeting under aggregations of roosting individuals to collect pooled fecal and urine samples to further capture population-level viral dynamics and diversity. During each sampling event, we will sample at least 50 individual bats and collect additional pooled samples.

Virus screening of newly collected and archived samples (Y1-OY2): We will use family-specific consensus PCRs and subsequent partial- and full-genome sequencing to detect and characterize pathogen diversity in viral groups known to include select agents and important zoonoses: CoVs, PMVs, and FVs. We will begin with screening archived samples (i.e. oral swabs, feces/rectal swabs) previously collected from 4,275 individual bats from 50 sites in seven Western Asian countries under our previous BTRP-funded project (2017-22). Archived samples have been tested for CoVs, thus we will begin screening archived samples in Y1 for PMVs using PCR (FVs will be screened using serology given low molecular detection rates). In Y1, we will also begin further molecular characterization (Spike gene or full-genome) of selected, previously-identified CoVs to better understand phylogenetic relationships and infection risk. In addition, we will establish and use Luminex-based *multiplex* serological assays (validated via other DTRA-funded projects) to screen for antibodies (IgG) against viruses in target viral families (e.g. all FVs, select PMVs (henipaviruses), and diverse CoVs). Serological data will allow us to model infection dynamics in the population and among subpopulations.

Data management & analysis (Y1-OY2): We will establish a secure data management system to integrate field and laboratory data, and train country partners on best practices in data management and quality control. We will use multivariate and spatio-temporal modeling approaches to test hypotheses of individual-, population-, and metapopulation-level factors on infection dynamics, and conduct training with country partners on using these analyses. EHA will develop data analysis pipelines and 'dashboards' that integrate directly with our data management system, which will allow for 'real-time' generation of reports and outputs for communicating project findings with in-country stakeholder, including local communities.

Capacity Building: We will: **a)** Provide hands-on field trainings for relevant government staff, university faculty, and students to expand capacity to implement a longitudinal surveillance program while following strict biosafety and biosecurity standards; **b)** Develop and share

laboratory protocols for partial- and full-genome sequencing and multiplex serological assays and coordinate a regional training workshop (Y1) for country partners to implement protocols; **c)** Host 2x/year virtual "hot topic" biosurveillance workshops to strengthen research skills of country partners and encourage regional sharing of best practices and lessons learned. **d)** Host annual WAB-Net meetings (hybrid, in-person/virtual) with project partners, multisectoral government and non-government stakeholders to discuss project results and facilitate cross-sectoral data sharing. Y1-2 focus will be on integrating project data with existing national surveillance platforms to enhance biological threat reduction strategies in the region. **e)** Modify, translate, and use EHA's "Living Safely with Bats" tool for community engagement at each site.



Partner Institutions and Roles: 1) EcoHealth Alliance (EHA, PI Olival and co-PI Phelps) will oversee all aspects of project implementation – study design, permissions, coordinate field and laboratory activities, and analyze and publish project findings with collaborators. EHA will be responsible for all contractual obligations, including tax liabilities, and maintaining regular communication with DTRA and project sub-awardees. EHA has worked with each partner institution for 5+ years. **2) R. Lugar Center, Georgia** will conduct longitudinal sampling at 2 roost sites for *R. ferrumequinum*; screen and conduct partial- and full-genome sequencing of viruses from 2,564 archived samples and newly collected samples. **3) Royal Scientific Society, Jordan** will screen and sequence viruses from 1,711 archived samples. **4) Jordan University for Science and Technology** will conduct full-genome sequencing on a subset of archived samples. **5) Boğaziçi University, Turkey** will conduct longitudinal sampling at 2 roost sites, 1 per species. **6) Izmir Biomedicine and Genome Center, Turkey** will screen newly collected samples using viral family-specific PCRs and partial- and full-genome sequencing. **7) Environment Authority, Oman** will conduct longitudinal sampling at 2 roost sites for *R. aegyptiacus*. **8) Sultan Qaboos University, Oman** will screen newly collected samples using viral family-specific PCRs and partial- and full-genome sequencing. **9) Uniformed Services University, USA** will lead in-country training, reagent development, and analysis for multiplex serological assays. Partner institutions will support cross-institution trainings for students and professionals. A **Scientific Advisory Board** with Western Asia representation will be established for overall project and scientific oversight.

Cost Estimates: Y1 ($950,000) + **Y2** ($985,000) + **Y3** ($985,000) = $2,920,000
OY1 ($980,000) + **OY2** ($950,000) = $1,930,000

We will allocate > 50% of funds to in-country partners via subawards and direct benefits.

References: 1. Olival, K.J et al. *Nature* 2017, 546, 646–650. **2.** Phelps, K.L. et al. *Viruses* 2019, 11. **3.** USAID PREDICT Legacy Report 2020. **4.** Chen L. et al. DBatVir: The Database of Bat-Associated Viruses, 2014. **5.** Towner, J.S. et al. *PLoS One* 2007, e764. **6.** Ruiz-Aravena, M. et al. *Nat. Rev. Microbiol.* 2021, doi:10.1038/s41579-021-00652-2.

DEFENSE THREAT REDUCTION AGENCY
BROAD AGENCY ANNOUNCEMENT
HDTRA1-14-24-FRCWMD-BAA
Amendment 17
December 2022

Research and Development Directorate (RD)
Enabling Capabilities Department (RD-EC)

Fundamental Research to Counter Weapons of Mass Destruction (C-WMD)

Original Posting Date: March 2015

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OVERVIEW INFORMATION

Agency Name:

Defense Threat Reduction Agency (DTRA)
Research and Development (RD) Directorate
Enabling Capabilities Department (EC)
8725 John J. Kingman Road, MS 6201
Fort Belvoir, VA 22060-6201

Funding Opportunity Title: Fundamental Research to Counter Weapons of Mass Destruction (FRCWMD) Broad Agency Announcement (BAA)

Announcement Type: This is an amended announcement of this funding opportunity. This BAA is in effect from March 2015 through September 2024. It is anticipated that a majority of the actions funded from this announcement will be in the form of grants; however, other instruments such as contracts, cooperative agreements (CAs) or other transactions agreements (OTAs) may also be awarded from this announcement. Submissions for this BAA may occur in two ways: 1) in response to the published topics detailed in Attachment 1 or 2) to a general thrust area as described in Section 1.5.

In general, all topic-specific and general thrust area submissions require pre-coordination in accordance with the guidelines in Section 1.5 and Section 4.2.1. DTRA reserves the right to waive the pre-coordination requirement for topics on a case-by-case basis; and will state the waiver applies within the individual topic description. If a pre-application white paper is received without prior coordination, DTRA may not review it. From the date of the disposition email the applicant has 63 days to submit the pre-application white paper. If the submission is not feasible within this 63-day window, the abstract must be coordinated again to ensure the interest in the white paper remains.

The evaluation of all submissions will be conducted in two phases. Phase I is for receipt and evaluation of pre-application white papers in direct response to a published topic or by invitation based on the assessment of the idea by the Technical POC. Phase II is for receipt and evaluation of invited proposal applications. Invitation to the Phase II, invited proposal submission, will be based on the evaluation results of the Phase I pre-application white paper.

Funding Opportunity Number: HDTRA1-14-24-FRCWMD-BAA

Catalog of Federal Domestic Assistance (CFDA) Number: 12.351

Dates: This BAA is open continuously from March 2015 through September 2024. Published topics will include instructions on any topic-specific opening and closing dates as well as any topic-specific limitations on award types, dollar values, and eligibility. Submissions to a general thrust area may occur at any time this BAA is in effect. Applicants should take care to note requirements for pre-coordination of an abstract.

ADDITIONAL OVERVIEW CONTENT

Research, educational program, or other effort proposals are sought from accredited degree-granting colleges and universities. Research, educational program, or other effort proposals are also sought from industrial, commercial (including small businesses), and not-for-profit research entities. DTRA strongly encourages and may give preference to pre-application white papers and proposals that demonstrate a significant contribution (significant is defined as a minimum of

30% of total value) by one (1) or more universities.

All submissions (pre-application white papers and invited proposals) must be made in accordance with the submission instructions in this BAA through www.grants.gov using the application packages linked with this BAA (under the "Package" tab) on www.grants.gov. Applicants are responsible for ensuring compliant and final submission of their pre-application white papers and proposal applications. Any submission that does not conform to the requirements outlined in the BAA and in the invitation for proposal may not be reviewed or considered further at the discretion of DTRA.

Pre-application white papers may be evaluated any time after receipt. Invitations for full proposal submission may occur any time after the pre-application white paper evaluation and will be limited to available program funds.

Efforts may be proposed for up to five (5) years. Awards may be for a base period of one (1) year with four (4) additional years as possible options, a base period of two (2) years with three (3) additional years as possible options, or a base period of three (3) years with two (2) additional years as possible options. Applicants should take care to propose the most logical mix of base and option years for the scope of work. Further, the base period should yield a logical completion point for the work. In cases where option years are proposed, decisions regarding exercising those options will be based on the evaluation of the work accomplished in the base period. Pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable; however, the Government reserves the right to invite option years for awards that originally only included a base period.

Grants may range from small dollar value (e.g., $25K) up to $1M annually (total, including both direct and indirect costs) depending on the nature and the scope of work. Payments on grants will be made in advance, subject to the conditions described in 2 CFR 200.305. Funding amounts for contracts, CAs, and other procurement instruments will be considered on a case-by-case basis. Thirty (30)-60 individual awards are anticipated each year.

Any assistance instrument awarded under this announcement will be governed by the award terms and conditions, which conform to DoD's implementation of OMB circulars applicable to financial assistance. This includes DoD implementation of OMB guidance in 2 CFR part 200, "Uniform Administrative Requirements, Cost Principles, and Audit Requirements for Federal Awards."

1. FUNDING OPPORTUNITY DESCRIPTION

1.1. DTRA safeguards America and its allies from weapons of mass destruction (WMD) and provides capabilities to reduce, eliminate, and counter the threat and effects from chemical, biological, radiological, nuclear, and high yield explosives. DTRA seeks to identify, adopt, and adapt emerging, existing and revolutionary sciences that may demonstrate high payoff potential to Counter-WMD (C-WMD) threats. This BAA is an extramural endeavor that combines the fundamental research, educational program, or other effort needs appropriate for basic or applied research funding of DTRA and other DoD interests.

This announcement solicits ideas and topic-based pre-application white papers for long-term challenges that offer a significant contribution: to the current body of knowledge, to the understanding of phenomena and observable facts, to significantly advance revolutionary technology, to new concepts for technology application, or that may have impact on future C-WMD threat reduction, expertise, or capabilities.

A portion of this effort is expected to be devoted to awards for science, technology, engineering and mathematics education programs with a C-WMD focus; such as, but not limited to postdoctoral fellowships, stipends, degrees, visiting scientist programs, student exchange programs, and development of accredited C-WMD curricula.

1.2. Fundamental research means basic and applied research in science and engineering, the results of which ordinarily are published and shared broadly within the scientific community, as distinguished from proprietary research and from industrial development, design, production, and product utilization, the results of which ordinarily are restricted for proprietary or national security reasons.

Contracted Fundamental Research includes research performed under grants, contracts (awards), or OTAs that are (a) funded by budget Category 6.1 (Basic Research), whether performed by universities or industry or (b) funded by budget Category 6.2 (Applied Research) performed on-campus at a university. Fundamental research provides for science and technology (S&T) research and early applied development. It seeks to lower performance risk to a manageable level and facilitate transition and funding to capability end-state programs.

1.3. Technology Readiness Levels (TRLs) provide a systematic metric/measurement system that supports assessments of the maturity of a particular technology and the consistent comparison of maturity between different types of technology. Fundamental research may be defined within the first four (4) TRLs.

1.4. This BAA seeks optimum approaches to meet DTRA fundamental research objectives. The Government encourages pre-application white papers and proposals that span a wide spectrum of research to expand fundamental scientific knowledge in response to specific topics and to the more general thrust areas. The Government reserves the right to award any combination of approaches which offer the best overall value to the Government and to oversee any and all processes and approaches once ongoing.

1.5. Thrust Areas 1-7 are described below. When a specific set of topics has been identified, these detailed needs will be described in Attachment 1 along with any topic-specific submission instructions, deadlines, anticipated award structure, and funding requirements. Otherwise, pre-application white papers and proposals may be written against one of the general thrust area descriptions.

DTRA may not review any pre-application white papers without prior coordination of the idea with the appropriate thrust area- e-mail address (Section 7). Applicants should note that there is extremely limited funding available for many of the thrust areas; Thrust Areas 1-5 are not currently accepting abstracts for pre-coordination. Pre-application white papers will only be accepted from the coordinated abstracts under limited circumstances.

1.5.1. **Thrust Area 1—*Science of WMD Sensing and Recognition:*** The science of WMD sensing and recognition investigates the fundamental understanding of materials that demonstrate measurable changes when stimulated by radiation or particles from WMD in the environment. This involves the exploration and exploitation of interactions between materials and various electromagnetic phenomena, molecules, nuclear radiation, and particles. Furthermore, these interactions and the specific form of recognition they offer are used for the subsequent generation of information, providing knowledge of the presence, identity, and quantity of material or energy in the environment that may be significant. The goal of this thrust area's portfolio is to advance the following capabilities: location, identification and characterization of radiological-nuclear (RN) materials; detection of RN materials at significant stand-off distances; and the reduction of the technical nuclear forensics timeline. Thrust Area 1 is currently not interested in research focusing on the sensing of explosives or the detection of Improvised Explosive Devices (IEDs). **Thrust Area 1 is NOT currently accepting abstracts for pre-coordination.**

1.5.2. **Thrust Area 2—*Network Sciences:*** The fundamental science of network sciences includes advancing knowledge of complex disparate but interdependent networks critical to military operations where WMD-related robustness, resiliency, recovery of, and informational and operational utility is required. It includes response, resilience, and recovery of interdependent, multi-layered physical networks after exposure from electromagnetic pulse and other nuclear weapons effects, rapid discovery and analyzing low-observable WMD-related information from large, disparate WMD-related data sets from multiple types of networks, and to develop theories and representations for low observable WMD-related radical ideation from social networks. **Thrust Area 2 is NOT currently accepting abstracts for pre-coordination.**

1.5.3. **Thrust Area 3—*Science for Protection:*** Fundamental science for protection involves advancing knowledge in physical, biological, and engineering sciences to protect personnel, sensitive electronic systems, and structural infrastructure from the effects of weapons of mass destruction. Protection includes both passive and active defense against threats. Approaches include advanced highly-ordered materials and nanomaterials to hardening infrastructure and facilities against blast, nuclear events, or other CBRNE effects; exploring new methods to experimentally and computationally simulate the effects of a nuclear event; investigations of the interaction of radiation with sensitive electronics and systems as well as development of novel materials and methods that are robust against radiation effects; novel methods to protect personnel from the physical, radiological, and nuclear effects of WMDs; and the study of biological systems, including intact structures, metabolic products, or discrete components and pathways, as applied to protection of U.S. Forces during operations in areas actually or potentially contaminated by radiation. For protection of personnel the areas of interest include development of radiation countermeasures to prevent biological damage associated with exposure to ionizing radiation and development of novel biologically-based or -produced detection systems for wide area surveillance to determine the nature, extent, and distribution of contamination. **Thrust Area 3 is NOT currently accepting abstracts for pre-coordination.**

1.5.4. **Thrust Area 4—*Science to Defeat WMD:*** Fundamental science for significantly improving energetic materials for use against WMD facilities and systems with minimal collateral effects from post-blast WMD release, for deeper penetration to deny the adversary sanctuary of WMD, and for predictable modeling of counter-WMD munitions and simulation of in-theater scenarios with accurate lethality calculations. **Thrust Area 4 is NOT currently accepting abstracts for pre-coordination.**

1.5.5. **Thrust Area 5—*Science to Secure WMDs:*** Fundamental science to support securing WMD includes: revolutionary means to safely handle, transport, control access, or eliminate WMD components and weapons; new physical or other methods to monitor compliance to support future agreements or treaties; and, exploring phenomena and means that facilitate reduction of nuclear or non-nuclear WMD proliferation pathways. This includes focus on: science principles to assist tagging, tracking, location to secure WMD; novel means to mark and read objects in order to secure inventories; remote or unattended monitoring to understand the nature of objects (e.g., is it nuclear, biological, chemical or conventional?); monitoring to detect intrusion, diversion or substitution, tampering, and other adverse activity; and, understanding of both physical and life science environmental signatures as witnesses of WMD-related activity. The ability to secure WMD may impact either verification of treaties, or control of WMD outside treaty regimes. **Thrust Area 5 is NOT currently accepting abstracts for pre-coordination.**

1.5.6. **Thrust Area 6—*Cooperative Counter WMD Research with Global Partners:*** Cooperative fundamental research to reduce the global threat of WMD in collaboration with a broad range of global research partners. This thrust area involves exploratory basic and applied research that will address opportunities to reduce, eliminate, and counter WMD across the Chemical, Biological, Radiological, Nuclear, and High Explosive (CBRNE) spectrum. Efforts in this area will develop strong international relationships which will foster a smooth transition of program ownership to the partnering country. The goal is to improve international collaboration to detect, characterize, and report WMD, and to advance partner nation sustainment through a culture of long-term cooperation and scientific responsibility for such programs. Multi-disciplinary, multinational research in science, technology, engineering, and mathematics development will be conducted to promote transparency through quality research publications and continual dialogue between scientists/engineers and young researchers.

The Cooperative Biological Engagement Program (CBEP), a component of the DoD Cooperative Threat Reduction (CTR) Program, recognizes the danger to U.S. and global health security posed by the risk of outbreaks of dangerous infectious diseases, whether natural or manmade. Consistent with the national and departmental strategies, CBEP strives to address this risk by promoting best practices in biological safety and security, improving partner country capability to safely and rapidly detect and report dangerous diseases, and establish and enhance international research partnerships that focus on informing the disease surveillance network. The desired end state for CBEP engagements is the development of sustainable partner country capabilities to:

- Employ responsible bio-risk management best practices and principles,
- Conduct a modern and effective disease surveillance mission,
- Independently sustain engagement with, and effectively compete for funding within, the international scientific community,

- Comply with World Health Organization (WHO) International Health Regulations (IHR) and World Organization for Animal Health (OIE)/U.N. Food and Agriculture Organization (FAO) reporting guidelines, and
- Promote the One Health Concept.

The goals and objectives of CBEP international research partnerships are to:

- Goal 1: Support Biosurveillance, Biosafety and Biosecurity (BS&S) Capability Building Efforts
    - Objective 1: Inform and enhance operational biosurveillance strategies and implementation through improved understanding of endemic disease burden and pathogen biology.
    - Objective 2: Institutionalize responsible biorisk management best practices with partner country scientists.
- Goal 2: Engage Partner Country Scientists in Hypothesis-Driven Research
    - Objective 1: Support local, national, and regional priorities for understanding and mitigating human and animal disease risk (e.g., small, focused projects within individual countries linked by broad, integrating projects that include regional partners).
    - Objective 2: Improve international collaborations to survey, detect, characterize, and report disease.
- Goal 3: Promote One Health Initiative
    - Objective 1: Emphasize the nexus of human health, animal health, and the environment, and seek to further understand the mechanisms and factors involved in disease transmission.
    - Objective 2: Advance partner country sustainment of global health security initiatives.
- Goal 4: Foster an International Culture of Responsible and Ethical Conduct in Biological Research
    - Objective 1: Transition to a culture of responsibility and ethical conduct in biological research through thoughtful experimental design and good laboratory practices that result in high-quality data, and active participation in professional societies and the peer-review process.
    - Objective 2: Train partner country researchers to think critically in the pursuit of ethical research and to be competitive in soliciting funding from the international scientific community.

Ultimately, the techniques, procedures, and approaches must be sustainable for the partner country and linked with appropriate training in order to promote global health security, reinforce norms of safe and responsible conduct, obtain timely and accurate insight on current and emerging infectious disease risks, and transform the international dialogue on biological threats.

CBEP research projects are not determined by or limited to specific biological agents, but must be plausibly linked to pathogens of security concern and aimed at measurably supporting threat reduction objectives that:

- Enhance partner country's/region's capability to identify, consolidate, and secure collections of pathogens and diseases of security concern in order to prevent the sale, theft, diversion, or accidental release of such pathogens and diseases.
- Enhance partner country's/region's capability to rapidly and accurately survey, detect, diagnose, and report biological terrorism and outbreaks of pathogens and diseases of security concern in accordance with international reporting requirements.

Region-specific areas of interest are described in CBEP Regional Science Plans. Examples of general CBEP research areas of interest include: Epidemiology (e.g. studies measuring disease prevalence and incidence), Pathogen Biology, Pathogen Characterization, Assay Adaptation and Optimization, Microbial Ecology within a Public Health Context, and Preventative Strategies and Countermeasures. For clarification, medical countermeasure development (i.e., development of diagnostic tools, vaccines, therapeutics) is supported by many other U.S. Government or international agencies and is generally not supported by CBEP; however, research projects may inform medical countermeasure development and support validation and verification testing (e.g., as part of proficiency testing, pilot studies/testing, or exercises, etc.). Additionally, CBEP does ***not*** generally support research with common disease agents such as HIV/AIDS, malaria, and tuberculosis where other U.S. agencies have dedicated missions to do so; however, the program may choose to capitalize on opportunities to leverage research on these diseases to further CBEP goals, for example by testing samples collected under the auspices of other programs. CBEP also **will not** support research which poses risks to the overall threat reduction mission of CBEP, Dual-Use Research of Concern, or related activities (i.e., *in vivo* pathogenicity studies, virulence studies, animal passaging, etc.).

CBEP is interested in collaborative research engagements with foreign partners in any one of the following regions: Countries of the Former Soviet Union (FSU) (specifically, Armenia, Azerbaijan, Georgia, Kazakhstan, and Ukraine), Africa (including, but not limited to, Kenya, Tanzania Uganda, South Africa), Southeast Asia (including, but not limited to, Cambodia, Indonesia, Laos, Malaysia, Philippines, Thailand), and Middle Eastern /South Asian countries (including, but not limited to, Afghanistan, Iraq, India and Pakistan). CBEP encourages proposers to develop projects in conjunction with foreign institutions in CBEP-engaged countries.

1.5.7. **Thrust Area 7—*Fundamental Science for Chemical and Biological Defense:*** Fundamental science for chemical and biological (CB) defense includes science and technology research that advances knowledge in physical and life sciences to defend and counter chemical and biological WMD that could be used against our Nation's warfighters. Fundamental research efforts enable capabilities such as development of improved detection devices for traditional and nontraditional chemical agents; development of diagnostics for existing and emerging infectious disease threats; increasing knowledge and improved capabilities for development of new or improved medical and material countermeasures to CB threats for both pre- and post-exposure scenarios; enhanced personal protection against, modeling of, prevention of, or decontamination of CB threats; and providing effective elimination strategies via non-kinetic approaches for threat agent destruction, neutralization and/or sequestration.

1.6. This BAA, in addition to any amendments issued in conjunction with this BAA, will be posted to the Grant Opportunities Website (https://www.grants.gov), the System for Award Management website (https://sam.gov/), and the DTRA website (https://www.dtra.mil). The

DTRA website is not the official sites; applicants are responsible for monitoring both sam.gov and www.grants.gov. Posted amendments supersede all previous versions of the BAA. Note that topics will be listed in Attachment 1 and will be added/closed with Amendments to this BAA.

1.7. All coordination and communication between applicants and the Government will be conducted using the e-mail address associated with this BAA, specified in Section 7. Applicants should include both the administrative email and the relevant thrust area email address. DTRA will not release employee personal contact information.

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2. AWARD INFORMATION

2.1. Award Types. The full range of flexible procurement instruments available to DTRA are possible results from this announcement, including but not limited to contracts, grants, CAs, and OTAs; however, grants will likely be the predominant procurement instrument. Each of the applicable procurement instruments offer different advantages, liabilities and responsibilities for applicants and the Government.

Applicants must specify in their submittal their recommended approach (e.g. contract, grant, CA, or OTA); however, the Government reserves the right to negotiate and award the types of procurement instruments determined most appropriate under the circumstances. If warranted, portions of resulting awards may be segregated into pre-priced options.

Except for OTAs, the Government actions under this BAA shall adhere to the requirements of the Federal Acquisition Regulation (FAR), Defense Federal Acquisition Regulation Supplement (DFARS) and/or DoD Grant and Agreement Regulations (DoDGARS), as appropriate for the type of instrument. DoDGARs can be accessed online at http://www.ecfr.gov/cgi-bin/text-idx?SID=e5d686f6760f3274b3368f36723fbb7e&mc=true&tpl=/ecfrbrowse/Title32/32CIsubchapC.tpl . See also 32 Code of Federal Regulations (CFR) 22, which can be accessed online at http://www.ecfr.gov/cgi-bin/text-idx?rgn=div5;node=32%3A1.1.1.3.16 . Any assistance instrument awarded under this announcement will be governed by the award terms and conditions, which conform to DoD's implementation of OMB circulars applicable to financial assistance.

On average, DTRA expects to award 30-60 individual awards each year. The predominance of awards will be grants. Payments on grants will be made in advance, subject to the conditions described in 2 CFR 200.305.

2.2. Scope of Awards. Awards may range from focused, exploratory projects with a high risk approach in innovative research in subjects with potential for high impact to C-WMD science to comprehensive programs of innovative research in an interdisciplinary area with potential for high impact.

Awards may have multiple Co-Principal Investigators (Co-PIs) and subawards. Authors of pre-application white papers and proposals should detail the contribution of all Co-PIs and any subawards to the C-WMD scientific impact.

Preference will be given to projects where undergraduate and/or graduate students, and/or postgraduate students are supported by the awards. Details regarding the participation of the students and the value of the research to the students as part of the pre-application white paper

and full proposal are expected. Additional guidance regarding student and/or postgraduate student participation may be provided in the published topics or in communications with the applicant to include the coordination of the abstract or in the debrief summary of the pre-application white paper. Any specific guidance provided in a topic or to an applicant supersedes the information provided herein.

2.3. Subawards. Subawards (subgrants and/or subcontracts) are permitted. Subawards may be used to carry out a portion of the research or efforts. Awards may have multiple subawards. Awards will be made by a single award, e.g., grant or contract, to the lead organization. All subawards are the responsibility of the award recipient; exceptions will not be made.

For submissions made to Thrust Area 6 and associated topics, there is no limitation on subawards. DTRA will review and consider the proposed subawards for all pre-application white papers and proposals on a case-by-case basis. The prime awardee will be responsible for transferring funds to the subawardee. Applicants are reminded that priority is given to projects with the main locus of activity in the region-of-interest, so budgets should be allocated accordingly. Preference will be given to proposals where the subaward component to the region-of-interest partner(s) represents more than half of the award value (as measured in U.S. dollars).

2.4. Award Values. Grants resulting from submissions to Thrust Areas 1-7, including topics associated with these thrust areas, may range from small dollar value (e.g., $25K) up to $1M annually (total, including both direct and indirect costs) depending on the nature and the scope of work. Contracts, CAs, and OTAs will be considered on a case-by-case basis. All awards are subject to the availability of funds. Additional guidance regarding award values may be provided in the published topics or in communications with the applicant to include the coordination of the abstract or in the debrief summary of the pre-application white paper. Any specific guidance provided in a topic or to an applicant supersedes the information provided herein. Funding for participation in this program is highly competitive and the cost of proposed research should strictly be maintained as detailed herein or as indicated in the invitation instructions.

2.5. Period of Performance and Award Structure. Efforts for Thrust Areas 1-7, including topics associated with these thrust areas, may be proposed for up to five (5) years. Awards may be for a base period of one (1) year with four (4) additional years as possible options, a base period of two (2) years with three (3) additional years as possible options, or a base period of three (3) years with two (2) additional years as possible options. Additional guidance regarding award structure may be provided in the published topics or in communications with the applicant to include the coordination of the abstract or in the debrief summary of the pre-application white paper. Any specific guidance provided in a topic or to an applicant supersedes the information provided herein.

Applicants should take care to propose the most logical mix of base and option years for the scope of work. Further, the base period should yield a logical completion point for the work. In cases where option years are proposed, decisions regarding exercising those options will be based on the evaluation of the work accomplished in the base period.

DTRA is flexible on the award structure unless otherwise specified in the published topics or in communications with the applicant to include the coordination of the abstract or in the debrief summary of the pre-application white paper. Applicants should take care to clearly label the tasks and anticipated outcomes for the base and option years in the pre-application white papers

and the proposals. Pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable; however, the Government reserves the right to invite option years for awards that were originally awarded with only a base period.

2.6. The Government Accountability Office, in its report GAO-16-14, WOMEN IN STEM RESEARCH: Better Data and Information Sharing Could Improve Oversight of Federal Grant-making and Title IX Compliance, December 3, 2015, recommended that the DoD collect certain demographic and career information to be able to assess the success rates of women who are proposed for key roles in applications in science, technology, engineering, or mathematics disciplines. To enable this assessment, DTRA will include with each Phase II application package the Research and Related Senior/Key Person Profile (Expanded) form and the Research and Related Personal Data form.

2.7. The Government does not anticipate the need to provide any hardware or software to execute the proposed research. However, DTRA will review and consider any hardware/software requests for all pre-application white papers and proposals on a case-by-case basis.

2.8. The Government reserves the right to fund all, some, or none of the proposals submitted; may elect to fund only part of any or all proposals; and may incrementally fund any or all awards under this BAA. The Government also reserves the right to request applicants make any changes necessary to submitted full proposals to increase the feasibility of making the proposal fundable. Applicants may decline to participate in any revisions to application packages requested by DTRA.

2.9. The Government may offer funding for any full proposals or portions of proposals at any time during the lifetime of this BAA.

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3. ELIGIBILITY INFORMATION

3.1. Pre-application white papers and proposals submitted for this BAA will be considered from the following U.S. and Foreign Institutions as follows:

- Accredited degree-granting colleges, universities, and academic institutions.
- Industrial and commercial entities, including small businesses.
- Not-for-profit entities with a portfolio predominantly in research and foreign government laboratories. Proof of 501(c)(3) status from the Internal Revenue Service may be required. For foreign-based establishments entirely based outside the U.S. and/or its territories, proof of not-for-profit status may be required. Foreign based government laboratory equivalents include those residing in the Ministry of Defense, Ministry of Health, Ministry of Agriculture, Ministry of Education and Science and Food Safety Agencies.

DTRA strongly encourages and may give preference to pre-application white papers and proposals that demonstrate a significant contribution (significant is defined as a minimum of 30% of total value) by one (1) or more universities. Applicants should note that university participation may be mandatory for some published topics. Additional guidance regarding university participation may be provided in the published topics or in communications with the

applicant to include the coordination of the abstract or in the debrief summary of the pre-application white paper. Any specific guidance provided in a topic or to an applicant supersedes the information provided herein.

The following entities may not participate as prime awardees nor furnish Principal Investigators (PIs) in awards made under this BAA but may act as collaborators, including as Co-PIs, and/or subawardees:

- Federal Academic organizations (e.g., Naval Postgraduate School).
- Federal laboratories (including DoD and Department of Energy (DOE)).
- U.S. Government agencies.
- DoD-sponsored Federally Funded Research and Development Centers (FFRDCs) specified in the Defense Federal Acquisition Regulation Supplement (DFARS) 235.017-1 (http://farsite.hill.af.mil/VFDFARA.HTM) and click on 'DFARS Part 35'.
- DOE-sponsored FFRDCs.

Note: Federal laboratories (including DoD and DOE) and FFRDCs are eligible to submit abstracts (when required), pre-application white papers, and proposals in response to the Government Call (HDTRA1-16-24-FRCWMD-Call). However, a FFRDC (other than the DoD FFRDCs specified in DFARS 235.017-1) must have authorization from its sponsoring agency in accordance with FAR 35.017-1. Eligibility requirements under the Call are subject to change. See http://www.dtrasubmission.net and after logging in, follow the link to the 'FY16-24 Fundamental Research to Counter Weapons of Mass Destruction (C-WMD) Government Call'.

3.2. Cost Sharing or Matching. In general, cost sharing or matching is not required for applications to this announcement. However, DTRA reserves the right to require cost sharing or matching on a case-by-case basis. Such instances will be specifically detailed in the published topics or in communications with the applicant to include the coordination of the abstract or in the debrief summary of the pre-application white paper.

3.3. Other. DTRA uses the System for Award Management (SAM) to exclude recipients ineligible to receive Federal awards. SAM can be accessed online at http://sam.gov (Reference 2 CFR 1125).

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4. APPLICATION AND SUBMISSION INFORMATION

4.1. Address to Request Application Package. This announcement contains all information required to submit a pre-application white paper and invited proposal.

4.1.1. The required application packages for the pre-application white papers and for the invited proposals are posted with this announcement. Note that each thrust area (as outlined in Section 1.6) and each topic (as outlined in Attachment 1) has a unique application package posted with this BAA. The application package corresponding to both: a.) the thrust area or topic of interest and b.) the phase, should be used for submission of pre-application white papers and invited full proposals.

4.1.2. The application packages posted to www.grants.gov consist of the forms as detailed in

Table 2.

Form Name	Phase I Pre-Application White Paper	Phase II Invited Proposal
SF-424 (R&R) Application for Federal Assistance Form	Required	Required
RR Budget Form	N/A	Required
R&R Subaward Budget Attachment(s) Form(s)	N/A	If Applicable
Research & Related Senior/Key Person Profile Form (Expanded)	N/A	Required
RR Personal Data	N/A	Required
Research & Related Other Project Information	N/A	Required
Disclosure of Lobbying Activities (SF-LLL)	N/A	If Applicable
Attachments Form	N/A	Required

Table 2: Forms. The instructions for completing each of these forms may be found online at the following web link: http://www.grants.gov/web/grants/form-instructions.html.

4.2. Content and Form of Application Submission. Submissions for this BAA will be conducted in two phases. Phase I is for receipt of pre-application white papers. Phase II is for receipt of invited proposal applications. Invitation to the Phase II proposal submission will be based on the evaluation results of the Phase I pre-application white paper.

4.2.1. The predominance of efforts, including all submissions to the thrust areas and some submissions to topics posted in Attachment 1, as noted within the topic, **must be** coordinated with the relevant technical point of contact (POC) for the appropriate thrust area prior to submission of a pre-application white paper; an e-mail for the DTRA technical POCs for Thrust Areas 1-7 are provided in Section 7. Applicants should note that Thrust Areas 1-5 are not currently accepting abstracts for pre-coordination. Coordination of research ideas and efforts must be accomplished via these email addresses, except in cases where a topic specifically states that pre-coordination is not required, and includes submission of an abstract (recommend less than 250 words) of the proposed project/effort or a paragraph description of the proposed project/effort to the email address in Section 7 and a reply email from the relevant email address in Section 7 with the disposition to the applicant. Pre-coordination may not be accomplished with email addresses other than those listed in Section 7. DTRA will not review white papers without prior coordination. Please note that attachments to e-mails may not be reviewed.

Applicants should note that there is extremely limited funding available for the general thrust areas. Pre-application white papers will only be accepted from the coordinated abstracts under very limited circumstances.

Topics may be posted in Attachment 1 of this announcement that may not require pre-coordination of an abstract. Please review the topics carefully.

4.2.2. Pre-application white papers and invited proposals **must be** submitted electronically

using www.grants.gov and the corresponding application packages linked with this BAA on www.grants.gov (under the "Packages" tab). All applications, including all supporting documents, must be submitted in the English language.

Applicants are responsible for ensuring compliant and final submission of their Phase I pre-application white paper and Phase II invited proposal application. Note that this also applies to applicants using third party systems to submit application packages and attachments. Any submission that does not conform to the requirements outlined in the BAA and in the invitation for proposal may not be reviewed or considered further at the discretion of DTRA.

4.2.3. DTRA will not review any of the following:

- Pre-application white papers that are not pre-coordinated as required
- Pre-application white papers and proposals that are not submitted in the English language.
- Pre-application white papers that are submitted to topics that have been previously closed via an amendment to the BAA.
- Application packages and proposals for Phase II submissions that were not invited.

Exceptions WILL NOT be made under any circumstances.

4.2.4. Phase I Pre-Application White Paper Submission and Content. Each pre-application white paper must address only one thrust area or topic. Each pre-application white paper must use the corresponding thrust area or topic application package.

Each Phase I application package contains the following forms:

Form	Attachment	Action
SF-424 (R&R) Application for Federal Assistance Form	Up to four (4) page white paper	Enter the appropriate information in data fields

Table 3: Phase I Pre-Application White Paper Package Chart.

Each Phase I application package contains the SF 424 (R&R) Application for Federal Assistance. To be considered a complete package, an up to four (4) page white paper is required to be uploaded as an attachment to the SF 424 (R&R).

DTRA-specific instructions for completing the SF 424 (R&R) Application for Federal Assistance are below, general application instructions can be found on www.grants.gov:

- Block 1 – Type of Submission. Applicants should indicate the Phase I submission is a "Pre-Application."
- Block 2.1 – Applicant Identifier. Not applicable.
- Block 3 – Date Received by State. Not applicable.
- Block 3.1 – State Application Identifier. Not applicable.
- Block 5 – Applicant Information. You must provide a Business Office Point of Contact (BPOC) with an e-mail address.
- Block 19 – Authorized Representative. The "signature of AOR" is not an actual signature and is automatically completed upon submission of the electronic application package. Hard copies or email attachments of applications will not be accepted.

- Block 20 – Pre-application. Must be used to attach an up to four (4) page white paper. The white paper itself should provide sufficient information on the research being proposed (e.g., the hypothesis, theories, concepts, approaches, data measurements and analysis, etc.) to allow for an assessment by a technical expert.

Any pages submitted for the white paper that exceed the limit of four pages will not be read or evaluated. A page is defined as 8 ½ x 11 inches, single-spaced, with one-inch margins in type not smaller than 12 point Times New Roman font. The white paper must be provided in portrait layout.

At minimum, the white paper should address the following:

- A project abstract, which should be concise (less than 250 words), provide a summary of the proposed work, and demonstrate relevance to the topic being addressed. The abstract should not contain any proprietary data or markings.
- Potential scientific impact to provide greater knowledge or understanding of the fundamental aspects of phenomena and of observable facts, including how the research contributes to the C-WMD science needs outlined in the thrust area or topic.
- The impact of the research on C-WMD science must be clearly delineated.
- Cost estimate by year and total dollars required to accomplish the research as presented in the white paper (no details or breakout of costs is required).
- Potential team and management plan, including details on student involvement.
- Multidisciplinary white papers should carefully detail each of the institutions/departments involved and the contribution that will be made by each of the investigators.
- Do NOT include corporate or personnel qualifications, past experience, or any supplemental information with the white paper. References may be included within the 4-page limit at the discretion of the applicant; however, extensive references are not required.
- Thrust Area 6 pre-application white papers must also include a description of the extent and duration of the relationship/collaboration between the universities/institutes/entities and/or scientists.
- The thrust area or the topic should be included as a header on the white paper attachment and referenced in the text of the white paper.

4.2.5. Phase I Pre-Application White Paper Re-Submission and Content. On a limited basis a second pre-application white paper may be submitted without pre-coordination of an abstract. These re-submissions will be based on the review of the original pre-application white paper and will be allowed when changes to the project scope, technical approach, and/or cost are envisioned for any potential full proposals. Revised pre-application white papers must conform to the standards for the pre-application white papers detailed in Section 4.2.4.

All submissions should be made with the appropriate Phase I application package which contains the following form:

Form	Attachment	Action
SF-424 (R&R) Application for Federal Assistance Form	Up to four (4) page white paper	Enter the appropriate information in data fields

Table 4: Phase I Pre-Application White Paper Package Chart.

Each Phase I application package contains the SF 424 (R&R) Application for Federal Assistance. To be considered a complete package, an up to four (4) page white paper is required to be uploaded as an attachment to the SF 424 (R&R).

The DTRA-specific instructions for completing the SF 424 (R&R) Application for Federal Assistance are the same as for the original pre-application white paper submission except for the following:

- Block 1 – Type of Submission. Applicants should indicate the Phase I re-submission is a "Changed/Corrected Application."
- Block 4c – Previous Grants.gov Tracking ID. Enter the Phase I Grant ID for the original submission.

At minimum, the revised white paper should address the issues and questions detailed in the debrief summary.

4.2.6. Phase II - Invited Proposal Submission and Content. Each proposal must address only the thrust area or topic for which it was invited. The application package corresponding to the thrust area or topic of interest should be used for submission of invited full proposals.

Each Phase II application package contains the following forms and attachments:

Form	Attachment	Action
SF-424 (R&R) Application for Federal Assistance Form	*N/A*	Enter the appropriate information in data fields
RR Budget Form	Budget Justification for entire performance period	Attach to Section K in budget period one
RR Sub-award Budget Attachment(s) Form (*if applicable*)	Individual sub-award budgets	Attach a separate budget with justification for each sub-award
Research & Related Senior/Key Person Profile Form	PI Biographical Sketch	Attach to Biographical Sketch field
	PI Current/Pending Support	Attach to Current & Pending Support field
	Key Personnel Biographical Sketches	Attach to Biographical Sketch field for each senior/key person
	Key Personnel Current/Pending Support	Attach to Current & Pending Support field for each senior/key person
RR Personal Data Form	*N/A*	Enter the appropriate information in data fields
Research & Related Other Project Information Form	Publically Releasable Proposal Summary/ Abstract	Attach to Block 7 Project Summary/ Abstract

	Project Narrative/Technical Proposal	Attach to Block 8 Project Narrative
Disclosure of Lobbying Activities (SF-LLL) *(if applicable)*	*N/A*	Enter the appropriate information
Attachments Form	Attachment 1 – SOW	Upload as Attachment 1
	Attachment 2 – Quad Chart	Upload as Attachment 2
	Attachment 3 – Supporting Documentation (Thrust Area 6 submissions only)	Upload as Attachment 3

Table 5: Phase II Proposal Package Forms and Attachments.

DTRA reserves the right to consider incomplete application packages and required attachments and to request any missing information via email. Should the applicant fail to provide all the requested information either as part of the www.grants.gov submission or in response to email requests from DTRA, at their discretion, DTRA may not consider the proposal further.

SF 424 (R&R) Application for Federal Assistance: DTRA-specific instructions for completing the SF 424 (R&R) are below. General application instructions can be found on www.grants.gov:

Block 1 – Type of Submission. Applicants should indicate the Phase II submission is an "Application."

Block 2.1 – Applicant Identifier. Not applicable.

Block 3 – Date Received by State. Not applicable.

Block 3.1 – State Application Identifier. Not applicable.

Block 4b – Agency Routing Identifier. Enter the corresponding Phase I Grant ID. If resubmissions were involved, enter the Grant ID for the last submission.

Block 5 – Applicant Information. You must provide a Business Office Point of Contact (BPOC) with an e-mail address.

Block 17 – Regarding Disclosure of Funding Sources. By checking "I Agree" you agree to abide by the following statement: "By signing this application, I certify the proposing entity is in compliance with Section 223(a) of the William M. (Mac) Thornberry National Defense Authorization Act for Fiscal Year 2021 which requires that: (a) the PI and other key personnel certify that the current and pending support provided on the proposal is current, accurate and complete; (B) agree to update such disclosure at the request of the agency prior to the award of support and at any subsequent time the agency determines appropriate during the term of the award; and (c) the PI and other key personnel have been made aware of the requirements under Section 223(a)(1) of this Act. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. code, Title 18, Section 1001)."

Block 19 – Authorized Representative. The "signature of AOR" is not an actual signature and is automatically completed upon submission of the electronic application package.

RR Budget Form: The Research and Related Budget Form provided as part of the application package for the Phase II submission should be filled out in its entirety for each project year proposed. Applicants are responsible for ensuring appropriate, approved rates are used in their budget forms. When notified of selection applicants will be requested to provide their current

rate agreement and the rate agreement of their subcontractor(s), if applicable. Applicants should note that in accordance with 32 CFR 22.205(b), grants shall not provide for the payment of fee or profit to the recipient. Applicants should also carefully review Section 4.5.4 to appropriately evaluate inclusion of Value Added Tax (VAT) or other taxes for assistance awards.

Applicants should plan and budget for travel to accommodate the two meetings outlined below:

- National Conferences/Workshops/Symposia: Applicants are strongly encouraged to attend a nationally recognized conference, workshop, or symposium in the field of research each calendar year (1 at minimum). Research should be presented as soon as adequate data are available to support posters and presentations. Conferences/workshops/symposia should be attended by the PI and students supporting the research, as appropriate.
- Annual Technical Review: Applicants should plan to attend an annual technical program review meeting. For planning purposes the review will be for five days and will be held in Northern Virginia.

Budget Justification: Applicants are required to submit a budget justification. The budget justification should be prepared as outlined in the instructions for the Research and Related Budget and uploaded as an attachment to Section K "Budget Justification" of the Research and Related Budget Form. The budget justification does not have a page limit, but should include sufficiently detailed information for meaningful evaluation. In addition, the budget justification must specifically address subaward costs and type to include the portion of work to be subawarded with a supporting rationale. The budget justification should include a discussion of how the subawardee(s) cost was determined to be fair and reasonable. The budget justification must specifically address VAT and other taxes in accordance with Section 4.5.4.

RR Subaward Budget Attachment(s) Form (if applicable): Detailed cost estimates are required for each proposed subaward. The cost estimate for the subawards should include sufficiently detailed information for meaningful evaluation, including labor rates and indirect cost rates.

Research and Related Senior/Key Person Profile Form (Expanded): The Research and Related Senior/Key Person Profile Form (Expanded) should be completed in its entirety for each of the PIs and Co-PIs on the project. The inclusion of additional personnel is at the discretion of the PI. The Degree Type and Degree Year fields will be used by DoD as the source for career information to assess the success rates of women. In addition to the required fields on the form, applicants should complete these two fields for all individuals that are identified as senior or key persons. For Thrust Area 6 submissions, the PI (and Co-PIs) in the region-of-interest should be included as key personnel.

A biographical sketch is required for each PI and Co-PI on the project. DTRA does not have a preference for the format of the biographical sketch; however, it should be limited to 1 page per person. The biographical sketch should be uploaded as an attachment to the corresponding field on the Research and Related Senior/Key Person Profile Form.

Additionally, a statement of current and pending support must be provided for each of the key personnel (e.g., PI and Co-PI) on the project. This statement must include the following items and requires disclosure of all grants and contracts through which each of the key personnel is currently receiving or may potentially receive financial support:

- A list of all current projects the individual is working on, in addition to any future support the

individual has applied to receive, regardless of the source.

- Title and objectives of the other research projects.
- The percentage per year to be devoted to the other projects.
- The total amount of support the individual is receiving in connection to each of the other research projects or will receive if other proposals are awarded.
- Name and address of the agencies and/or other parties supporting the other research projects.
- Period of performance for the other research projects.

Applicants should note that in accordance with the instructions for completion of the SF 424, checking of Block 17 is required. Further, applicants should note that by checking block 17 and submitting an application package, you agree to abide by the following statement: "By signing this application, I certify the proposing entity is in compliance with Section 223(a) of the William M. (Mac) Thornberry National Defense Authorization Act for Fiscal Year 2021 which requires that: (a) the PI and other key personnel certify that the current and pending support provided on the proposal is current, accurate and complete; (B) agree to update such disclosure at the request of the agency prior to the award of support and at any subsequent time the agency determines appropriate during the term of the award; and (c) the PI and other key personnel have been made aware of the requirements under Section 223(a)(1) of this Act. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. code, Title 18, Section 1001)."

RR Personal Data Form: This form will be used by DoD as the source of demographic information, such as gender, race, ethnicity, and disability information for the PI and Co-PI(s). Each application must include this form with the name fields of the PI and any Co-PI(s) completed; however, provision of the demographic information in the form is voluntary. The demographic information, if provided, will be used for statistical purposes only and will not be made available to merit reviewers. Applicants who do not wish to provide some or all of the information should check or select the "Do not wish to provide" option.

Research and Related Other Project Information Form:

Block 7 – Project Summary/Abstract. To fulfill the requirements of Section 8123 of the Defense Appropriations Act, which states: "The Secretary of Defense shall post grant awards on a public Web site in a searchable format," DTRA will collect and post via the Defense Technical Information Center (DTIC) basic information about all awards made under this BAA. The information posted will include the abstract submitted to Block 7 of this form.

The uploaded project abstract should be less than one page and provide a summary of the proposed work and demonstrate relevance to the topic being addressed. Most importantly, the abstract **must be** written such that the general public may easily understand the potential scientific contribution and the impact of the research. The header of this uploaded document must contain the following statement:

> *"This publically releasable abstract is provided to DTRA for use in fulfillment of Section 8123 of the Defense Appropriations Act and future versions of the same."*

The abstract absolutely must not contain any proprietary data or markings.

Block 8 – Project Narrative (Technical Proposal). The uploaded technical proposal must not exceed 20 pages (including references). If the proposal exceeds 20 pages, only the first 20 pages will be reviewed. A page is defined as 8 ½ x 11 inches, single-spaced, with one-inch margins in type not smaller than 12 point Times New Roman font. The technical proposal must be provided in portrait layout.

The project narrative (technical proposal) must include the following components:

- ***Abstract.*** Should be a technical project abstract that is distinct from the Project Summary/Abstract that is attached to Block 7.
- ***Scope.***
- ***Objective.*** A clear and concise objective of the proposed project.
- ***Background.*** Provide the necessary technical and scientific background to support the scientific and/or technical merit of the proposed project.
- ***Programmatics.*** Describe your organization's management plan for the proposed project; list supporting and collaborating centers, and the roles/responsibilities of each identified academic and/or industrial subcontractor supporting the project. Authors of multidisciplinary proposals must take great care to clearly outline the impact to C-WMD science that is to be gained from the investment and justify the scientific contribution from each investigator.

  Thrust Area 6 narratives must also describe of the extent and duration of the relationship/collaboration between the universities/institutes/entities and/or scientists. Teams with pre-existing collaborative research relationships and those which propose to establish new collaborations will be considered, provided teams can supply documentation to demonstrate that an operational framework exists to support the proposed work. Please see Attachment 3 below for information on the submission of this documentation.
- ***Relevance.*** Describe the relevance of the proposed project in terms of advancing the state of the science and the anticipated scientific impact on capabilities to potentially reduce, eliminate, counter, provide greater knowledge or understanding of the threat, and mitigate the effects of WMD fundamental aspects of phenomena and of observable facts.
- ***Credentials.*** Describe the PI's qualifications and the organization's qualifications to perform the proposed work. Summarize the credentials of the primary performing center, and supporting academic and industrial partners to perform the work. Describe specific examples of equipment and/or facilities available to perform the proposed work. Focus on information directly relevant to the proposed work.
- ***Work to be Performed.*** Provide details of the work to be performed by task and subtask. Tasks must be grouped by project year; base and option years should be clearly labeled. Additional details that are required include the following:
  - ***Sample Repository.*** Thrust Area 6 narratives must also clearly identify how the applicant plans to maintain samples collected during the proposed research effort, along with relevant metadata, for at least 12 months after the project end date. Note that annual sample repository information must be submitted using a DTRA-specified format that will be made available to awardees.
  - ***Protection of Human Subjects.*** For full discussion, see Section 6.2.2. If the proposed

work involves human subjects or the use of human anatomical substances (e.g., biospecimens, blood, tissue, cell lines), either living or post-mortem, applicants are required to: a) justify and b) outline the use, and c) include the source of the human subjects, human biospecimens and/or human data involved in the research. The DTRA Research Oversight Board (ROB) will provide ongoing oversight throughout the duration of the effort to ensure proper approvals are in place. Further information will be required if the proposal is selected for award.

- ***Animal Use.*** For full discussion, see Section 6.2.3. If the proposed work involves the use of animals, applicants are required to: a) justify and b) include detailed information on the use of animals, and c) include the location(s) of where the animal work is to be performed. The DTRA Research Oversight Board (ROB) will provide ongoing oversight throughout the duration of the effort to ensure proper approvals are in place. Further information will be required if the proposal is selected for award.

- ***Performance Schedule.*** Provide a table of tasks and sub-tasks and the duration of performance of each in a Gantt or other suitably formatted chart.
- ***References.*** List any relevant documents referenced.

Disclosure of Lobbying Activities (SF-LLL) Form: The Disclosure of Lobbying Activities Standard Form-LLL, if applicable, should be completed.

Attachments Form: The attachments form should be used to include the following three items with the application:

Attachment 1 – SOW. The SOW does not have a page limit, but should be approximately 3-5 pages in length for incorporation into an award document. The SOW should not contain any proprietary data or markings. Pages should be numbered and the initial page should have a date (document date) shown under the title (the title of the SOW should match that of the proposal).

The proposed SOW must accurately describe the research to be performed. The proposed SOW must also contain a summary description of the technical methodology as well as the task description, but not in so much detail as to make the SOW inflexible. The SOW format/guidance is as follows:

- ***Objective:*** Brief overview of the specialty area. Describe why the research is being pursued and what knowledge is being sought.
- ***Scope:*** Include a statement of what the SOW covers including the research area to be investigated, objectives/goals, and major milestones and schedule for the effort.
- ***Background:*** The applicant must identify appropriate documents, including publications that are applicable to the research to be performed. This section includes any information, explanations, or constraints that are necessary in order to understand the hypothesis and scientific impact on capabilities needed to reduce, eliminate, and counter the threat, and also mitigate the effects of WMD. It may also include previously performed relevant research and preliminary data.
- ***Tasks/Scientific Goals:*** This section contains the detailed description of tasks which represent the research to be performed that are contractually binding. Thus, this portion of the SOW should be developed in an orderly progression and presented in sufficient detail to

establish the methodology and feasibility of accomplishing the overall program goals. The work effort should be segregated by performance period for all tasks to be performed and anticipated milestones realized in that year (e.g., Year 1, Year 2, etc., should be detailed separately). Identify the major tasks in separately numbered sub-paragraphs. Each major task should delineate, by subtask, the research to be performed by year and number each task using the decimal system (e.g., 4.1, 4.1.1, 4.1.1.1, 4.2, etc.). The sequence of performance of tasks and achievement of milestones must be presented by project year and task in the same sequence as in the Project Narrative/Technical Proposal. The SOW must contain every task to be accomplished to include a detailed schedule.

- The tasks must be definite, realistic, and clearly stated. Use "the awardee shall" whenever the work statement expresses a provision that is binding. Use "should" or "may" whenever it is necessary to express a declaration of purpose. Use active voice in describing work to be performed. Do not use acronyms or abbreviations without spelling out acronyms and abbreviations at the first use; place the abbreviation in parenthesis immediately following a spelled-out phrase. If presentations/meetings are identified in your schedule, include the following statement in your SOW: "Conduct presentations/meetings at times and places specified in the grant schedule."
- ***Deliverables:*** Thrust Area 6 **requires** several additional items be included in the SOW. These items are as follows:
    - Submission of annual sample repository information using a DTRA-specified format.
    - Access to all samples collected and data generated during the course of the project, up to and including at least 12 months after the project end date.

Attachment 2 – Quad Chart. The quad chart must be presented on one (1) page. The quad chart must not contain any proprietary data or markings. The quad chart must be provided in landscape layout. The quad chart should be uploaded as "Attachment 2" of the Attachments Form.

Attachment 3 – Supporting Documentation. For Thrust Area 6 proposals ONLY. Thrust Area 6 narratives must also describe an operational framework to support the proposed work. This includes, but is not limited to the following: the extent and duration of the relationship/collaboration between the universities/institutes/entities and/or scientists. Teams with pre-existing collaborative research relationships and those which propose to establish new collaborations will be considered, provided teams can supply documentation to demonstrate that an operational framework exists to support the proposed work. Each of the following should be concatenated into a single document, in the order specified:

- Specific identification of foreign Principal Investigators (PIs) and number of/job title for other members of the foreign research team.
- Detailed description of the relationship between the proposed research project and current research efforts at the foreign institution.
- Description of facilities and any other evidence of suitability of foreign collaborators and sites. In the event that the foreign research component will involve human or other vertebrate animal use, appropriate facilities compliance and certifications documents must be provided. Refer to Section 6.2.2 and Section 6.2.3 for specific information on required

approvals and documentation.

- Foreign PI letter of collaboration describing, at minimum, the suitability of the proposed work with respect to ongoing research efforts at the foreign institution, merit of the proposed collaboration, and the expected mutual benefits.

Protocol Risk Assessment Tool (PRAT). For Thrust Area 6 proposals ONLY. Applicants will be provided a copy of the PRAT file following their invitation to submit a Phase II full proposal and complete it in its entirety for **each** foreign institution participating in the project. Additional instructions for completing the PRAT may be found within the file. The completed PRAT file(s) should be emailed as a Portable Document File (PDF) format to HDTRA1-FRCWMD-A@mail.mil within two (2) weeks of the full proposal submission. **DO NOT** attempt to attach the PRAT(s) to the www.grants.gov submission.

4.2.7. Phase II - Additional Information Requests by DTRA. A revised proposal may be requested based on the review of the original proposal. Revised proposals will be requested when changes to the project scope, technical approach, and/or cost are required before the proposal could be further considered for an award. Applicants whose proposals are of interest to DTRA may be contacted to provide additional information or to make requested revisions prior to the final decision on funding. This request for further information may include revised budgets or budget explanations, revised SOWs, and other information, as applicable, to the proposed award. Additional instructions may be provided in the request for a revised proposal. Applicants who are not responsive to Government requests for information in a timely manner, defined as meeting Government deadlines established and communicated with the request and not making satisfactory updates as requested, may be removed from award consideration. Applicants may also be removed from award consideration if the applicant and the Government fail to negotiate mutually agreeable terms within a reasonable period of time.

Re-submissions should be made with the appropriate Phase II application package for the thrust area or topic of interest and should be completed in accordance with the instructions provided in the notification email.

The DTRA-specific instructions for completing a proposal re-submission are the same as for the original submission, except the SF 424 (R&R) Application for Federal Assistance should be marked as follows:

- Block 1 – Type of Submission. Applicants should indicate the Phase II submission is a "Changed/Corrected Application."
- Block 4b – Agency Routing Identifier. Enter the corresponding Phase I Grant ID.
- Block 4c – Previous Grants.gov Tracking ID. Enter the Phase II Grant ID for the original Phase II submission.

4.2.8. File Format. Documents should be uploaded as a Portable Document File (PDF) format. Perform a virus check before uploading any files to www.grants.gov as part of your application package. If a virus is detected, it may cause rejection of the file.

Do not lock or encrypt any files you upload to www.grants.gov as part of your application package. Movie and sound file attachments will not be accepted.

4.2.9. All submissions must be completely UNRESTRICTED and UNCLASSIFIED;

submissions must not contain Controlled Unclassified Information (CUI), other Proprietary information or export controlled information or be marked as such.

4.2.10. Confirmed Proposal Expiration Date. Applicants requesting contracts must provide written confirmation that holds the proposal, to include proposed costs, firm for 180 days after the submission due date, as included in the invitation to submit a full proposal. This information must be included in the text of the technical proposal.

4.2.11. Withdrawal of Proposals. Proposals may be withdrawn by written notice received at any time before award. Withdrawals are effective upon receipt of notice by the Grants/Contracting Officer via the administrative e-mail address listed in Section 7.

4.3. Submission Dates and Times.

Coordination of abstracts may be accomplished at any time that this BAA is in effect, unless otherwise stated as part of a specific topic. Once an applicant has been notified that a pre-application white paper is welcomed, the white paper should be submitted within 60 days. If the white paper is not submitted within 60 days, DTRA reserves the right to require the applicant to re-initiate the process with another abstract coordination.

Pre-application white papers may be submitted anytime that this BAA is in effect (as long as it occurs within the 60 day window following pre-coordination of the abstract), unless otherwise stated as part of a specific topic. Pre-application white papers may be evaluated at any time after submission and invitations for full proposal submission may occur any time after pre-application white paper evaluation. Note that proposal invitations may be limited to available program funds.

The due date for the Phase II invited proposal submissions will be provided in the letter of invitation. The applicant will not be allowed less than 45 days to prepare a full proposal submission; there is no penalty for early submissions. An extension for submission of the Phase II proposal submission may be requested by emailing the administrative email address in Section 7 prior to the deadline for the proposal submission. Full proposals may be evaluated at any time after submission.

Applicants are responsible for submitting all materials to www.grants.gov. When sending electronic files, the applicant should allow for potential delays in file transfer from the originator's computer server to the www.grants.gov website/computer server, as well as the delay associated with the www.grants.gov validation of applications, which may be up to 48 hours. Applicants are encouraged to submit their proposals early to avoid issues with file transfers, rejection of applications by www.grants.gov, and delays due to high website demand.

Acceptable evidence to establish the time of receipt at the Government office includes documentary and electronic evidence of receipt maintained by DTRA. Applicants should also print, and maintain for their records, the electronic receipt following submission of a proposal to www.grants.gov.

Applicants should note that DTRA uses a system that pulls applications from www.grants.gov en masse, but this system does not mark applications as "retrieved" on www.grants.gov. As a result, when applicants check the status on www.grants.gov the applications will always look like they have not been retrieved by DTRA. Should you require confirmation of receipt by the Agency, you may request such via the administrative email address provided in Section 7. Note that such requests will generally be treated with low priority by the Agency.

Please note 15 U.S.C. 260a establishes daylight saving time as the standard time during the daylight saving period.

If the application package and required attachments are submitted to www.grants.gov after the exact time and date specified in this announcement or in any written communications provided by DTRA, the application may be considered "late" and may not be reviewed.

If an emergency or unanticipated event interrupts normal Government processes so that proposals cannot be submitted to www.grants.gov by the exact time specified by DTRA correspondence, the time specified for receipt of applications will be deemed to be extended to the same time of day specified in the BAA or in the letter of invitation on the first work day on which normal Government processes resume.

4.4. Intergovernmental Review. Not Applicable.

4.5. Other Submission Requirements.

4.5.1. Organizations must have an active System for Award Management (SAM) registration, and Grants.gov account to apply for grants. Creating a Grants.gov account can be completed online in minutes, but SAM registrations may take additional time. Therefore, an organization's registration should be done in sufficient time to ensure it does not impact the entity's ability to meet required application submission deadlines.

All organizations applying online through Grants.gov must register with the SAM and will receive a unique entity identifier (UEI) number. Failure to register with SAM will prevent your organization from applying through Grants.gov. SAM registration must be renewed annually. For more detailed instructions for registering with SAM, refer to: https://www.grants.gov/web/grants/applicants/organization-registration/step-2-register-with-sam.html. Additional information may be found on Grants.gov here: https://www.grants.gov/web/grants/applicants/organization-registration.html

4.5.2. Compliance with Appendix A to 32 CFR 28. All awards require certifications of compliance with Appendix A to 32 CFR 28 regarding lobbying. Proposers are certifying compliance with this regulation by submitting the invited proposal. It is not necessary to include the certification text with your invited proposal. If applicable, proposers should submit the Disclosure of Lobbying Activities (SF-LLL) Form in accordance with Section 4.2.6.

4.5.3. Marking Guidance for Pre-Application White Paper and Invited Proposal and Disclosure of Proprietary Information other than to the Government. The pre-application white papers and invited proposals submitted in response to this BAA may contain technical and other data that the applicant does not want disclosed to the public or used by the Government for any purpose other than application evaluation. Public release of information in any pre-application white paper and invited proposal submitted will be subject to existing statutory and regulatory requirements.

If proprietary information which constitutes a trade secret, proprietary commercial or financial information, confidential personal information, or data affecting national security, is provided by an applicant in a pre-application white paper and/or invited proposal, it will be treated in confidence, to the extent permitted by law, provided that the following legend is included on the front page of the pre-application white paper and/or invited proposal:

“For any purpose other than to evaluate the pre-application white paper and/or proposal, this data

shall not be disclosed outside the Government and shall not be duplicated, used, or disclosed in whole or in part, provided that if an award is made to the applicant as a result of or in connection with the submission of this data, the Government shall have the right to duplicate, use or disclose the data to the extent provided in the agreement. This restriction does not limit the right of the Government to use information contained in the data if it is obtained from another source without restriction. The data subject to this restriction is contained in page(s) _____ of this pre-application white paper and/or proposal."

Any other legend may be unacceptable to the Government and may constitute grounds for removing the pre-application white paper and/or invited proposal from further consideration without assuming any liability for inadvertent disclosure.

The Government will limit dissemination of properly marked information to within official channels. In addition, the pages indicated as restricted must be marked with the following legend:

> *"Use or disclosure of the pre-application white paper and/or proposal data on lines specifically identified by asterisk (*) are subject to the restriction on the front page of this pre-application white paper and/or proposal."*

The Government assumes no liability for disclosure or use of unmarked data and may use or disclose such data for any purpose.

In the event that properly marked data contained in a pre-application white paper and/or invited proposal submitted in response to this BAA is requested pursuant to the Freedom of Information Act (FOIA), 5 U.S.C. § 552, the applicant will be advised of such request and prior to such release of information, will be requested to expeditiously submit to DTRA a detailed listing of all information in the pre-application white paper and/or invited proposal which the applicant believes to be exempt from disclosure under the Act. Such action and cooperation on the part of the applicant will ensure that any information released by DTRA pursuant to the Act is properly identified.

By submission of a pre-application white paper and/or invited proposal, the applicant understands that proprietary information may be disclosed outside the Government for the sole purpose of technical evaluation. DTRA will obtain a non-disclosure agreement from the evaluator that proprietary information in the pre-application white paper and/or invited proposal will only be used for evaluation purposes and will not be further disclosed or utilized.

4.5.4. VAT and Other Taxes in Assistance Awards. Prior to proposal submission, the applicant will require any supplier of goods or services to assess and verify potential VAT, excise duties, and other tax implications to avoid the imposition of such charges with respect to the goods and/or services in question to the maximum extent possible.

In instances where the supplier of goods or services is exempt from the VAT, excise duties, or other taxes or is entitled to claim reimbursement thereof, the taxes must not be included in the proposed cost of the award.

In instances where the supplier of goods or services is not exempt from the VAT, excise duties, or other taxes or is not entitled to claim reimbursement thereof, the applicant must itemize the VAT and/or other taxes in the proposal. Further, applicants are advised that prior to the award of any grant or cooperative agreement, DTRA and the recipient will mutually agree upon the use of DTRA funds for the VAT, excise duties, or other taxes, and project activities may be revised

accordingly. All applicants may include costs in their proposal to pay for VAT costs associated with lodging, meals, and transportation for travel.

4.6. Applicants that Propose Use of Contracts or OTAs.

4.6.1. Recommended Procurement Instrument and Pricing Arrangement. Applicants that propose use of contracts or OTAs must provide a summary of their recommended procurement instrument and pricing arrangement as part of the Phase II proposal. However, the Government reserves the right to negotiate and award the types of instruments determined most appropriate under the circumstances. It is anticipated that most instruments will be grants.

4.6.2. Representations and Certifications. Representations and Certifications must be completed at the time of Phase II submission. The applicant must complete the annual representations and certifications electronically via the System for Award Management (SAM) website at https://www.sam.gov/portal/SAM/#1#1. After reviewing their information, the applicant verifies by submission of the application that the representations and certifications currently posted electronically have been entered or updated within the last 12 months.

4.6.3. Organization Conflict of Interest Advisory. Certain post-employment restrictions on former federal officers and employees may exist, including special Government employees (including but not limited to 18 U.S.C § 207, the Procurement Integrity Act, 41 U.S.C. § 2101 *et.seq*). If a prospective applicant believes that a conflict of interest exists, the situation should be raised to the DTRA Contract/Grant Officer before time and effort are expended in preparing a proposal. All applicants and proposed sub-contractors must therefore affirmatively state whether they are providing scientific, engineering and technical assistance (SETA), advisory and assistance services (A&AS) or similar support, through an active contract or subcontract, to any DoD technical office to include, but not limited to, the Joint Program Executive Office (JPEO), the Office of the Assistant Secretary of Defense for Nuclear, Chemical, and Biological Defense Programs (ASD-NCB), or the Office of the Special Assistant for Chemical and Biological Defense and Chemical Demilitarization Programs (OSA (CBD&CDP)). This information must be included in Technical Proposal of the Phase II full submission. All affirmations must state which office(s) the applicant(s) supports, and identify the prime contract number. Affirmations must be furnished at the time of Phase II full proposal submission. All facts relevant to the existence or potential existence of organizational conflicts of interest, including but not limited to those arising out of activities with the above-referenced organizations, must be disclosed. The disclosure must include a description of the action the applicant has taken or proposes to take to avoid, neutralize, or mitigate such conflict.

4.6.4. Contracts with Subcontracts. Any applicant, other than small businesses, submitting a proposal that exceeds $750,000.00 must submit a subcontracting plan in accordance with FAR 19.704(a) (1) and (2). This information must be included in Technical Proposal of the Phase II full submission. The plan format is outlined in FAR 19.704. Pursuant to Section 8(d) of the Small Business Act (15 U.S.C. § 637(d)), it is the policy of the Government to enable small business and small disadvantaged business concerns to be considered fairly as subcontractors to contractors performing work or rendering services as prime contractors or subcontractors under Government contracts, and to assure that prime contractors and subcontractors carry out this policy.

4.6.5. Limitations on OTAs. Applicants are advised that an Other Transaction for Research Agreement (10 U.S. Code § 2371) or an Other Transaction for Prototype Agreement (10 U.S.

Code § 2371b) will only be awarded if the use of a standard contract or CA is not feasible or appropriate. Applicants are advised that an OTA may only be awarded if there is:

a. At least one nontraditional defense contractor participating to a significant extent in the prototype project, or

b. All significant participants in the transaction other than the Federal Government are small businesses or nontraditional defense contractors; or

c. At least one-third of the total cost of the prototype project is to be paid out of funds provided by the parties to the transaction other than the Federal Government. The cost share should generally consist of labor, materials, equipment, and facilities costs (including allocable indirect costs).

d. Exceptional circumstances justify the use of a transaction that provides for innovative business arrangements or structures that would not be feasible or appropriate under a procurement contract.

e. Although use of one of these options is required to use an Other Transaction for Prototype agreement as the procurement vehicle, no single option is encouraged or desired over the others.

NOTE: For purposes of determining whether or not a participant may be classified as a nontraditional defense contractor or a small business and whether or not such participation is determined to be participating to a significant extent in the prototype project, the following definitions are applicable:

a. "Nontraditional defense contractor" means an entity that is not currently performing or has not performed, for at least the one-year period preceding this solicitation, any of the following for the Department of Defense: any contract or subcontract that is subject to full coverage under the cost accounting standards prescribed pursuant to section 26 of the Office of Federal Procurement Policy Act (41 USCS §§ 1501 et seq.) and the regulations implementing such section; or any other contract in excess of $500,000 under which the contractor is required to submit certified cost or pricing data under section 2306a of this title (10 USCS § 2306a).b. "Small business" means a small business concern as defined under Section 3 of the Small Business Act (15 U.S.C. § 632).

"Participating to a significant extent in the prototype project" means that the nontraditional defense contractor or small business is supplying a new key technology or product, is accomplishing a significant amount of the effort wherein the role played is more than a nominal or token role in the research effort, or in some other way plays a significant part in causing a material reduction in the cost or schedule of the effort or an increase in performance of the prototype in question. NOTE: Applicants are cautioned that if they are classified as a traditional defense contractor, and propose the use of an Other Transaction for Prototype Agreement, the Government will require submittal of both a cost proposal under the guidelines of the FAR/DFARS, and a cost proposal under the proposed Other Transaction for Prototype Agreement, so that an evaluation may be made with respect to the cost tradeoffs applicable under both situations. The Government reserves the right to negotiate either a FAR based procurement contract, or Other Transaction for

Prototype Agreement as it deems is warranted under the circumstances.

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5. APPLICATION REVIEW INFORMATION

5.1. Evaluation Criteria. The four evaluation criteria to be used for responses received to this BAA are as follows:

1. Scientific and Technical Merit. The objective of this criterion is to assess the extent to which the applicant presents ideas that are innovative and/or unique with the potential for high payoff in the science area and details a comprehensive technical approach based on sound scientific principles. Innovation will be judged contextually against the white paper's/proposal's scope, goals, and setting. To the extent possible, the technical risks, including those of biosafety and security, to accomplish the research or project should be identified with appropriate mitigation/management details.

   For Thrust Area 6 white papers/proposals, innovation will also be considered with respect to partner country capabilities.

2. Value to Mission Goals. The objective of this criterion is to assess the extent to which the applicant demonstrates an understanding of the C-WMD research or mission challenges and the contribution to the C-WMD research or mission needs of that thrust area/topic. White papers/proposals must detail research or a project that is responsive to the thrust area/topic as presented in this solicitation. This criterion also addresses the benefit of the proposed effort on enabling knowledge, technology, or capabilities over current methods and/or practices and on the transition potential that is appropriate to the proposed effort. Applicants must also demonstrate an impact of the proposed effort on the institution's ability to perform research relevant to reducing the global WMD threat; and/or to train, through the proposed effort, students and/or partner scientists in science, technology, engineering and/or mathematics.

   Thrust Area 6 white papers/proposals must demonstrate an understanding of the CBEP priorities and mission. As such, the degree to which the proposed collaborations may lead to long-term partner country self-sufficiency and sustainment of the jointly developed capabilities will be considered.

3. Capability of the Personnel and Facilities to Perform the Proposed Effort. The objective of this criterion is to assess the extent to which the applicant's team has the requisite expertise, skills and resources necessary to perform the proposed program. This includes an assessment of the team's management construct, key personnel, facilities and past technical experience in conducting similar efforts of the proposed scope. Applicants must demonstrate that their team has the necessary background and experience to perform this project. Facilities should be detailed with discussion of any unique capabilities pertinent to the research. Subcontractors may include Government facilities or Agencies; however the unique expertise or specialized facilities provided through their inclusion must be clearly presented and the validity of the proposer-Governmental relationship must be clearly documented.

4. Cost Realism Evaluation. The objective of this criterion is to establish that the proposed costs are reasonable, realistic, and justified for the technical approach offered and to assess the applicant's practical understanding of the scope of the proposed effort.

5.2. Review and Selection Process. The pre-application white paper and proposal selection

process will be conducted based upon a technical review as described in the DoDGARs (32 CFR 22.315(c)) and includes the use of non-Government peer-reviewers.

Each pre-application white paper and invited proposal submitted to a general TA will be reviewed on a rolling basis; topic-based submissions will be reviewed as a batch following receipt deadlines. All applications will be reviewed based on the merit and relevance of the specific pre-application white paper/proposal as it relates to the DTRA program, rather than against other pre-application white papers/proposals for research in the same general area.

Pre-application white paper (Phase I) evaluation will be based on the two (2) equally weighted criteria of (1) Technical/Scientific Merit and (2) Value to Mission Goals. The criteria will be scored as Outstanding (O), Good (G), Acceptable (A), Marginal (M) or Unacceptable (U). Any criterion scored as "Unacceptable (U)" will render the pre-application white paper "Not Selectable," and the pre-application white paper will not be considered further.

The full proposal evaluation will be based on the four criteria listed above. Of these, the first two (2) criteria of (1) Technical/Scientific Merit and (2) Value to Mission Goals are equally weighted and more important than the third criterion of (3) Capability of the Personnel and Facilities to Perform the Proposed Effort. These first three criteria will be scored Outstanding (O), Good (G), Acceptable (A), Marginal (M) or Unacceptable (U). The fourth criterion of Cost Realism will be scored as either Acceptable (A) or Unacceptable (U). Any criterion scored as "Unacceptable (U)" will render the proposal "Not Selectable," and the proposal will not be considered further.

Other factors that may be considered are duplication with other research, program balance, past performance and budget limitations. Prior to award, the Government reserves the right to perform a review of past performance. Sources that may be used for past performance review may include the Past Performance Information Retrieval System (PPIRS) and the Federal Awardee Performance and Integrity Information System (FAPIIS). The Government will also evaluate the impact of any proposed limitations to the use of intellectual property (e.g. asserted technical data/computer software restrictions or patents) during the selection and/or negotiation process, and may request additional information from the applicant, as may be necessary, to evaluate the applicant's assertions. Accordingly, proposals may be selected for funding which are not reviewed as highly as others, which are of higher risk and/or which may be of a higher cost.

The Government reserves the right to select all, some, or none of the proposals, or any part of any proposal received in response to this BAA and to make awards without discussions with applicants; however, the Government reserves the right to conduct discussions if determined necessary.

5.3. DTRA anticipates that the total Federal share of awards made under this announcement will be greater than the simplified acquisition threshold over the period of performance (see §200.88 Simplified Acquisition Threshold). Therefore, in accordance with Appendix I to 2 CFR Part 200, Section E.3, this section serves to inform applicant:

i. That DTRA, prior to making a Federal award with a total amount of Federal share greater than the simplified acquisition threshold, is required to review and consider any information about the applicant that is in the designated integrity and performance system accessible through SAM (currently Federal Awardee Performance and Integrity

Information System (FAPIIS)) (see 41 U.S.C. 2313);

ii. That an applicant, at its option, may review information in the designated integrity and performance systems accessible through SAM and comment on any information about itself that a Federal awarding agency previously entered and is currently in the designated integrity and performance system accessible through SAM;

iii. That DTRA will consider any comments by the applicant, in addition to the other information in the designated integrity and performance system, in making a judgment about the applicant's integrity, business ethics, and record of performance under Federal awards when completing the review of risk posed by applicants as described in §200.205 Federal awarding agency review of risk posed by applicants.

iv. For awards that exceed $500,000 over the period of performance, DTRA will employ the additional post-award reporting requirements reflected in Appendix XII—Award Term and Condition for Recipient Integrity and Performance Matters of 2 CFR 200.

5.4. Technical and Administrative Support by Non-Government Personnel. It is the intent of DTRA to use both Government and non-Government personnel to assist with the review and administration of submittals for this BAA. All pre-application white papers and invited proposals may be reviewed by subject matter experts, including, but not limited to, peer reviewers from across the academic and industrial community, as applicable to the research proposed.

Further, participation in this BAA requires DTRA support contractors to have access to pre-application white paper and invited proposal information including information that may be considered proprietary or otherwise marked with restrictive legends Each contract contains organizational conflict of interest provisions and/or includes contractual requirements for non-disclosure of proprietary contractor information or data/software marked with restrictive legends. The applicant, by submitting a white paper or proposal, is deemed to have consented to the disclosure of its information to the aforementioned contractors under the conditions and limitations described herein.

All individuals—including subject matter experts and support contractors—having access to any proprietary data must certify that they will not disclose any information pertaining to this BAA including any submittal, the identity of any submitters, or any other information relevant to this BAA. All applicants to this BAA consent to the disclosure of their information under these conditions.

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6. AWARD ADMINISTRATION INFORMATION

6.1. Award Notices. Applicants will be notified regarding the status of their applications (invitation/non-invitation for full proposals, re-submission of white papers, selection/non-selection for award, etc.) via e-mail to the BPOC listed in Block 5 of the SF-424 and the PI listed in Block 14 of the SF-424 provided at the time of submission. A debrief summary will be provided as part of all notification e-mails.

A notice of selection should not be construed as an obligation on the part of the Government; only duly authorized procurement personnel may commit resources, this will be done by issuing a grant or contract document to the selected applicant. Also, this notification must not be used as

a basis for accruing costs to the Government prior to award. Selected applicants are not authorized to begin work, as any award is subject to successful negotiations (if determined necessary by DTRA) between the DTRA contracting division and the selected organization, and to the availability of funds.

All notifications will be made from notification@dtrasubmission.net. **E-mails to this e-mail address will not be answered or forwarded.**

Applicants must be aware that it is their responsibility to ensure: (1) correct e-mail addresses are provided at the time of submission, (2) this e-mail notification reaches the intended recipient(s), and (3) the e-mail is not blocked by the use of 'spam blocker' software or other means that the recipient's Internet Service Provider may have implemented as a means to block the receipt of certain e-mail messages.

If for any reason there is a delivery failure of these e-mail notices, DTRA will not further attempt to contact the applicants.

6.2. Administrative and National Policy Requirements. All awards require certifications of compliance with national policy requirements. Statutes and Government-wide regulations require some certifications to be submitted at the time of proposal submission. See Section 4.5.2 and Section 4.6.2 for the certification(s) required at the time of submission.

This BAA focuses on fundamental research in a DoD contractual context, which was defined in Section 1.2 of this BAA. Per DoD policy[1], "…products of fundamental research are to remain unrestricted to the maximum extent possible." Furthermore, "The DoD will place no other restrictions on the conduct or reporting of unclassified fundamental research, except as otherwise required by statue [sic], regulation, or Executive Order." As such, fundamental research is normally exempt from controls under the International Traffic in Arms Regulation (ITAR) (22 CFR Parts 120-130) and/or the Department of Commerce regarding the Export Administration Regulations (15 CFR Parts 730-774), but the DoD rule recognizes that there are "rare" situations where export-controlled information or technology may be used in fundamental research that may require a license(s) or restrictions on products.

6.2.1. Export Control Notification. Applicants are responsible for ensuring compliance with any export control laws and regulations that may be applicable to the export of and foreign access to their proposed research. Applicants may consult with the Department of State with any questions regarding the International Traffic in Arms Regulation (ITAR) (22 CFR Parts 120-130) and/or the Department of Commerce regarding the Export Administration Regulations (15 CFR Parts 730-774). Please note that the prime awardee is responsible for monitoring ITAR compliance of all subawardees.

6.2.2. Protection of Human Subjects. If the proposed work involves human subjects or the use of human anatomical substances (e.g., biospecimens, blood, tissue, cell lines), either living or post-mortem, applicants are required to: a) justify and b) outline the use, and c) include the source of the human subjects, human biospecimens and/or human data involved in the research,

[1] Under Secretary of Defense for Acquisition, Technology and Logistics Memorandum, SUBJECT: Contracted Fundamental Research, dated 26 Jun 2008

hereafter referred to as "research."

The DTRA Research Oversight Board (ROB) will provide ongoing oversight throughout the duration of the effort to ensure proper approvals are in place. Further information will be required if the proposal is selected for award. Further information will be required if the proposal is selected for award.

DTRA PMs responsible for the research are required to complete and submit Section A of the DTRA Form 156, available through the DTRA1 Forms Library, to the DTRA Research Oversight Board (ROB) through the ROB Central Mailbox, dtra.belvoir.rd.mbx.research-oversight-board@mail.mil.

Through an Agreement with DTRA and the U.S. Army Medical Research Development Command, Office of Human and Animal Research Oversight(MRDC OHARO), OHARO must review and approve all DTRA funded or supported research prior to the start of the proposed work. This review requirement is in addition to the DTRA ROB review. Therefore, along with the DTRA Form 156, the DTRA PM/STM must complete and submit the MRDC OHARO form titled "USAMRDC_ORP_Proposal Submission_Form" to the DTRA ROB for review of the proposed work. These forms are available through the ROB DTRA1 Sharepoint site, https://dtra1portal.unet.dtra.mil/RD/ROB/default.aspx. Allow up to four months, from date award is submitted to the DTRA ROB, for regulatory review and approval processes. Applicants are to build the review time into their project schedules.

All work under any award made under this BAA involving research must be conducted in accordance with 32 CFR 219, 10 U.S.C. § 980, and DoD Instruction (DoDI) 3216.02, DTRA Instruction (DTRAI) 3216.01, and, as applicable, 21 CFR parts 11, 50, 56, GCP, the International Council for Harmonization (ICH) as well as other applicable federal and state regulations. Contracts must include DFARS clause 252.235-7004 and DTRA Clause 252.223-9002. Other funding vehicles (e.g., grant, OTA) must include similar language. Non-compliance with any provision of this clause may result in withholding of payments under the contract pursuant to the terms and conditions. The Government shall not be responsible for any costs incurred for research involving human subjects prior to protocol approval by the MRDC OHRO and ROB.

It is the responsibility of the PM to ensure performers are cognizant of and abide by the additional restrictions and limitations imposed by the DoD regarding research involving human subjects and human anatomical substances, specifically in regards to vulnerable populations (32 CFR 219 modifications to subparts B-D of 45 CFR 46), recruitment of military research subjects (32 CFR 219), and surrogate consent (10 U.S.C. § 980).

Through the Component Management Plan (CMP), reviewed and approved by USD(R&E), the DTRAI 3216.01 establishes the DTRA Human Research Protection Program (HRPP), and sets forth the policies, defines the applicable terms, and delineates the procedures necessary to ensure DTRA compliance with federal and DoD regulations and legislation governing human subject research, and is managed by the DTRA ROB. The regulations mandate that all DoD activities, components, and agencies protect the rights and welfare of human subjects in DoD funded or supported research, development, test and evaluation, and related activities.

The DTRAI 3216.01 requires that research involving human subjects or human anatomical substances may not begin or continue until the DTRA ROB and MRDC OHRO have reviewed

and approved the proposed work. The requirement to comply with the regulations applies to new starts and continuing research for the life of the project, until closure. The completion of a research project requires closure document (e.g., IRB Final Review submission) submitted to the DTRA ROB and/or the MRDC OHRO.

A study is considered to involve human research subjects if: 1) there is interaction with the subject (even simply talking to the subject qualifies; no needles are required); and 2) if the study involves collection and/or analysis of personal/private information about an individual, or if material used in the study contains links to such information.

A study is considered to use human anatomical substances if it involves human biospecimens such as peripheral blood mononuclear cells, primary cells, blood, saliva, tissue, etc. Commercially available sources (e.g., a vendor, medical facility's discarded materials, research collaborators, biobanks, repositories) of human anatomical substances require review. This includes cadaveric specimens and substances.

Commercially available cell lines are exempt from this definition and do not require review (note: commercially available embryonic cell lines are not exempt, and must be reviewed).

Approval to begin research or to subcontract under the proposed protocol will be provided in writing from the MRDC OHRO and the DTRA ROB Executive Secretary (ES) or Program Manager, in absence of the ROB ES. Both the contractor and the Government must maintain a copy of this approval. Any proposed modifications or amendments to the approved research must be submitted to the DTRA ROB and/or the MRDC OHRO for review and approval. Examples of modifications or amendments to the approved work that would require a new review of the project include, but are not limited to:

- a change of the Principal Investigator (PI);
- a change or addition of an institution (note: review and approval of institution is required),
- elimination or alteration of the informed consent process,
- a change in the human subjects study population (e.g., adding children, active duty, etc.) has regulatory implications
- changes in duration or intensity of exposure to some stimulus or agent;
- changes in the information requested of volunteers, or changes to the use of specimens or data collected;
- changes in perceived or measured risks or benefits to volunteers that require changes to the study,
- a change in the IRB of record;
- a change that could potentially increase risk to human subjects
- significant change in study design (i.e., would prompt significant additional scientific review).

Research pursuant to such modifications or amendments must not be initiated without IRB and OHRO approval except when necessary to eliminate apparent and immediate hazards to the subject(s). All unanticipated problems involving risk to subjects or others (UPIRTSOs),

suspensions, clinical holds (voluntary or involuntary), or terminations of the research by the IRB or regulatory agencies, the institution, the sponsor, or any instances of serious or continuing noncompliance with the federal regulation or IRB requirements, must be promptly reported to the DTRA ROB and/or MRDC OHRO.

Greater than minimal risk research projects lasting more than one year require IRB and OHRO review at least every 365 days, or more frequently as required by the responsible IRB. ROB review and approval is required annually from the date of Section A of the DTRA Form 156, through recertification of the DTRA Form 156. The awardee must provide documentation of continued IRB review of protocols for MRDC OHRO review and approval. Research must not continue without renewed OHRO and ROB approval unless necessary to eliminate apparent and immediate hazards to the subject(s).

6.2.3. Animal Use. If the proposed work involves the use of animals, applicants are required to: a) justify and b) include detailed information on the use of animals, and c) include the location(s) of where the animal work is to be performed. The DTRA Research Oversight Board (ROB) will provide ongoing oversight throughout the duration of the effort to ensure proper approvals are in place. . Further information will be required if the proposal is selected for award.

DTRA PMs responsible for the research are required to complete and submit Section A of the DTRA Form 156, available through the DTRA1 Forms Library, to the DTRA Research Oversight Board (ROB) through the ROB Central Mailbox, dtra.belvoir.rd.mbx.research-oversight-board@mail.mil.

Through an Agreement with DTRA, the Animal Care and Use Review Office (ACURO), a component of the USAMRDC Office of Human and Animal Research Oversight (MRDC OHARO) must review and approve all DTRA funded or supported research involving animal use prior to the start of the proposed work. This review requirement is in addition to the DTRA ROB review. Therefore, along with the DTRA Form 156, the DTRA PM must complete and submit the MRDC OHARO form titled "USAMRDC_ORP_Proposal Submission_Form" to the DTRA ROB for review of the proposed work. This form is available through the ROB DTRA1 Sharepoint site, https://dtra1portal.unet.dtra.mil/RD/ROB/default.aspx. Allow up to four months, from date award is submitted to the DTRA ROB, for regulatory review and approval processes. Applicants are to build the review time into their project schedules.

All work under any award made under this BAA involving the use of animals must be conducted in accordance with DoD Instruction (DoDI) 3216.01, DTRA Instruction (DTRAI) 3216.01, and Army Regulation (AR) 40-33. Provisions include rules on animal acquisition, transport, care, handling, and use in: (i) 9 CFR parts 1-4, Department of Agriculture rules that implement the Laboratory Animal Welfare Action of 1966 (U.S.C. 2131-2156); and (ii) the "Guide for the Care and Use of Laboratory Animals," National Institutes of Health Publication No. 86-23. Contracts must include DFARS Clause 252.235-7002 and DTRA Clause 252.235-9001. Other funding vehicles (e.g., grant, OTA) must include similar language. Non-compliance with any provision of this clause may result in withholding of payments under the contract pursuant to the terms and conditions. The Government shall not be responsible for any costs incurred for research involving animal use prior to protocol approval by the MRDC ACURO and ROB. It is the responsibility of the PM to ensure performers are cognizant of and abide by the additional restrictions and limitations imposed by the DoD regarding animal-use research.

The DTRAI 3216.01 requires that research using animals not begin or continue until the DTRA

ROB and MRDC ACURO have reviewed and approved the proposed work.

Through the DTRA Component Animal Use Management Plan (CAUMP), reviewed and approved by the USD(R&E), the DTRAI 3216.01 establishes the DTRA Animal Use Oversight Program (AUOP), and sets forth the policies, defines the applicable terms, and delineates the procedures necessary to ensure DTRA compliance with federal and DoD regulations and legislation governing research involving animal use, and is managed by the DTRA ROB. The regulations mandate that all DoD activities, components, and agencies protect the care and welfare of animals in DoD funded or supported research, development, test and evaluation and training, and related activities. The requirement to comply with the regulations applies to new starts and continuing research for the life of the project, until closure. The completion of a research project requires closure document (e.g., IACUC Final Review submission) submitted to the DTRA ROB and/or the MRDC ACURO.

The DoD definition of animal is "any living or dead vertebrate animal, including birds, cold blooded animals, rats of the genus rattus and mice of the genus mus." "Dead" is defined as animals killed for the direct purpose of conducing RDT&E or training.

Approval to begin research or to subcontract under the proposed protocol will be provided in writing from the MRDC ACURO and the DTRA ROB Executive (ES) Secretary or the ROB PM, in the absence of the ROB ES. Both the awardee and the Government must maintain a copy of this approval. Any proposed modifications or amendments to the approved research must be submitted to the DTRA ROB and/or the MRDC ACURO for review and approval. Examples of modifications or amendments to the approved protocol that would require a new review of the project include, but are not limited to:

- a change of the Principal Investigator (PI),
- a change or addition of an institution (note: review and approval of institutions is required),
- a change in the duration or intensity of exposure to a stimulus or agent,
- a change in the animal model and/or numbers of animals used,
- a change in the IACUC of record, or
- a significant change to in study design (i.e., would prompt significant additional scientific review).

Research pursuant to such modifications or amendments must not be initiated without IACUC and ACURO approvals.

6.2.4. Biological Defense Research Program (BDRP) Requirements: BioSurety and Select Agent Use.

Proposals must specify what Select Agent work will be conducted at the applicant's facility and what Select Agent work will be performed in other facilities. Proposals also must provide the source of the Select Agent(s), any appropriate registration information for the facilities, and specify the Laboratory Bio-safety Level. All Select Agent work is subject to verification of information and certifications. Further information may be required if the proposal is successful.

For those institutions in which PI's are conducting research with Bio-safety Levels 3 and 4 material, a Facility Safety Plan must be prepared and made available during the project award

phase in accordance with 32 CFR 626.18. For grants awarded to foreign institutions, you must follow either local or U.S. laws (as stated above) depending on which laws provide stronger protection. (DTRA requires that research using Select Agents not begin or continue until DTRA has reviewed and approved the proposed agent use. See URL: https://www.gpo.gov/fdsys/pkg/CFR-2002-title32-vol3/pdf/CFR-2002-title32-vol3-sec626-18.pdf for a copy of 32 CFR 626.18, Biological Defense Safety Program.)

For projects that will employ the use of chemical agents, either neat agent or dilute agent, the offeror must provide approved Facility Standard Operating Procedures that conform to Federal, State and local regulations and address the storage, use and disposition of these chemical materials.

6.2.5. Dual-Use Potential. In accordance with National Science Advisory Board for Biosecurity (NSABB) recommendations, DTRA will not support research that, based on current understanding, can reasonably be anticipated to provide knowledge, information, products, or technologies that could be directly misapplied to pose a significant threat with broad potential consequences to public health and safety, agricultural crops and other plants, animals, the environment, materiel, or national security. Research involving select agents and toxins is within scope of the DTRA mission; however, the use of select agents and toxins in certain experimental categories is considered "dual-use research of concern" (DURC) according to U.S. policy. (http://www.phe.gov/s3/dualuse/Documents/us-policy-durc-032812.pdf) Proposals that contain DURC will not be funded. Dual-use potential will be assessed based on application of the following criteria:

- Use of select agents or toxins. This factor evaluates whether the proposed research involves use of one or more select agents or toxins [as identified by the Select Agent Program under Federal Law (7 C.F.R. part 331, 9 C.F.R. part 121, and 42 C.F.R. part 73)] which pose significant risk of deliberate misuse with potential for mass casualties or devastating effects to the economy, critical infrastructure, or public confidence.
- Scope of proposed experiments. This factor evaluates whether the proposed research involves experiments that will produce, aim to produce, or is reasonably anticipated to produce: (a) Enhanced harmful consequences of the agent or toxin; (b) Disruption of immunity or effectiveness of an immunization against the agent or toxin without clinical or agricultural justification; (c) Conferred resistance by the agent or toxin to clinically or agriculturally useful prophylactic or therapeutic interventions against the agent or toxin, or facilitated ability to evade detection methodologies; (d) Increased stability, transmissibility, or dissemination ability of the agent or toxin; (e) Altered host range or tropism of the agent or toxin; (f) Enhanced susceptibility of a host population to the agent or toxin; or (g) Eradicated or extinct select agents or toxins.

6.2.6. Military Recruiting. This is to notify potential applicants that each award under this announcement to an institution of higher education, with exception of any grants awarded to institutions of higher education entirely located outside the United States and/or its territories, must include the following term and condition: "As a condition for receipt of funds available to DoD under this award, the recipient agrees that it is not an institution of higher education (as defined in 32 CFR 216) that has a policy of denying, and that it is not an institution of higher education that effectively prevents, the Secretary of Defense from obtaining the following for military recruiting purposes: (A) entry to campuses or access to students on campuses; or (B)

access to directory information pertaining to students. If the recipient is determined, using procedures in 32 CFR 216 to be such an institution of higher education during the period of performance of this agreement, and therefore to be in breach of this clause, the Government will cease all payments of DoD funds under this agreement and all other DoD grants and CAs, and it may suspend or terminate such grants and agreements unilaterally for material failure to comply with the terms and conditions of award." 32 CFR 216 may be accessed electronically at http://www.ecfr.gov/cgi-bin/text-idx?SID=ee45add5e352854b7089ce420c7fd0a6&mc=true&tpl=/ecfrbrowse/Title32/32cfr216_main_02.tpl . If your institution has been identified under the procedures established by the Secretary of Defense to implement Section 558 of Public Law 103-337, then: (1) no funds available to DoD may be provided to your institution through any grant, including any existing grant; and (2) your institution is not eligible to receive a grant in response to this BAA. This is to notify potential applicants that each award under this announcement to an institution of higher education, with exception of any grants awarded to institutions of higher education entirely located outside the United States and/or its territories, must include the following clause: 32 CFR 22.520 (DoDGARS 22.520), Military Recruiting and Reserve Officer Training Corps Program Access to Institutions of Higher Education.

6.2.7. Combating Trafficking in Persons. The recipient agrees to comply with the trafficking in persons requirement in Section 106(g) of the Trafficking Victims Protection Act of 2000 (TVPA), as amended (22 U.S.C. 7104(g)).

6.2.8. Reporting Subawards and Executive Compensation. The recipient agrees to ensure they have the necessary processes and systems in place to comply with the reporting requirements of the Transparency Act, as defined at 2 CFR 170.320, unless they meet the exception under 2 CFR 170.110(b).

6.2.9. Representation Regarding the Prohibition on Using Funds under Grants and Cooperative Agreements with Entities that Require Certain Internal Confidentiality Agreements. By submission of its proposal or application, the applicant represents that it does not require any of its employees, contractors, or subrecipients seeking to report fraud, waste, or abuse to sign or comply with internal confidentiality agreements or statements prohibiting or otherwise restricting those employees, contractors, or subrecipients from lawfully reporting that waste, fraud, or abuse to a designated investigative or law enforcement representative of a Federal department or agency authorized to receive such information. Note that: (1) the basis for this representation is a prohibition in section 743 of the Financial Services and General Government Appropriations Act, 2015 (Division E of the Consolidated and Further Continuing Appropriations Act, 2015, Pub. L. 113-235) and any successor provision of law on making funds available through grants and cooperative agreements to entities with certain internal confidentiality agreements or statements; and (2) section 743 states that it does not contravene requirements applicable to Standard Form 312, Form 4414, or any other form issued by a Federal department or agency governing the nondisclosure of classified information.

6.2.10. Prohibition on Covered Telecommunications Equipment or Services. Section 889 of the National Defense Authorization Act (NDAA) for Fiscal Year (FY) 2019 (Public Law 115-232) prohibits the head of an executive agency from obligating or expending loan or grant funds to procure or obtain, extend, or renew a contract to procure or obtain, or enter into a contract (or extend or renew a contract) to procure or obtain the equipment, services, or systems prohibited systems as identified in section 889 of the NDAA for FY 2019.

(a) In accordance with 2 CFR 200.216 and 200.471, a recipient and subrecipient are prohibited from obligating or expending grant funds to:

1. Procure or obtain;
2. Extend or renew a contract to procure or obtain; or
3. Enter into a contract (or extend or renew a contract) to procure or obtain equipment, services, or systems that use covered telecommunications equipment or services as a substantial or essential component of any system, or as critical technology as part of any system. Covered telecommunications equipment is telecommunications equipment produced by Huawei Technologies Company or ZTE Corporation (or any subsidiary or affiliate of such entities).
    - For the purpose of public safety, security of government facilities, physical security surveillance of critical infrastructure, and other national security purposes, video surveillance and telecommunications equipment produced by Hytera Communications Corporation, Hangzhou Hikvision Digital Technology Company, or Dahua Technology Company (or any subsidiary or affiliate of such entities);
    - Telecommunications or video surveillance services provided by such entities or using such equipment; or
    - Telecommunications or video surveillance equipment or services produced or provided by an entity that the Secretary of Defense, in consultation with the Director of the National Intelligence or the Director of the Federal Bureau of Investigation, reasonably believes to be an entity owned or controlled by, or otherwise connected to, the government of a covered foreign country.

(b) In implementing the prohibition under Public Law 115-232, section 889, subsection (f), paragraph (1), heads of executive agencies administering loan, grant, or subsidy programs shall prioritize available funding and technical support to assist affected businesses, institutions and organizations as is reasonably necessary for those affected entities to transition from covered communications equipment and services, to procure replacement equipment and services, and to ensure that communications service to users and customers is sustained.

(c) See Public Law 115-232, section 889 for additional information.

COVERED FOREIGN COUNTRY means the People's Republic of China.

6.3. Reporting. General requirements are provided below; however, each awardee should check the award agreement and its contract data requirements list (CDRLs) and/or terms and conditions to determine the requirements for that specific award.

6.3.1. Annual Reports. Annual Reports will be due no later than 1 July of each year. Awards effective after 31 January will not require an Annual Report until 1 July of the following year. The Annual Report is *not* a cumulative report.

6.3.2. Final Technical Reports. A comprehensive final technical report is required prior to the end of an effort, due on the date specified in CDRLs and/or the terms and conditions of the award document. The purpose of the Final Report is to document the results of the effort. The Final Report *is* a cumulative report.

The final report will always be sent to the Defense Technical Information Center (DTIC) and reports may be available to the public through the National Technical Information Service (NTIS).

6.3.3. Financial Reports. Federal Financial Reports (SF-425) are due no later than 1 July of each year. Grants effective after 31 January will not require a Federal Financial Report until 1 July of the following year.

6.3.4. Foreign Travel Reports. Within thirty (30) days after returning to the United States from foreign travel, the PI may be required to submit an acceptable trip report summarizing the highlights of the trip. For grants, contracts, or OTAs awarded to institutions entirely located outside the United States and/or its territories, this is not required.

6.4. After-the-Award Requirements for *Grants*. Closeout, subsequent adjustments, continuing responsibilities, and collection of amounts due are subject to requirements found in 32 CFR 32.71 – 73 (Institutions of Higher Education, Hospitals, and Other Non-Profit Organizations) and 32 CFR 34.61 – 63 (For-Profit Organizations).

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7. AGENCY CONTACTS

Administrative Correspondence and Questions	HDTRA1-FRCWMD-A@mail.mil
Thrust Area 1: *Science of WMD Sensing and Recognition*	HDTRA1-FRCWMD-TA1@mail.mil
Thrust Area 2: *Network Sciences*	HDTRA1-FRCWMD-TA2@mail.mil
Thrust Area 3: *Science for Protection*	HDTRA1-FRCWMD-TA3@mail.mil
Thrust Area 4: *Science to Defeat WMD*	HDTRA1-FRCWMD-TA4@mail.mil
Thrust Area 5: *Science to Secure WMD*	HDTRA1-FRCWMD-TA5@mail.mil
Thrust Area 6: *Cooperative Counter WMD Research with Global Partners*	HDTRA1-FRCWMD-TA6@mail.mil
Thrust Area 7: *Fundamental Science for Chemical and Biological Defense*	HDTRA1-FRCWMD-TA7@mail.mil

Table 6: Agency Contacts.

7.1. Questions regarding administrative content of this BAA must be addressed to the administrative e-mail address listed above. Applicants should include the relevant thrust area email address.

7.2. Questions regarding technical content of this BAA must be referred to the thrust area email listed above.

DTRA will not release employee personal contact information.

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8. OTHER INFORMATION

Topics from previous periods may or may not be repeated. DTRA will not provide additional information regarding the posting of future topics, including dates for posting, the potential for a topic to be repeated in out years, the potential for similar topics to be posted, and/or topic details in advance of issuance of an amended BAA.

The Post Doc-Topic B spans the technical areas covered by Thrust Areas 1-7 and is ***NO longer accepting pre-application white paper submission. Submissions to the general thrust area descriptions for Thrust Areas 1-7 are closed; abstracts and pre-application white papers submitted to the general descriptions for Thrust Areas 1-7 will NOT be reviewed.

DTRA anticipates that the award(s) made under Post Doc-Topic B will be contracts. Pre-application white papers and proposals submitted to Post Doc-Topic B must have a single lead organization and single submission for the pre-application white paper and the invited proposal. Awards will be made by a single award to the lead institution. Subawards, including all grants and/or contracts, are the responsibility of the award recipient; exceptions will not be made.

Post Doc-Topic B: Postdoctoral Scholars Program (Thrusts 1-7)

*****PRE-APPLICATION WHITE PAPERS FOR THIS TOPIC ARE NO LONGER BEING ACCEPTED.**

Background: The Defense Threat Reduction Agency (DTRA) Postdoctoral Scholars Program started more than 20 years ago and has a proven track-record of success—measured by program participation by excellent professionals and follow-on career decisions. The future of the Postdoctoral Scholars Program remains consistent with the initial program goals of fostering and strengthening long-term strategic partnerships with the scientific community while leveraging the best and the brightest professionals to address critical science and technology challenges of interest to DTRA. This topic seeks a contracted partner(s) to continue the Postdoctoral Program at DTRA.

The benefits to the contracted partner(s) are that of enhanced institutional ability to effectively combat the threat posed by WMD through strengthened relationships with DTRA as well as workforce development. The benefit for the individual program participants is also tremendous. Each Postdoctoral Scholar is exposed to a breadth of information and gains knowledge of current capabilities, critical national security challenges, and the business of government—areas of skill development that are not readily available through many other early career opportunities.

Impact: The overall purpose of this programmatic initiative is to provide advanced research support, technical expertise, and execution capabilities in scientific, technical, and engineering disciplines relevant to the DTRA mission, specifically those that directly enhance the institutional ability to effectively combat the threat posed by WMD.

Objective: To find a contracted partner(s) organization that is capable of providing up to eight Postdoctoral Scholars (as an initial estimate of the program). The overall program contract period of performance will be 60 months (5 years).

Postdoctoral Scholars will possess doctoral degree credentials. They will be American citizens, capable of obtaining security clearances at the Secret level; clearances at higher levels will be considered on a case-by-case basis. They will exhibit such academic, research, and/or professional credentials as to demonstrate a disciplinary “state of the art” focus, flexibility, and innovation in methodology and approach, which will ultimately enhance the mission capabilities of DTRA.

Requirements and needs for the provision and assignment of such Postdoctoral Scholars, as may be selected to participate under this program, will be defined and prioritized by Agency

leadership, as deemed appropriate. The successful applicant(s) to this topic will provide an appropriate process for the provision of suitable candidates with advanced educational credentials and capabilities responsive to the stated requirements and needs.

Science, technology, engineering, and mathematical skills particularly critical and highly desirable to the enhancement of the Agency mission include: nuclear and radiation physics; weapons engineering; structural, electrical, and mechanical engineering; broad-based nano-technological engineering and applications; weapons effects and system response technologies; physics, chemistry, and biological sciences related to detection, characterization, and destruction of WMD materials; medical and pharmaceutical sciences; information technology, modeling, technical editing and publication, data visualization, data science, and advanced computational sciences; social, adversarial, and behavioral modeling, science, and analysis. This is not an exclusive listing, and DTRA reserves the right to amend this skill list as mission requirements warrant.

Postdoctoral Scholars will be assigned to DTRA functions, missions, and projects according to their respective specialties and at such places and locations determined to be in the best interests of the Agency (taking into practicable account individual career and professional needs). These locations may include DTRA facilities, national and/or DoD laboratories, other government facilities, military facilities, and contractor sites. They will serve as independent technical advisors and professional subject matter experts (SME) on scientific, technical, and engineering issues related to the execution of the DTRA mission.

Each Postdoctoral Scholar will be assigned a DTRA Mentor to guide, lead, and ensure the optimal utilization of each participant, ensuring that all assigned Postdoctoral Scholars' duties maximize individual intellectual contributions to the overall enhancement of the Agency's capabilities. The Postdoctoral Scholars will report to these individuals on a regular basis to provide informal status reports, to present feedback, and to obtain guidance on current and future activities. Institutionally provided online library services is an important aspect to the success of the Postdoctoral Scholar.

To ensure the goals of the program are met to the maximum extent possible, as well as minimize disruption on academic and professional careers, a minimum commitment of one calendar year of effort per Scholar appointment is required. Two additional one calendar year extensions may be made available as determined by the DTRA manager and on the desire of the Postdoctoral Scholar to extend.

Postdoctoral Scholars will be expected to perform some travel (both local and non-local) approximately once a month during their assignment to DTRA to maintain their technical proficiency. This travel will include, but is not limited to, national conferences, symposia, workshops, interfacing with their home organizations, and site visits for outreach. Travel may also include, but is not required, one or more international conferences.

Thrust Area 7 has ten (10) topics —Topics M1-M10 —detailed below. Submissions to the general thrust area descriptions for this thrust area in accordance with the requirements detailed in this BAA are also welcome.

- If NOT submitting to one of the specific topic numbers detailed below, use one of the **Thrust Area NO TOPIC** application packages
- If you ARE submitting to one of the specific topic numbers detailed below, use the applicable **Basic Research-Thrust Area 7-Topic M1 to M10** application package

Great care must be taken to use the appropriate application package on www.grants.gov, as the package selection dictates how each submission will be reviewed:

*****BASIC RESEARCH TOPICS M1-M10*****

In accordance with Section 4.2.1, the requirement for abstract pre-coordination is waived for Topics M1-M10; these topics do NOT require pre-coordination of an abstract prior to the submission of pre-application white papers. All other pre-coordination requirements remain in effect.

The pre-application white paper deadline for Topics M1-M10 is 3 February 2023. **PRE-APPLICATION WHITE PAPERS FOR THESE TOPICS MUST BE SUBMITTED BY 11:59 PM (MIDNIGHT) EST ON 3 February 2023.** White papers submitted to Topics M1-M10 may not be considered if they are received after this deadline.

Topics M1-M10 are interested in research projects that span from those that focus on exploratory aspects of a unique problem or approaches to those that involve a comprehensive program with interdisciplinary areas. Consistent across all proposals should be the focus on innovative research with the potential for high impact to C-WMD science.

The following topics are Basic Research topics, and proposals should not be solely written with or marketed to a DoD centric application; the offerer should also present a description of the broader implications of their work to our Nation and the whole of society.

DTRA anticipates that the predominance of awards made under Topics MI-M10 will be grants. Pre-application white papers and proposals submitted to Topics M1-M10 must have a single lead organization and single submission for the pre-application white paper and the invited proposal. Awards will be made by a single award to the lead institution. Sub-awards, including all grants and/or contracts, are the responsibility of the award recipient; exceptions will not be made.

Thrust Area 7, Topic M1: Host Response to Emerging Viral Threats: Discovery of Common Mechanisms for Therapeutic Intervention

Award Amounts for this topic are anticipated to be up to $500,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation.

The preferred award structure for this topic is a base period of two (2) years with up to three (3) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white

papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: Direct acting antivirals deployed during the COVID-19 pandemic have helped mitigate the effects of the viral infection when administered early in the course of disease; however, the disease processes targeted and induced by SARS-COV2 have been persistent and have contributed to long periods of morbidity in several subsets of the population. The COVID-19 pandemic highlights the need for additional therapeutic strategies, such as host-targeted therapeutics, that are effective in mitigating or reversing symptoms caused by viral infection.

This solicitation seeks research to understand host processes and mechanisms involved in viral infection, replication and disease progression for members of viral families Filoviridae (e.g. Sudan, Marburg, Ebola), Hantaviridae (e.g. Hantaan), Arenaviridae (e.g. Lassa, Machupo), and/or Togaviridae (e.g. VEEV, EEV), which can leveraged to identify common host targets for broad-spectrum (i.e., multi-pathogen) therapeutic intervention.

Impact: If successful, this effort will result in the discovery and validation of host processes and mechanisms common among/within viral families that can be targeted to develop broad-spectrum, host-directed therapeutic medical countermeasures for emerging threats.

Objective: Pre-application white papers and proposals will describe technical approaches to characterize and identify host processes and mechanisms common among/within viral families of interest to the ChemBio Defense Program that are involved in the propagation of viral pathogens and/or are linked to the progression of disease in an infected patient. The approach proposed must include methods for confirming the role of identified targets in viral infection or pathogenesis in more than one orthogonal assay. Experimental methods may include cell cultures, micro-physiological systems, *ex-vivo* systems, and small animal models of disease. The data collected must be documented and formatted such that it can be used by potential collaborators for future development of therapeutic medical countermeasures.

References:

1. Geraghty RJ, Aliota MT, Bonnac LF (2021) Broad-Spectrum Antiviral Strategies and Nucleoside Analogues, Viruses, 13(4):667. doi: 10.3390/v13040667.
2. Lu L, Su S, Haitao Yang H, Shibo Jiang S. (2021) Antivirals with common targets against highly pathogenic viruses, Cell 184(6):1604-1620.
3. García-Cárceles J, Caballero E, Carmen Gil C, Martínez A,(2022) Kinase Inhibitors as Underexplored Antiviral Agents J. Med Chem 65(2):935-954

Thrust Area 7, Topic M2: Exploration of Consumer Non-invasive Brain-Computer Interface (BCI) Technologies for Chemical and Biological Defense (CBD) Applications

Award Amounts for this topic are anticipated to be up to $500,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation. Award amounts for this topic are anticipated to be commensurate with the

proposed work involved in exploring consumer non-invasive brain-computer interface for CBD applications, evaluating materials, and other aspects of this program outlined in the metrics below. It is anticipated that teams with varying expertise are required to meet the metrics outlined below.

The preferred award structure for this topic is a base period of one (1) years with up to four (4) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Note: For this topic, awardees will be required to grant the US Government a licensing agreement to all software and or hardware in perpetuity for all Chemical and Biological Defense Program (CBDP) uses.

Background: The battlespace is a complex environment with the six fundamental variables of combat represented by the military acronym METT-TC (Mission, Enemy, Troops, Terrain and weather, Time available, and Civilian considerations). For CBRN operators, the operation variables are further complicated by the threat of operating in contaminated environments. To protect our Warfighters, Mission Oriented Protective Posture (MOPP) is the standard protective gear used when operating in contaminated environments. However, it is known that wearing MOPP gear can cause physical and mental stress as well as reduction in mobility, sensory awareness, attention, alertness, and both gross and fine dexterity [1-3]. The situational uncertainties, physical limitations, and the associated risks can impact awareness of the situation while operating in such a complex and dynamic environment. Yet, effective decision making is built off of situational awareness and the ability to recognize the essence of a given problem and the creative ability to devise a practical solution. These abilities are the products of experience, education, and intelligence [4]. Therefore, the Joint Science and Technology Office Digital Battlespace Management Division (JSTO CBI) is seeking methods that can allow easier access to information while operating to enable comprehensive decision making, and promote the inheritance of knowledge and experience.

JSTO CBI has identified brain-computer interface and the related neurotechnologies as the potential solution. The human brain, formed by nearly 100 billion neurons and 100 trillion connections, is one of the greatest mysteries in science. With the dedication of neuroscientists, it is now common knowledge that these neurons and connections work to process and transmit electrophysiological signals. Jacque Vidal asked in his publication Toward Direct Brain-computer Communication nearly half a century ago [5], “Can these observable electrical brain signals be put to work as carriers of information in man-computer communication or for the purpose of controlling such external apparatus as prosthetic devices or spaceships?”. In this topic, JSTO CBI would like to ask a similar question—can these observable electrical brain

signals be put to use in order to serve our Warfighters in Chem/Bio defense missions and tasks.

A brain-computer interface (BCI) is a communication system that allows human interactions with surroundings without the involvement of peripheral nerves and muscles. Such interaction is achieved by leveraging control signals generated from electroencephalographic activity [6]. BCI can serve to record data from the brain for further decoding to transform the data into meaningful outputs for purposes such as control of external devices (e.g., robotic limb) [7]. Because of the possible external device control, BCI applications in medical fields for restoration purposes are often seen. However, the advancements in neuroscience and BCI is shining light on other applications. This expansion toward other applications is evident with an unprecedented amount of corporate investments in neurotechnologies, such as Nissan's brain-to-vehicle [8], Valve Software's focus on BCI for virtual reality (VR) interaction [9], Facebook's optical BCI investment [10], and Neuralink's focus of bringing neurotechnology to the mass market. Although nascent, this trend is expanding toward applications other than restoration of physical functions.

The combination of BCI, Team Awareness Kit (TAK), and heads-up display technologies are potential tools that can provide enhanced situational awareness to the Warfighters while operating in highly dynamic and hazardous environment as an alternative to commonly seen hand gesture, eye-tracking, or voice controls, allowing Warfighters to have hands on tasks, eyes on target, and minimal risk of exposing their locations. It is desired to explore whether consumer non-invasive BCI can be exploited for human interest detection (HID) [11] and decision making [12, 13] to support CBD situational awareness and mission readiness. JSTO CBI is seeking to investigate the possibility of leveraging consumer non-invasive BCI for human interest detection (HID) in combination with artificial intelligence (AI) or machine learning (ML) models to help reduce cognitive load by reducing amount of information to be projected on head-up displays (HUD). It is also of interest to explore if and how the detected interest can be leveraged to enhance or facilitate decision making. Additionally, we seek to answer the question whether consumer non-invasive BCI can assist understanding the key responses that lead to successful completion of tasks in hazardous and dynamic environments.

Impact: The research in this topic seeks to explore the potential and possibilities of leveraging signals from consumer non-invasive BCI for detecting human interest toward the applications of HUD content control, CBD knowledge inheritance, and decision making. The results of the studies are envisioned to reduce cognitive load and enhance decision making when operating in high-stress, dynamic, and hazardous environments as well as to promote the preservation of critical CBD operational knowledge.

Objective: Pre-application white papers and proposals should describe the development and demonstration of the potential to leverage signals from consumer non-invasive BCI to detect human interest for the purposes of HUD content control to reduce cognitive load, enhance decision making, and preserve and extract critical knowledge or responses from experienced

CBD operators.

Applicants should consider methods that leverage consumer non-invasive BCI and related neurotechnologies for the above mentioned 3 areas. Proposed work focusing on selecting any number of the above listed areas will be considered. Proposed work should employ a phased approach with the following recommended stages of applications:

- Demonstrate capabilities in controlled indoor settings to allow explorations and baselining
- Demonstrate capabilities in controlled outdoor settings to explore influences from outdoor environment
- Demonstrate capabilities in a stressed outdoor environment that simulates a dynamically changing battlefield
- Demonstrate capabilities in a stressed outdoor environment with tasks to simulate the level of complexity to carry out a mission in dynamically changing battlefield

The order and stages of milestones are open to modifications that best support the proposed studies. The number of subjects to be involved in phases should be considered. Applicant should consider IRB and HRPO processes when proposing the research schedule.

Research areas may include, but are not limited to:

- Exploration of applicability of one or multiple consumer non-invasive BCIs for signal extraction
- Exploration of different modalities of consumer non-invasive BCI (e.g. EEG-based, in-ear BCI)
- Development of necessary AI/ML algorithms or models to achieve the goals of HID for HUD content control, achieve enhanced decision making, or preserve critical CDB knowledge

Proposed work may leverage a combination of multiple consumer non-invasive BCI devices. It should be noted that minimum modifications to consumer non-invasive BCI devices are acceptable. However, this topic is not aimed at hardware development and should focus on making use of signals available from consumer non-invasive BCI devices. Applicants are encouraged to consider leveraging TAK variants when reasonable.

It is anticipated that this topic will require teams of researchers with different expertise in brain-computer interface, neurotechnologies, AI/ML, computer science modeling, and extended reality. A justification of the budget will need to be provided to supplement the costs proposed for this effort.

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Thrust Area 7, Topic M3: Leveraging quantum effects to improve diagnostic and detection capabilities for threat agents

Award Amounts for this topic are anticipated to be up to $500,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation. Award amounts for this topic are anticipated to be commensurate with proposed research seeking to characterize, control, or exploit quantum mechanical effects and properties involved in biological interactions for the purpose of improving detection and diagnostic tools. It is anticipated that teams with varying expertise are required to meet the metrics outlined below.

The preferred award structure for this topic is a base period of two (2) years with up to three (3) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable.

Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: Current diagnostic and detection technologies are largely based on our understanding of biological processes at the macromolecular scale. In order to develop transformative solutions that improve our ability to identify threats and approach the ultimate limit of single cell and single molecule detection in handheld assays, we must seek to understand and leverage knowledge of the processes that occur in the quantum realm. There are numerous ways quantum mechanics can be exploited to improve portable, handheld diagnostic and detection assays including leveraging knowledge gained through quantum biological processes, exploitation of quantum light properties, and the use of quantum materials that interact with biological molecules.

The burgeoning field of Quantum biology seeks to achieve a better understanding of quantum mechanics in biological processes. It involves the study of the influence of non-trivial quantum phenomena which can be explained by reducing the biological process to fundamental physics. Quantum measurements in noisy biological environments pose a formidable challenge, but work in this field has already begun leveraging higher resolution tools and even synthetic biology methodologies to elucidate biological activities that involve quantum effects such as tunneling, coherence, and entanglement. Is it possible that knowledge gained from this work could be leveraged to increased signal amplification or reaction speeds for biological assays?

Diagnostic and detection technologies could also be improved by exploiting the quantum properties of light. Many biological assays, such as optical microscopy, cellular histology, and fluorescence immunoassays use classical light for detection/readout. In contrast to classical light, quantum light can have unique properties, such as temporal/spatial coherence or entanglement that can provide unique advantages, including increased sensitivity, lower light dose, superior spatial resolution, or wider and novel spectral windows of observation. Recent work has brought together quantum physics and biology to show that quantum light can be used to track enzyme reactions in real time. This made it possible to use low illumination without disrupting the enzymes, with the potential to achieve a better sensitivity. This type of work is an important step toward development of quantum sensors for biomedical applications.

Another potential area of exploration is the use of quantum materials to improve sensitivity of assay read out. Here, we define quantum materials as systems where key material properties (such as discrete and quantized fluorescence emission or altered/enhanced electrical conductivity properties) stem from quantum effects such as quantum confinement. This confinement may come in zero dimensional materials (e.g. quantum dots or vacancy centers), 1D materials (such as carbon nanotubes or nanowires) or 2D quantum materials such as graphene. The small and reduced dimension of these materials generally makes them sensitive to their local nano-environments, which have the potential to be used as ultra-sensitive reporters of chem/bio agents and their effects on cellular and human health.

This topic seeks to leverage quantum mechanics to gain better understanding of biological processes and to exploit quantum mechanical effects or properties to develop transformative

solutions for the diagnosis of disease and detection of threat agents.

Impact: This topic encompasses broad topics related to applying quantum mechanics and quantum tools and materials, as well as exploitation of quantum effects to improve diagnostic and detection capabilities for chemical/biological threats. Successful efforts from this topic will provide novel solutions for improving the sensitivity or specificity of diagnostic and detection capabilities.

Objective: Proposals for this topic should address the broad objective of using quantum mechanics to improve diagnostic and detection capabilities. Quantum material proposals for this call should focus on novel materials and synthesis methods, therefore, proposals including established commercial off-the-shelf (COTS) materials will not be considered. Examples of specific research questions that may be addressed include but are not limited to the following:

- What quantum effects play a non-trivial role in biological processes and how can these effects be exploited to improve point of care diagnostic and detection capabilities?
- Can quantum mechanical effects or properties be used to enhance the sensitivity, resolution, or specificity of biological assays in areas important to biological health or chemical/biological agent detection?
- Can quantum mechanical effects or properties be used to extend bio-imaging and sensing to important novel spectral windows difficult to obtain via classical methods?
- Can quantum mechanical effects or properties be used to extend the observation period or increase the spatial resolution of visualizing host-pathogen or host-agent interactions and interventions?
- Can quantum mechanical effects or properties be used to reduce the cost or SWAP (Size, Weight, and Power) requirements for standard biological assays, chemical/biological agent detection, or imaging methods?
- Are there key biological molecule and quantum materials interactions of relevance to chemical and biological agent interactions that could be shaped/manipulated/detected because of quantum interactions?
- Can new quantum materials be developed and exploited to measure parameters of interest to biological health in response to chemical/biological agents?
- Can new quantum materials be developed for chem/bio detection and sensing, measuring key biomolecular properties on spatial or temporal scales un-obtainable via classical methods?

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Thrust Area 7, Topic M4: Let's Get Moving: Surface Agitation and Self-propellant Materials in Liquid Films for Decontamination

Award Amounts for this topic are anticipated to be up to $500,000 per year (total dollar value = direct and indirect costs). Award Amounts for this topic are anticipated to be indicative of the amount of work involved to comprehensively address the objectives of this program outlined below. It is anticipated based on this topic that there will be teams with varying expertise that is required. In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation.

The preferred award structure for this topic is a base period of two (2) years with up to three (3) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: Decontamination and protection rely on diffusion of toxic chemicals (for example GD, HD, and VX) to active/catalytic sites that will then detoxify those materials. Although highly efficient towards toxic chemical decomposition these active/catalytic materials have the rate limiting step of being controlled by diffusion of the toxic chemical to active sites. Having a reactive or catalytic decontamination technology with the capacity to be "mobile" could impact the ability for the decontaminant to improve efficacy with respect to time (increasing the amount of analyte decontaminated in a specific time) and/or allow for migration of the decontaminant into small spaces where the current dominant mechanism is reliant on chemical diffusion out of these small spaces such as rivets or metal joints on vehicles.

Current research in micromotors, active droplets, small scale/micro-swimmers, and propelled metal organic frameworks (as examples) have explored these "mobile" materials for toxic chemical remediation, detection and neutralization, improved mixing to reduce increase reaction efficiency and also including wastewater remediation of various chemical and biological contaminants. Researchers have also studied mechanisms of particulate self-propellancy and interactions with other particles (e.g. swarm behaviors) and different geometries and chemistries to introduce additional functionalities for detection, monitoring, and decontamination. These technologies have the potential to allow for self-propellancy of the decontamination technology such that the decontaminant could maneuver across surfaces and/or into complex features to allow for self-propellant behavior to/from contaminated areas or surfaces increasing rates of decontamination and getting into complex features where current diffusion based mechanisms

are dominant.

This topic seeks to develop a fundamental understanding of how to develop and integrate components for both self-propellant and decomposition of toxic chemicals (surrogates, simulants or model systems) into one material. Initial studies can be initially demonstrated in solution, with the ultimate goal to minimize the solvent to a liquid film on a surface. This topic also seeks self-propellant behavior through utilizing internal or external stimuli (chemical reactions, attraction/repellency, enzymatic, biologically inspired, light, pH, magnetism, electrostatic, ultrasound, predator/prey relationships or other mechanism) without requiring mechanical stirring or other direct intervention.

Impact: If successful the methods and materials developed during this effort could be developed into a new decontamination technology or integrated with existing platforms to increase rates of surface decontamination and increased diffusion/decontamination rates in areas that are difficult to decontaminate.

Objective: This topic seeks to develop a fundamental understanding of how to develop and integrate components for both self-propellant and decomposition of toxic chemicals (surrogates, simulants or model systems) into one material capable of neutralizing toxic chemicals. Proposals should describe the approach for investigation of the components and integration of these materials for eventual use in or on surfaces or in complex features that will be self-propellant with internal or external additives or other triggers (e.g. chemicals, catalytically propelled, light, pH, magnetic particles, Marangoni effect, thermal, concentration gradients, other particle attraction/repulsion interactions) and reactive or catalytic towards toxic chemicals decomposition/destruction. Proposal designs should include the following:

- Base Period Goals:
    - Proof-of-concept (iterative design, development, fabrication, and characterization) of an active/self-propellant material or self-propellant phase (micromotor, active droplet, etc.) and a catalytic or reactive component for decomposition of surrogates/simulants or a model system for one or more toxic chemicals.
    - Initial studies of mechanisms and kinetics of decomposition with these materials in the model system or for simulants/surrogates including determination of efficacy of static versus self-propellant version of the material in liquid films
    - Initial studies of multiple materials in close proximity (e.g. aggregation potential)
    - Preliminary fundamental studies of self-propellancy and development/use of methods to measure self-propellancy of these materials in liquid films
    - Demonstration of relative efficacy for simulants of static and self-propellant version of the offerors concept and demonstration of self-propellancy of the initial designs in liquid films to adjudicate potential for future work
- Option Period(s) Goals
    - Year 3: Refined studies of mechanisms and kinetics of decomposition with these materials in the model system or for simulants/surrogates ; continued advancement of decontamination rates and improved kinetics of diffusion to/from

these integrated materials on surfaces; continued study of efficacy rates relative to static materials

- Year 4: Determine stability (mechanical, chemical); formal study of behavior of multiple self-propellant materials in close proximity; determination of initial ability for use on actual surface materials or in complex features
- Year 5: Determination of ability to maneuver, traverse longer distances and encounter other materials; determination of efficacy of reaction of simulant/surrogates and efficacy relative to time, determination of chemical decomposition rates relative to static material using DMMP, DIMP, CEES or other simulants.

The selected providers must provide appropriate model systems/reactions for CB defense applications showing promise for compatibility with potential protection and hazard mitigation technologies.

Proposals should focus on one or more toxic chemicals and associated model compounds. It is expected that the awardees will work only with simulant/surrogates and/or model compounds as part of this research. Actual chemical agents should be considered but not proposed as part of this work.

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Thrust Area 7, Topic M5: Medical Countermeasures Against the Aging of Acetylcholinesterase Complexed with Organophosphate Agents

Award Amounts for this topic are anticipated to be up to $500,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation. Award amounts for this topic are anticipated to be commensurate with the proposed work involved in developing novel in vitro models. It is anticipated that teams of collaborating organizations with varying expertise maybe required to meet the metrics outlined below.

The preferred award structure for this topic is a base period of three (3) years with up to two (2) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: Organophosphate agents (OP) continue to be a threat to the Armed Forces and

agricultural workers, and there are no broad spectrum medical countermeasures for treatment of OP intoxication. The management of OP exposure is further compounded by the phenomenon of "aging," during which the OP-ChE conjugate is dealkylated, and the enzyme inhibition becomes irreversible. Once aging has occurred, existing medical countermeasures become ineffective. The rate of onset of aging is variable **across** OP agents, but can occur fast enough to severely limit treatment options post-exposure.

The structural basis of the aging process for OP-ChE conjugate has been investigated, and protein structural effects seem to be critical to the irreversibility of aging. As early as 2008, X ray diffraction and other analysis have shown that the aging process involves agent O-dealkylation resulting in a negatively charged oxygen "salt bridge" to a proximal histidine in AChE, as well as conformational changes. These insights have not, however led, to progress against the problem of aging.

Since then, there are multiple lines of evidence that the aging phenomenon both is dependent on particular enzyme structural features in order to occur, and imposes structural effects on the enzyme that prevent reactivation. Specifically, aging can be inhibited by both mutations to the AChE and interactions with other molecules that affect protein structural dynamics. On the other hand it is shown that aged OP-AChE conjugate has increased rigidity compared to the native enzyme, which is thought to prevent countermeasure access to the active site and interaction to restore function.

Separately the science of structural biology has advanced significantly since the time of these findings. There are now theoretical and analytical tools for structural analysis that have not yet been fully investigated for their relevance to this problem. Therefore, the intent of this topic is to support novel approaches using emerging structural tools and insights to defeat the problem of OP-AChE aging and to expand the utility of existing OP countermeasures.

Impact: Successful execution of this effort will establish the foundation for novel therapeutic approaches using the prevention of OP-AChE aging as an adjunct or alternative to the standard of care, reactivation of AChE from OP inhibition. As a result, the utility of existing countermeasures will be expanded to a larger therapeutic window after exposure, and exposures to fast-aging OPs that were previously not treatable in practical terms will become more available to medical intervention.

Objective: This program seeks to leverage the known structural aspects of the OP-AChE aging process and the emerging technologies in the prediction, study, and control of protein structure to establish a basis for preventing OP-AChE aging.

Pre-application white papers and proposals should describe the development and demonstration of novel insight into the role of protein structure changes in the OP-AChE aging process as well as approaches to modulating structural effects to prevent or reverse OP-AChE aging.

- Considerations for a responsive proposal can include the following:
- The investigation of a structural basis for restoring catalytic function to aged OP-AChE conjugate.
- The investigation of a structural basis for preventing formation of aged OP-AChE conjugate.
- The targeting of OPs, e.g. with small molecules, to prevent them from being able to age with AChE.

- Mechanistic understanding of any of the above processes.
- Ensuring the mechanisms developed are broadly applicable to a spectrum of OP agents.
- Use of AI/ML and/or microphysiological systems (MPS) (e.g. organ-on-a-chip or human-on-a-chip) to establish and characterize rational approaches to these solutions.
- Integrating the issue of physiological relevance early in the development of these medical solutions, to support their ability to eventually transition to human use.

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Thrust Area 7, Topic M6: In Vitro Model Development of Alphavirus Infection

Award Amounts for this topic are anticipated to be up to $500,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation. Award amounts for this topic are anticipated to be commensurate with the proposed work involved in developing novel in vitro models. It is anticipated that teams of collaborating organizations with varying expertise maybe required to meet the metrics outlined below.

The preferred award structure for this topic is a base period of three (3) years with up to two (2) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: Neuroinflammation is present in nearly all pathological conditions in the central nervous system (CNS) by either being the primary cause of the condition or as a response to the disruption of homeostasis following disease progression. Microglia and astrocytes become

activated following insult or injury to the CNS. The resulting crosstalk between neurons, astrocytes, and microglia has been shown to play a significant role in the observed neuroinflammatory response including dysregulation of endothelial signaling pathways.

For alphaviruses the pathology surrounding brain inflammation is a significant factor of the disease and is therefore a target for medical countermeasure development. For example, Severe Combined Immunodeficient (SCID) mice survive longer than immune-competent mice suggesting that it is the inflammation that is the major contributor to morbidity, rather than any immune deficiency.

Alphaviruses poorly elicit an innate immune response in cell culture which leads to mischaracterization of in vivo tropisms. In addition, current cell culture models are limited in their ability to observe the effects of membrane-bound or cell proximity-dependent mechanisms and such innate responses. Recent advances in the manipulation of individual cell types and culture systems offers new possibilities to develop cell, tissue, and 3-D models to better represent animal model systems.

Impact: The research explored in this topic seeks to develop new neurotropic cell and tissue based models that better mimic the natural response to infection. Few current neuroinflammatory models are able to capture the important interplay between neurons, astrocytes, and microglia. Thus, there is a need for new, multicellular culture systems that are capable of modeling the impact of crosstalk between different cells in the CNS. This could result in an enhanced understanding of the fundamental regulators of the CNS, increase target identification, better prediction and screening for promising medical countermeasures.

Objective: Pre-application white papers and proposals should describe how their proposal would be used in drug discovery.

Research areas may include, but are not limited to:

- Neurovascular tissues and alphaviruses (VEEV/EEEV/WEEV)
- Characterization of molecular pathways and/or mechanisms of action for alphavirus-induced inflammation.
- Identify common molecular pathways and/or mechanisms of action for alphavirus-induced host response that may be suggestive of novel targets for therapeutic intervention.
- Scaffold based techniques such as hydrogel-based support, polymeric hard material-based support, hydrophilic glass fiber, and organoids
- Scaffold free hanging drop microplates, magnetic levitation, and spheroid microplates with ultra-low attachment coating
- The development of CNS organoids, spheroids, 3D printed microfluidics, methods of improving imaging, automation of liquid handling, and other innovative technologies.

It is anticipated that this topic will require teams of researchers with different expertise in modeling, synthesis and characterization of these materials. A justification of the budget will

need to be provided to supplement the costs proposed for this effort.

Pre-application white papers and proposals may focus on incremental and high risk attempts to develop realistic in vitro models.

References:

1. Agata Matejuk, A., Ransohoff R. (2020) Crosstalk between Astrocytes and Microglia: An Overview. doc 10.3389/fimmu.2020.01416
2. Jensen C., Youg T. (2020) Is It Time to Start Transitioning From 2D to 3D Cell Culture? 06 March 2020, doi: 10.3389/fmolb.2020.00033
3. Adriani, G., Ma, D., Pavesi, A., Kamm, R. D., and Goh, E. L. (2017). A 3D neurovascular microfluidic model consisting of neurons, astrocytes and cerebral endothelial cells as a blood–brain barrier. Lab Chip 17, 448–459. doi: 10.1039/c6lc00638h

Thrust Area 7, Topic M7: Correlating Multi-omics Changes with Measurable Physiological Responses

Award amounts for this topic are anticipated to be commensurate with the proposed work involved in elucidating the underlying principles for multi-omics and other biomarkers in response to chemical and biological (CB) agent exposure and the ability to use them to quantify human physiological responses. It is anticipated that a multi-disciplinary team is required to meet the metrics outlined below.

The preferred award structure for this topic is a base period of one (1) year with up to two (2) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of three (3) years will not be considered.

Background: Infectious disease pathogenesis is often first recognized at the onset of symptoms in an infected individual. A cascade of chemical mediators and cellular pathways trigger the inflammatory response to infection and cause measureable physiological symptoms, such as increased temperature and a change in cardiac rhythms, which are felt by the individual as having a fever and feeling generally unwell.[i] Throughout this response to infection, changes in specific biomarkers have been characterized, to include total white blood cell count (WBC), C-reactive protein (CRP), and procalcitonin (PCT).

Beyond vital signs and biomarkers, multi-omics (transcriptomic, proteomic, and metabolomics) have been increasingly studied to advance the molecular understanding of host-pathogen interactions during viral and bacterial infections. Specifically, multi-omic approaches have been

used to characterize virulence, pathogenicity, and metabolic pathway regulation after infection.[ii]

Similarly, exposure to chemical agents has been shown to cause substantial omics changes, notably significant downregulation of multiple proteins. A proteomic-based study examined the impact of the never agent soman on brain tissue in small animals. The soman-induced proteomic changes impacted key pathways for cell inflammation, metabolism, neurodegeneration, and cell death, among others, and demonstrated that persistent proteomic changes in the brain can cause multiple neurological effects.[iii]

While research on both physiological effects and multi-omics data related to CB agent exposure continue to advance alongside one another, there is limited evidence correlating the two research areas. This topic seeks to determine the feasibility of correlating multi-omics from the course of infection or chemical exposure with specific physiological responses such as *changes* in heart rate, temperature, respiratory rate, and blood pressure.

Impact: DTRA continues to invest in leveraging data collected from wearable devices to provide early warning of CB threat agent exposure[iv], as well as in advancing research on understanding multi-omics effects of CB exposure to aid in improved diagnostics, therapeutics, and medical intervention to minimize warfighter casualties. However, physiological data collected from wearable devices is limited to more common infections, such as influenza and COVID, and does not cover the full breadth of CB threats. By answering fundamental questions on the feasibility of correlating multi-omics data with the physiological response to infection/exposure, it may be possible to extend the current state-of-the-art for wearables-based early warning to encompass additional threat agents. Organ-on-a-chip (OOC) technology provides an opportunity to explore multi-omics changes across a wide array of tissue types in response to different CB threats, which may allow for data collected from multi-omics OOC studies to be leveraged to improve predictive wearable based algorithms specific to CB agents.

Objective: Pre-application white papers and proposals should describe an approach for inferring changes in, and quantifying, if possible, specific physiological responses (e.g., vital signs) as they relate to biomarkers and multi-omics data collected during biological pathogenesis or chemical agent exposure. Applicants should propose access to datasets that include **both** physiological and multi-omics data for the same patient during course of infection or chemical exposure, and may consider leveraging additional Sponsor-provided datasets.

Applicants should demonstrate expertise in the areas below and propose methodology to assess the following:

- Commercial-off-the-shelf (COTS) wearable-based data analysis
    - Understand changes in the physiological response after exposure to CB agents.
    - Propose methodology to compare COTS wearable data with varying temporal resolutions, varying devices, and varying features.
    - Hypothesize key feature importance and probability for correlation to host response to infection.
- Multi-omics data analysis
    - Demonstrate experience with analyzing multi-omics data (e.g., proteomics,

metabalomics, transcriptomics, epigenomics) from blood, serum, interstitial fluid, saliva, or breath for biomarkers in response to disease/infection or exposure to chemical agents
    - Propose methodology for identifying key markers and/or specific cellular pathways following infection or chemical exposures and the intended approach for analyzing the impact of multi-omics changes on physiological response.
- Machine Learning (ML) approaches for analyzing disparate datasets
    - Propose intended ML approaches and demonstrate experience in selecting ideal approaches depending on dataset parameters and intended algorithm development.
- Hypothesis on correlating multi-omics and other biomarkers to vital sign data
    - Propose methodology and anticipated capability to leverage multi-omics data to qualitatively correlate it with changes in physiological responses (e.g., temperature, heart rate).
    - If feasible, expand the qualitative correlation between multi-omics data and the physiological responses to explore if changes in vital signs can be quantified (e.g., actual temperature or heart rate) based on multi-omics data alone.
    - Hypothesize the granularity of any correlation and methodology to assess this.

Final output of this topic should be a report on the feasibility of correlating multi-omics data with the physiological response (e.g., changes in vital signs) to a CB threat exposure. If a qualitative or quantitative correlation can be demonstrated, the methodology and underlying data analysis to support the correlation is an expected deliverable.

Data collections are not a part of this topic, and data proposed must already exist. Responses should discuss availability of or access to in-vivo datasets of omics and vital sign data, and/or expect to be able to Sponsor-provided datasets which include in-vivo omics data and vital sign data for all individuals.

It is anticipated that this topic will require teams of researchers with expertise spanning data analytics, ML and algorithm development, physiology, CB threat agents, and immunology / molecular cell biology. A justification of the budget will need to be provided to supplement the costs proposed for this effort.

1. El-Radhi, A. Sahib (2019). Pathogenesis of Fever. PubMedCentral <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7122269/>
2. Cristea, Ileana M. (2017). Proteomics and integrative omic approaches for understanding host-pathogen interactions and infectious diseases. Molecular Systems Biology. <https://www.embopress.org/doi/full/10.15252/msb.20167062>.
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4. McFarlane, Daniel C. (2022). Real-time infection prediction with wearable physiological monitoring and AI to aid military workforce readiness during COVID-19. Nature Scientific. <https://www.nature.com/articles/s41598-022-07764-6>

Thrust Area 7, Topic M8: Project MAGNETO, lowering SWaP for Microsensors

Award Amounts for this topic are anticipated to be up to $500,000 per year (total dollar value = direct and indirect costs). In all cases, the proposed award value should be clearly substantiated by the scope of the effort. Further guidance on scope and cost may be provided in each full proposal invitation. Award amounts for this topic are anticipated to be commensurate with the proposed work involved in elucidating the underlying principles for multiferroic materials, evaluating materials, integrating materials into a sensor prototype, computational models to understand the properties of proposed materials, and other aspects of this program outlined in the metrics below. It is anticipated that teams with varying expertise are required to meet the metrics outlined below.

The preferred award structure for this topic is a base period of three (3) years with up to two (2) additional years as possible options. However, pre-application white papers and proposals that outline scope and effort for only the base period and do not propose options are also acceptable. Pre-application white papers and proposals that outline scope and effort for different base period and option combinations may also be considered; however, note that pre-application white papers and proposals that outline scope and effort that exceed a total of five (5) years will not be considered.

Background: A variety of sensors are being researched and developed to detect chemical and biological (CB) threats in ambient conditions. Recently, there has been a paradigm shift in the field towards developing lower size, weight, and power (SWaP) microsensors to detect CB threats. As sensors are being studied to possibly use for detection applications of CB threats, there is a need to address how to smartly store the data gathered from the sensor. Employing a data storage capability that enables magnetoelectrically-coupled multiferroic materials to write electrically (faster, low power) and read magnetically (nondestructive), this integration into a sensor could provide a means to advance innovation into this area, resulting in a smaller bit size and reducing the applied external fields required within the circuity of the sensor. By having a lower demand on power, this will also help reduce the battery power needed to operate the sensor. Collectively, this could lower SWaP to advance microsensor development, enabling technologies that can operate longer in the battlefield due to lower power consumption and smarter data storage. This would equip the Warfighter with state-of-the-art innovative microsensors to support their mission and increase their situational awareness.

However, the challenge is to find single-phase materials exhibiting multiferroic properties, i.e. more than one type of ferroic property, at room temperature or composites merging ferroelectric and ferromagnetic materials. A major step toward making viable magnetoelectric technology would be the ability to alternate ferroelectric states with relatively minor voltages. The discovery of more magnetoelectrically-coupled materials or composites would help in the potential development of such sensors and modernizing technology.

This topic seeks to develop a fundamental understanding of multiferroic materials or composites (characterizing bulk materials and thin films), computational models to investigate the structural, magnetic, and electrical properties of materials and to compare to experimental studies, and

integration of the magnetoelectrically-coupled materials or composites into a sensor prototype. This topic also seeks to evaluate these materials integrated into a sensor prototype during year 5, and if milestones are achieved, this technology will be transitioned into a 6.2 program, supporting the Unconventional Detection Modalities Thrust Area listed in Division CBA's portfolio. This project also seeks to potentially develop a microsensor prototype to detect CWAs, emerging threats, and/or simulants of CWAs, and utilize data storage capacity to collect measurements. Measurements collected by the sensor can be AC impedance, infrared, Raman, photovoltaics, but not limited to.

While the ultimate intention is to integrate these materials into suit-based technologies that detect CB threats when exposed to low levels of emerging threats, CWAs, and/or simulants, the basic research should be focused solely on the magnetoelectrically-coupled multiferroic materials or composites to use as data storage for potential optimization and development of the next generation of microsensors.

Impact: The research explored in this topic seeks to develop a fundamental understanding of multiferroic materials (characterizing bulk materials and thin films), computational models to investigate the structural, magnetic, and electrical properties of materials and to compare to experimental studies, and integration of the magnetoelectrically-coupled materials or composites into a sensor prototype. This could be integrated into suit technology for microsensors that could be used to detect agents and other toxic emerging threats, mitigating risk for operational deployment and increasing the Warfighter's situational awareness. Thus, this technology can help unburden the Warfighter and promote integrated layer defense to support the mission.

Objective: Pre-application white papers and proposals should describe the research, development, and demonstration of the technology. Applicants should keep in mind the technology must be operationally relevant to support the Warfighter's mission and ability to tolerate varying environmental conditions in the battlefield.

Applicants should propose magnetoelectrically-coupled multiferroic materials or composites merging ferroelectric and ferromagnetic materials that will be able to have adequate activity to meet metrics including:

- Multiferroic properties –
    - More than one ferroic property: ferromagnetism, ferroelectricity, ferroelasticity, ferrotoroidicity
        - Antiferromagnetism and weak ferroelectricity will also be considered
    - Minimally magnetoelectric materials
- Characterization profile of the structural, magnetic, and electrical properties –
    - X-ray diffraction for crystal structure identification, phase verification, and Rietveld structure refinement
    - Neutron diffraction for crystal structure and magnetic structure identification as well as determining the atomic positions and bond lengths in the unit cell
    - Zero-field and field cooled susceptibility curves and hysteresis loops to provide information about the magnetism
    - Electrical polarization hysteresis loop to monitor the current density and

ferroelectricity
    - Dielectric constant as a function of temperature to provide insights toward the ferroelectric behavior and magnetoelectric coupling
- Fabrication of thin films –
    - Characterization profile of the structural, magnetic, and electrical properties
    - Investigation of films for data storage capability
- Computational models to help understand properties of materials
- Integration of materials into a sensor prototype as an optional year –
    - Demonstration that sensor can operate for at least an hour (threshold) and up to 12 hours (objective) after powered on.
        - Maintain ability to detect emerging threats, CWAs, and/or simulants for 1 to 12 hours but not necessarily continuously monitoring of agents or simulants
        - Ability to operate in varying environmental conditions, such as low and high relative humidity conditions, low and room temperature conditions, and salty, sandy, and smoky conditions
        - Demonstration of threshold metrics (above) within base period (3 years from award) or prove significant progress towards those goals
    - Require little or no external resources required to operate
- Live warfare agent testing should be strongly considered but not limited to as part of this effort or proposed work.

Ultimately, these materials should be cost-realistic and deployment in varying environmental conditions should be strongly considered.
Research areas may include, but are not limited to:

- Determination (theoretical and experimental) of magnetoelectrically-coupled materials or composites that work in ambient conditions, using external fields, that are capable of storing the data over time to allow for measurements to continue for 1 to 12 hrs.
    - Experimental synthesis and characterization of the materials (bulk materials and thin films) and measurements at the laboratory scale
    - Applicants are encouraged to submit magnetoelectrically-coupled materials or composites for data storage capability and will function at ambient conditions.
    - Applicants are encouraged to evaluate technologies including the potential for detection capability of emerging threats, CWAs, and/or simulants (as vapors or aerosols).
    - Applicants should propose determination of environmental conditions (e.g., light, temperature, relative humidity, salty, sandy, smoky), preferably ambient conditions, for operational relevance of technology.
    - Applicants should propose evaluation of emerging threats, CWAs, and/or simulants (as vapors or aerosols) with these magnetoelectrically-coupled materials or composites.

It is anticipated that this topic will require teams of researchers with different expertise in modeling, synthesis, and characterization of these materials (bulk materials and thin films). A justification of the budget will need to be provided to supplement the costs proposed for this

effort.

Pre-application white papers and proposals should focus on a fundamental understanding of research, development, integration, and demonstration of magnetoelectrically-coupled multiferroic materials or composites to use as data storage and integrate these materials into a sensor prototype aimed to detect emerging threats, chemical warfare agent, and/or simulants for potential use with Warfighter suit of technologies.

References:

1. Eerenstrein, W.; Mathur, N.D.; Scott, J.F. Nature 2006, 442, 759-765.
2. Zvezdin, A.K.; Logginov, A.S.; Meshkov, G.A.; Pyatakov, A.P. Bulletin of the Russian Academy of Sciences: Physics 2007, 71, 1561-1562.
3. Spaldin, N.A.; Fiebig, M. Science 2005, 309, 391-392.
4. Du, Y.; Cheng, Z.X.; Dou, S.X.; Wang, X.L.; Zhao, H.Y.; Kimura, H. Applied Physics Letters 2010, 97, 122502.
5. Azuma, M.; Kanda, H.; Belik, A.A.; Shimakawa, Y.; Takano, M. Journal of Magnetism and Magnetic Materials 200

Thrust Area 7, Topic M9: "Next" Next-Generation Material(s) for Chemical and Biological (CB) Protection and Decontamination

Award Amounts for this topic are anticipated to be up to $200,000 (total dollar value = direct and indirect costs) for a maximum of one (1) year effort.

The preferred award structure for this topic is a base period of a maximum of one (1) year with no option years. Phase I white papers and proposals that outline scope and effort for periods beyond the base period will not be considered.

Award Amounts for this topic are anticipated to be indicative of the amount of work involved in demonstrating feasibility of highly innovative basic research areas that may serve as the basis for advancement of new concepts for chemical and biological protection and decontamination, and other aspects of this program outlined below.

Background: DTRA/JSTO's recent basic research investments relevant to chemical and biological (CB) protection and decontamination have identified materials that demonstrate adsorption, reactivity, selective permeability or a combination thereof. Examples include metal-organic frameworks, metal oxide nanoparticles, reactive polymers, and biomimetic and bioengineered systems. While studies with these material classes continue to evolve, new foundational materials research and discoveries are essential to shape future technological capabilities for the Warfighter.

In order to realize new materials with extraordinary function, the exploration of novel scientific opportunities is desired. These studies should seek to identify scientific breakthroughs that may begin to address technical or logistical challenges associated with materials utilized in current

protection and hazard mitigation technologies. For example, conventional adsorbent materials function by means of both physical adsorption and chemisorption; however the lack of selectivity and reactivity limits the chemical protection that can be attained. Elastomers provide excellent barrier properties but at the trade-off of reduced tactility and moisture vapor transport. Polyfluoroalkyl (PFAS)-based coatings provide a high level of surface repellency, but due to environmental and health impacts, efforts are shifting towards non-PFAS material alternatives. Decontaminants have proven to reduce hazard levels, but materials that can turnover agent efficiently and are compatible with complex surfaces are needed.

Impact: This topic will foster innovative ideas to advance the scientific state-of-the-art, with potential of identifying creative material solutions to modernize CB filtration, decontamination, and protective suit technologies.

Objective: This topic seeks short-term, fundamental research investigations focused on proof-of-concept studies and collection of preliminary data of highly innovative research initiatives in support of the "*next"* next-generation material(s) and concepts for CB protection and decontamination. Example research areas *include but are not limited to:*

- Catalysis and reaction engineering to generate concepts that demonstrate rapid, efficient turnover
- Polymer and soft materials to generate concepts that demonstrate stretchable, repellent barriers
- Hybrid or multifunctional materials to generate concepts that demonstrate a combination of adsorptive, catalytic, and/or repellent properties
- Interface and colloid science to generate concepts that demonstrate the ability to enhance reaction rates, diffusion, transport, and/or mechanical properties
- Computational approaches that provide a new means of screening novel materials, developing quantitative structure-activity relationships and other models, predicting the properties of materials, and performing other materials-related studies

Additional considerations:

- Research must be completed within 12 months of award of the agreement.
- No capital equipment may be purchased under this award.
- Due to the relatively small dollar amount and short-term nature of this award, applicants are encouraged to maximize the benefit derived from this funding by prioritizing labor and employing other cost-saving measures in support of the effort. In particular, applicants are strongly encouraged to contribute as a cost-share or significantly reduce the indirect costs associated with the proposed effort.
- The Phase II project narrative (technical proposal) should reflect the level of work to be performed within 12 months, and emphasize the key tasks leading to proof of idea. Due to the short-term nature of this award, the Phase II project narrative (technical proposal) should not exceed 10 pages (including references), and does NOT require the following components/attachments: programmatics, performance schedule, and quad chart. For budgeting purposes, there is no travel requirement for an annual technical review.
- Under this reward, reporting requirements are reduced to a final technical report only.

References:

1. Mondloch, J.E.; Katz,M.J.; Isley, W.C.; Ghosh, P.; Liao,P.; Bury,W.; Wagner, G.W.; Hall, M.G.; DeCoste, J.B.; Peterson, G.W.; Snurr, R.Q.; Cramer, C.J.; Hupp, J.T.; Farha, O.K. Destruction of chemical warfare agents using metal–organic frameworks. *Nat. Mater.* **2015,** *14*, 512-516.
2. Lasseuguette, E.; Malpass-Evans, R.; Casalini, S.; McKeown, N.B.; and Ferrari, M. Optimization of the fabrication of amidoxime modified PIM-1 electrospun fibres for use as breathable and reactive materials. *Polymer* **2021** *213*, 123205.
3. Jung, D.; Su, S.; Syed, Z.H.; Atilgan, A.; Wang, X.; Sha, F.; Lei, Y.; Gianneschi, N.C.; Islamoglu, T.; Farha, O.K. A Catalytically Accessible Polyoxometalate in a Porous Fiber for Degradation of a Mustard Gas Simulant. *ACS Appl. Mater. Interfaces* **2022**, *14*, 14 16687-16693.
4. Tu,Y.; Samineni, L.; Ren, T.; Schantz, A.B.; Song, W.; Sharma, S.; Kumar; M. Prospective applications of nanometer-scale pore size biomimetic and bioinspired membranes. *J. Membr. Sci.* **2021**, *620*, 118968.
5. McEntee, M.; Gordon, W.O.; Balboa, A.; Delia, D.J.; Pitman, C.L.; Pennington, A.M.; Rolison, D.R.; Pietron, J.J.; DeSario, P.A. Mesoporous Copper Nanoparticle/TiO2 Aerogels for Room-Temperature Hydrolytic Decomposition of the Chemical Warfare Simulant Dimethyl Methylphosphonate. *ACS Appl. Nano Mater.* 2020 *3* (4), 3503-3512.
6. Li, T.; Tsyshevsky,R.;McEntee, M.; Durke, E.M.; Karwacki, C.; Rodriguez, E.E.; Kuklja, M.M. Titania Nanomaterials for Sarin Decomposition: Understanding Fundamentals. *ACS Appl. Nano Mater.* 2022**,** *5* (5), 6659-6670.
7. S.1605-117th Congress (2021-2022): National Defense Authorization Act for Fiscal Year 2022, Section 347. (2021, December 27). https://www.congress.gov/bill/117th-congress/senate-bill/1605/text

Thrust Area 7, Topic M10: Leveraging Protein Structure Insights and Allosteric Effectors of MCM Targets

Award Amounts for this topic are anticipated to be up to $500,000 per year (total dollar value = direct and indirect costs). Award amounts for this topic are anticipated to be indicative of the amount of work involved to comprehensively address the objectives of this program outlined below. It is anticipated based on this topic that there will be teams with varying expertise that is required.

The preferred award structure for this topic is a base period of three (3) years with up to two (2) additional years as possible options with options being dependent on meeting benchmarks established in the proposal and statement of work. Phase I white papers and proposals that outline scope and effort for only the base period and do not propose options are acceptable, but note if

option(s) are not proposed they will not be granted at a later date. Note that efforts that propose exceeding a total of five (5) years will not be considered.

Background: Development of safe and effective pre-exposure prophylactic or post-exposure therapeutic medical countermeasures (MCMs) to counter the adverse effects of CWAs (including blister agents, nerve agents, pharmaceutical based agents and other emerging chemical threats) is necessary to protect the warfighter and maintain Force Lethality in CWA-contested environments.

The full complement of protein receptors, metabolic pathways, and tissue distribution of receptor targets of chemicals of concern are not well understood. The improved understanding of the identity, abundance, and tissue-specific distribution of receptors in humans and animal models affected by various chemicals of concern will provide a significantly improved profile to facilitate enhanced medical countermeasure development and identify receptors that are impacted by more than one class of existing and emerging chemical threats.

In order to develop more effective and broadly acting medical countermeasures there is a need to identify host-related biomarkers and biochemical pathways that overlap amongst various threat exposures.

There is also a need to implement a state-of-the-art computational approach, including artificial-intelligence (AI) and machine-learning (ML) based predictive methods that will prioritize specific protein targets for further experimental validation. Use of experimental and/or predicted protein structures to discover novel receptors for CWAs (including blister agents, nerve agents, pharmaceutical based agents and other emerging chemical threats) will help to determine the basis for potential new CWA -protein interactions that can include active sites, ligand binding sites and/ or allosteric sites.

Impact: This approach can leverage protein structure prediction capabilities to identify novel protein targets that are most likely to be functionally impacted by CWA interactions. There are breakthroughs happening in the understanding of protein structure, through both analytical methods and AI- and ML-based predictive methods.

An in-depth understanding of the nature of the molecular recognition/interaction is also of great importance in facilitating the discovery, design, and development of new MCMs.

This approach also enables prioritization of the most biologically meaningful proteins for further study and development of MCMs.

Objective: CWAs continue to be a threat to the warfighter, against which there are no broad spectrum medical countermeasures.

Identification of overlapping biomarkers and biochemical pathways that are affected by exposure to various types of chemical agent threats can help with development of broad-spectrum, potentially cross-toxidromic, medical countermeasures.

The proposal should include one or several of the following approaches, as applicable:

- Enable discovery and quantification of both known and novel (e.g., off-target) receptors of CWAs to allow for development of novel MCMs for broad-spectrum prophylactic and/or therapeutic interventions.

- Use of multi-omic approaches and other pathway analysis to identify nodes of convergence for identifying targets for cross-toxidromic MCM development.
- Use of AI/ML and/or microphysiological systems (MPS) (e.g. organ-on-a-chip or human-on-a-chip) to identify MCMs that can be used to treat multiple chemical toxidromes, allowing for broad-spectrum chemical agent MCMs to enable a suite of MCMs that addresses CWAs (including blister agents, nerve agents, pharmaceutical based agents and other emerging chemical threats).
- Leverage novel protein structure prediction capabilities to identify novel protein targets that are likely to be impacted by CWA interactions to help prioritize specific protein targets for further experimental validation.
- Identify common molecular pathways and/or mechanisms of action for adverse effects resulting from exposure to a broad range of chemicals (e.g., blister agents, organophosphorus compounds, opioids, non-opioid sedatives and other emerging chemical threats) in order to identify new areas for broad-spectrum, cross-toxidromic pre-exposure prophylactic and/or post-exposure therapeutic interventions.
- Include mapping of receptors and enzymes across various CWA-relevant laboratory animal models to allow for more relevant animal model selection in medical countermeasure development.

The CWAs (including blister agents, nerve agents, pharmaceutical based agents and other emerging chemical threats) of interest may include, but are not limited to, organophosphorus compounds, opioids and non-opioid sedatives.

References:

1. Convention on the Prohibition of the Development, Production, Stockpiling and use of Chemical Weapons and Destruction, Technical Secretariat of Organization for Prohibition of Chemical Weapons, The Hague, accessible through internet. 1997, https://www.opcw.org/about-us/history.
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3. Prentiss AM. Chemicals in warfare. New York: McGraw-Hill Book Company; 1937. p. 579
4. May LT, Leach K, Sexton PM, Christopoulos A (2007). "Allosteric modulation of G protein-coupled receptors". Annual Review of Pharmacology and Toxicology. 47: 1–51.
5. Jianlin Cheng, Allison N Tegge, Pierre Baldi (2008). “Machine learning methods for protein structure prediction”. IEEE Reviews in Biomedical Engineering (Volume: 1): 41-49
6. Sheela Kolluri, Jianchang Lin, Rachael Liu, Yanwei Zhang, and Wenwen Zhang (2022). “Machine Learning and Artificial Intelligence in Pharmaceutical Research and Development: a Review”. AAPS J. 24(1): 19.
7. Abdel-Magid AF (2015). "Allosteric modulators: an emerging concept in drug discovery". ACS Medicinal Chemistry Letters. 6 (2): 104–7.

ATTACHMENT 2: INTELLECTUAL PROPERTY

(Applies to FAR Contracts & OTAs)

Applicants must describe any limitations on the use of any intellectual property (patents, inventions, trade secrets, copyrights, trademarks, technical data or computer software) that will impact the offeror's performance of the contract or impact the Government's subsequent use of any deliverable under the contract. In particular, the applicant must describe the intellectual property in sufficient detail and describe the limitations on its use (potential patent licenses required by the Government, data assertions of the offeror or any subcontractor, etc.) and describe how the Government can accomplish the stated objectives of this BAA with the limitations described or proposed by the offeror.

Patents. Applicants must list any known patents, patent applications, or inventions which the offeror may be required to license in order to perform the work described in the Applicant's proposal, or which the Government may be required to license to make or use the deliverables of

the contract should the Applicant's proposal be selected for award. For any patent, patent application or invention listed, the Offeror must provide the invention title, a summary of the invention, patent number, patent application publication number, or provisional patent application number, and indicate whether the offeror is the patent or invention owner. If a patent or invention is in-licensed by the offeror, identify the licensor.

If any listed patent, patent application or invention is owned or licensed by the applicant, the applicant must provide a statement, in writing, confirming that it either owns or possesses the appropriate licensing rights to patent, patent application or invention to perform the work described in the proposal and/or to grant the Government a license to make or use the deliverables for this program. If any listed patent, patent application or invention is not owned or licensed by the applicant, then the applicant must explain how it will obtain a license, how the Government may obtain a license and/or whether the offeror plans to obtain these rights on behalf of the Government.

Be advised that no patent, patent application, or invention disclosure will be accepted if identified in the Data Rights Assertion list. **The list of patents, patent applications, and inventions of this section must be a separate list from the Data Rights Assertion list.**

Government rights in any technology that might be invented or reduced to practice under the contract are addressed in the patent rights clause to be included in the contract.

Data Rights. Applications submitted in response to this BAA shall identify, to the extent known at the time an offer is submitted to the Government, the technical , the technical data, or computer software that the Offeror, its subcontractors or suppliers, or potential subcontractors or suppliers assert should be furnished to the Government with restrictions on use, release, or disclosure, in accordance with DFARS 252.227-7017, Identification and Assertion of Use, Release or Disclosure Restrictions, and DFARS 252.227-7028, Technical Data or Computer Software Previously Delivered to the Government. The applicant's assertions, including the assertions of its subcontractors or suppliers, or potential subcontractors or suppliers, shall be submitted in the following format, dated and signed by an official authorized to contractually obligate the applicant. If the applicant will deliver all technical data and computer software to the Government without restrictions, enter "NONE" in this table under the heading "Technical Data or Computer Software to be Furnished with Restrictions."

Identification and Assertion of Restrictions on the Government's Use, Release, or Disclosure of Technical Data or Computer Software.

The applicant asserts for itself, or the persons identified below, that the Government's rights to use, release, or disclose the following technical data or computer software should be restricted:

Technical Data or Computer Software to be Furnished with Restrictions*	Basis for Assertion**	Asserted Rights Category***	Name of Person Asserting Restrictions****
(LIST)*****	(LIST)	(LIST)	(LIST)

*For technical data (other than computer software documentation) pertaining to items,

components, or processes developed at private expense, identify both the deliverable technical data and each such item, component, or process. For computer software or computer software documentation identify the software or documentation.

**Generally, development at private expense, either exclusively or partially, is the only basis for asserting restrictions. For technical data, other than computer software documentation, development refers to development of the item, component, or process to which the data pertain. The Government's rights in computer software documentation generally may not be restricted. For computer software, development refers to the software. Indicate whether development was accomplished exclusively or partially at private expense. If development was not accomplished at private expense, or for computer software documentation, enter the specific basis for asserting restrictions.

***Enter asserted rights category (e.g., government purpose license rights from a prior contract, rights in SBIR data generated under another contract, limited, restricted, or government purpose rights under this or a prior contract, or specially negotiated licenses).

****Corporation, individual, or other person, as appropriate.

*****Enter "none" when all data or software will be submitted without restrictions.

Date	_____
Printed Name	_____
Printed Title	_____
Signature	_____

Applicants responding to this BAA requesting an Other Transaction or Other Transaction for Prototype shall specifically identify any asserted restrictions on the Government's use of intellectual property contemplated under those award instruments. For this purpose, applicants must propose specific Intellectual Property terms and conditions and a data deliverable list. Further, the applicants must explain why an Other Transaction is necessary and, in particular, how the intellectual property terms and conditions proposed will meet the objectives of this BAA.

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[i] El-Radhi, A. Sahib (2019). Pathogenesis of Fever. *PubMedCentral* *<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7122269/>*

[ii] Cristea, Ileana M. (2017). Proteomics and integrative omic approaches for understanding host-pathogen interactions and infectious diseases. *Molecular Systems Biology.* *<https://www.embopress.org/doi/full/10.15252/msb.20167062>.*

[iii] Palit, Meehir (2020). Quantitative proteomic changes after organophosphorus nerve agent exposure in the rat hippocampus. *ACS Publications.* *<https://pubs.acs.org/doi/10.1021/acschemneuro.0c00311>.*

[iv] McFarlane, Daniel C. (2022). Real-time infection prediction with wearable physiological monitoring and AI to aid military workforce readiness during COVID-19. *Nature Scientific.* *<https://www.nature.com/articles/s41598-022-07764-6>*

From:	Luke Hamel on behalf of Luke Hamel <hamel@ecohealthalliance.org>
To:	Boxley, Kimberly
Cc:	Eric Laing; Kevin Olival
Subject:	Re: Internal USU review deadline for WAB-Net 2 proposal?
Date:	Wednesday, February 8, 2023 5:12:05 PM
Attachments:	WABNet2-whitepaper_7Sep2022.pdf FRBAA14-24-Amendment 17.pdf

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Thank you, Kim! We'll let you know if we have any questions.

I've attached our White Paper as well as the grant guidelines (the Broad Agency Announcement). Please let me know if you need any additional information.

Best,

Luke Hamel
Program Coordinator and Research Assistant

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

1.212.380.4476 (direct)
www.ecohealthalliance.org

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

On Wed, Feb 8, 2023 at 3:29 PM Boxley, Kimberly <kimberly.boxley.ctr@usuhs.edu> wrote:

> Hello Luke and Dr Laing,
>
> Attached is the budget. If it is OK - we will proceed with the rest of the documentation.
>
> Can you also send me a copy of the proposal/guidelines? It is needed for the review process.
>
> Thanks much .... Kim
>
> On Tue, Feb 7, 2023 at 7:54 AM Luke Hamel <hamel@ecohealthalliance.org> wrote:
>
> > Thanks for letting me know, Kim!
> >
> > **Luke Hamel**
> > *Program Coordinator and Research Assistant*
> >
> > EcoHealth Alliance
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> > ***EcoHealth Alliance develops science-based solutions to prevent pandemics and promote***

conservation

On Mon, Feb 6, 2023 at 5:01 PM Boxley, Kimberly <kimberly.boxley.ctr@usuhs.edu> wrote:

> Hello Luke,
>
> We do but it does not involve paperwork needed from EcoHealth.
>
> Thanks much .... Kim
>
> On Fri, Feb 3, 2023 at 1:20 PM Luke Hamel <hamel@ecohealthalliance.org> wrote:
>
> > Hi Kimberly,
> >
> > I was just curious if USU has an internal review deadline for our WAB-Net 2 proposal that I should be aware of? Is there a certain date by which you need to submit all of USU's components of the proposal? Please let me know, and happy Friday!
> >
> > Best,
> >
> > **Luke Hamel**
> > *Program Coordinator and Research Assistant*
> >
> > EcoHealth Alliance
> > 520 Eighth Avenue, Ste. 1200
> > New York, NY 10018
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> > 1.212.380.4476 (direct)
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> > ***EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation***
>
> --
> **Kim N. Boxley l Program Manager 3 l Henry M Jackson Foundation**
> Uniformed Services University of the Health Sciences l Microbiology and Immunology
> 4301 Jones Bridge Road, Room B4122 l Bethesda, Maryland 20814
> Phone: **301-295-1941** l Fax: **301-295-1545** l Cell: **301-793-1883**
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>
>

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

Vertebrate Animals (Laing, Uniformed Services University)

1. Description of Procedures

New Zealand whites are used for polyclonal antibody development, aged 4 to 6 months, and housed and supplied by Noble Life Sciences (formerly Spring Valley Laboratories, Inc.). We use only three rabbits per any one immunogen. Rabbits will be immunized with purified recombinant glycoproteins. The adjuvant used will be the Ribi-Adjuvant-System (RAS). Animals will be monitored for an immune response to recombinant protein by an immunoblot procedure. We will provide the necessary protein antigen (400 ug per rabbit) for the immunizations. Whole blood will be collected and sera processed from immunized rabbits at four time points, a pre-bleed, and then after the first, second, and third immunizations spaced 21 days apart. Following the third immunization, a terminal bleed will be performed. We anticipate making protein-specific polyclonal antiserum to 28 glycoproteins. For henipaviruses, the F and RBP glycoproteins will be immunized in combination, thereby limiting the requirement to have two distinct sets of rabbits receiving either F or G proteins, which decreases the number of animals required.

2. Justifications

The goals of this project are to characterize the magnitude and breadth of the polyclonal antibody response to filovirus and henipavirus envelope glycoprotein, in order to establish the expected antigenic distances and space of each virus species, and examine glycoprotein-based serogroups. For this work, we must use an experimental animal model to allow the course of virus protein antigen-specific polyclonal antibodies development to be studied. Rabbits are a standard choice for the production of polyclonal antibodies. We intend on using only three rabbits per any one purified recombinant protein immunogen (e.g., EBOV GP, NiV RBP+F). We aim to examine how multiple immunizations with the same virus antigen shapes the magnitude and breadth of the antibody response to the immunizing antigen and those derived from closely-related viruses. We have chosen to utilize rabbits due to their ease of care, the prior use of rabbits as models for polyclonal antibody studies, and our preliminary data henipavirus RBP immunizations of these rabbit models performed in by my former lab. Rabbits are required because larger volumes of specific antisera are required to evaluate the antigenic relationships detailed in the grant application. The total number of required rabbits is 75, split over years 1 and 2 of the proposal study.

3. Minimization of Pain and Distress

Noble Life Sciences is fully certified and licensed by the US Department of Agriculture as a research facility; accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International); Their Assurance of Compliance with Public Health Service Policy on Human Care and Use of Laboratory Animals is filed with the OPRR, National Institutes of Health, #A3731-01; registered for the veterinary and research use of schedules 2 through 4 controlled substances by the Drug Enforcement Agency; and their IACUC approval #SVL 002 is for antibody production in rabbits. Attending veterinary care will be provided by SVL. Animals are routinely observed at least twice daily and additional observations are made following procedures.

Bleeding will be performed with hypodermic needle (19 ga.) from auricular artery. One or two prebleed blood draws will be performed prior to immunizations. Blood samples will be drawn no more than once every three weeks. The maximum volume of blood obtained will be 30 ml. Rabbits will be placed in restraining cages. However, rabbits are given Ketaset, 10.0mg/lb. body weight and Rompun, 2.0mg/lb body weight (intramuscularly; hind leg/ thigh; with 25 ga. needle) prior to exsanguination by cardiac puncture

4. Method of Euthanasia

Rabbits will be euthanized by intravenous barbiturate overdose, i.e. approx. 40 mg/kg body weight according to label instructions. Both of these methods are consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA).

SPECIFIC AIMS

Serosurveillance provides powerful measures of the infectome of wildlife and humans that can shed light on the disease ecology of zoonotic viruses. Yet **serological data is plagued with ambiguities that limit our ability to use it to identify zoonotic reservoirs or identify populations at risk from exposure to emerging viruses.** For example, the wildlife source of Ebola virus (EBOV), a continued ~~source~~cause of deadly outbreaks, has eluded researchers since the 1970s. Molecular surveillance has yielded extremely few detections, but serology paints a muddied picture as EBOV antisera are extensively cross-reactive with antigens from other filoviruses. Human seroprevalence estimates vary widely and positive EBOV detections in wildlife hosts are not distinguishable signals of other known or uncharacterized related filoviruses. Similarly, Nipah virus (NiV) a zoonotic henipavirus that has caused repeated outbreaks in Malaysia, Bangladesh and India with fatality rates greater than 70%, has a known host in flying foxes. Yet many other reservoir species test positive for NiV ~~pah~~ antibodies without neutralization or molecular confirmation. Further, ~~r~~Recent ~~expansion~~ description of ~~of known~~ henipavirus~~eal diversity~~s, such as Langya virus in acutely ill patients and shrews, suggests ~~these serologic signals are footprints of other,~~ the serologic footprints of potentially zoonotic henipaviruses have gone unnoticed by current serological testing strategies.

Our previous work has developed native-like antigens of filoviruses and henipaviruses for multiplex microsphere-based immunoassays, detected those viruses in humans and wildlife hosts, and used these data to model the circulation of zoonotic disease in wildlife and humans. **We propose to extend our methods into new laboratory and statistical techniques that can definitively identify the species of viruses ~~from~~ through serology,~~antibodies~~ and characterize the properties and epidemiology of novel viruses only detectable~~ed~~ by their antigenic ~~signals – *shadow viruses*~~shadows.**

Our specific aims are to:

1. **Create multiplex serological panels optimized for discovery and characterization of novel viruses.** We will generate serological *discovery panels* of native-like filovirus and henipavirus proteins with mixtures of sensitive and specific antibody binding properties that collectively can distinguish between the signatures of targeted known viruses and unknown~~shadow~~ virus shadows~~es in antisera~~. We will measure their cross-reactive properties via experimental immunizations, including the effects of repeat exposure and multiple-virus interactions. We will test consistency of results in model organisms against reference sera for humans (NiV, EBOV, Marburg virus) and sera collected from bat species with serological profiles consistent with exposure to ~~wild-type samples with known specific serologic exposure to select viruses (NiV,~~ Bundibugyo virus~~)~~, yet the authenticity of this specificity being unclear. ~~and that are reactive despite highly unlikely exposure.~~
2. **Develop novel statistical methods to extract viral signatures from discovery panel data and integrate rich multidimensional serology into epidemiology.** Will extend *spectral mixture* methods from image analysis to develop statistical models that use discovery panel data to disentangle the signals of multiple viruses and extract the antigenic signature of shadow viruses as well as quantify immune response. We will measure the discriminatory power of our models via simulation and validate them via a leave-one-out strategy, testing if we can identify viruses omitted from our panels. We will integrate these methods with cross-scale immuno-epidemiological models, improving the accuracy and precision of both individual- and population-level serology measures.
3. **Apply these methods to characterize novel and emerging viruses and their epidemiology in wildlife and humans.** We have identified wildlife and human populations demonstrating exposure to potentially novel but indeterminate filoviruses and henipaviruses. **This work will leverage investment from active NIH and DTRA-BTRP surveillance projects** in South Africa, Thailand, and Bangladesh by further testing and analyzing samples from these projects under our new protocols to antigenically characterize sera and distinguish known viruses from shadow viruses. We will use our cross-scale epidemiological models to determine the risk factors and spatiotemporal patterns of exposure and host-level immunological response to identify reservoirs and likely exposure interfaces.

This work has broad significance, addressing NIAID's Pandemic Preparedness Plan priorities of immunology and assay development, reagents and resources, and epidemiology and pathogen discovery. **It will produce biological resources, published protocols, and reusable statistical software to enable the rapid dissemination and adoption of these methods.**

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RESEARCH STRATEGY

I. SIGNIFICANCE

Estimates of the cost of global virus discovery range in the billions of dollars [1]. One driving factor is the exceedingly low detection rate of viruses of concern, especially those in the *Ebolavirus, Marburgvirus, and Henipavirus* genera**.** The wildlife hosts of Ebola virus (EBOV), a continued source of deadly outbreaks in West and Centra Africa, have eluded researchers since the 1970s despite considerable effort. In 2005, EBOV RNA was detected in three bat species [2]. Then in 2019 the USAID-funded Ebola Host Project sampled over 45,000 specimens from 10,000 bats discovering the complete genome of one novel ebolavirus (Bombali virus, BOMV), and a one-fifth genome of EBOV [3]. Wildlife reservoirs for related ebolaviruses, Sudan virus (SUDV), Bundibugyo (BDBV) virus, and Tai Forest virus (TAFV), have not been identified. Nipah virus (NiV), ~~a zoonotic henipavirus that has caused repeated outbreaks in Malaysia, Bangladesh and India with fatality rates greater than 70%~~responsible for annual outbreak of fatal encephalitis in Bangladesh and southern India with some outbreak fatalities exceeding 90% [4-7], is known to circulate in flying foxes (*Pteropus* spp.). Even in a flying fox colony with known circulation, estimates of viremia are <2%, and a study of 3,600 bats over five years, including a period where an outbreak occurred, yielded only eight molecular detections [8]. The recent discovery of Langya virus (LayV) in patients with acute febrile illness and shrews in eastern China [9], indicates that unknown and undetected henipaviruses are also a global health concern [10].

In contrast to molecular surveillance, targeted and broad **serology can provide aa more cost-effective approach to detecting emerging viruses in wildlife hosts and humans, and ~~to~~ measuring e exposure rates** to identify at-risk populations [11-13]. Viral shedding occurs over a short window, with most shedding often occurring over a period of only days, while viral antibodies can last the lifetime of an organism. In the study of NiV circulation above, 3020% of individuals had NiV antibodies, and NiV seropositivity ranges from 11-40% across populations and species of flying foxes in South and Southeast Asia [PMID: 33139552, PMID: 31855143, PMID: 34218820ERIC-cite]. Using the adaptive humoral memory to indirectly measure prior infection and **identifying the serologic footprints left behind after virus infections has the potential to vastly improve our understanding of zoonotic virus diversity, distributions, and dynamics.**

Since serology is an indirect measurement of virus infection, wildlife and human zoonotic virus **serosurveillance faces limitations in the interpretation of data**, especially when attempting to identify natural hosts of viruses [14]. Expected and unexpected **antibody cross reactions frequently confound conclusions from single-antigen assays.** EBOV-positive antisera is extensively cross-reactive with protein antigens from heterotypic ebolaviruses [15]. Antisera from survivors infected with EBOV, BDBV, and RESTV is highly cross-reactive with heterotypic virus antigens; furthermore, RESTV antisera preferentially interacted with EBOV antigens [16]. ~~This is likely the reason for many bewildering serologic observations: seroprevalence for EBOV was found in humans without a history of Ebola Virus Disease (EVD)~~ This likely contributes to the challenges of estimating EBOV spillover [PMID: 31194734] and bewildering serologic observations: EBOV seroprevalence in humans without a history of Ebola Virus Disease (EVD) [17]. In non-outbreak settings people who had contact with wildlife were found seropositive or indeterminate against EBOV, SUDV, BDBV [18, 19]. Community health volunteers with indirect contact of EVD patients were also ebolavirus seropositive [20]. Bush meat hunters sampled in Guinea had serologic evidence of infection by ebolaviruses, but seropositivity was detected by measuring immunoreactivity against several antigens and neutralization tests [21]. It is unclear whether assay performance are sensitive or specific enough to draw meaningful conclusions on the role of wildlife contact on human EBOV seropositivity. Antigen-based serosurveillance studies of fruit bats have also yielded ambiguous conclusions about wildlife reservoir statuses [22-24]. With >1200 species of bats and an unknown diversity of uncharacterized filoviruses the challenges of distinguishes EBOV-positive antisera from other ebolaviruses is daunting.

Similar challenges impede serosurveillance of henipaviruses. Since 1999, NiV surveillance has remained a top priority in south and southeast Asia. After the first documented cases of NiV emergence in Peninsular Malaysia~~,~~ and an understanding of the factors that lead to transmission to humans [25, 26], government regulations on agricultural practices have limited further spillovers in Malaysia. The current epicenter of NiV disease is a region in western Bangladesh termed the *Nipah Belt,* where annual outbreaks are linked to the consumption of NiV contaminated unpasteurized date palm sap [27]. Flying foxes (*Pteropus* spp.) are considered the wildlife reservoirs of NiV, and in south and southeast Asia, NiV has been detected and

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isolated from many species of pteropodid bats sampled in India, Bangladesh, Malaysia, Cambodia, and Thailand [28-33]. Despite isolation of NiV from flying foxes, detection of neutralizing NiV neutralizing antibodies, and experimental challenge models of NiV in flying foxes to confirm reservoir status, other bats across Asia test seropositive for NiV and their role in inter-species transmission remains unclear [34-36]. Rousettes and mouse-eared bats collected in China, Vietnam, and Kerala, Inida tested seropositive for NiV via a combination of nucleocapsid protein (NP), envelope receptor-binding glycoprotein (RBP) and cell lysate ELISA, but tested negative via neutralization tests. [37-39]. It is unclear whether seropositivity in these rousette bats is a factor of sensitivity and specificity issues with the immunoassay or the presence of another similar henipavirus. In sub-Saharan Africa, NiV-like neutralizing antibodies were detected in pigs in Ghana and Uganda, [40, 41] and humans sampled in Cameroon [42]. Genomic assemblages of two henipaviruses, Ghana virus and Angavokeely virus, were detected in closely related fruit bat species (genus *Eidolon*) [43, 44]. Recently ~~found we~~found we genetic evidence of novel henipaviruses detected in Egyptian rousette bats [45],~~.~~ which adds to conclusions that rousette bats may be in fact harboring henipaviruses that cross-react with single antigen NiV ELISA.

Serology is also dependent on the quality and qualification of protein antigen. The EBOV envelope attachment glycoprotein (GP), ~~nucleocapsid protein (~~NP~~)~~, and matrix protein (VP40) have been utilized by several groups in antigen-based serology PMID: 2948188, PMID: 34748613, PMID: 33827978]~~[ERIC cite]~~. In one longitudinal study of Ebola virus disease survivors, ~~seroconversion against~~anti-~~-~~GP and VP40 IgG ~~occurred at XX/XX%, respectively~~,increased with days post-infection along with affinity maturation of GP-positive B cells ~~[~~PMID: 32053790~~ERIC-cite~~]. However, studies that detail the frequency of seroconversion against ~~the the nucleocapsid protein (~~NP~~)~~ after EBOV infection, or the frequency of seroconversion against GP, VP40, and NP with non-EBOV ebolaviruses have not been verified.

Most statistical approaches to interpreting serosurveillance data are limited to single antigen analysis and unrealistically simple models of host immunology and epidemiology. The state of the art approach to dealing with serological assay data in wildlife or with viruses without standardized controls has been clustering approaches [46] that assume seropositive and seronegative individuals separate into two distinct groups by a threshold antibody titer. Yet most real data is far less distinct, due a mix of processes including cross reactions, host heterogeneity, and antibody waning and nonlinearities in the physio-chemical processes of antibody assays [47] ~~(Figure HOTEL)~~. More sophisticated multi-antigen, *multiplex*, analyses [48] can account for cross reactions to distinguish amongst known viruses, but **fundamentally current serological laboratory and statistical approaches are not designed to deal with the challenges of detecting and characterizing novel viruses.**

The major goal of this proposal is to dramatically improve the utility of antigen-based serosurveillance for new viral discovery and epidemiology of emerging zoonotic diseases by addressing the challenges of cross-reaction and statistical rigor. We will create *discovery panels* - qualified and optimized panels of multiplexed immunoassays designed to detect and characterize shadow viruses, and distinguish known from novel viruses through mixtures of sensitive and specific binding properties. (Aim 1). We will develop new statistical approaches to extract the signals of novel viruses from those of known viruses or mixtures of multiple previous viral exposures, as well as integrating quantitative measures of immune response into epidemiological models (Aim 2). Finally, we will apply these techniques to identifying and characterizing filoviruses and henipaviruses in bat populations in South Africa and at-risk human populations in Thailand, and capturing immune-epidemiological dynamics of Nipah virus ~~V~~ circulation in Bangladesh (Aim 3).

This work has broad significance, addressing NIAID's Pandemic Preparedness Plan priorities of immunology and assay development, reagents and resources, and epidemiology and pathogen discovery. **It will produce biological resources, published protocols, and reusable statistical software to enable the rapid dissemination and adoption of these methods.**

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II. INNOVATION

We propose a unique and innovative approach to characterizing antibodies induced by prior infections to discover the signals of novel viruses – *shadow viruses* – in wildlife and human hosts through antigen-based multiplex serology. While serology is an increasingly common technique in viral surveillance, multiplex panels have hitherto been composed of several proteins each designed specifically to bind antibodies of individual antigens, optimizing for specificity to individual viruses. We will instead design and test *discovery panels* composed of proteins with a mixture of sensitivities and specificities to a range of viral antigens within a family. This will simultaneously cover *antigenic space*, enabling detection of previously unknown viruses, and enable definitively ruling out or confirming the signal of known pathogens via their unique *antigenic signatures*. We will build these panels on a multiplex microsphere-based immunoassays (MMIA) platform ~~[ERIC-cite]~~. This enables cost-effective testing of dozens of antigens simultaneously and is easily updated with new reagents. We have successfully deployed this assay~~it~~ to~~in~~ laboratories for surveillance projects ~~in low-resource environments~~ [PMID: 32034942, PMID: 31671094, PMID: 29260678]~~[ERIC-cite]~~. Through the multiplex discovery approach, we can achieve accuracy far more readily adoptable than low-throughput panels such as ~~indirect~~ ELISA [48], or neutralization tests requiring containment and virus isolates or verified pseudovirus systems.

Terms

shadow virus: a novel or unidentified virus only detected in a population via serology of infected individuals

discovery panel: a multiplexed immunoassay designed to detect shadow viruses and distinguish them from known viruses

antigenic space: the phenotypic range of antigens of known and unknown viruses in a family

antigenic signature: a combination of responses of an antiserum against a panel of antigens that identifies a virus

reaction/cross reaction matrix: the full combination of response curves of all antisera to all antigens in a multiplexed immunoassay

cross-binding interaction: the change in response of antisera to an antigen in the presence of another antigen due to preferential binding

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We further innovate by adapting statistical methods from image-processing – nonlinear spectral unmixing models, implemented in a Bayesian framework [49-51] **– to analyze multiplex serological data as *joint* rather than individual response signals.** This flexible approach allows us to incorporate nonlinearities in the interaction of viruses and antigens, as well as those introduced in the physiochemical processes of the assay. **It will enable us to disambiguate data that could represent a variety of exposure histories**, including exposure to multiple viruses, known viruses with cross-reactions to multiple antigens, or novel viruses with an intermediate antigenic profile between known viruses, assigning probabilities to each hypothesis **This framework is applicable to serological methods beyond MMIA** such quantitative LFIA and ELISA, as well as large-scale peptide multiplexing via PhIP-seq [52], which currently lacks unifying computational or analytical frameworks [53].

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We will integrate our statistical framework with population-level epidemiological models to capture cross-scale immunological patterns and dynamics. The dominant threshold-based approach to seroepidemiology determines serostatus at the individual level from antibody titer or assay response, then describes population-level patterns as frequencies of seronegative/positive states. This discards the rich quantitative information on antibody levels and the role of processes such as individual immune variation, antibody waning, and inheritance that can be informative of population processes. **By linking the estimation of individual- and population-level quantities, we increase the accuracy and precision of both.** We will develop cross-scale statistical models that model individuals' complex binary and continuous immune state, and jointly model the variation of this complex serostatus across time, space, and groupings (species, populations, and demographic groups).

Finally, this proposal leverages active federal investments in global viral surveillance programs by partnering with them to rapidly validate these techniques. We will partner with an NIH Centers for Research in Emerging Infectious Disease (CREID) study of high wildlife-contact individuals in Thailand, a DTRA Biological Threat Reduction Program survey of bat virus diversity in Southern Africa, and an NIH NIAID research program on Ni~~pah virus~~V circulation and spillover in Bangladesh. In each, serosurveillance has generated preliminary but ambiguous evidence of novel virus circulation. We will test archived samples to corroborate these data and characterize the suspected shadow viruses and their epidemiology.

III. APPROACH

RESEARCH TEAM AND MANAGEMENT

We have assembled a team with a unique combination of laboratory, analytic, and field expertise and a history of successful collaboration in zoonotic disease surveillance. Project PIs Laing and Ross are early-stage investigators with methodological expertise on laboratory and statistical methods for serological analysis, respectively. Other Co-Investigators lead viral discovery surveillance projects across Bangladesh (Epstein), Southern Africa (Markotter, Epstein), and Southeast Asia (Wacharapluesadee). PI Laing and other significant contributor (OSC), Brook, are collaborators on other surveillance projects; and OSC Munster is an established lab chief with a history of collaboration and whose research interests in virus ecology align with project PIs Laing and Ross.

The PIs began working together in 2017 on a project titled, Serological Biosurveillance for Spillover of Henipaviruses and Filoviruses at Agricultural and Hunting Human-Animal Interfaces in Peninsular Malaysia (HDTRA11710037). Both PIs remain respective methodological experts for active surveillance projects, Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa (HDTRA12010025), and CREID EID-SEARCH.

We have designed our program so that laboratory and mathematical methods development are complementary but can proceed independently. Each benefit from data and innovations of the other. Statistical innovations enhance interpretation of discovery panel data and the data in turn can be used to parameterize and increase power of statistical techniques. However, we will be able to make progress on both in parallel, test their success on their own terms and apply outputs to the surveillance studies even if their success and completion time differ.

See the **MULTIPLE PD/PI LEADERSHIP PLAN** for additional team and project management detail.

AIM 1: OPTIMIZATION OF ANTIGEN-BASED MULTIPLEX SEROLOGY FOR DISCOVERY AND DETECTION

We have developed, expressed, and purified ~~envelope attachment glycoprotein (GP) of~~ filoviruses GP and ~~envelope receptor binding glycoprotein (RBP) of~~ henipaviruses RBP antigens in~~expressed in~~ mammalian cell culture systems as native-like ectodomain trimers and tetramers, respectively, retaining conformation-dependent epitopes [54-58]. We have used these antigens to create MMIAs focused on the detection of presently described ebolaviruses, marburgviruses, and henipaviruses. These have been successfully transferred to laboratories globally and used in completed and ongoing biosurveillance projects including DARPA PREEMPT, our CREID center EID-SEARCH (Co-I Wacharapluesadee), our DTRA BTRP-funded project in southern Africa at the University of Pretoria (Co-I Markotter), and for collaborative projects with OSCs Munster and Brook. Through these projects, these panels have been used to identify wildlife hosts of filoviruses and henipaviruses, and detect evidence of prior infection of high-risk human populations [59, 60]. The panels are continually expanding with each novel virus discovery, e.g., ~~Bombali virus~~BOMV and La~~ngya virus~~yV. However, without standardized protein antigens for antigen-based serosurveillance and sera standards for each virus, demonstrating specificity in wildlife reservoirs and human populations without outbreak histories remains a challenge. When screening samples collected from wildlife, it remains unclear if we are capturing antibodies induced by known virus infections or by antigenically related uncharacterized viruses. Here, we propose a strategy to overcome this fundamental limitation.

Designing the discovery panel with a two-protein approach

Preliminary Data: Cross reaction among filoviruses and henipaviruses. Antibodies induced by ebolavirus infections are cross-reactive with GP antigens, likely the ~~resulf~~result of several contributing factors such as amino acid sequence similarity, conserved ~~eptitopes~~epitopes, and ~~glyco~~glycoeptipopesepitopes or glycosylations in the glycan gap and mucin-like domain [61, 62]. Previous groups have attempted to distinguish homotypic and heterotypic known ebolavirus IgG binding to GP antigens by performing multiple ELISAs for each known ebolavirus [48]. This technique conferred specificity but at a great cost to throughput, and has unclear applications for the detection of unknown ebolaviruses.

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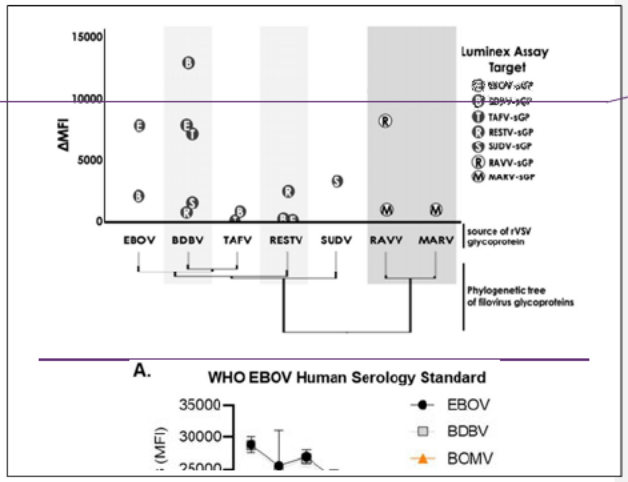
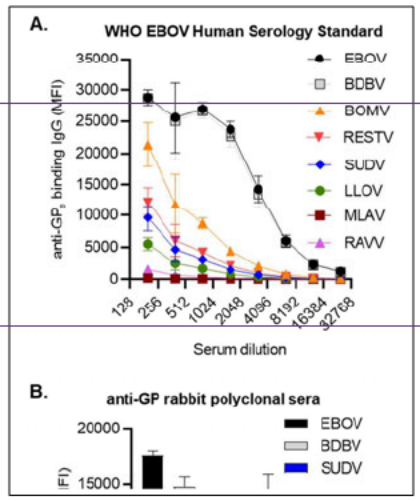
~~In our GPe-based MMIA, the WHO EBOV antibody standard reacted equally against both EBOV and BDBV GPe antigens (Figure FOXTROT A).~~ Although ebolavirus antisera are cross-reactive with heterotypic ebolaviruses, through a GP-based multiplex approach we have been able to specifically identify the homotypic infecting ebolavirus after a single infection by a VSV-filoGP in a bat infection model despite observable cross reactions (~~preliminary data, not shown~~Figure 1). This indicated that pooled polyclonal antisera from human EVD survivors may include more cross-reactive antibodies than those generated after single infections in a putative wildlife host. ~~Futher~~Further, preliminary data from polyclonal antisera from rabbits ~~s hyper~~immunized with EBOV GP corroborated data with the WHO EBOV serology standard that EBOV and BDBV specific antibodies are cross-reactive with GP trimers, though preferential binding to the homotypic EBOV antigen was noted. However, the GP1 subunit protein improved specificity (~~Figure FOXTROT B~~Figure 2B).





Cross-reaction among the other henipaviruses is less described. HeV antisera is known to display a fair degree of cross-reaction with NiV, however the inverse does not hold true ~~...~~[PMID: 18556094, PMID: 22875827 ~~CITE~~]. We have examined henipavirus cross-reactivity with polyclonal antisera from rabbits immunized with recombinant RBP antigens and observed little cross-reactivity outside of HeV-positive antisera with NiV G (~~Figure CHARLIE~~Figure 3).

Approach: Protein development. Here, we propose to design antigen-based *discovery panels* that can distinguish between targeted and cross-reactive signals using a two-protein approach, selecting a highly specific (GP1) and a broadly sensitive (GP trimer) antigen for each filovirus in the panel. This approach draws from our successful approach to large-scale COVID-19 serosurveillance, which used SARS-CoV S glycoprotein expressed as a trimeric prefusion stabilized spike ectodomain and a monomeric receptor-binding domain antigens, providing an optimal balance of assay sensitivity and specificity. We note this indicates that a similar strategy to succeed for surveillance and discovery of novel and zoonotic coronaviruses in the future.

Thus, our two-protein panel approach requires a sensitive protein antigen that could be the cross-reactive target for antibodies induced by shadow henipaviruses. Since the henipavirus RBP provides the *specific* antigen, we plan to develop the soluble native-like trimeric fusion (F) glycoproteins into the *sensitive* antigen. HeV and CedV RBP share ~~23~~30% sequence identity while the F proteins share ~44% amino acid expressed, and purified and has been extensively studied as a target for monoclonal antibodies [57, 63-65]. In its role to mediate virus-cellular fusion, these F proteins share higher sequence identity and similarity across henipaviruses than the RBP sequence identity. The F protein has been similarly

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designed, ~~sequence identity. The F protein has been similarly designed, expressed, and purified and has been extensively studied as a target for monoclonal antibodies [57, 63-65]. In its role to mediate virus-cellular fusion, these proteins share higher sequence identity and similarity across henipaviruses than the RBP.~~ Thus, we plan to explore the ability of the henipavirus F protein as to function as a *sensitive antigen* for antigenic discovery of novel henipaviruses. We will develop GP/GP1 antigen pairs for each of the filoviruses and RBP/F antigens comprising the range of currently known mammalian filoviruses and henipavirus~~henipaviruses (Table ALPHA).~~

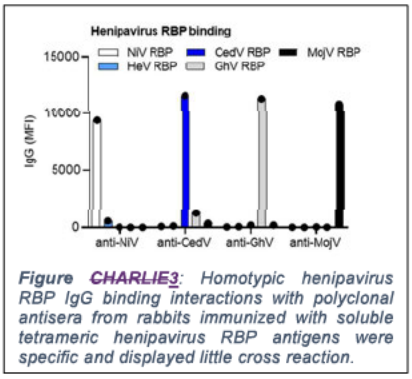

***Figure ~~CHARLIE3~~**: Homotypic henipavirus RBP IgG binding interactions with polyclonal antisera from rabbits immunized with soluble tetrameric henipavirus RBP antigens were specific and displayed little cross reaction.*

~~...Filoviruses~~	~~Sensitive antigen~~	~~Specific antigen~~
~~Ebola virus (EBOV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Bundibugyo virus (BDBV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Taï Forest virus (TAFV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Bombali virus (BOMV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Sudan virus (SUDV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Reston virus (RESTV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Marburg virus (MARV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Ravn virus (RAVV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Lloviu virus (LLOV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Mengla virus (MLAV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Henipaviruses~~		
~~Hendra virus (HeV)~~	~~RBP (tetramer)~~	~~F (trimer)~~
~~Nipah virus (NiV)~~	~~RBP (tetramer)~~	~~F (trimer)~~
~~Cedar virus (CedV)~~	~~RBP (tetramer)~~	~~F (trimer)~~
~~Ghana virus (GhV)~~	~~RBP (tetramer)~~	~~F (trimer)~~
~~Angavokely virus (AngV)~~	~~RBP (tetramer)~~	~~F (trimer)~~
~~Mojiang virus (MojV)~~	~~RBP (tetramer)~~	~~F (trimer)~~
~~Langya virus (LayV)~~	~~RBP (tetramer)~~	~~F (trimer)~~
~~Gamak virus (GamV)~~	~~RBP (tetramer)~~	~~F (trimer)~~
~~Daeryong virus (DarV)~~	~~RBP (tetramer)~~	~~F (trimer)~~

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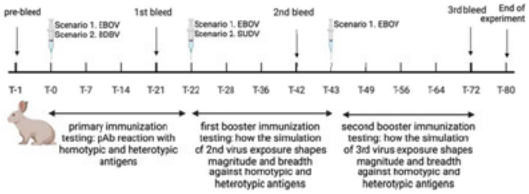
Approach: Quantifying cross-reactions: To optimize a discovery panel for discovery and detection, we first need to fully qualify the pairwise cross-reactions amongst proteins, generating a comprehensive *reaction/cross reaction matrix* via protein immunization experiments in rabbits. This will establish and qualify (a) the homotypic virus reactivity, (b) heterotypic cross-reactivity across other viruses in the family, and (c) how multiple immunogen exposures shape homotypic antigen binding and the cross-reactive breadth against heterotypic viruses. Since wildlife reservoirs are naturally exposed to these viruses throughout their lives, repeated exposures shape the magnitude, durability, and breadth of humoral responses. Data from (c) will support use be used to estimate time-from-infection and antigenic-relatedness. Using our filovirus GP antigens and henipavirus RBP & F antigens, we will sequentially immunize rabbits (n = 3 rabbits per virus antigen) with up to two immunogen boosts (100 µug of protein antigen per injection), and whole blood will be collected three times, 21 days



after each immunization;~~.~~ experimentation will end after the final bleed of day 72 (Figure 4, Scenario 1)~~followed by a terminal bleed after 84 days~~.

Sera will be tested against all antigens within the panel at a range of dilutions to find the optimal dilution factors within the range of the linear slope of the MMIA median fluorescence intensity (MFI) output signal. For the prototypical viruses, EBOV, NiV, and MARV, we will include WHO human serology standards developed by the National Institute for Standards and Control for interpolations of MFI into IgG concentrations. For each pair in the matrix, we will quantify cross-reaction by fitting logistic functions to the reactivity vs (log) dilution and estimating the slope parameter as the measure of reaction. Utilizing MFI values from optimal dilution factors, we will apply antigenic mapping [66], projecting the heterotypic cross-reactivity matrix into *antigenic space* for both filoviruses and henipaviruses, separately for the *specific* (GP1/RBP) and *sensitive* (GP/F) antigen of each virus. We expect the mappings of sensitive antigens, e.g., filo GP and henipa F, to cluster closely together and for some closely related viruses to broadly overlap as serogroups in antigenic space, while specific antigens, e.g., filo GP1 and henipa G will not. Gaps in antigenic space will indicate areas unlikely to be detected by the panel, while overlaps from specific antigens will indicate likely ambiguities. The *antigenic signature* data of viruses will serve as priors for our statistical models (Aim 2).

Determining how virus interactions shape antigenic signatures

The wildlife reservoirs of EBOV, BDBV, TAFV, and SUDV remain unknown. Outbreaks of ~~Ebola virus disease~~EVD caused by EBOV, SUDV, and BDBV have all occurred either in the Democratic Republic of Congo or nearby in Sudan and Uganda. The close spatial relationships of these outbreaks suggest the possibility that these viruses may co-circulate in certain bat species, populations, or multi-host communities. Furthermore, the degree of antigen-specific cross reactions between EBOV, BDBV, SUDV, TAFV may confound current serologic testing studies that rely on a single antigen or only investigate EBOV immunoreactivity. Thus, antibodies induced by BDBV or SUDV infections may seemingly be interpreted as evidence of prior EBOV infections. We aim to determine separate three distinct scenarios that may shape serological profiles, **(a) exposure to one virus and cross-reaction with the second, (b) independent exposures to both viruses, or (c) exposure to a third, uncharacterized virus with cross-reaction to both viruses.**

Approach: Multiple Immunization: As described in Figure 4, w~~W~~e will sequentially immunize ~~a~~ sets of rabbits with a GP ~~against~~ antigens from EBOV, BDBV, and SUDV ~~two viruses from the same family~~ to simulate multiple exposures to different ebolaviruses, determine the additivity of spectral signatures, and test our ability to extract the~~ir~~s signature of each via our statistical models (Aim 2). For example, rabbits will be first immunized with BDBV GP (100 µg) then EBOV GP (100 µg), EBOV then BDBV, or SUDV then BDBV. The immunization pairings simulate hypotheses of common circulation in wildlife such as ambiguous EBOV and BDBV serologies, and overlapping EVD outbreak regions where BDBV and SUDV are known to circulate. ~~fe...~~

We will apply a similar approach to address these three scenarios for henipaviruses, focusing on the more recently described rodent-associated henipaviruses. Mojiang virus (MojV)~~V~~ was detected in the southern province of Yunnan, China, whereas LayV has been detected in eas~~s~~tern Shangdong and Henan provinces. Reports of shrew-associated henipaviruses in South Korea, Gamak virus and Daeryong virus, have contributed to an expansion of rodent-associated henipaviruses discovered since 2020. In Thailand we have detected serologic evidence infection by a MojV-like henipavirus (see Aim 3). As the field does not yet understand the antigenic-relationships among these rodent-associated henipaviruses we cannot confidently interpret serologic evidence of MojV-like in Southeast Asia. Thus, similar to the experiments described for the ebolaviruses, we will sequentially immunize rabbits with multiple Asiatic rodent-henipaviruses to examine how imprinted antibody memory and heterologous boosting shapes reaction and cross reactions to homotypic and heterotypic ebolavirus GP and GP1, and henipavirus RBP and F antigens. The effects of heterologous boosts will be utilized to refine antigenic shape modeling in Aim 2.~~:~~

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~~A~~*pproach: Measuring Cross-Binding Interactions:* Antigen-antibody binding is context-dependent; the degree of binding may vary due to the presence of other antigens to which antibodies exhibit preferential binding.~~-~~ We will measure the extent of this *cross-binding interaction* by conducting *in vitro* "leave-one-out"

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assays, where the virus-specific **antigens matching each antiserum** (both specific and sensitive), are removed from the panel and ~~only~~ forcing only cross-reactions ~~can~~ to occur. This simulates the detection conditions ~~of~~ antibodies ~~to~~ induced previously by uncharacterized viruses – shadow viruses – in the antisera. We will measure the difference in cross-reaction strength in the presence and absence of the ~~with and without the~~ target antigen, and determine the dilution levels that minimize this effect.

Approach: Testing consistency in non-model organisms: To further test the consistency of cross-binding interactions beyond our rabbit animal models ~~organisms~~, we will also conduct leave-one-out experiments ~~within~~ wild-type sera collected from~~in~~ bats and WHO polyclonal reference sera for EBOV, MARV, and NiV. Through a collaboration with Dr. Vincent Munster (~~other significant contributor,~~ OSC; see Letter of Support), we identified BDBV-like ebolavirus immunoreactivity in populations of hammer-headed fruit bats (unpublished data). Hammer-headed fruit bats have been cited as putative EBOV reservoirs [PMID: 17848072, PMID: 31574111~~cite~~], yet in our GP-based MMIA we detected greater immunoreactivity to BDBV. The distribution of these bats in southern Uganda is consistent with BDBV-driven EVD outbreaks. In addition, through ongoing research with Dr. Cara Brook (OSC; see Letter of Support), we identified 16% seroprevalence for a BDBV-like ebolavirus in populations of Madagascan rousette bats (~~Figure 5, ECHO~~). The result was surprising for two reasons, a) rousette bats are a reservoir of marburgviruses, not ebolaviruses [PMID: 19649327, PMID: 33219802~~cite~~] and b) Madagascar is >2500 km from Uganda where all confirmed BDBV outbreaks have been reported [PMID: 34467242, PMID: 21122234, PMID: 25910637~~cite~~]. These two bat species do not share overlapping geographic distribution, so how do we interpret seemingly similar serological profiles suggesting BDBV circulation? We propose to test the hammer-headed fruit bat and Madagascan rousette bat sera in multiplexes that leave-out the BDBV GP antigens, and to examine how the polyclonal antibodies shift binding patterns to other ebolavirus GP antigens. These shifts in serological profiles in the absence of BDBV GP antigens will be compared to the cross-reaction data established by the protein immunized rabbit antisera standards. We expect that the hammer-headed fruit bat sera would increase preferential binding to EBOV GP in the absence of BDBV GP, consistent with phylogenetic data, providing compelling data that these bats have a high probability of hosting BDBV. Whereas we may expect the Madagascan rousette bat sera to bind to ebolavirus GP in serologic pattern that was not predicted in polyclonal rabbit antisera standards. We anticipate that unexpected serological profiles, which break the ~~serolgoicalserological~~ standards, will help us to identify unique clusters of serogroups that would indicate unknown ebolaviruses.

Risks, limitations, and mitigating factors

We have proposed to establish antibody responses and antisera cross reactions via protein immunization and rabbits as our animal model. In the absence of WHO serology standard for all ebola-, marburg-, and henipaviruses, the development of antigen immunized rabbit polyclonal standards is a feasible goal. The uniqueness of the bat immune response among mammals has been an area of research interest for over a decade. The growing body of literature suggests that bats as an Order may have a uniquely adapted immune response including dampened inflammatory responses, and heavily favored innate immunity [67-70]. Thus, there may be distinctions unique to the bat humoral response to viral infections that we will not be reflected by protein immunizations in a rabbit model. However, outside of immunoglobulin ~~(Ig)~~ repertoire sequencing [PMID: 20816694, PMID: 20162414~~eric cite~~], the adaptive response has been less readily investigated, and questions about the functional role of cellular and humoral immunity for viral infection control remains. From field-collected bat sera, we observe robust virus-specific IgG responses, and do anticipate that conservation of humoral responses between rabbits and bats will reflect our ability to accurately determine serogroups through antigenic cartography. Further, we can verify selected rabbit-protein immunization results in a nascent bat infectious disease model being developed by ~~application OSC,~~ Dr. ~~Vincent~~ Munste~~r,r (see Letter of Support),~~W~~e~~ ~~We~~ have previously collaborated with Dr. Munster to investigate whether we can differentiate homotypic and heterotypic ebolavirus antibodies collected from captive Egyptian fruit bats experimentally infected with replication-competent vesicular stomatitis viruses expressing transmembrane bound ebola- and marburgvirus GP (Figure ~~MIKE~~1). Additionally, it is possible that immunological imprints after the first immunization may be recalled following heterologous henipavirus G+F/ebolavirus GP booster immunization, and affect the development of *de novo* antibodies against the booster immunizing antigen. In this case, the order of protein antigens used for multiple exposures is important and we will increase the redesign the sequential strategy of immunizations if additional experiments are necessary.

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AIM 2: NOVEL STATISTICAL MODELS FOR MULTIPLEX SEROLOGICAL ASSAYS

Preliminary data: We and others have used clustering approaches [46] to establish virus-specific seropositivity thresholds relying on our knowledge of confirmed virus-host interactions [8, 60, 71, 72]. However, we find that in many cases the required assumptions of this approach – that individuals separate out into distinct positive and negative groups, and that clusters conform to expected, usually symmetric distributions – break down. Rather, large numbers of antibody responses fall into an indeterminate range without distinct breaks, and nonlinear assay response breaks down expectations at the high and low end of the response range (Fig. Figure 5~~6HOTEL~~). As a result, many results, are classified as indeterminate or assigned a serostatus with high uncertainty. On the other hand, we have extended these cluster methods to multidimensional panel data and found that they are more successful at disentangling groups of sera by their ratio of response to different antigens, indicating the *kind* of antigenic response had distinct differences, while magnitude of response was a more continuous measure (Fig ~~BO~~Bure ~~6~~7). This initial work indicates that clustering can detect distinct signals of samples that react to viral antigens in different ratios, indicating exposure to different viruses in intermediate antigenic space between known filoviruses. However, these multidimensional models conflate two components of these data – distinct reaction ratios as signals of exposure to different viruses, and clustering of the strength of serological response between seropositive and seronegative individuals. We propose a new class of multidimensional models for serology panel data that take a mechanistic approach to representing these two processes, disentangling the different types of clustering and deal with their separate complexities

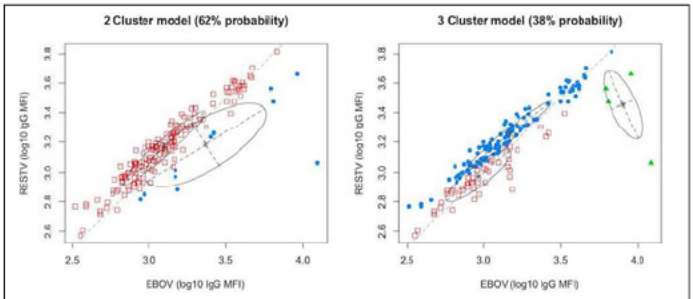

Figure ~~7~~5~~BOB~~: Clustering of serological responses of Liberian Hipposideros spp. Best-fit indicates clusters binding to both RESTV and EBOV glycoproteins in clusters with distinct ratios of response, suggesting the signal of two two viruses, one primarily reacting to EBOV, the other to both RESTV and EBOV similarly, and possibly a set of individuals exposed to both.

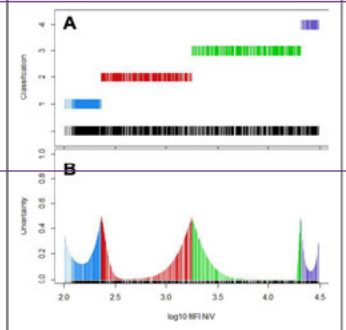

Figure ~~6~~6~~HOTEL~~: Degenerate clustering in real antigen data. (A) Latent cluster models divide data into four rather than two clusters due to lack of distinct breaks, with central clusters interpretation ambiguous. (B) Measure of cluster uncertainty shows that distinction between clusters is weak over a wide range.

Detecting shadow viruses in panel data via antigenic signatures

Model structure: Our minimal model of serological mixing is

$$R_i = \sum_{j=1}^{M} f_{ij}(t_j) + \varepsilon_{ij}$$

Here R_i ,the *response* (measured in as the log_{10} mean fluorescent intensity), is the sum of the responses f_{ij} of assay i against each antibody titer t_j that binds to the antigens presented on that assay, plus measurement error ε_{ij}, for all M antigens present in the sample. If the response curve f_{ij} is linear this model resembles that of Schuh [48], as well as the structure of approaches to signal extraction from remote sensing imagery [49]. but due to saturation of the biochemical or instrument response, it is typically sigmoidal.

Cross-binding interactions in the multiplex assay, where antigens in the same solution attract the same antibodies, modify the additive framework of the spectral mixing model. In the absence of these interactions, we could fully test the ability of the multiplex panel to identify the signal of a novel "shadow virus" *in silico* simply by removing the response data from one target antigen and determininge if it could be identified via cross-reactions of antibodies against closely related viruses. However, with these

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interactions, the response of antigen j against antibody i depends on the presence of the other antigens present. We can decompose this effect in our model into the core ~~titer~~antibody-response function $f_{ij}(t_j)$ and an additional interaction term

$$R_i = \sum_{j=1}^{J} f_{ij}(t_j) + \sum_{j=1}^{J}\sum_{i=1}^{I} g_{ij}(t_i, t_j) + \varepsilon_{ij}$$

Fully estimating $g_{ij}(t_i, t_j)$, especially with nonlinearities in the response, would not be realistic. But nonlinear models from image processing provide reasonable approximations where these interactions take a set of reduced forms [50, 51]. Moreover, we can constrain the model with priors based on our experimental data. Our leave-one out experiments will estimate the size, consistency and linearity of the cross-binding interaction effects in both model and wild organisms and optimize our procedures to minimize it.

~~*Experimental a*~~*Approach:* We will develop a Bayesian representation of this model and conduct simulation-based experiments to determine the conditions under which it can distinguish between different immunological conditions. We will test the ability to distinguish between a target virus and related shadow virus response as a function of antigenic distance, breadth of cross-reaction of both the target and shadow virus, sample and individual level variation, and sample size. We will use data from our challenge experiments to parameterize the models, initially using preliminary data so that we can conduct these aims in parallel. We will use the data from our experimental replicates to simulate sample-level variation and data from previous surveillance work (see Aim 3) to simulate individual variation in response within seropositive and seronegative groups. We will also simulate a range of scenarios for cross-reactive binding interactions, ranging from small, consistent effects to highly variable interactions amongst different viral pairings. We will conduct both *in silico* and *in vitro* validation tests to determine if the method can extract the antigenic signal of a shadow virus. We will create simulated leave-one-out tests by using data from our rabbit immunization experiments, with data from each virus in the panel removed in turn, and determine if the model identifies the signal of a non-target virus from cross-reactions alone. We will repeat this with the data from our *in vitro* leave-one-out experiments.

Cross-scale immuno-epidemiological modeling

Zoonotic serosurveillance, in wildlife and human populations, faces the challenge of a lack of controls. Surveillance requires testing many understudied host species, and by definition no standard controls can be created for novel viruses. Thus, standard cut-offs cannot be pre-determined and inference of individual immune states and population seroprevalence and risk factors is best conducted jointly, modeling individual serostatuses as continuous or probabilistic states informed by population measures.

Model structure: To build this cross-scale framework, we extend our mixing model above to the population scale. Titer t_j becomes t_{jk}, a sample k drawn from a population of individuals x. In this population with a seroprevalence $E(\omega_j)$, the distribution t_{jk} is a mixture of titer values ($F(\mu, \sigma)$) in both seronegative and seropositive individuals.

$$E(t_{jk}) \sim \big(1 - E(\omega_j)\big)F\big(\mu_{j-}, \sigma_{j-}\big) + E(\omega_j)F\big(\mu_{j+}, \sigma_{j+}\big)$$

Both the individual probability of seropositivity ω_{jk} and the distributions $F()$ of seronegative and seropositive distributions are affected by host traits, infectious dosage, and environmental factors. Immunological state has both binary (serostatus) and continuous (antigen-reactive IgG levels, ~~*concentrations*~~~~titer~~) components that are intertwined. In the simple case assuming $F()$ are normal distributions and those traits and risk factors have linear effects, these can be represented as the relationships

$$\rho \sim \text{Probit}\big(\beta_{\rho 0 j} + \beta_{\rho j} x_k\big);\ \ \mu_{j-} \sim \text{Normal}\big(\beta_{\mu 0 j-} + \beta_{\mu j-} x_k\big);\ \mu_{j+} \sim \text{Normal}\big(\beta_{\mu 0 j+} + \beta_{\mu j+} x_k\big);$$

with seroprevalence ρ, seronegative ~~titer~~ concentration μ_{j-}, and seropositive ~~titer~~ concentration μ_{j+} modeled as a joint distribution, and the β values the marginal effect of risk factor on both seropositivity and antibody ~~titer~~concentration.

This allows us to integrate estimation of the factors affecting the probability of individual seropositivity (demographics, geography, host species), along with how those factors modify the ~~titer~~ antibody concentration or response within seropositive or seronegative groups. For instance, if juvenile hosts have stronger responses than adults, but also are less likely to be exposed to the virus of interest, these will

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separate out under this approach. Under a traditional threshold-based approach these effects would mask each other.

One application of this joint modeling of the continuous and binary components is robust cross-species seroprevalence comparisons through combining mixed-effect structures [73] with this model framework. In many serosurveillance studies, large numbers of species are screened for evidence of antigen response to determine likely viral reservoirs, but species sample sizes vary due the difficulty of capture. This poses a challenge in comparing seroprevalence among species. Also, baseline or seropositive response binding levels may vary along with seroprevalence, and for some species sample numbers are not sufficient to establish species-level baselines. The joint approach will enable partial-pooling of species-level effects on both seroprevalence and ~~titer~~antibody concentration, reducing noise from small size groups while capturing important differences.

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~~*Experimental a*~~*Approach:* We will simulate application of this model for two common surveillance inference problems. In each, we will compare this joint estimation approach to two-step threshold-based approaches based on outlier detection or simple clustering.

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First, we simulate determining seroprevalence in a single group (species) of hosts, the probability of seropositivity and the strength of seropositive response are correlated. We will determine how seroprevalence, degree of correlation, sample size affect the accuracy of each method.

Second, we will perform studies of the case of sampling many species with mixed baseline and seropositive response rates to compare seroprevalence rates. Will simulate and fit from a mixed-effect model of the form

$$\begin{bmatrix} \mu_{-} \\ \mu_{+} \\ \text{Probit}(\rho) \end{bmatrix} \sim MVN(\beta_0 + \beta_s \text{species} \ ) + \epsilon; \quad \beta_s \sim \text{MVN}(\mu_s, \sigma_s)$$

where the response distributions and seroprevalence vary as joint random effects by species. We will compare the mixed-model structure to a form where each species is evaluated independently and models where all species are pooled. In both cases we also compare two-step threshold-based approaches to the joint approach. We will use realistic sample and group sizes based on previous multispecies serosurveys [74], varying the size of interspecies differences and the evenness of sampling across groups to determine where the mixed-effect joint approach has the greatest differences from the alternatives.

Statistical approach and implementation:

All our simulation experiments will used a principled Bayesian framework [75] of simulating data from a structured model and testing the ability of the model as well as alternatives to retrieve the original parameters. We will implement models in the Stan probabilistic programming language [76], using Hamiltonian Markov Cain Monte Carlo to estimate model parameters. The R language [77] and will be used for data and model manipulation, workflows, and visualization.

Creating reusable software tools

We will develop an open-source software package for fitting, visualizing, and interpreting these models, written in the R computer language [77]. To maximize usability, we will test reduced-forms of all models, as well as multiple model-fitting algorithms including HMCMC, variational inference or maximum-likelihood optimization. For each we will measure model performance differences and model runtimes on both standard laptop on high-performance environments. The software package will include the ability to select amongst these options and include sensible defaults and guidance on trade-offs so that users can run tests in their compute environment. Reaction-cross reaction curve data and interaction will be packaged together with the software for users to use in their own model calibration.

Software will follow community best practices for software usability, maintenance and algorithmic testing and correctness as developed under the rOpenSci Project [78, 79] . We expect conduct training on software use for staff across all the cooperating surveillance projects as well as teach at least one workshop at an applicable conference in Year 4 or 5 of the projects. See RESOURCE SHARING PLAN and DATA MANAGEMENT AND SHARING PLAN for additional detail on software standards and dissemination.

Risks, limitations, and mitigating factors

Severals factor may limit the scope of applicability of these approaches. First, the high dimensionality and thus parameter size of these models may limit their use to large sample-size studies with moderate to high

seroprevalence levels. Our pilot studies (Aim 3), have sample sizes of hundreds to thousands, with initial estimates of seroprevalence in the range of 15-20%. Second, if cross-binding interactions are strong between antigens in our panels as well as ~~validation~~ verification tests in wild-type bat ser~~a~~um, it will make it difficult to distinguish cross-binding from a shadow virus signal within close antigenic distance. This will effectively increase the minimum antigenic distance needed to separate. However, even if strong, cross-binding is expected to predictably reduce with antigenic distance. That can be exploited to incorporate into explicit structures that predict associated uncertainty in the model structure or in model diagnostics.

AIM 3: APPLYING NOVEL METHODS TO OPEN QUESTIONS IN SPILLOVER IMMUNOLOGY

Our discovery panels and statistical models have direct applications to current open questions in serological surveillance. We will apply Aims 1 and 2 and leverage active serosurveillance projects where the identities of known or shadow viruses in sera from wildlife and humans could not be determined with current multiplex assays and statistical analyses. As testing will be conducted by and with local project teams, these projects will also serve as mechanisms for transfer and training and to refine protocols for publishing.

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Henipaviruses in human community surveillance in Thailand

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Preliminary Data: As part of the NIH CREID EID-SEARCH in Thailand, team members have been conducting clinical and community surveillance of humans in contact with wild bats cave guano mining. We implemented our standard (non-discovery) filovirus and henipavirus glycoprotein antigen-based panels for MMIA testing. During these research activities, we identified that 19% (54/284) of study participants sampled near the Khao Chong Phran Non-hunting Area in Ratchaburi Province, were specifically immunoreactive with the MojV G (Figure 7~~8XXX~~). We also identified one participant with a distinct serological pattern that was had cross reactive and preferential binding to GhV, HeV, and CedV antigens. These data provide preliminary evidence that study participants may have been infected with two serologically distinct henipaviruses, one that is MojV-like and one that is ancestral to HeV/CedV and GhV-like. However, their identities indeterminate, and other hypotheses such as exposure to multiple viruses are plausible.

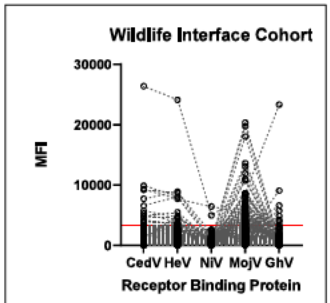

Figure 7~~8~~: Response of sera from Thai guano harvesters to henipavirus ~~G proteins~~RBP antigens, including a group with anti-~~Mòjiāng virus~~MojV IgG ~~response~~ and another with a broad sero-re~~re~~sponse across ~~both~~

Approach: In Year 3 of this project, we will re-test sera from individuals with high wildlife contact currently being collected under the EID-SEARCH project using our full discovery 38plex antigen panels developed in Aim 1. With current archival sera and planned collection in the next two years, we estimate we will test 550 samples. Using the spectral mixture approach developed in Aim 2, we will fit models to determine (a) whether individuals with response to MojV were exposed to MojV or a closely henipavirus, and (b) whether individuals with response to GhV, CedV, and HeV were exposed one of these with cross-reaction to the other, multiple henipavirus with cross reaction to both or a new shadow virus. Using the antigenic mapping approaches, we will determine the relatedness of any shadow viruses to known henipaviruses. We will use our multi-scale models to determine how the presence and strength of response to each of these known or shadow viruses varies with sex, age, and occupational exposures.

~~*Expected results and limitations: ….*~~

Filoviruses in Egyptian fruit bats in South Africa

Preliminary Data: Through ongoing research activities with co-investigator Dr. Wanda Markotter (University of Pretoria), our team has detected seroprevalances of 17.5% (77/440) for an unknown ebolavirus that is most antigenically-like EBOV/BOMV, as well a 4.5% (20/440) for RAVV marburgvirus ~~(Figure KILO A)~~ in *R. aegyptiacus* (Figure 89A-B). ~~This degree of co-positivity is unexpected based on the WHO EBOV antisera, which favored preferential cross-reaction between EBOV and BDBV (Fig igure 2AFOXTRA A).~~ This data is highly suggestive of the existence of a~~n~~ novel intermediate ebolavirus that is antigenically equidistant from both EBOV and BOMV.

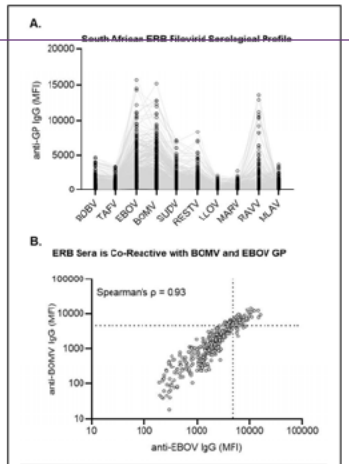

Figure ~~89~~: A) Sera from Egyptian fruit bats (ERB) were screened for filovirus-reactive antibodies. Anti-GPe IgG peaks were observed for EBOV, BOMV, and RAVV. B) BOMV and EBOV IgG correlations were strong, and the same seropositive serum samples were equally reactive with both ebolavirus GP antigens.

Approach: Years 3 and 4 of this project we propose to test *R. aegyptiacus* and other bat sera collected from across s~~S~~outhern Africa using our panel and methods. Under the SABRENET project, over 15,000 samples are expected to be collected via longitudinal and cross-sectional sampling across Southern Africa Based on current seropositive rates and planned sampling, we estimate 3,000 samples will have nonzero signals of a filovirus. Using a case-control approach we will select matching seronegative samples from collections for a total of approximately 6,000 samples. We will adapt our multi-scale models to a spatiotemporal framework to model the dynamics of these shadow viruses across different bat colonies.

Expected results and limitations: We expect that the sera IgG from Egyptian rousette bats reactive with BOMV and EBOV GP would be minimally reactive with the GP1 antigen from both viruses. This expectation would fit our reasoning that GP1 subunit antigen provide specificity to for the homotypic infecting virus and minimize cross reaction potentials. ….

We expect that sera IgG collected from humans who participate in guano mining and have evidence of cross reaction with a MojV-like henipavirus would be reactive with the F protein of MojV as well as the F proteins of LayV, which is closely related to MojV. We expect that this anti-MojV-like virus IgG response will form a unique serogroup based on both RBP and F proteins and would provide strong evidence that a novel henipavirus that is antigenically-distinct from those detected in southern and eastern China, and South Korea is present in Thailand and is able to cross the species barrier.

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Linked serological-epidemiological models: Viral circulation in NiV reservoir* Pteropus *colonies

Preliminary data: In our previous and ongoing work studying NiV in Indian flying fox (*Pteropus medius*) colonies in Bangladesh, we found cyclical but non-seasonal patterns in seropositivity and viral shedding in a colony complex, a study we are now repeating across several colonies to understand geographic variation in seasonal shedding. We also found evidence of annual circulation of other filoviruses a, based on antibodies binding to EBOV and Menangle virus (genus *Rubulavirus*) envelope protein antigens. Data collected in these serological studies was analyzed using a Bayesian cluster modeling to determine a threshold IgG level for serostatus, and threshold-based serostatus used as a binary variable in subsequent epidemiological analyses. However, this approach masks considerable complexity and uncertainty in the relationship between the individual binding levels in actual serostatus. For instance, for filovirus responses, the cluster of "baseline" responses of presumed seronegative bats increases with age, while in pre-weaned bats, an upper cluster of "seropositive" individuals has considerably weaker responses (Figure ~~9~~10, publication *in prep*). Binding response also appears to change both seasonally and interannually.

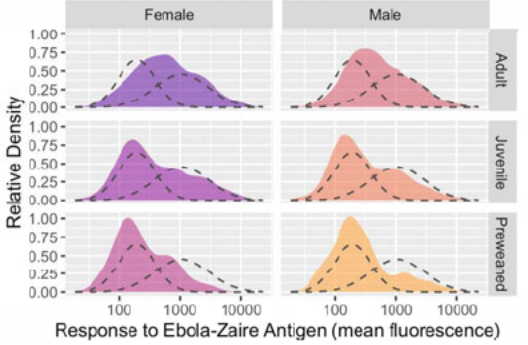

Figure ~~9~~10: Clustering of binding response of Bangladeshi Pteropus *sera to Ebola-Zaire antigen (filled densities), with clusters distributions from a Bayesian mixture model (dotted lines). Model thresholds do not capture within-cluster changes across demographic groups, which confound measures of seroprevalence.*

Approach: We will use the antigenic relationships developed in Aim 1, and the cross-scale modeling approach developed in Aim 2, to re-analyze longitudinal and spatial extensive data on NiV, filo-, and rubulavirus binding response in *Pteropus* colonies. We will model the changes in baseline response binding (μ_-), seropositive response binding (μ_+),, and seropositive binding probability (ρ , i.e. seroprevalence) as joint responses of a spatio-temporal model

$$\begin{bmatrix}\mu_- \\ \mu_+ \\ \rho\end{bmatrix} \sim \text{Multivariate}(f(\beta_0 + \beta_1 \text{sex} + \beta_2 \text{age} + \text{s}(\text{time}[\text{annual}, \text{seasonal}], \text{location}))) + \epsilon$$

Where $\text{Multivariate}(\dots)$ is a joint multivariate normal-probit distribution representing the correlated response of all three outcomes, $f(\dots)$ is the pseutotiter-response relationship, and $s(\dots)$ is a term with a spatio-temporal correlation structure. We will test several joint hypotheses by determining the consistency of posterior model results: Does baseline serological response increase with age, and how much does this vary across viral types? Does average seropositive response (as well as seroprevalence) decline following peaks of seroprevalence, consistent with antibody decline following an outbreak? Do maternal traits determine the strength of vertical transmission of antibody titer?

Expected results and limitations: ....

RESOURCE SHARING PLAN

I. REAGENTS

In-house expressed and purified ...pProtein antigens and commercially sourced polyclonal rabbit antisera will be made available upon reasonable request.

II. PROTOCOLS

Protocols for generating reagents, performing assays, etc....will be published on https://www.protocols.io/

III. SOFTWARE

As part of activities in statistical model development we will create a software package to enable researchers to implement their methods. The program will be implemented in the R language (with Stan and C++ components as needed). It will be written to be compatible with Windows, macOS, and Linux platforms and tested on each. Algorithm implementation, code style, user and documentation will all follow rOpenSci standards for statistical software implementations (https://stats-devguide.ropensci.org/). It will be released under an Open Source Initiative-compatible license.

The software will include both reference documentation and tutorials on implementing the software on example data. We will test both software usability and documentation quality by distributing alpha versions to researcher, students, and postdoctoral scholars at PI and co-I institutions and collecting feedback. Beginning with alpha versions, the software will be publicly released on a platform inviting user reports.

The software will be published on several platforms allowing easy retrieval and installation (CRAN, R-Universe, GitHub, Zenodo). A project website will be created for documentation. We will maintain the software, including bug fixes, and updates to maintain compatibility with changing dependencies and platforms, for 5 years after the end of the funding period. Containerized versions of the software at any major release, packaged together with all dependencies and compute environment, will also be archived so that archival analyses using the software may be run even after the end of maintenance.

See DATA MANAGEMENT AND SHARING PLAN for further details on publication, archiving and standards.

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VERTEBRATE ANIMALS (LAING, USUHS)

1. Description of Procedures

New Zealand whites are used for polyclonal antibody development, aged 4 to 6 months, and housed and supplied by Noble Life Sciences (formerly Spring Valley Laboratories, Inc.). We use only three rabbits per any one immunogen. Rabbits will be immunized with purified recombinant glycoproteins. The adjuvant used will be the Ribi-Adjuvant-System (RAS). Animals will be monitored for an immune response to recombinant protein by an immunoblot procedure. We will provide the necessary protein antigen (400 ug per rabbit) for the immunizations. Whole blood will be collected and sera processed from immunized rabbits at four time points, a pre-bleed, and then after the first, second, and third immunizations spaced 21 days apart. Following the third immunization, a terminal bleed will be performed. We anticipate making protein-specific polyclonal antiserum to 28 glycoproteins. For henipaviruses, the F and RBP glycoproteins will be immunized in combination, thereby limiting the requirement to have two distinct sets of rabbits receiving either F or G proteins, which decreases the number of animals required.

2. Justifications

The goals of this project are to characterize the magnitude and breadth of the polyclonal antibody response to filovirus and henipavirus envelope glycoprotein, in order to establish the expected antigenic distances and space of each virus species, and examine glycoprotein-based serogroups. For this work, we must use an experimental animal model to allow the course of virus protein antigen-specific polyclonal antibodies development to be studied. Rabbits are a standard choice for the production of polyclonal antibodies. We intend on using only three rabbits per any one purified recombinant protein immunogen (e.g., EBOV GP, NiV RBP+F). We aim to examine how multiple immunizations with the same virus antigen shapes the magnitude and breadth of the antibody response to the immunizing antigen and those derived from closely-related viruses. We have chosen to utilize rabbits due to their ease of care, the prior use of rabbits as models for polyclonal antibody studies, and our preliminary data henipavirus RBP immunizations of these rabbit models performed in by my former lab. Rabbits are required because larger volumes of specific antisera are required to evaluate the antigenic relationships detailed in the grant application. The total number of required rabbits is 75, split over years 1 and 2 of the proposal study.

3. Minimization of Pain and Distress

Noble Life Sciences is fully certified and licensed by the US Department of Agriculture as a research facility; accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International); Their Assurance of Compliance with Public Health Service Policy on Human Care and Use of Laboratory Animals is filed with the OPRR, National Institutes of Health, #A3731-01; registered for the veterinary and research use of schedules 2 through 4 controlled substances by the Drug Enforcement Agency; and their IACUC approval #SVL 002 is for antibody production in rabbits. Attending veterinary care will be provided by SVL. Animals are routinely observed at least twice daily and additional observations are made following procedures.

Bleeding will be performed with hypodermic needle (19 ga.) from auricular artery. One or two prebleed blood draws will be performed prior to immunizations. Blood samples will be drawn no more than once every three weeks. The maximum volume of blood obtained will be 30 ml. Rabbits will be placed in restraining cages. However, rabbits are given Ketaset, 10.0mg/lb. body weight and Rompun, 2.0mg/lb body weight (intramuscularly; hind leg/ thigh; with 25 ga. needle) prior to exsanguination by cardiac puncture.

4. Method of Euthanasia

Rabbits will be euthanized by intravenous barbiturate overdose, i.e. approx. 40 mg/kg body weight according to label instructions. Both of these methods are consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (AVMA).

DATA MANAGEMENT AND SHARING PLAN

I. DATA TYPES

Types and amount of scientific data expected to be generated in the project

This project will produce data on antigen binding responses via multiplex bead array assays, as generated from Bio-Rad Bio-Pplex and Luminex MAGPIX multiplexing Sysstems. These assays will be performed on sera from hyperimmunization challenge experiments in rabbits, human serology a standards for select viruses, and archival sera samples from wildlife surveillance studies in South Africa, Republic of Congo, Madagascar, and Thailand as well as human surveillance studies in Thailand. Calibration data, including measures from blank and mock samples, will also be generated.

The project will produce sample-level metadata for challenge experiments and standard dilutions. It will aggregate copies of metadata associated with archival samples from other research projects projects. The latter will include host species, traits, time and location of collection of animals sampled, and demographic information of humans sampled.

The Bio-Pplex and MAGPIX systems will generate raw data in XML and XLSX files, with XML files being comprehensive logs of run metadata, machine calibration, and all measurements per sample, and XLSX being sample-level total measurements. Sample-level metadata will be collected in CSV and XSLX form.

Across experimental and archival samples, we expect to generate approximately 7,000 tests on 90 plates, totaling approximately 3.5MB of processed XLSX files and 440MB of raw XML files.

The project will also produce synthetic data from simulations and performance data from experiments. These will be generated in RDS (R Object files), and parts computationally intensive or difficult to reproduce will also be exported as CSV files. The project will produce computer code for both analyses and as a primary product in the form of reusable software packages. These will be in standard UTF-8 text of computer languages including, R, Stan, and C++, as well as compiled executable binary files.

Scientific data that will be preserved and shared

Excepting identifiable data associated with human samples, all data produced in the course of this project will be publicly shared. Final sample-level cleaned and joined data and metadata will be shared as CSVs, as well as raw Bio-Pplex or MAGPIX software XML data. All computer code created will be shared. Synthetic data and summaries of simulation experiments will be shared in summarized form, with raw data shared as reproducible code that can regenerate the exact results, excepting data that is computationally-intensive and therefore more efficient to share in raw form.

For data associated with human samples personally identifying information will be not be shared. Personal individual-level data will be removed, demographic and location data will be binned to prevent re-identification, with location information binned to the province (ADM-2) level and age aggregated to bins of 10 years or more. Other fields (e.g. occupation) will be analyzed to determine potential for re-identification and removed or binned as necessary.

Metadata, other relevant data, and associated documentation

This project will also produce as primary outputs standard operating procedures and instructions for performing the assays under development, documentation to reproduce simulations and statistical analyses from provided code, as well as user documentation and tutorials for the statistical software that will be created. Descriptive and structural metadata will be included in deposits for each data set.

II. RELATED TOOLS, SOFTWARE AND/OR CODE

All produced data formats (CSV, JSON, XML, XLSX) can be read by multiple, widely available free and open-source tools, with only RDS files specifically requiring the free and open-source R language.

III. STANDARDS

In general, all data will be reported using the Frictionless data standard, as both an "umbrella" framework and a general approach to data sets without domain-specific standards. The Frictionless data standard provides a framework for consistently packaging data stored in most inoperable file formats. We will use accompanying Frictionless JSON files to describe CSV metadata, with additional standardized fields specific to the data repository used (e.g., Zenodo). Where applicable, we will use the NIH Common Data Elements (CDE, https://cde.nlm.nih.gov) for relevant fields.

Formal standards for non-target and quantitative serological data have not yet been widely adopted, and the development of essential reporting quantities is an anticipated output of this project. However, we will aim to ensure the data reported is, where applicable, compatible with standards for serological studies, notably the NIH ImmPort data model and its recommended ontologies for proteins and diseases.

Spatiotemporal and taxonomic metadata on wildlife samples will follow the Darwin Core Standard.

Computer code will be released using two standards. Software packages for reuse will be in the format of an R packages following the rOpenSci standards for statistical software implementations (https://stats-devguide.ropensci.org/) and CodeMeta standards for software descriptions (https://codemeta.github.io/), under an Open Source Initiative-compatible license. For computer code to reproduce simulations and statistical analyses, we will use git version controlled code repositories, the "Make" and "targets" frameworks to define dependencies between steps of analysis, the "renv" and "venv" frameworks for recording software dependencies, and Docker containers to capture the machine computing environments. Binary machine images of the containers will be stored alongside the source code.

IV. DATA PRESERVATION, ACCESS, AND ASSOCIATED TIMELINES

Repository where scientific data and metadata will be archived

We will archive all data and metadata generated, as well as all computer code, on the Zenodo archive. A subset of data generated is appropriate for NIH's ImmPort repository and will be cross-deposited there with appropriate semantic references to describe the relationships between data. Protocols will also be deposited on protocols.io.

How scientific data will be findable and identifiable

All data on Zenodo, NIH ImmPort, and protocols.io will be identifiable DOIs and have searchable metadata deposited with DataCite and indexed by search engines. In addition, we will mirror data on non-archival sites that are popular and accessible for their features and search optimization: DoltHub for data, GitHub for source code, and R-Universe for installable R software binaries.

When and how long the scientific data will be made available

Data associated with publications will be shared in its processed and cataloged form upon release of associated products (preprints, software, or publications upon acceptance), or the end of the period of performance (inclusive of any approved extensions), whichever comes first. The duration of preservation and sharing of the data will be a minimum of 10 years after the funding period.

V. ACCESS, DISTRIBUTION, OR REUSE CONSIDERATIONS

Factors affecting subsequent access, distribution, or reuse of scientific data

There are no anticipated factors or limitations that will affect the access, distribution or reuse of the scientific data generated by the proposal.

Whether access to scientific data will be controlled

All data generated by this project will be released in full excepting data aggregated from other projects with personally identifiable data.

Protections for privacy, rights, and confidentiality of human research participants

Where data used by this project from other projects is linked with personally identifiable information, only de-identified information will be released. Identifiable data will not be retained. Requests for the identifiable data will be referred to the original projects and institutions.

VI. OVERSIGHT OF DATA MANAGEMENT AND SHARING

PI Ross (ORCiD 0000-0002-2136-0000) will be responsible for oversight of project data management and release of data. The project research assistant at EcoHealth Alliance will be responsible for management of the project database, maintenance of project metadata, aggregation of data from project partners, and releasing data to appropriate repositories under appropriate standards. A data librarian at EcoHealth Alliance will support this role and act as an organizational fallback for data maintenance. Data release will take place after agreement amongst PIs from all contributing institutions. State of data management and release will be a standing topic at project quarterly and annual meetings.

PROJECT NARRATIVE

This project develops new methods to identify emerging and zoonotic viruses by analyzing the virus-induced antibodies present in wildlife and human populations in contact with wildlife. It will create both new laboratory techniques to test for those antibodies as markers of infection and new mathematical models to analyze data to identify the trace of those viruses. It will test these methods in partnership with international virus surveillance projects in Bangladesh, Thailand, and South Africa.

PROJECT SUMMARY/ABSTRACT

Serology remains under-utilized as a reliable surveillance tool for the detection of emerging zoonotic viruses. Detection and estimation of Ebola virus spillovers is dependent on outbreak scale and thus may not be caught through early warning systems. The geographies at-risk for Nipah virus, and bat-borne and rodent-borne henipaviruses spill over across sub-Saharan Africa, Asia and Oceania remain under-surveyed. Limitations of serosurveillance in addressing virus emergence cacan be found at both the front-end in serological assay design and development, and back-end in the statistical frameworks used to analyze data. Both of these need to be adapted to the unique challenges associated with known emergent viruses and novel unknown viruses not yet g~~, as opposed to known pathogenic viruses already g~~enetically characterized. These challenges include cross-reactivity of antigens and antibodies and complex, nonbinary immune responses. The major goal of this proposal is to dramatically improve the utility of antigen-based serosurveillance for new viral discovery and epidemiology of emerging zoonotic ~~diseases~~ viruses by addressing the challenges of cross-reaction and statistical rigor. We will create discovery panels - qualified and optimized panels of multiplexed immunoassays designed to identify a characterize previously unknown viruses only detected through the ~~antibody response of~~serologically profiling wildlife and human hosts. We will develop new statistical approaches to extract the signals of novel viruses from those of known viruses or mixtures of virus-specific antibodies from complex histories multiple previous viral exposures, as well as integrate these into models into population-scale epidemiological models. Finally, we will partner with international viral surveillance projects to apply these techniques to identifying and characterizing filoviruses and henipaviruses in bat populations in South Africa and ~~at-risk wildlife~~ workers at-risk for exposure to wildlife viruses in Thailand, and to modeling immune-epidemiological dynamics of Nipah virus circulation in bats and people in Bangladesh.

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FOREIGN JUSTIFICATION

This project concerns zoonotic infectious diseases found in wildlife and human populations globally and with the potential to spread and cause outbreaks in the United States. Study populations of interest – high animal-contact workers in Thailand, and known filovirus host bat species in South Africa – were selected because prior results indicated the presence of antibodies to novel viruses that could be better characterized using the methods developed in this proposal. In both cases, the systems are already being studied under U.S. federally-funded disease surveillance projects: The NIH CREID Emerging Infectious Diseases - South East Asia Research Collaboration Hub (U01 AI151797), the DTRA BTRP South Africa Bat Research Network (HDTRA12010025).

In the case of surveillance in Thailand, the lead expert in the disease ecology and epidemiology of the system of interest is Co-I Wacharapluesadee. Archival sera from study of this population are stored at her institution, King Chulalongkorn Memorial Hospital, and she and her team are best equipped to analyze these specimens and interpret the results. Dr. Wacharapluesadee and King Chulalongkorn Memorial Hospital have a history of collaboration with U.S. institutions under USAID and NIH programs.

In the case of surveillance in South Africa, the lead expert in the disease ecology and epidemiology of the system of interest is Co-I Markotter. Archival sera from study of this population are stored at her institution, University of Pretoria, and she and her team are best equipped to analyze these specimens and interpret the results. Dr. Markotter and University of Pretoria under DTRA and NIH programs.

CONSORTIUM/CONTRACTUAL ARRANGEMENTS

This project is a multi-institutional collaboration led by EcoHealth Alliance, New York (PI Ross, Co-I Epstein) that will subcontract funds in accordance to NIH Institutional guidelines to three other institution:

1. **Uniformed Services University of the Health Sciences, Bethesda, MD** (PI Laing)
2. **University of Pretoria** (Co-I Markotter)
3. **King Chulalongkorn Memorial Hospital, Thailand** (Co-I Wacharapluesadee)

The applicant organization (EcoHealth Alliance) is justified in taking the lead on this project because it specializes in understanding the ecological and virological processes underlying zoonotic disease emergence, and has conducted international, multi-disciplinary and multi-partner research around the world for more than 30 years, including joint projects with the listed institutions EHA administers technological and data-management platforms used by partners as part of these project.

PI Ross oversees modeling, statistical design, and data systems fat EcoHealth Alliance. Dr. Ross, a mathematical disease ecologist, is an expert in complex semiparametric modeling approaches and the role of host and pathogen heterogeneity in disease dynamics. He leads multiple multi-institution projects in scientific and statistical software development [80, 81], disease forecasting (Wellcome 226061/Z/22/Z). He has had core roles in quantitative methods for multi-institution projects under USAID, DTRA, and NIAID, including collaborations with all partner institutions on this project.

EcoHealth Alliance will be the lead for administrative components and oversight of this project, and also be the center of development and testing of quantitative methods, software development, data integration, and overall synthesis. Co-I Epstein at EcoHealth Alliance will provide expertise in the virology, disease ecology, and epidemiology of the populations of interest in Bangladesh.

The subcontract institutions will work on specific issues and areas in which they have proven expertise. These areas are:

- Wildlife virology and immunology, Novel serological and assay development; generation of reagents for novel assays; training and technology for serological and molecular protocol development, of Thailand and South Africa laboratory staff for technology transfer for serological and molecular protocol development (**Uniformed Services University of the Health Sciences,** PI Laing).
- Wildlife and human community surveillance and specimen collection, human clinical or hospital syndromic surveillance, screening of serum specimens using serological assays, disease ecology and epidemiology of zoonotic diseases specific to populations in Southern Africa (**University of Pretoria,** Markotter), and Thailand (**King Chulalongkorn Memorial Hospital,** Wacharapluesadee)

PROJECT LEADERSHIP PLAN

I. BACKGROUND AND MULTI-PI RATIONALE

This research program, *Characterizing unknown emerging viruses through targeted serological profiling*, is inherently interdisciplinary, jointly developing *in vitro* and *in silico* methods for serological viral discovery, drawing from the expertise, teams, networks, and resources of both PIs. This project will be co-led by PIs Dr. Noam Ross (EcoHealth Alliance, New York, NY), and Dr. Eric Laing (Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences (USU), Bethesda, MD). The laboratory and computational components, as represented in Aim 1 and Aim 2, are complementary and co-equal. Each PI brings methodological expertise and experience to their component. Dr. Ross, a mathematical disease ecologist, is an expert in complex semiparametric modeling approaches and the role of host and pathogen heterogeneity in disease dynamics. He leads multiple projects in scientific and statistical software development [80, 81]. Dr. Laing, a virologist, is an experienced researcher in the field of infectious diseases, serological application and profiling, and cellular immunology. He is a co-investigator and associate investigator on multiple virus surveillance projects including Epidemiology, Immunology, and Clinical Characteristics of Emerging Infectious Diseases with Pandemic Potential (EpICC-EID), Prospective Assessment of SARS-CoV-Seroconversion, EID-SEARCH, SABRENET, and along with OSC Munster is a co-investigator on a pending project, Establishment of a Bat Resource for Infectious Disease Research. The PIs have a history of collaboration as part of multiple projects in disease surveillance, including serological surveillance of wildlife under DTRA-funded surveillance projects in Malaysia (HDTRA11710037) and South Africa (SABRENET, HDTRA12010025), and NIH-funded research on Nipah virus circulation in Bangladesh (U01 AI153420-01). In each they work(ed) jointly on interpretation of serological data and epidemiological models, serving as the methodological experts within those teams.

Co-Investigators Epstein, Markotter, and Wacharapluesadee all lead active federally funded projects in zoonotic viral surveillance and discovery that make use of serological methods developed by PI Laing. Each provide system-specific expertise in the ecology, epidemiology, and virology of the regions and species in those projects, which are the source of samples and data for our applications for Aim 3.

II. ROLES AND RESPONSIBILITIES

PI Ross will be the contact PI of the project, responsible for funder communication, budgeting, and reporting, and oversight of sub-awardees. He will be responsible for project data management, statistical methods and development under Aim 2, and analyses under Aim 3. Dr. Ross will be supported by EHA's administrative staff, including organizational-level finance and human resources directors, a federal grants administrator, a data librarian and computing cluster systems administrator. PI Laing will be the lead laboratory processes and development and site lead for USU. He will be responsible for protein and panel design, challenge experiments, and other laboratory experiments under Aim 1. Both PIs will participate equally in interpretation of analyses and manuscript writing.

Co-Is Markotter and Wacharapluesadee will be oversee the testing of samples from South Africa and Thailand at their institutions, respectively, and act as consortium leads for University of Pretoria and will liaise with PI Laing for logistics and implementation of protocols. SPs Epstein, Markotter, and Wacharapluesadee will all participate in the (re-)analyses and interpretation of results in Aim 3.

III. PROGRAM MANAGEMENT

COMMUNICATION

PIs will coordinate via monthly video calls. These calls will include other Co-Is quarterly, and more frequently as needed starting with Aim 3 activities in Year 3. At the discretion of PI and Co-Is, other laboratory staff from each institution and OSCs will participate in these meetings. The purpose of these meetings will be to keep all parties informed of the status, progress, and plans. Quarterly meetings will be extended to include presentation of preliminary results. Calls will be coordinated by PIs Ross and Laing with monthly rotation of leadership and responsibility of making agendas for and disseminating notes from each meeting.

PIs will use AirTable and GitHub project management boards, administered by PI Ross and EHA administrative staff, to track task status, including laboratory and computational experiments, testing, administrative tasks, manuscript preparation and publication. EHA's internal content sharing hub (secure.eha.io), will be used for sharing interim reports and preliminary results across project participants.

IN-PERSON MEETINGS

Project PIs will meet in-person at least annually at EHA, USU, or at jointly attended conferences in the last quarter of each project year. Following annual meetings PIs will generate annual reports, using this period to communicate and discuss program status and any possible changes to research directions should information indicate failure of certain directions or the possibility of new opportunities. Decision making will be made by all program's PIs. If issues/topics cannot be resolved at this level then PIs (unlikely) will consult with NIH program staff on future allocation of research funds pending any replacement or revision of the program's goals.

We have budgeted additional funds for laboratory exchanges of staff. The modeling Postdoc at EHA will travel to USU to train in Year 2 to train in laboratory methods to inform mathematical work. A USU graduate student will travel to EHA to in Year 2-3 to study modeling and statistical methods. A PI or postdoc the project will travel to the University of Pretoria and Bangkok sites in Year 4 to work with other project members and staff on methods training and interpretation of results.

In addition, we will take advantage of existing collaborations to liaise with Co-Is. A USU project staff member is visiting scientist at the CKMH and PI Laing travels to CKMH under EID-SEARCH. PI Ross, PI Laing, and co-I Epstein travel regularly to UP for collaboration under other projects (HDTRA12010025, Wellcome 226061/Z/22/Z).

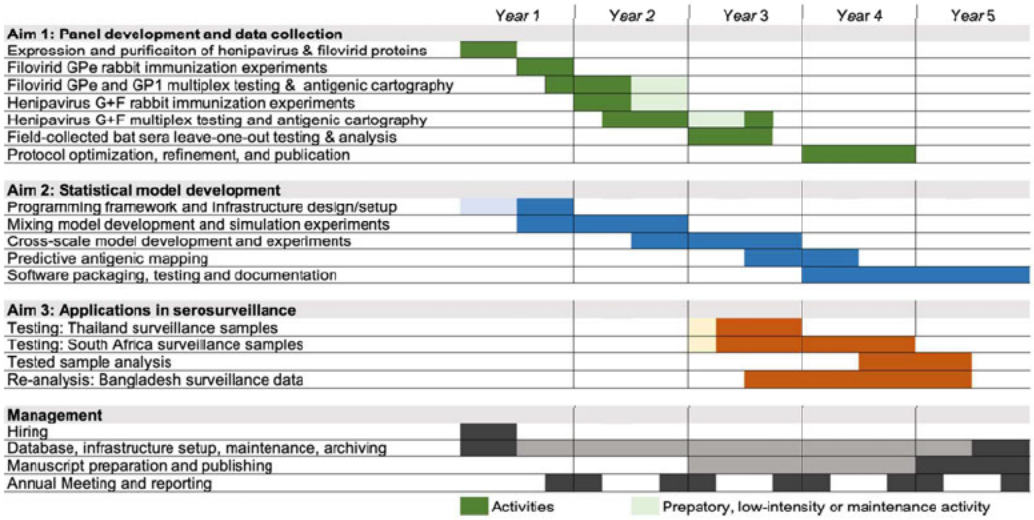
DATA MANAGEMENT

All data and code will be managed in a project database overseen by PI Ross and administered by the project research assistant. All other consortium members will upload data to this database and also maintain local copies in their local information management systems. See DATA MANAGEMENT AND SHARING PLAN for additional details on dat dissemination.

COMPUTATIONAL RIGOR AND REPRODUCIBILITY

Development of model code will follow our framework of *deployment-ready research,* an approach to developing scientific code that can be integrated into reusable software with little additional effort. Code will be maintained in a version control system (git), with versions of models linked to the versions of data they were run against in a versioned database (dolt). Changes to model design or implementation will require code review by a team member. Review will include diagnostics of code quality and comparing output and performance of each version of models, implemented automatically by continuous integration (CI) on our code-sharing platform (GitHub).

PROJECT TIMELINE



COVER LETTER

National Institutes of Health (NIH)
9000 Rockville Pike
Bethesda, Maryland 20892

February 5, 2021

Application for the NIH Research Grant Program Announcement number PA-20-185, entitled NIH Research Project Grant (Parent R01 Clinical Trial Not Allowed), dated May 5, 2020

To whom it may concern:

We are pleased to submit our grant proposal titled "**Characterizing unknown emerging viruses through targeted serological profiling**" for consideration.

Please assign our proposal to the following:

Institutes/Centers: National Institute of Allergy and Infectious Diseases - NIAID

Scientific Review Groups: Transmission of Vector-Borne and Zoonotic Diseases (TVZ)

We know of no individuals (e.g. competitors) who should not review the application.

Our project is aligned with the mission of NIAID because it focuses on detection and characterization of pathogens with potential for emergence, including near neighbors of Ebola virus and Nipah virus, which are priority pathogens. This project addresses NIH's priorities of Immunology and Assay Development, Reagents and Resources, and Epidemiology and Pathogen Discovery under the NIAID Pandemic Preparedness Plan (https://www.niaid.nih.gov/sites/default/files/pandemic-preparedness-plan.pdf).

Two of our subawards, for University of Pretoria (UP) and King Chulalongkorn Memorial Hospital (KCMH), are not active for the first two budget periods of the project. This grant concerns the development, testing, and validation of new methods, with initial development occurring at the main site, EcoHealth Alliance, and consortium partner Uniformed Services University. Testing and validation will involve all consortium partners and begin in Year 3 of the project.

Please note that the proposed work is NOT a Select Agent study because we work only with synthesized antigens and archived sera.

Thank you very much for your consideration.

Sincerely,

(b) (6)

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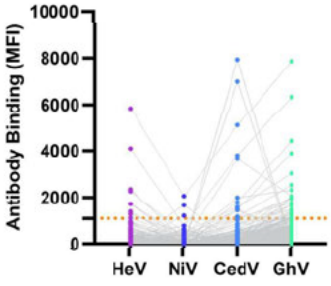
NOTES AND STUFF FROM OLD PROPOSALS

The majority of antibodies induced by virus infections are specific to conformational dependent epitopes found on virus proteins. Antibodies raised against envelope glycoprotein of henipaviruses and ebolaviruses undergo affinity maturation and are the targets of vaccine design and development. However, the confounding effects of cross-reactivity against native-like proteins expressed to retain quaternary structures, and a lack of true controls in understudied wildlife species or for yet-uncharacterized viruses stymies use of rich qualitative data that they produce.

Old World frugivorous bats have been largely considered the wildlife reservoirs of henipaviruses, the discovery of four Asiatic henipaviruses in rodents has come as a suprise [cite]. implies that serosurveillance measures thus far have been unable to appropriately detect the diverse serological profile of extant henipaviruses amongst widlife, livestock, and human hosts. In fact, PI Laing's research team detected a unique serological profile in an archived sera bank of acutely febrile patients in Cambodia that were XX% (44/1400) seropositive for a henipavirus that is seemingly antigenically-distinct and -ancestral to Ghana, Hendra, and Cedar viruses (**Figure JULIET**).



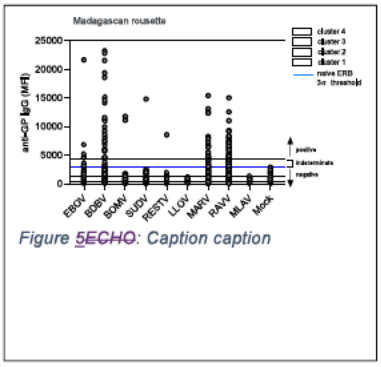
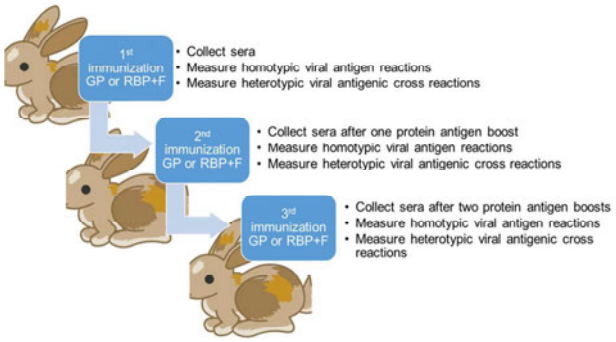
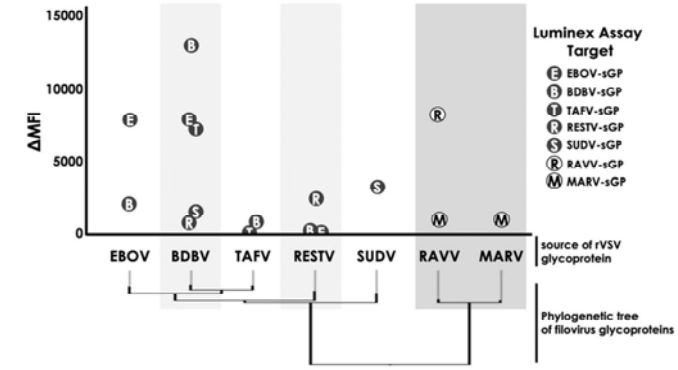


Figure 5~~ECHO~~: Caption caption

The shape of this serological profile if protein antigens from LayV and the other rodent associated henipaviruses had been included will remain unknown, but the serologic data suggests that there are other serologic footprints left by unknown zoontoic henipaviruses in southeast Asia, the shadows of which can be measured through serological approaches.





IV. SIGNIFICANCE

Serological tests frequently viruses outside their known host range or geographic range (Filovirus, Singapore, etc.).

Serological surveys can capture usable info PCR cannot [11]. Serological screening can identify novel strains that would otherwise be missed, such as this Hendra virus variant [82].

Bayesian clustering approaches [83] are useful but have largely been applied to single-assay surveillance. At higher dimensions, the clustering in high/low expected values confounds with clustering of different viruses.

Outlier-based detection is inherently problematic for rare conditions. Rare values can occur at a rate less than statistically expected outliers.

V. INNOVATION

This project is innovative in in its multi-scale approach, integrating laboratory method development and population-scale surveillance data through modeling to develop.

Estimate the probability of a multiplex panel response to represent exposure to novel coronaviruses. This approach has several statistical antecedents: spectral mixture analysis in remote imaging, antigenic cartography.

Paper on Nipah Panel: [84]

These measures will produce novel insights via *antigenic mapping*. The mixture of high- and low-specificity (RBD and IgG) will allow us to better understand the degree to which phylogenetic similarity between viral species is reflected in antigenic properties.

We will extend techniques of antigenic mapping,[66] 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.

Model 1: mixed effects for unknown responses in wildlife species. Account for variation in wildlife, use phylogenetic information to inform immune response.

Model 2: "spectral mixing" / antigenic mapping approach. Determine the probability that an individual has an exposure to a single virus.

Experiments: test sera exposed to multiple viruses rather than single viruses only, determine additivity. Additive allow for straight spectral mixing, non-additivity requires models with covariance components.

Model 3: Multiple outcome (multivariate probit) models for joint seroprevalence.

In wildlife serological surveillance, we generally lack important controls, including PCR-based or symptom-based confirmation. We lack negative controls because we don't know the wild-caught status. In the few cases where there are captive-raised animals, these negative controls aren't representative of wild types that may have had other exposures. In any case the number of species of interests for serological monitoring far exceeds those it would be possible to establish captive models.

Many multi-species surveillance studies.

Analyses need to distinguish between *correlation in assay response*, which may be driven by cross reactions, and *correlation in exposure*, driven by epidemiological and ecological processes.

Pooling data from multiple studies can establish standardized responses for some species, while simultaneously giving a base of evidence for predicting to other species.

Pooling factors allow us to evaluate the level of species- or geography-level information incoroporated into estimates

Making studies comparable by extracting raw data on machine calibration, sample dilution, etc.

Mixed models will allow us to estimate both intra- and interspecific variation in wild-type responses, and develop predictors of this using both phylogenetic and trait-based species data.

Phylogenetic models – assessing phylo-immuno relationship across the panels, allowing for prediction in phylogenetic space

Incorporate measures of variation by test

Benefits – quantitative and scalable interpretation, researchers don't all have access to the statistical or virological expertise which is required to interpret beyond specific recipes

Nonlinear relationship of cross-reactivity https://link.springer.com/article/10.1007/s10875-021-00997-6

We will screen serum from all subjects for IgG antibodies against Nipah and all other known henipaviruses (Hendra, Cedar, Ghanavirus & Mojiang) using a Luminex multiplexed sphere-based assay developed by the Broder lab and the CSIRO Australian Animal Health Laboratory in Geelong (10).

are acquiring new laboratory experimental data on titer-response curves and cross-reaction against non-target antibodies for Luminex assays.

The core of this proposal is the development of statistical models and the ability to test these models against field data from a variety of systems that has been collected, curated, and interpreted by our team.

Our team has been involved in 17+ years of research in host-pathogen dynamics and spillover processes for zoonotic viruses across continents including Hendra, Nipah, SARS-CoV, SARS-CoV-2, MERS-CoV, and Ebola viruses, as well as development of state-of the-art multiplex assays for serological testing for these viruses. Our data from multiple NIH R01s, as well as other federally-funded work, includes paired individual data on both antibody levels and PCR detection of viral RNA, multiplex data from multiple systems, and serochip data. We will enable broader use of these models in epidemiology through the development of software for both model-fitting and visual diagnostics.

Our team has experience in turning research code into generalizable and sustainably maintainined. scientific software, including designing for user needs, recruiting open-source communities.

Our 3 specific aims are to:

1. Create procedures to detect signals of new viruses from multiplex serological assays.

2. Discovering real signals in high-dimensional serochip data.

and .

Creation of a library of priors: While there are missing baselines for a wide array of serological assays, an accumulating body of evidence can provide input to future routines. We will use data accumulated from a series of projects to build a database of priors. This data will help us answer question such as: how applicable are thresholds and models across species? How consistent are immune responses within and

across families of viruses? How do distributions of responses, when normalized, compare across quantitative methods that are related but not linearly proportional with antibody titers?

We will use this database to identify laboratory calibrations that will provide the greatest ability to improve statistical discrimination among responses.

Cross calibration of methods: We have both Luminex data, serochip data, and a spectrum of VNT or pseudo-neutralization assays. All are related to antibody titer but have different mechanisms, and levels of specificity and nonlinearity in response. We will perform cross-calibrations. We will integrate these into our model designs so that mechanisms can be swapped out and serology can be made comparable across populations.

1. We will validate these models with a "leave-one-out" strategy – testing whether a novel virus is identified in sera from a population with a known exposures to a virus, with protiens specific to that protien removed. We will repeat this via both *in silica* and *in vitro* approaches. *In silica*, we will simply remove data from the target viral protiens from multiplex results from a full panel. *In vitro*, we will omit beads for the target viruses from the panel. Comparison of these two approaches will quantify the extent that competitive binding affects the validation technique.

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BIOGRAPHICAL SKETCH

NAME: Brook, Cara E.

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

POSITION TITLE: Assistant Professor

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)	END DATE MM/YYYY	FIELD OF STUDY
Stanford University, Stanford, CA	BS	06/2010	Earth Systems
Princeton University, Princeton, NJ	MS	09/2014	Ecology and Evolutionary Biology
Princeton University, Princeton, NJ	PHD	09/2017	Ecology and Evolutionary Biology
University of California, Berkeley, Berkeley, California	Postdoctoral Fellow	08/2020	Miller Postdoctoral Fellow
University of California, Berkeley, Berkeley, California	Postdoctoral Fellow	07/2021	Branco Weiss Fellowship

A. Personal Statement

My interdisciplinary research background spans the disciplines of field biology, molecular immunology, and mathematical modeling of infectious disease transmission. Across the course of my PhD and postdoc, I founded and developed a longitudinal field study deciphering the transmission dynamics of potentially zoonotic pathogens infecting wild fruit bats in Madagascar, which forms the backbone of the bulk of my research program. This work also highlights my commitment to scientific outreach, development, and capacity-building for Malagasy scientists in biology and medicine, most clearly manifest in the R-based programming workshop (E2M2: Ecological and Epidemiological Modeling in Madagascar), which I inaugurated and teach annually, and in the R-based mentoring program in quantitative ecology (C4C: Coding for Conservation), which I launched in 2022. In addition to my expertise in population-level transmission processes, from both an empirical and computational perspective, I also have extensive experience in developing within-host models to elucidate the role of the unique bat immune system in controlling viral infections, as well as a strong background in carrying out in vitro experiments to collect data against which to test the predictions of these models. Though just beginning my second year as an Assistant Professor in the Department of Ecology and Evolution at the University of Chicago, I have been independently funding my international research program since its inauguration with the start of my PhD in 2012. In total, I have amassed over $5 million in grant money to support this work since its origination, resulting in 14 first-authored and 10 senior-authored peer-reviewed publications, in addition to a suite of co-authored papers, which span a range of subjects reflecting my wide-ranging interests in ecological theory, conservation, and public health. In addition to my own work, I have funded and mentored four Malagasy scientists in part with this research, including Dr. Hafaliana Christian Ranaivoson, a Malagasy biologist who is now a postdoctoral scholar in my lab at the University of Chicago. In addition, I have trained and mentored three other University of Chicago postdoctoral scholars, two University of Chicago PhD students, two University of Chicago undergraduates, and nine postbaccalaureate research scholars in my lab.

B. Positions, Scientific Appointments and Honors

Positions and Scientific Appointments

2021 -	Assistant Professor, Dept of Ecology & Evolution, University of Chicago, Chicago, IL
2020 -	Branco Weiss Fellow, Branco Weiss Society in Science Fellowship, ETH Zurich, Zurich
2017 - 2020	Miller Postdoctoral Fellow, Miller Institute for Basic Research, UC Berkeley, CA
2013 - 2017	NSF Graduate Research Fellow, Dept Ecology & Evol Biol, Princeton Univ, Princeton, NJ

Honors

2020 - 2025	Branco Weiss 'Society in Science' Fellowship, ETH-Zurich
2020 - 2021	L'Oréal USA For Women in Science Fellowship, AAAS
2017 - 2020	Miller Postdoctoral Fellowship, Miller Institute for Basic Research, UC Berkeley
2013 - 2017	Graduate Research Fellowship, National Science Foundation
2013	National Defense, Science, and Engineering Graduate Fellowship, Dept of Defense
2010	Firestone Medal for Undergraduate Research Excellence, Stanford University
2010	Earth Systems Award for Senior Thesis Excellence, Stanford University

C. Contribution to Science

1. **Population-level bat virus transmission dynamics:** At a broad scale, one major theme of my work seeks to decipher transmission mechanisms underpinning the persistence of potentially zoonotic pathogens circulating in bat reservoir hosts, with an eye towards addressing the drivers of seasonal pulses of virus shedding that, globally, coincide with zoonotic spillover. Across the course of my PhD and postdoc, I established a long-term field system dedicated to deciphering these mechanisms for disparate pathogen types infecting wild, fruit bats in Madagascar. Through this work, my research team and I undertake monthly capture and collection of biological samples from longitudinally-monitored roosts for three endemic fruit bat species. To date, we have successfully identified, described, and analyzed the ecology of potentially zoonotic bat-borne henipaviruses, filoviruses, and coronaviruses, *Babesia* spp. protozoa, and *Bartonella* spp. bacteria infecting these bats. Our work points to mechanisms of waning immunity and a role for viral recrudescence and reinfection in viral persistence in this system.

    a. Gentles AD, Guth S, Rozins C, **Brook CE**. A review of mechanistic models of viral dynamics in bat reservoirs for zoonotic disease. Pathog Glob Health. 2020 Dec;114(8):407-425. PubMed Central PMCID: PMC7759253.
    b. **Brook CE**, Ranaivoson HC, Broder CC, Cunningham AA, Héraud JM, Peel AJ, Gibson L, Wood JLN, Metcalf CJ, Dobson AP. Disentangling serology to elucidate henipa- and filovirus transmission in Madagascar fruit bats. J Anim Ecol. 2019 Jul;88(7):1001-1016. PubMed Central PMCID: PMC7122791.
    c. Ranaivoson HC, Héraud JM, Goethert HK, Telford SR 3rd, Rabetafika L, **Brook CE**. Babesial infection in the Madagascan flying fox, Pteropus rufus É. Geoffroy, 1803. Parasit Vectors. 2019 Jan 23;12(1):51. PubMed Central PMCID: PMC6343336.

2. **Evolution of cross-species virulence in bat-borne zoonoses:** A second major theme of my work explores the within-host processes which enable bats to host pathogens that cause extreme pathology in other mammals without, themselves, experiencing significant disease. My work in this arena has demonstrated quantitatively that bats do source zoonotic viruses which cause higher case fatality rates in human populations than those derived from other mammalian hosts and also established an evolutionary mechanism to explain this phenomenon. Using a theoretical, within-host / population-level nested model, I

showed how unique anti-inflammatory mechanisms in bat physiology, believed to have evolved to mitigate the accumulation of oxidative stress incurred during flight, result in a high degree of bat tolerance to immunopathology. These mechanisms, in turn, lift the evolutionary cap on virus growth rates otherwise set by the classic virulence-transmission tradeoff, resulting in the evolution of faster-replicating pathogens that, while not pathological to a bat, cause extreme virulence in other mammals that lack unique bat physiology. My *in vitro* work infecting bat cells with virus in tissue culture offers an empirical validation of this phenomenon, suggesting that viruses replicating under constitutively antiviral innate bat immune conditions do, indeed, demonstrate faster growth rates than under more typical induced mammalian immune defenses.

a. Guth S, Mollentze N, Renault K, Streicker DG, Visher E, Boots M, **Brook CE**. Bats host the most virulent-but not the most dangerous-zoonotic viruses. Proc Natl Acad Sci U S A. 2022 Apr 5;119(14):e2113628119. PubMed Central PMCID: PMC9168486.
b. **Brook CE**, Rozins C, Guth S, Boots M. Reservoir host immunology and life history shape virulence evolution in zoonotic viruses. bioRxiv : the preprint server for biology. 2021. Available from: https://doi.org/10.1101/2021.10.06.463372 DOI: 10.1101/2021.10.06.463372
c. **Brook CE**, Boots M, Chandran K, Dobson AP, Drosten C, Graham AL, Grenfell BT, Müller MA, Ng M, Wang LF, van Leeuwen A. Accelerated viral dynamics in bat cell lines, with implications for zoonotic emergence. Elife. 2020 Feb 3;9 PubMed Central PMCID: PMC7064339.
d. Guth S, Visher E, Boots M, **Brook CE**. Host phylogenetic distance drives trends in virus virulence and transmissibility across the animal-human interface. Philos Trans R Soc Lond B Biol Sci. 2019 Sep 30;374(1782):20190296. PubMed Central PMCID: PMC6711300.

3. **Expanding public health capacity in Madagascar:** An additional major theme of my research focuses on capacity-building for public health in Madagascar. I have continuously worked to support the quantitative training of Malagasy scientists in biology and medicine, co- and senior-authoring numerous publications with Malagasy collaborators to promote research into infectious disease dynamics. These initiatives have ranged in subject from guiding the roll-out of rubella vaccine, to deciphering the dynamics of COVID-19, to understanding the landscape for potential recombination in bat-borne and human-circulating coronaviruses. In addition, this work has resulted in the inauguration of the first-ever Illumina Next Generation Sequencing platform in Madagascar, used for SARS-CoV-2 genomic surveillance.

a. Tegally H, San JE, Cotten M…**Brook CE…** Wilkinson E (397 authors). The evolving SARS-CoV-2 epidemic in Africa: Insights from rapidly expanding genomic surveillance. *Science.* 2022. Oct 7;378(6615):eabq5358. PubMed Central PMCID: PMC9529057.
b. Andriamandimby SF*, **Brook CE***, Razanajatovo N, Randriambolamanantsoa TH, Rakotondramanga JM, Rasambainarivo F, Raharimanga V, Razanajatovo IM, Mangahasimbola R, Razafindratsimandresy R, Randrianarisoa S, Bernardson B, Rabarison JH, Randrianarisoa M, Nasolo FS, Rabetombosoa RM, Ratsimbazafy AM, Raharinosy V, Rabemananjara AH, Ranaivoson CH, Razafimanjato H, Randremanana R, Héraud JM, Dussart P. Cross-sectional cycle threshold values reflect epidemic dynamics of COVID-19 in Madagascar. Epidemics. 2022 Mar;38:100533. PubMed Central PMCID: PMC8628610. **joint first authorship*
c. Wilkinson E, Giovanetti M, Tegally H… **Brook CE** de Oliveira T (291 authors). A year of genomic surveillance reveals how the SARS-CoV-2 pandemic unfolded in Africa. Science. 2021 Oct 22;374(6566):423-431. PubMed Central PMCID: PMC7613315.
d. Ravelomanantsoa NAF, Guth S, Andrianiaina A, Andry S, Gentles A, Ranaivoson HC, **Brook CE**. The zoonotic potential of bat-borne coronaviruses. Emerg Top Life Sci. 2020 Dec 11;4(4):353-369. PubMed PMID: 33258903.

e. Wesolowski A, Mensah K, **Brook CE**, Andrianjafimasy M, Winter A, Buckee CO, Razafindratsimandresy R, Tatem AJ, Heraud JM, Metcalf CJ. Introduction of rubella-containing-vaccine to Madagascar: implications for roll-out and local elimination. J R Soc Interface. 2016 Apr;13(117) PubMed Central PMCID: PMC4874430.

4. **Viral discovery:** Another scientific contribution of my work has revolved around viral discovery and characterization of novel, potentially zoonotic viruses of fruit bats in Madagascar. In recent studies, we have described and characterized novel genomes of important bat coronaviruses and henipaviruses which fill critical gaps in global phylogenetic trees. Additionally, we have work-in-progress characterizing novel picornaviruses and astroviruses from these bats, as well.

   a. Madera S, Kistler A, Ranaivoson HC, Ahyong V, Andrianiaina A, Andry S, Raharinosy V, Randriambolamanantsoa TH, Ravelomanantsoa NAF, Tato CM, DeRisi JL, Aguilar HC, Lacoste V, Dussart P, Heraud JM, **Brook CE**. Discovery and Genomic Characterization of a Novel Henipavirus, Angavokely Virus, from Fruit Bats in Madagascar. J Virol. 2022 Aug 30; PubMed PMID: 36040175.
   b. Kettenburg G, Kistler A, Ranaivoson HC, Ahyong V, Andrianiaina A, Andry S, DeRisi JL, Gentles A, Raharinosy V, Randriambolamanantsoa TH, Ravelomanantsoa NAF, Tato CM, Dussart P, Heraud JM, **Brook CE**. Full Genome *Nobecovirus* Sequences From Malagasy Fruit Bats Define a Unique Evolutionary History for This Coronavirus Clade. Front Public Health. 2022;10:786060. PubMed Central PMCID: PMC8873168.

5. **Conservation biology:** Finally, my work has helped shape an understanding of population viability for threatened taxa, mostly with a Madagascar focus. My early work demonstrated the life history traits that predispose threatened Malagasy lemurs to extinction or survival, highlighting the critical influence of the age of first reproduction and the adult lemur survival rate across each interbirth interval in safeguarding species into the future. Later studies recapitulated some of these predictions for Malagasy fruit bats, highlighting extreme population vulnerability in threatened *Pteropus rufus*, while others still demonstrated a role for population resilience to hunting pressure in rapidly-reproducing tenrecs.

   a. Andrianiaina A, Andry S, Gentles A, Guth S, Héraud J, Ranaivoson H, Ravelomanantsoa N, Treuer T, **Brook CE**. Reproduction, seasonal morphology, and juvenile growth in three Malagasy fruit bats. Journal of Mammalogy. 2022 August 30;gyac072. Available from: https://doi.org/10.1093/jmammal/gyac072. DOI: 10.1093/jmammal/gyac072.
   b. Annapragada A, **Brook CE**, Luskin M, Rahariniaina R, Helin M, Razafinarivo O, Ambinintsoa Ralaiarison R, Randriamady H, Olson L, Goodman S, Golden C. Evaluation of tenrec population viability and potential sustainable management under hunting pressure in northeastern Madagascar. Animal Conservation. 2021; 24(6):1059-1070. Available from: https://doi.org/10.1111/acv.12714 issn: 1367-9430
   c. **Brook CE**, Ranaivoson HC, Andriafidison D, Ralisata M, Razafimanahaka J, Héraud JM, Dobson AP, Metcalf CJ. Population trends for two Malagasy fruit bats. Biol Conserv. 2019 Jun;234:165-171. PubMed Central PMCID: PMC6959543.
   d. **Brook CE**, Herrera JP, Borgerson C, Fuller EC, Andriamahazoarivosoa P, Rasolofoniaina BJR, Randrianasolo JLRR, Rakotondrafarasata ZRE, Randriamady HJ, Dobson AP, Golden CD. Population viability and harvest sustainability for Madagascar lemurs. Conserv Biol. 2019 Feb;33(1):99-111. PubMed PMID: 29896899.

SPECIFIC AIMS

Serosurveillance provides powerful measures of the infectome of wildlife and humans that can shed light on the disease ecology of zoonotic viruses. Yet **serological data is plagued with ambiguities that limit our ability to use it to identify zoonotic reservoirs or identify populations at risk from exposure to emerging viruses**. For example, the wildlife source of Ebola virus (EBOV), a continued ~~source~~cause of deadly outbreaks, has eluded researchers since the 1970s. Molecular surveillance has yielded extremely few detections, but serology paints a muddied picture as EBOV antisera are extensively cross-reactive with antigens from other filoviruses. Human seroprevalence estimates vary widely and positive EBOV detections in wildlife hosts are not distinguishable signals of other known or uncharacterized related filoviruses. Similarly, Nipah virus (NiV) a zoonotic henipavirus that has caused repeated outbreaks in Malaysia, Bangladesh and India with fatality rates greater than 70%, has a known host in flying foxes. Yet many other reservoir species test positive for NiV ~~pah~~ antibodies without neutralization or molecular confirmation. Further, ~~r~~Recent ~~expansion~~ description of ~~of known~~ henipavir~~use~~al diversity~~s~~, such as Langya virus in acutely ill patients and shrews, suggests ~~these serologic signals are footprints of other,~~ the serologic footprints of potentially zoonotic henipaviruses have gone unnoticed by current serological testing strategies.

Our previous work has developed native-like antigens of filoviruses and henipaviruses for multiplex microsphere-based immunoassays, detected those viruses in humans and wildlife hosts, and used these data to model the circulation of zoonotic disease in wildlife and humans. **We propose to extend our methods into new laboratory and statistical techniques that can definitively identify the species of viruses ~~from~~ through serology,~~antibodies~~ and characterize the properties and epidemiology of novel viruses only detectable~~ed~~ by their antigenic ~~signals – *shadow viruses*~~shadows.**

Our specific aims are to:

1. **Create multiplex serological panels optimized for discovery and characterization of novel viruses.** We will generate serological *discovery panels* of native-like filovirus and henipavirus proteins with mixtures of sensitive and specific antibody binding properties that collectively can distinguish between the signatures of target~~ed~~ known viruses and unknown~~shadow~~ virus shadow~~ses in antisera~~. We will measure their cross-reactive properties via experimental immunizations, including the effects of repeat exposure and multiple-virus interactions. We will test consistency of results in model organisms against reference sera for humans (NiV, EBOV, Marburg virus) and sera collected from bat species with serological profiles consistent with exposure to ~~wild-type samples with known specific serologic exposure to select viruses (NiV,~~ Bundibugyo virus~~)~~, yet the authenticity of this specificity being unclear. ~~and that are reactive despite highly unlikely exposure.~~
2. **Develop novel statistical methods to extract viral signatures from discovery panel data and integrate rich multidimensional serology into epidemiology**. Will extend *spectral mixture* methods from image analysis to develop statistical models that use discovery panel data to disentangle the signals of multiple viruses and extract the antigenic signature of shadow viruses as well as quantify immune response. We will measure the discriminatory power of our models via simulation and validate them via a leave-one-out strategy, testing if we can identify viruses omitted from our panels. We will integrate these methods with cross-scale immuno-epidemiological models, improving the accuracy and precision of both individual- and population-level serology measures.
3. **Apply these methods to characterize novel and emerging viruses and their epidemiology in wildlife and humans.** We have identified wildlife and human populations demonstrating exposure to potentially novel but indeterminate filoviruses and henipaviruses. **This work will leverage investment from active NIH and DTRA-BTRP surveillance projects** in South Africa, Thailand, and Bangladesh by further testing and analyzing samples from these projects under our new protocols to antigenically characterize sera and distinguish known viruses from shadow viruses. We will use our cross-scale epidemiological models to determine the risk factors and spatiotemporal patterns of exposure and host-level immunological response to identify reservoirs and likely exposure interfaces.

This work has broad significance, addressing NIAID's Pandemic Preparedness Plan priorities of immunology and assay development, reagents and resources, and epidemiology and pathogen discovery.

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It will produce biological resources, published protocols, and reusable statistical software to enable the rapid dissemination and adoption of these methods.

RESEARCH STRATEGY

I. SIGNIFICANCE

Estimates of the cost of global virus discovery range in the billions of dollars [1]. One driving factor is the exceedingly low detection rate of viruses of concern, especially those in the *Ebolavirus, Marburgvirus, and Henipavirus* genera. The wildlife hosts of Ebola virus (EBOV), a continued source of deadly outbreaks in West and Centra Africa, have eluded researchers since the 1970s despite considerable effort. In 2005, EBOV RNA was detected in three bat species [2]. Then in 2019 the USAID-funded Ebola Host Project sampled over 45,000 specimens from 10,000 bats discovering the complete genome of one novel ebolavirus (Bombali virus, BOMV), and a one-fifth genome of EBOV [3]. Wildlife reservoirs for related ebolaviruses, Sudan virus (SUDV), Bundibugyo (BDBV) virus, and Tai Forest virus (TAFV), have not been identified. Nipah virus (NiV), ~~a zoonotic henipavirus that has caused repeated outbreaks in Malaysia, Bangladesh and India with fatality rates greater than 70%~~responsible for annual outbreak of fatal encephalitis in Bangladesh and southern India with some outbreak fatalities exceeding 90% [4-7], is known to circulate in flying foxes (*Pteropus* spp.). Even in a flying fox colony with known circulation, estimates of viremia are <2%, and a study of 3,600 bats over five years, including a period where an outbreak occurred, yielded only eight molecular detections [8]. The recent discovery of Langya virus (LayV) in patients with acute febrile illness and shrews in eastern China [9], indicates that unknown and undetected henipaviruses are also a global health concern [10].

In contrast to molecular surveillance, targeted and broad **serology can provide a~~a~~ more cost-effective approach to detecting emerging viruses in wildlife hosts and humans, and ~~to~~ measuring ~~e~~ exposure rates** to identify at-risk populations [11-13]. Viral shedding occurs over a short window, with most shedding often occurring over a period of only days, while viral antibodies can last the lifetime of an organism. In the study of NiV circulation above, 3020% of individuals had NiV antibodies, and NiV seropositivity ranges from 11-40% across populations and species of flying foxes in South and Southeast Asia [PMID: 33139552, PMID: 31855143, PMID: 34218820~~ERIC cite~~]. Using the adaptive humoral memory to indirectly measure prior infection and **identifying the serologic footprints left behind after virus infections has the potential to vastly improve our understanding of zoonotic virus diversity, distributions, and dynamics.**

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Since serology is an indirect measurement of virus infection, wildlife and human zoonotic virus **serosurveillance faces limitations in the interpretation of data**, especially when attempting to identify natural hosts of viruses [14]. Expected and unexpected **antibody cross reactions frequently confound conclusions from single-antigen assays**. EBOV-positive antisera is extensively cross-reactive with protein antigens from heterotypic ebolaviruses [15]. Antisera from survivors infected with EBOV, BDBV, and RESTV is highly cross-reactive with heterotypic virus antigens; furthermore, RESTV antisera preferentially interacted with EBOV antigens [16]. This is likely the reason for many bewildering serologic observations: seroprevalence for EBOV was found in humans without a history of Ebola Virus Disease (EVD) [17]. In non-outbreak settings people who had contact with wildlife were found seropositive or indeterminate against EBOV, SUDV, BDBV [18, 19]. Community health volunteers with indirect contact of EVD patients were also ebolavirus seropositive [20]. Bush meat hunters sampled in Guinea had serologic evidence of infection by ebolaviruses, but seropositivity was detected by measuring immunoreactivity against several antigens and neutralization tests [21]. It is unclear whether assay performance are sensitive or specific enough to draw meaningful conclusions on the role of wildlife contact on human EBOV seropositivity. Antigen-based serosurveillance studies of fruit bats have also yielded ambiguous conclusions about wildlife reservoir statuses [22-24]. With >1200 species of bats and an unknown diversity of uncharacterized filoviruses the challenges of distinguishes EBOV-positive antisera from other ebolaviruses is daunting.

Similar challenges impede serosurveillance of henipaviruses. Since 1999, NiV surveillance has remained a top priority in south and southeast Asia. After the first documented cases of NiV emergence in Peninsular Malaysia~~,~~ and an understanding of the factors that lead to transmission to humans [25, 26], government regulations on agricultural practices have limited further spillovers in Malaysia. The current epicenter of NiV disease is a region in western Bangladesh termed the *Nipah Belt,* where annual outbreaks are linked to the consumption of NiV contaminated unpasteurized date palm sap [27]. Flying foxes (*Pteropus* spp.)

are considered the wildlife reservoirs of NiV, and in south and southeast Asia, NiV has been detected and isolated from many species of pteropodid bats sampled in India, Bangladesh, Malaysia, Cambodia, and Thailand [28-33]. Despite isolation of NiV from flying foxes, detection of neutralizing NiV neutralizing antibodies, and experimental challenge models of NiV in flying foxes to confirm reservoir status, other bats across Asia test seropositive for NiV and their role in inter-species transmission remains unclear [34-36]. Rousettes and mouse-eared bats collected in China, Vietnam, and Kerala, Inida tested seropositive for NiV via a combination of nucleocapsid protein (NP), envelope receptor-binding glycoprotein (RBP) and cell lysate ELISA, but tested negative via neutralization tests. [37-39]. It is unclear whether seropositivity in these rousette bats is a factor of sensitivity and specificity issues with the immunoassay or the presence of another similar henipavirus. In sub-Saharan Africa, NiV-like neutralizing antibodies were detected in pigs in Ghana and Uganda, [40, 41] and humans sampled in Cameroon [42]. Genomic assemblages of two henipaviruses, Ghana virus and Angavokeely virus, were detected in closely related fruit bat species (genus *Eidolon*) [43, 44]. Recently ~~found wefound~~ we genetic evidence of novel henipaviruses detected in Egyptian rousette bats [45], ~~-~~ which adds to conclusions that rousette bats may be in fact harboring henipaviruses that cross-react with single antigen NiV ELISA.

Serology is also dependent on the quality and qualification of protein antigen. The Ebola virus envelope attachment glycoprotein (GP), ~~nucleocapsid protein (~~NP~~)~~, and matrix protein (VP40) have been utilized by several groups in antigen-based serology PMID: 2948188, PMID: 34748613, PMID: 33827978][~~ERIC cite~~]. In one longitudinal study of Ebola virus disease survivors, ~~seroconversion against~~anti- GP and VP40 IgG ~~occurred at XX/XX%, respectively,~~increased with days post-infection along with affinity maturation of GP-positive B cells [PMID: 32053790~~ERIC cite~~]. However, studies that detail the frequency of seroconversion against the ~~the nucleocapsid protein (~~NP~~)~~ after EBOV infection, or the frequency of seroconversion against GP, VP40, and NP with non-EBOV ebolaviruses have not been verified.

Most statistical approaches to interpreting serosurveillance data are limited to single antigen analysis and unrealistically simple models of host immunology and epidemiology. The state of the art approach to dealing with serological assay data in wildlife or with viruses without standardized controls has been clustering approaches [46] that assume seropositive and seronegative individuals separate into two distinct groups by a threshold antibody titer. Yet most real data is far less distinct, due a mix of processes including cross reactions, host heterogeneity, and antibody waning and nonlinearities in the physio-chemical processes of antibody assays [47] ~~(Figure HOTEL)~~. More sophisticated multi-antigen, *multiplex*, analyses [48] can account for cross reactions to distinguish amongst known viruses, but **fundamentally current serological laboratory and statistical approaches are not designed to deal with the challenges of detecting and characterizing novel viruses.**

The major goal of this proposal is to dramatically improve the utility of antigen-based serosurveillance for new viral discovery and epidemiology of emerging zoonotic diseases by addressing the challenges of cross-reaction and statistical rigor. We will create *discovery panels* - qualified and optimized panels of multiplexed immunoassays designed to detect and characterize shadow viruses, and distinguish known from novel viruses through mixtures of sensitive and specific binding properties. (Aim 1). We will develop new statistical approaches to extract the signals of novel viruses from those of known viruses or mixtures of multiple previous viral exposures, as well as integrating quantitative measures of immune response into epidemiological models (Aim 2). Finally, we will apply these techniques to identifying and characterizing filoviruses and henipaviruses in bat populations in South Africa and at-risk human populations in Thailand, and capturing immune-epidemiological dynamics of Nipah virus V circulation in Bangladesh (Aim 3).

This work has broad significance, addressing NIAID's Pandemic Preparedness Plan priorities of immunology and assay development, reagents and resources, and epidemiology and pathogen discovery. **It will produce biological resources, published protocols, and reusable statistical software to enable the rapid dissemination and adoption of these methods.**

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II. INNOVATION

We propose a unique and innovative approach to characterizing antibodies induced by prior infections to discover the signals of novel viruses – *shadow viruses* – in wildlife and human hosts through antigen-based multiplex serology. While serology is an increasingly common technique in viral surveillance, multiplex panels have hitherto been composed of several proteins each designed specifically to bind antibodies of individual antigens, optimizing for specificity to individual viruses. We will instead design and test *discovery panels* composed of proteins with a mixture of sensitivities and specificities to a range of viral antigens within a family. This will simultaneously cover *antigenic space*, enabling detection of previously unknown viruses, and enable definitively ruling out or confirming the signal of known pathogens via their unique *antigenic signatures*. We will build these panels on a multiplex microsphere-based immunoassays (MMIA) platform ~~[ERIC cite]~~. This enables cost-effective testing of dozens of antigens simultaneously and is easily updated with new reagents. We have successfully deployed this assay~~it~~ to~~in~~ laboratories for surveillance projects ~~in low resource environments~~ [PMID: 32034942, PMID: 31671094, PMID: 29260678]~~[ERIC cite]~~. Through the multiplex discovery approach, we can achieve accuracy far more readily adoptable than low-throughput panels such as ~~indirect~~ ELISA [48], or neutralization tests requiring containment and virus isolates or verified pseudovirus systems.

Terms

shadow virus: a novel or unidentified virus only detected in a population via serology of infected individuals

discovery panel: a multiplexed immunoassay designed to detect shadow viruses and distinguish them from known viruses

antigenic space: the phenotypic range of antigens of known and unknown viruses in a family

antigenic signature: a combination of responses of an antiserum against a panel of antigens that identifies a virus

reaction/cross reaction matrix: the full combination of response curves of all antisera to all antigens in a multiplexed immunoassay

cross-binding interaction: the change in response of antisera to an antigen in the presence of another antigen due to preferential binding

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We further innovate by adapting statistical methods from image-processing – nonlinear spectral unmixing models, implemented in a Bayesian framework [49-51] – **to analyze multiplex serological data as *joint* rather than individual response signals.** This flexible approach allows us to incorporate nonlinearities in the interaction of viruses and antigens, as well as those introduced in the physiochemical processes of the assay. **It will enable us to disambiguate data that could represent a variety of exposure histories**, including exposure to multiple viruses, known viruses with cross-reactions to multiple antigens, or novel viruses with an intermediate antigenic profile between known viruses, assigning probabilities to each hypothesis **This framework is applicable to serological methods beyond MMIA** such quantitative LFIA and ELISA, as well as large-scale peptide multiplexing via PhIP-seq [52], which currently lacks unifying computational or analytical frameworks [53].

We will integrate our statistical framework with population-level epidemiological models to capture cross-scale immunological patterns and dynamics. The dominant threshold-based approach to seroepidemiology determines serostatus at the individual level from antibody titer or assay response, then describes population-level patterns as frequencies of seronegative/positive states. This discards the rich quantitative information on antibody levels and the role of processes such as individual immune variation, antibody waning, and inheritance that can be informative of population processes. **By linking the estimation of individual- and population-level quantities, we increase the accuracy and precision of both.** We will develop cross-scale statistical models that model individuals' complex binary and continuous immune state, and jointly model the variation of this complex serostatus across time, space, and groupings (species, populations, and demographic groups).

Finally, this proposal leverages active federal investments in global viral surveillance programs by partnering with them to rapidly validate these techniques. We will partner with an NIH Centers for Research in Emerging Infectious Disease (CREID) study of high wildlife-contact individuals in Thailand, a DTRA Biological Threat Reduction Program survey of bat virus diversity in Southern Africa, and an NIH NIAID research program on Ni~~pah virus~~V circulation and spillover in Bangladesh. In each, serosurveillance

has generated preliminary but ambiguous evidence of novel virus circulation. We will test archived samples to corroborate these data and characterize the suspected shadow viruses and their epidemiology.

III. APPROACH

RESEARCH TEAM AND MANAGEMENT

We have assembled a team with a unique combination of laboratory, analytic, and field expertise and a history of successful collaboration in zoonotic disease surveillance. Project PIs Laing and Ross are early-stage investigators with methodological expertise on laboratory and statistical methods for serological analysis, respectively. Other Co-Investigators lead viral discovery surveillance projects across Bangladesh (Epstein), Southern Africa (Markotter, Epstein), and Southeast Asia (Wacharapluesadee). PI Laing and other significant contributor (OSC), Brook, are collaborators on other surveillance projects; and OSC Munster is an established lab chief with a history of collaboration and whose research interests in virus ecology align with project PIs Laing and Ross.

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The PIs began working together in 2017 on a project titled, Serological Biosurveillance for Spillover of Henipaviruses and Filoviruses at Agricultural and Hunting Human-Animal Interfaces in Peninsular Malaysia (HDTRA11710037). Both PIs remain respective methodological experts for active surveillance projects, Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa (HDTRA12010025), and CREID EID-SEARCH.

We have designed our program so that laboratory and mathematical methods development are complementary but can proceed independently. Each benefit from data and innovations of the other. Statistical innovations enhance interpretation of discovery panel data and the data in turn can be used to parameterize and increase power of statistical techniques. However, we will be able to make progress on both in parallel, test their success on their own terms and apply outputs to the surveillance studies even if their success and completion time differ.

See the **MULTIPLE PD/PI LEADERSHIP PLAN** for additional team and project management detail.

AIM 1: OPTIMIZATION OF ANTIGEN-BASED MULTIPLEX SEROLOGY FOR DISCOVERY AND DETECTION

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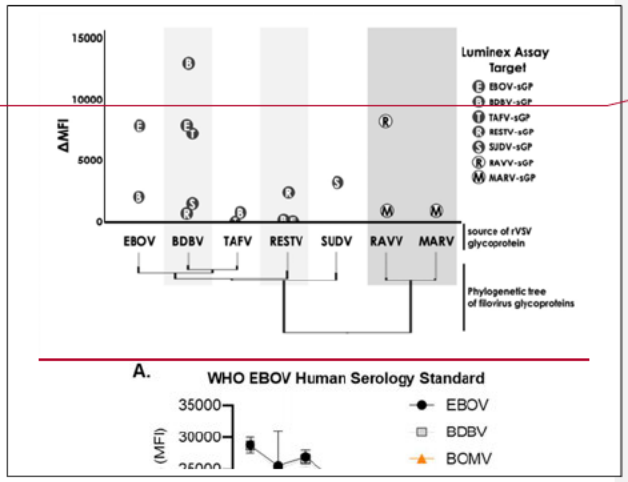
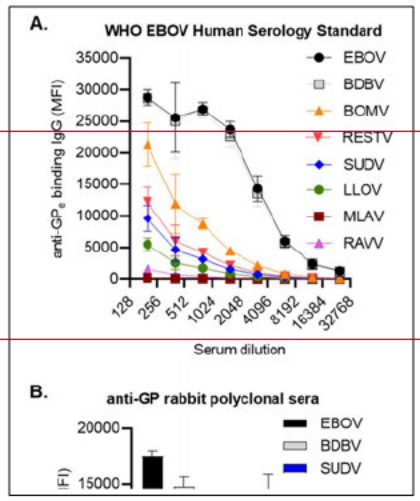
We have developed, expressed, and purified ~~envelope attachment glycoprotein (GP) of~~ filoviruses GP and ~~envelope receptor binding glycoprotein (RBP) of~~ henipaviruses RBP antigens in~~expressed in~~ mammalian cell culture systems as native-like ectodomain trimers and tetramers, respectively, retaining conformation-dependent epitopes [54-58]. We have used these antigens to create MMIAs focused on the detection of presently described ebolaviruses, marburgviruses, and henipaviruses. These have been successfully transferred to laboratories globally and used in completed and ongoing biosurveillance projects including DARPA PREEMPT, our CREID center EID-SEARCH (Co-I Wacharapluesadee), our DTRA BTRP-funded project in southern Africa at the University of Pretoria (Co-I Markotter), and for collaborative projects with OSCs Munster and Brook. Through these projects, these panels have been used to identify wildlife hosts of filoviruses and henipaviruses, and detect evidence of prior infection of high-risk human populations [59, 60]. The panels are continually expanding with each novel virus discovery, e.g., ~~Bombali virus~~BOMV and La~~ngya virus~~yV. However, without standardized protein antigens for antigen-based serosurveillance and sera standards for each virus, demonstrating specificity in wildlife reservoirs and human populations without outbreak histories remains a challenge. When screening samples collected from wildlife, it remains unclear if we are capturing antibodies induced by known virus infections or by antigenically related uncharacterized viruses. Here, we propose a strategy to overcome this fundamental limitation.

Designing the discovery panel with a two-protein approach

Preliminary Data: Cross reaction among filoviruses and henipaviruses. Antibodies induced by ebolavirus infections are cross-reactive with GP antigens, likely the ~~resulf~~result of several contributing factors such as amino acid sequence similarity, conserved ~~optitopose~~epitopes, and ~~glyco~~glyco~~eptipopose~~epitopes or glycosylations in the glycan gap and mucin-like domain [61, 62]. Previous groups have attempted to distinguish homotypic and heterotypic known ebolavirus IgG binding to GP antigens by performing multiple ELISAs for each known ebolavirus [48]. This technique conferred specificity but at a great cost to throughput, and has unclear applications for the detection of unknown ebolaviruses.

~~In our GPe based MMIA, the WHO EBOV antibody standard reacted equally against both EBOV and BDBV GPe antigens (Figure FOXTROT A).~~ Although ebolavirus antisera are cross-reactive with heterotypic ebolaviruses, through a GPe-based multiplex approach we have been able to specifically identify the homotypic infecting ebolavirus after a single infection by a VSV-filoGP in a bat infection model despite observable cross reactions (~~preliminary data, not shown~~Figure 1). This indicated that pooled polyclonal antisera from human EVD survivors may include more cross-reactive antibodies than those generated after single infections in a putative wildlife host. ~~Futher~~Further, preliminary data from polyclonal antisera from rabbits ~~s hyper~~immunized with EBOV GPe corroborated data with the WHO EBOV serology standard that EBOV and BDBV specific antibodies are cross-reactive with GPe trimers, though preferential binding to the homotypic EBOV antigen was noted. However, the GP1 subunit protein improved specificity (~~Figure FOXTROT B~~Figure 2B).





Cross-reaction among the other henipaviruses is less described. HeV antisera is known to display a fair degree of cross-reaction with NiV, however the inverse does not hold true. ~~...~~[PMID: 18556094, PMID: 22875827 ~~CITE~~]. We have examined henipavirus cross-reactivity with polyclonal antisera from rabbits hyperimmunized with recombinant sGtet protein antigens and observed little cross-reactivity outside of HeV-positive antisera with NiV G (~~Figure CHARLIE~~Figure 3).

Approach: Protein development. Here, we propose to design antigen-based *discovery panels* that can distinguish between targeted and cross-reactive signals using a two-protein approach, selecting a highly specific (GP1) and a broadly sensitive (GPe trimer) antigen for each filovirus in the panel. This approach draws from our successful approach to large-scale COVID-19 serosurveillance, which used SARS-CoV S glycoprotein expressed as a trimeric prefusion stabilized spike ectodomain and a monomeric receptor-binding domain antigens, providing an optimal balance of assay sensitivity and specificity. We note this indicates that a similar strategy to succeed for surveillance and discovery of novel and zoonotic coronaviruses in the future.

Thus, our two-protein panel approach requires a sensitive protein antigen that could be the cross-reactive target for antibodies induced by shadow henipaviruses. Since the henipavirus RBP provides the *specific* antigen, we plan to develop the soluble native-like trimeric fusion (F) glycoproteins into the *sensitive* antigen. HeV and CedV RBP share ~~23~~30% sequence identity while the F proteins share ~44% amino acid expressed, and purified and has been extensively studied as a target for monoclonal antibodies [57, 63-65]. In its role to mediate virus-cellular fusion, these F proteins share higher sequence identity and similarity across henipaviruses than the RBP sequence identity. The F protein has been similarly

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designed. ~~sequence identity. The F protein has been similarly designed, expressed, and purified and has been extensively studied as a target for monoclonal antibodies [57, 63-65]. In its role to mediate virus-cellular fusion, these proteins share higher sequence identity and similarity across henipaviruses than the RBP.~~ Thus, we plan to explore the ability of the henipavirus F protein as to function as a *sensitive antigen* for antigenic discovery of novel henipaviruses. We will develop GPe/GP1 antigen pairs for each of the filoviruses and RBP/F antigens comprising the range of currently known mammalian filoviruses and henipavirus~~henipaviruses (Table ALPHA).~~

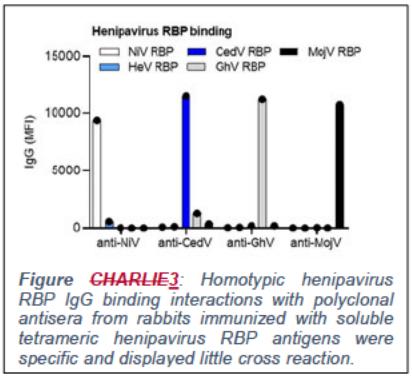

Figure ~~CHARLIE~~3: Homotypic henipavirus RBP IgG binding interactions with polyclonal antisera from rabbits immunized with soluble tetrameric henipavirus RBP antigens were specific and displayed little cross reaction.

~~...Filoviruses~~	~~Sensitive antigen~~	~~Specific antigen~~
~~Ebola virus (EBOV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Bundibugyo virus (BDBV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Tai Forest virus (TAFV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Bombali virus (BOMV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Sudan virus (SUDV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Reston virus (RESTV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Marburg virus (MARV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Ravn virus (RAVV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Lloviu virus (LLOV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Mengla virus (MLAV)~~	~~GPe (trimer)~~	~~GP1 subunit~~
~~Henipaviruses~~		
~~Hendra virus (HeV)~~	~~RBP (tetramer)~~	~~F (trimer)~~
~~Nipah virus (NiV)~~	~~RBP (tetramer)~~	~~F (trimer)~~
~~Cedar virus (CedV)~~	~~RBP (tetramer)~~	~~F (trimer)~~
~~Ghana virus (GhV)~~	~~RBP (tetramer)~~	~~F (trimer)~~
~~Angavokely virus (AngV)~~	~~RBP (tetramer)~~	~~F (trimer)~~
~~Mojiang virus (MojV)~~	~~RBP (tetramer)~~	~~F (trimer)~~
~~Langya virus (LayV)~~	~~RBP (tetramer)~~	~~F (trimer)~~
~~Gamak virus (GamV)~~	~~RBP (tetramer)~~	~~F (trimer)~~
~~Daeryong virus (DarV)~~	~~RBP (tetramer)~~	~~F (trimer)~~

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Approach: Quantifying cross-reactions: To optimize a discovery panel for discovery and detection, we first need to fully qualify the pairwise cross-reactions amongst proteins, generating a comprehensive *reaction/cross reaction matrix* via protein immunization experiments in rabbits. This will establish and qualify (a) the homotypic virus reactivity, (b) heterotypic cross-reactivity across other viruses in the family, and (c) how multiple immunogen exposures shape homotypic antigen binding and the cross-reactive breadth against heterotypic viruses. Since wildlife reservoirs are naturally exposed to these viruses throughout their lives, repeated exposures shape the magnitude, durability, and breadth of humoral responses. Data from (c) will support use be used to estimate time-from-infection and antigenic-relatedness. Using our filovirus GPe antigens and henipavirus RBP & F antigens, we will sequentially immunize rabbits (n = 3 rabbits per virus antigen) with up to two immunogen boosts (100 µg of protein antigen per injection), and whole blood will be collected 21 days after each immunization; followed by a terminal bleed after 84 days.

Sera will be tested against all antigens within the panel at a range of dilutions to find the optimal dilution factors within the range of the linear slope of the MMIA median fluorescence intensity (MFI) output signal. For the prototypical viruses, EBOV, NiV, and MARV, we will include WHO human serology standards developed by the National Institute for Standards and Control for interpolations of MFI into IgG concentrations. For each pair in the matrix, we will quantify cross-reaction by fitting logistic functions to the reactivity vs (log) dilution and estimating the slope parameter as the measure of reaction. Utilizing MFI values from optimal dilution factors, we will apply antigenic mapping [66], projecting the heterotypic cross-reactivity matrix into *antigenic space* for both filoviruses and henipaviruses, separately for the *specific* (GP1/RBP) and *sensitive* (GPe/F) antigen of each virus. We expect the mappings of sensitive antigens, e.g., filo GPe and henipa F, to cluster closely together and for some closely related viruses to broadly overlap as serogroups in antigenic space, while specific antigens, e.g., filo GP1 and henipa G will not. Gaps in antigenic space will indicate areas unlikely to be detected by the panel, while overlaps from specific antigens will indicate likely ambiguities. The *antigenic signature* data of viruses will serve as priors for our statistical models (Aim 2).

Determining how virus interactions shape antigenic signatures

The wildlife reservoirs of EBOV, BDBV, TAFV, and SUDV remain unknown. Outbreaks of ~~Ebola virus disease~~EVD caused by EBOV, SUDV, and BDBV have all occurred either in the Democratic Republic of Congo or nearby in Sudan and Uganda. The close spatial relationships of these outbreaks suggest the possibility that these viruses may co-circulate in certain bat species, populations, or multi-host communities. Furthermore, the degree of antigen-specific cross reactions between EBOV, BDBV, SUDV, TAFV may confound current serologic testing studies that rely on a single antigen or only investigate EBOV immunoreactivity. Thus, antibodies induced by BDBV or TAFV infections may seemingly be interpreted as evidence of prior EBOV infections. We aim to determine separate three distinct scenarios that may shape serological profiles, **(a) exposure to one virus and cross-reaction with the second, (b) independent exposures to both viruses, or (c) exposure to a third, uncharacterized virus with cross-reaction to both viruses.**

Approach: Multiple Immunization: As described above, ~~W~~we will sequentially immunize ~~a~~ sets of rabbits with a GPe ~~against~~ antigens from EBOV, BDBV, and TAFV; or BDBV and SUDV, ~~two viruses from the same family~~ to simulate multiple exposures, determine the additivity of spectral signatures, and test our ability to extract the~~ir~~ signature of each via our statistical models (Aim 2). For example, rabbits will be first immunized with BDBV GPe (100 µg) then EBOV GPe (100 µg), EBOV then BDBV, or TAFV then EBOV. The immunization pairings simulate hypotheses of common circulation in wildlife such as ambiguous EBOV and BDBV serologies, and overlapping EVD outbreak regions where BDBV and SUDV are known to circulate. ~~fo…~~

We will apply a similar approach to address these three scenarios for henipaviruses, focusing on the more recently described rodent-associated henipaviruses. Mo~~j~~jiang virus (MojV)~~V~~ was detected in the southern province of Yunnan, China, whereas LayV has been detected in ea~~s~~stern Shangdong and Henan provinces. Reports of shrew-associated henipaviruses in South Korea, Gamak virus and Daeryong virus, have contributed to an expansion of rodent-associated henipaviruses discovered since 2020. In Thailand we have detected serologic evidence infection by a MojV-like henipavirus (see Aim 3). As the field does not yet understand the antigenic-relationships among these rodent-associated henipaviruses we cannot confidently interpret serologic evidence of MojV-like in Southeast Asia. Thus, similar to the experiments described for the ebolaviruses, we will sequentially immunize rabbits with multiple Asiatic rodent-henipaviruses to examine how imprinted antibody memory and heterologous boosting shapes reaction and cross reactions to homotypic and heterotypic ebolavirus GPe and GP1, and henipavirus RBP and F antigens. The effects of heterologous boosts will be utilized to refine antigenic shape modeling in Aim 2.;

Approach: Measuring Cross-Binding Interactions: Antigen-antibody binding is context-dependent; the degree of binding may vary due to the presence of other antigens to which antibodies exhibit preferential binding. We will measure the extent of this *cross-binding interaction* by conducting *in vitro* "leave-one-out" assays, where the virus-specific **antigens matching each antiserum** (both specific and sensitive), are removed from the panel and ~~only~~ forcing only cross-reactions ~~can~~ to occur. This simulates the detection conditions of antibodies ~~to~~ induced previously by uncharacterized viruses – shadow viruses – in the

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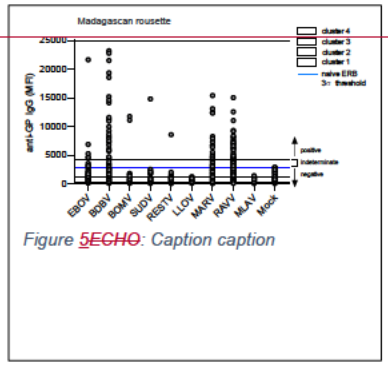
antisera. We will measure the difference in cross-reaction strength in the presence and absence of the ~~with and without the~~ target antigen, and determine the dilution levels that minimize this effect.

Approach: Testing consistency in non-model organisms: To further test the consistency of cross-binding interactions beyond our rabbit animal model~~s organisms~~, we will also conduct leave-one-out experiments within wild-type sera collected from bats and WHO polyclonal reference sera for EBOV, MARV, and NiV. Through a collaboration with Dr. Vincent Munster (~~other significant contributor,~~ OSC; see Letter of Support), we identified BDBV-like ebolavirus immunoreactivity in populations of hammer-headed fruit bats (unpublished data). Hammer-headed fruit bats have been cited as putative EBOV reservoirs [PMID: 17848072, PMID: 31574111~~cite~~], yet in our GPe-based MMIA we detected greater immunoreactivity to BDBV. The distribution of these bats in southern Uganda is consistent with BDBV-driven EVD outbreaks. In addition, through ongoing research with Dr. Cara Brook (OSC; see Letter of Support), we identified 16% seroprevalence for a BDBV-like ebolavirus in populations of Madagascan rousette bats (~~Figure 5 ECHO~~). The result was surprising for two reasons, a) rousette bats are a reservoir of marburgviruses, not ebolaviruses [PMID: 19649327, PMID: 33219802~~cite~~] and b) Madagascar is >2500 km from Uganda where all confirmed BDBV outbreaks have been reported [PMID: 34467242, PMID: 21122234, PMID: 25910637~~cite~~]. These two bat species do not share overlapping geographic distribution, so how do we interpret seemingly similar serological profiles suggesting BDBV circulation? We propose to test the hammer-headed fruit bat and Madagascan rousette bat sera in multiplexes that leave-out the BDBV GP antigens, and to examine how the polyclonal antibodies shift binding patterns to other ebolavirus GP antigens. These shifts in serological profiles in the absence of BDBV GP antigens will be compared to the cross-reaction data established by the protein immunized rabbit antisera standards. We expect that the hammer-headed fruit bat sera would increase preferential binding to EBOV GP in the absence of BDBV GP, consistent with phylogenetic data, providing compelling data that these bats have a high probability of hosting BDBV. Whereas we may expect the Madagascan rousette bat sera to bind to ebolavirus GP in serologic pattern that was not predicted in polyclonal rabbit antisera standards. We anticipate that unexpected serological profiles, which break the ~~serolgoical~~serological standards, will help us to identify unique clusters of serogroups that would indicate unknown ebolaviruses.



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Risks, limitations, and mitigating factors

We have proposed to establish antibody responses and antisera cross reactions via protein immunization and rabbits as our animal model. In the absence of WHO serology standard for all ebola-, marburg-, and henipaviruses, the development of antigen immunized rabbit polyclonal standards is a feasible goal. The uniqueness of the bat immune response among mammals has been an area of research interest for over a decade. The growing body of literature suggests that bats as an Order may have a uniquely adapted immune response including dampened inflammatory responses, and heavily favored innate immunity [67-70]. Thus, there may be distinctions unique to the bat humoral response to viral infections that we will not be reflected by protein immunizations in a rabbit model. However, outside of immunoglobulin ~~(Ig)~~ repertoire sequencing [PMID: 20816694, PMID: 20162414~~eric cite~~], the adaptive response has been less readily investigated, and questions about the functional role of cellular and humoral immunity for viral infection control remains. From field-collected bat sera, we observe robust virus-specific IgG responses, and do anticipate that conservation of humoral responses between rabbits and bats will reflect our ability to accurately determine serogroups through antigenic cartography. Further, we can verify selected rabbit-protein immunization results in a nascent bat infectious disease model being developed by ~~application OSC,~~ Dr. ~~Vincent~~ Munster ~~(see Letter of Support). W e We~~ have previously collaborated with Dr. Munster to investigate whether we can differentiate homotypic and heterotypic ebolavirus antibodies collected from captive Egyptian fruit bats experimentally infected with replication-competent vesicular stomatitis viruses expressing transmembrane bound ebola- and marburgvirus GP (Figure ~~MIKE~~1).

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AIM 2: NOVEL STATISTICAL MODELS FOR MULTIPLEX SEROLOGICAL ASSAYS

Preliminary data: We and others have used clustering approaches [46] to establish virus-specific seropositivity thresholds relying on our knowledge of confirmed virus-host interactions [8, 60, 71, 72]. However, we find that in many cases the required assumptions of this approach – that individuals separate out into distinct positive and negative groups, and that clusters conform to expected, usually symmetric distributions – break down. Rather, large numbers of antibody responses fall into an indeterminate range without distinct breaks, and nonlinear assay response breaks down expectations at the high and low end of the response range (~~Fig~~ Figure 6~~HOTEL~~). As a result, many results, are classified as indeterminate or assigned a serostatus with high uncertainty. On the other hand, we have extended these cluster methods to multidimensional panel data and found that they are more successful at disentangling groups of sera by their ratio of response to different antigens, indicating the *kind* of antigenic response had distinct differences, while magnitude of response was a more continuous measure (Fig ~~BOB~~ure 7). This initial work indicates that clustering can detect distinct signals of samples that react to viral antigens in different ratios, indicating exposure to different viruses in intermediate antigenic space between known filoviruses. However, these multidimensional models conflate two components of these data – distinct reaction ratios as signals of exposure to different viruses, and clustering of the strength of serological response between seropositive and seronegative individuals. We propose a new class of multidimensional models for serology panel data that take a mechanistic approach to representing these two processes, disentangling the different types of clustering and deal with their separate complexities

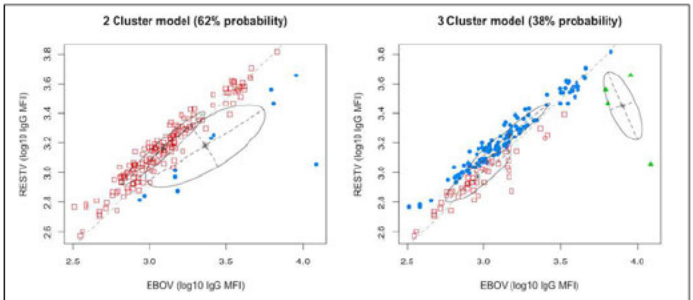

Figure 7~~BOB~~: Clustering of serological responses of Liberian Hipposideros spp. Best-fit indicates clusters binding to both RESTV and EBOV glycoproteins in clusters with distinct ratios of response, suggesting the signal of two two viruses, one primarily reacting to EBOV, the other to both RESTV and EBOV similarly, and possibly a set of individuals exposed to both.

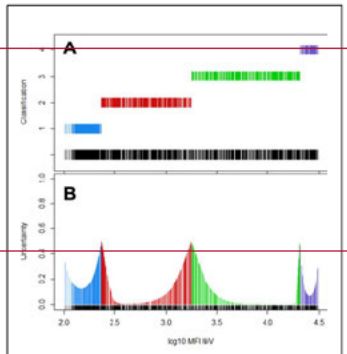

Figure 6~~HOTEL~~: Degenerate clustering in real antigen data. (A) Latent cluster models divide data into four rather than two clusters due to lack of distinct breaks, with central clusters interpretation ambiguous. (B) Measure of cluster uncertainty shows that distinction between clusters is weak over a wide range.

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Detecting shadow viruses in panel data via antigenic signatures

Model structure: Our minimal model of serological mixing is

$$R_i = \sum_{j=1}^{M} f_{ij}(t_j) + \varepsilon_{ij}$$

Here R_i ,the *response* (measured in as the log_{10} mean fluorescent intensity), is the sum of the responses f_{ij} of assay i against each antibody titer t_j that binds to the antigens presented on that assay, plus measurement error ε_{ij}, for all M antigens present in the sample. If the response curve f_{ij} is linear this model resembles that of Schuh [48], as well as the structure of approaches to signal extraction from remote sensing imagery [49]. but due to saturation of the biochemical or instrument response, it is typically sigmoidal.

Cross-binding interactions in the multiplex assay, where antigens in the same solution attract the same antibodies, modify the additive framework of the spectral mixing model. In the absence of these

interactions, we could fully test the ability of the multiplex panel to identify the signal of a novel "shadow virus" *in silico* simply by removing the response data from one target antigen and determininge if it could be identified via cross-reactions of antibodies against closely related viruses. However, with these interactions, the response of antigen j against antibody i depends on the presence of the other antigens present. We can decompose this effect in our model into the core ~~titer~~antibody-response function $f_{ij}(t_j)$ and an additional interaction term

$$R_i = \sum_{j=1}^{J} f_{ij}(t_j) + \sum_{j=1}^{J}\sum_{i=1}^{I} g_{ij}(t_i, t_j) + \varepsilon_{ij}$$

Fully estimating $g_{ij}(t_i, t_j)$, especially with nonlinearities in the response, would not be realistic. But nonlinear models from image processing provide reasonable approximations where these interactions take a set of reduced forms [50, 51]. Moreover, we can constrain the model with priors based on our experimental data. Our leave-one out experiments will estimate the size, consistency and linearity of the cross-binding interaction effects in both model and wild organisms and optimize our procedures to minimize it.

~~*Experimental a*~~*Approach:* We will develop a Bayesian representation of this model and conduct simulation-based experiments to determine the conditions under which it can distinguish between different immunological conditions. We will test the ability to distinguish between a target virus and related shadow virus response as a function of antigenic distance, breadth of cross-reaction of both the target and shadow virus, sample and individual level variation, and sample size. We will use data from our challenge experiments to parameterize the models, initially using preliminary data so that we can conduct these aims in parallel. We will use the data from our experimental replicates to simulate sample-level variation and data from previous surveillance work (see Aim 3) to simulate individual variation in response within seropositive and seronegative groups. We will also simulate a range of scenarios for cross-reactive binding interactions, ranging from small, consistent effects to highly variable interactions amongst different viral pairings. We will conduct both *in silico* and *in vitro* validation tests to determine if the method can extract the antigenic signal of a shadow virus. We will create simulated leave-one-out tests by using data from our rabbit immunization experiments, with data from each virus in the panel removed in turn, and determine if the model identifies the signal of a non-target virus from cross-reactions alone. We will repeat this with the data from our *in vitro* leave-one-out experiments.

Cross-scale immuno-epidemiological modeling

Zoonotic serosurveillance, in wildlife and human populations, faces the challenge of a lack of controls. Surveillance requires testing many understudied host species, and by definition no standard controls can be created for novel viruses. Thus, standard cut-offs cannot be pre-determined and inference of individual immune states and population seroprevalence and risk factors is best conducted jointly, modeling individual serostatuses as continuous or probabilistic states informed by population measures.

Model structure: To build this cross-scale framework, we extend our mixing model above to the population scale. Titer t_j becomes t_{jk}, a sample k drawn from a population of individuals $\boldsymbol{x}$. In this population with a seroprevalence $E(\omega_j)$, the distribution t_{jk} is a mixture of titer values ($F(\mu, \sigma)$) in both seronegative and seropositive individuals.

$$E(t_{jk}) \sim (1 - E(\omega_j))F(\mu_{j-}, \sigma_{j-}) + E(\omega_j)F(\mu_{j+}, \sigma_{j+})$$

Both the individual probability of seropositivity ω_{jk} and the distributions $F()$ of seronegative and seropositive distributions are affected by host traits, infectious dosage, and environmental factors. Immunological state has both binary (serostatus) and continuous (antigen-reactive IgG levels, *concentrations*~~titer~~) components that are intertwined. In the simple case assuming $F()$ are normal distributions and those traits and risk factors have linear effects, these can be represented as the relationships

$$\rho \sim \text{Probit}(\beta_{\rho 0 j} + \beta_{\rho j} x_k);\ \mu_{j-} \sim \text{Normal}(\beta_{\mu 0 j-} + \beta_{\mu j-} x_k);\ \mu_{j+} \sim \text{Normal}(\beta_{\mu 0 j+} + \beta_{\mu j+} x_k);$$

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with seroprevalence ρ, seronegative ~~titer~~ concentration μ_{j-}, and seropositive ~~titer~~ concentration μ_{j+} modeled as a joint distribution, and the β values the marginal effect of risk factor on both seropositivity and antibody ~~titer~~concentration.

This allows us to integrate estimation of the factors affecting the probability of individual seropositivity (demographics, geography, host species), along with how those factors modify the ~~titer~~ antibody concentration or response within seropositive or seronegative groups. For instance, if juvenile hosts have stronger responses than adults, but also are less likely to be exposed to the virus of interest, these will separate out under this approach. Under a traditional threshold-based approach these effects would mask each other.

One application of this joint modeling of the continuous and binary components is robust cross-species seroprevalence comparisons through combining mixed-effect structures [73] with this model framework. In many serosurveillance studies, large numbers of species are screened for evidence of antigen response to determine likely viral reservoirs, but species sample sizes vary due the difficulty of capture. This poses a challenge in comparing seroprevalence among species. Also, baseline or seropositive response binding levels may vary along with seroprevalence, and for some species sample numbers are not sufficient to establish species-level baselines. The joint approach will enable partial-pooling of species-level effects on both seroprevalence and ~~titer~~antibody concentration, reducing noise from small size groups while capturing important differences.

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~~*Experimental a*~~*Approach:* We will simulate application of this model for two common surveillance inference problems. In each, we will compare this joint estimation approach to two-step threshold-based approaches based on outlier detection or simple clustering.

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First, we simulate determining seroprevalence in a single group (species) of hosts, the probability of seropositivity and the strength of seropositive response are correlated. We will determine how seroprevalence, degree of correlation, sample size affect the accuracy of each method.

Second, we will perform studies of the case of sampling many species with mixed baseline and seropositive response rates to compare seroprevalence rates. Will simulate and fit from a mixed-effect model of the form

$$\begin{bmatrix} \mu_{-} \\ \mu_{+} \\ \text{Probit}(\rho) \end{bmatrix} \sim MVN(\beta_0 + \beta_s \text{species} \ ) + \epsilon; \quad \beta_s \sim \text{MVN}(\mu_s, \sigma_s)$$

where the response distributions and seroprevalence vary as joint random effects by species. We will compare the mixed-model structure to a form where each species is evaluated independently and models where all species are pooled. In both cases we also compare two-step threshold-based approaches to the joint approach. We will use realistic sample and group sizes based on previous multispecies serosurveys [74], varying the size of interspecies differences and the evenness of sampling across groups to determine where the mixed-effect joint approach has the greatest differences from the alternatives.

Statistical approach and implementation:

All our simulation experiments will used a principled Bayesian framework [75] of simulating data from a structured model and testing the ability of the model as well as alternatives to retrieve the original parameters. We will implement models in the Stan probabilistic programming language [76], using Hamiltonian Markov Cain Monte Carlo to estimate model parameters. The R language [77] and will be used for data and model manipulation, workflows, and visualization.

Creating reusable software tools

We will develop an open-source software package for fitting, visualizing, and interpreting these models, written in the R computer language [77]. To maximize usability, we will test reduced-forms of all models, as well as multiple model-fitting algorithms including HMCMC, variational inference or maximum-likelihood optimization. For each we will measure model performance differences and model runtimes on both standard laptop on high-performance environments. The software package will include the ability to select amongst these options and include sensible defaults and guidance on trade-offs so that users can run tests in their compute environment. Reaction-cross reaction curve data and interaction will be packaged together with the software for users to use in their own model calibration.

Software will follow community best practices for software usability, maintenance and algorithmic testing and correctness as developed under the rOpenSci Project [78, 79] . We expect conduct training on software use for staff across all the cooperating surveillance projects as well as teach at least one workshop at an applicable conference in Year 4 or 5 of the projects. See RESOURCE SHARING PLAN and DATA MANAGEMENT AND SHARING PLAN for additional detail on software standards and dissemination.

Risks, limitations, and mitigating factors

Several factor may limit the scope of applicability of these approaches. First, the high dimensionality and thus parameter size of these models may limit their use to large sample-size studies with moderate to high seroprevalence levels. Our pilot studies (Aim 3), have sample sizes of hundreds to thousands, with initial estimates of seroprevalence in the range of 15-20%. Second, if cross-binding interactions are strong between antigens in our panels as well as ~~validation~~ verification tests in wild-type bat seraum~~,~~ it will make it difficult to distinguish cross-binding from a shadow virus signal within close antigenic distance. This will effectively increase the minimum antigenic distance needed to separate. However, even if strong, cross-binding is expected to predictably reduce with antigenic distance. That can be exploited to incorporate into explicit structures that predict associated uncertainty in the model structure or in model diagnostics.

AIM 3: APPLYING NOVEL METHODS TO OPEN QUESTIONS IN SPILLOVER IMMUNOLOGY

Our discovery panels and statistical models have direct applications to current open questions in serological surveillance. We will apply Aims 1 and 2 and leverage active serosurveillance projects where the identities of known or shadow viruses in sera from wildlife and humans could not be determined with current multiplex assays and statistical analyses. As testing will be conducted by and with local project teams, these projects will also serve as mechanisms for transfer and training and to refine protocols for publishing.

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Henipaviruses in human community surveillance in Thailand

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Preliminary Data: As part of the NIH CREID EID-SEARCH in Thailand, team members have been conducting clinical and community surveillance of humans in contact with wild bats cia guano mining. We implemented our standard (non-discovery) filovirus and henipavirus glycoprotein antigen-based panels for MMIA testing. During these research activities, we identified that 19% (54/284) of study participants sampled near the Khao Chong Phran Non-hunting Area in Ratchaburi Province, were specifically immunoreactive with the MojV G (Figure 8~~XXX~~). We also identified one participant with a distinct serological pattern that was had cross reactive and preferential binding to GhV, HeV, and CedV antigens. These data provide preliminary evidence that study participants may have been infected with two serologically distinct henipaviruses, one that is MojV-like and one that is ancestral to HeV/CedV and GhV-like. However, their identities indeterminate, and other hypotheses such as exposure to multiple viruses are plausible.

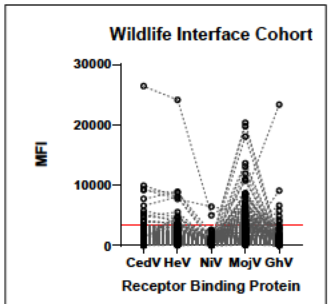

Figure 8: Response of sera from Thai guano harvesters to henipavirus ~~G proteins~~RBP antigens, including a group with anti-~~Mòjiāng virus~~MojV IgG ~~response~~ and another with a broad sero-re~~re~~sponse across ~~both~~

Approach: In Year 3 of this project, we will re-test sera from individuals with high wildlife contact currently being collected under the EID-SEARCH project using our full discovery 38plex antigen panels developed in Aim 1. With current archival sera and planned collection in the next two years, we estimate we will test 550 samples. Using the spectral mixture approach developed in Aim 2, we will fit models to determine (a) whether individuals with response to MojV were exposed to MojV or a closely henipavirus, and (b) whether individuals with response to GhV, CedV, and HeV were exposed one of these with cross-reaction to the other, multiple henipavirus with cross reaction to both or a new shadow virus. Using the antigenic mapping approaches, we will determine the relatedness of any shadow viruses to known henipaviruses. We will use our multi-scale models to determine how the presence and strength of response to each of these known or shadow viruses varies with sex, age, and occupational exposures.

Expected results and limitations: ….

Filoviruses in Egyptian fruit bats in South Africa

Preliminary Data: Through ongoing research activities with co-investigator Dr. Wanda Markotter (University of Pretoria), our team has detected seroprevalances of 17.5% (77/440) for an unknown ebolavirus that is most antigenically-like EBOV/BOMV, as well a 4.5% (20/440) for RAVV marburgvirus ~~(Figure KILO A)~~ in *R. aegyptiacus* (Figure 9A-B). This degree of co-positivity is unexpected based on the WHO EBOV antisera, which favored preferential cross-reaction between EBOV and BDBV (F~~ig~~igure 2A~~FOXTRA A~~). This data is highly suggestive of the existence of a~~n~~ novel intermediate ebolavirus that is antigenically equidistant from both EBOV and BOMV.

Approach: Years 3 and 4 of this project we propose to test *R. aegyptiacus* and other bat sera collected from across Southern Africa using our panel and methods. Under the SABRENET project, over 15,000 samples are expected to be collected via longitudinal and cross-sectional sampling across Southern Africa Based on current seropositive rates and planned sampling, we estimate 3,000 samples will have nonzero signals of a filovirus. Using a case-control approach we will select matching seronegative samples from collections for a total of approximately 6,000 samples. We will adapt our multi-scale models to a spatiotemporal framework to model the dynamics of these shadow viruses across different bat colonies.

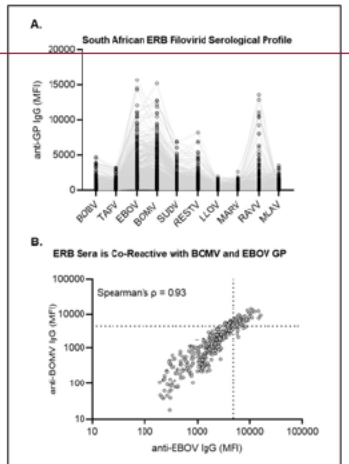

Figure 9: A) Sera from Egyptian fruit bats (ERB) were screened for filovirus-reactive antibodies. Anti-GPe IgG peaks were observed for EBOV, BOMV, and RAVV. B) BOMV and EBOV IgG correlations were strong, and the same seropositive serum samples were equally reactive with both ebolavirus GP antigens.

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Expected results and limitations: ….

Linked serological-epidemiological models: Viral circulation in NiV reservoir* Pteropus *colonies

Preliminary data: In our previous and ongoing work studying NiV in Indian flying fox (*Pteropus medius*) colonies in Bangladesh, we found cyclical but non-seasonal patterns in seropositivity and viral shedding in a colony complex, a study we are now repeating across several colonies to understand geographic variation in seasonal shedding. We also found evidence of annual circulation of other filoviruses a, based on antibodies binding to EBOV and Menangle virus (genus *Rubulavirus*) envelope protein antigens. Data collected in these serological studies was analyzed using a Bayesian cluster modeling to determine a threshold IgG level for serostatus, and threshold-based serostatus used as a binary variable in subsequent epidemiological analyses. However, this approach masks considerable complexity and uncertainty in the relationship between the individual binding levels in actual serostatus. For instance, for filovirus responses, the cluster of "baseline" responses of presumed seronegative bats increases with age, while in pre-weaned bats, an upper cluster of "seropositive" individuals has considerably weaker responses (Figure 10, publication *in prep*). Binding response also appears to change both seasonally and interannually.

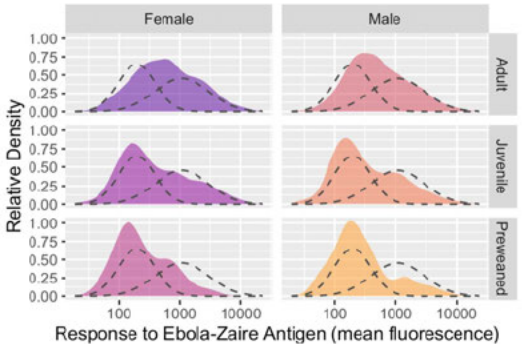

Figure 10: Clustering of binding response of Bangladeshi Pteropus *sera to Ebola-Zaire antigen (filled densities), with clusters distributions from a Bayesian mixture model (dotted lines). Model thresholds do not capture within-cluster changes across demographic groups, which confound measures of seroprevalence.*

Approach: We will use the antigenic relationships developed in Aim 1, and the cross-scale modeling approach developed in Aim 2, to re-analyze longitudinal and spatial extensive data on NiV, filo-, and rubulavirus binding response in *Pteropus* colonies. We will model the changes in baseline response binding (μ_-), seropositive response binding (μ_+),, and seropositive binding probability (ρ , i.e. seroprevalence) as joint responses of a spatio-temporal model

$$\begin{bmatrix}\mu_- \\ \mu_+ \\ \rho\end{bmatrix} \sim \text{Multivariate}(f(\beta_0 + \beta_1 \text{sex} + \beta_2 \text{age} + \text{s}(\text{time}[\text{annual}, \text{seasonal}], \text{location}))) + \epsilon$$

Where $\text{Multivariate}(\dots)$ is a joint multivariate normal-probit distribution representing the correlated response of all three outcomes, $f(\dots)$ is the pseutotiter-response relationship, and $s(\dots)$ is a term with a spatio-temporal correlation structure. We will test several joint hypotheses by determining the consistency of posterior model results: Does baseline serological response increase with age, and how much does this vary across viral types? Does average seropositive response (as well as seroprevalence) decline following peaks of seroprevalence, consistent with antibody decline following an outbreak? Do maternal traits determine the strength of vertical transmission of antibody titer?

Expected results and limitations: ….

RESOURCE SHARING PLAN

I. REAGENTS

~~---~~Protein antigens and commercially sourced polyclonal rabbit antisera will be made available upon reasonable request.

II. PROTOCOLS

Protocols for generating reagents, performing assays, etc….will be published on https://www.protocols.io/

III. SOFTWARE

As part of activities in statistical model development we will create a software package to enable researchers to implement their methods. The program will be implemented in the R language (with Stan and C++ components as needed). It will be written to be compatible with Windows, macOS, and Linux platforms and tested on each. Algorithm implementation, code style, user and documentation will all follow rOpenSci standards for statistical software implementations (https://stats-devguide.ropensci.org/). It will be released under an Open Source Initiative-compatible license.

The software will include both reference documentation and tutorials on implementing the software on example data. We will test both software usability and documentation quality by distributing alpha versions to researcher, students, and postdoctoral scholars at PI and co-I institutions and collecting feedback. Beginning with alpha versions, the software will be publicly released on a platform inviting user reports.

The software will be published on several platforms allowing easy retrieval and installation (CRAN, R-Universe, GitHub, Zenodo). A project website will be created for documentation. We will maintain the software, including bug fixes, and updates to maintain compatibility with changing dependencies and platforms, for 5 years after the end of the funding period. Containerized versions of the software at any major release, packaged together with all dependencies and compute environment, will also be archived so that archival analyses using the software may be run even after the end of maintenance.

See DATA MANAGEMENT AND SHARING PLAN for further details on publication, archiving and standards.

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VERTEBRATE ANIMALS

DATA MANAGEMENT AND SHARING PLAN

I. DATA TYPES

Types and amount of scientific data expected to be generated in the project

This project will produce data on antigen binding responses via multiplex bead array assays, as generated from Bio-Rad Bio-Pplex and Luminex MAGPIX multiplexing Sysstems. These assays will be performed on sera from hyperimmunization challenge experiments in rabbits, human serology a standards for select viruses, and archival sera samples from wildlife surveillance studies in South Africa, Republic of Congo, Madagascar, and Thailand as well as human surveillance studies in Thailand. Calibration data, including measures from blank and mock samples, will also be generated.

The project will produce sample-level metadata for challenge experiments and standard dilutions. It will aggregate copies of metadata associated with archival samples from other research projects projects. The latter will include host species, traits, time and location of collection of animals sampled, and demographic information of humans sampled.

The Bio-Pplex and MAGPIX systems will generate raw data in XML and XLSX files, with XML files being comprehensive logs of run metadata, machine calibration, and all measurements per sample, and XLSX being sample-level total measurements. Sample-level metadata will be collected in CSV and XSLX form.

Across experimental and archival samples, we expect to generate approximately 7,000 tests on 90 plates, totaling approximately 3.5MB of processed XLSX files and 440MB of raw XML files.

The project will also produce synthetic data from simulations and performance data from experiments. These will be generated in RDS (R Object files), and parts computationally intensive or difficult to reproduce will also be exported as CSV files. The project will produce computer code for both analyses and as a primary product in the form of reusable software packages. These will be in standard UTF-8 text of computer languages including, R, Stan, and C++, as well as compiled executable binary files.

Scientific data that will be preserved and shared

Excepting identifiable data associated with human samples, all data produced in the course of this project will be publicly shared. Final sample-level cleaned and joined data and metadata will be shared as CSVs, as well as raw Bio-Pplex or MAGPIX software XML data. All computer code created will be shared. Synthetic data and summaries of simulation experiments will be shared in summarized form, with raw data shared as reproducible code that can regenerate the exact results, excepting data that is computationally-intensive and therefore more efficient to share in raw form.

For data associated with human samples personally identifying information will be not be shared. Personal individual-level data will be removed, demographic and location data will be binned to prevent re-identification, with location information binned to the province (ADM-2) level and age aggregated to bins of 10 years or more. Other fields (e.g. occupation) will be analyzed to determine potential for re-identification and removed or binned as necessary.

Metadata, other relevant data, and associated documentation

This project will also produce as primary outputs standard operating procedures and instructions for performing the assays under development, documentation to reproduce simulations and statistical analyses from provided code, as well as user documentation and tutorials for the statistical software that will be created. Descriptive and structural metadata will be included in deposits for each data set.

II. RELATED TOOLS, SOFTWARE AND/OR CODE

All produced data formats (CSV, JSON, XML, XLSX) can be read by multiple, widely available free and open-source tools, with only RDS files specifically requiring the free and open-source R language.

III. STANDARDS

In general, all data will be reported using the Frictionless data standard, as both an “umbrella” framework and a general approach to data sets without domain-specific standards. The Frictionless data standard provides a framework for consistently packaging data stored in most inoperable file formats. We will use accompanying Frictionless JSON files to describe CSV metadata, with additional standardized fields

specific to the data repository used (e.g., Zenodo). Where applicable, we will use the NIH Common Data Elements (CDE, https://cde.nlm.nih.gov) for relevant fields.

Formal standards for non-target and quantitative serological data have not yet been widely adopted, and the development of essential reporting quantities is an anticipated output of this project. However, we will aim to ensure the data reported is, where applicable, compatible with standards for serological studies, notably the NIH ImmPort data model and its recommended ontologies for proteins and diseases.

Spatiotemporal and taxonomic metadata on wildlife samples will follow the Darwin Core Standard.

Computer code will be released using two standards. Software packages for reuse will be in the format of an R packages following the rOpenSci standards for statistical software implementations (https://stats-devguide.ropensci.org/) and CodeMeta standards for software descriptions (https://codemeta.github.io/), under an Open Source Initiative-compatible license. For computer code to reproduce simulations and statistical analyses, we will use git version controlled code repositories, the "Make" and "targets" frameworks to define dependencies between steps of analysis, the "renv" and "venv" frameworks for recording software dependencies, and Docker containers to capture the machine computing environments. Binary machine images of the containers will be stored alongside the source code.

IV. DATA PRESERVATION, ACCESS, AND ASSOCIATED TIMELINES

Repository where scientific data and metadata will be archived

We will archive all data and metadata generated, as well as all computer code, on the Zenodo archive. A subset of data generated is appropriate for NIH's ImmPort repository and will be cross-deposited there with appropriate semantic references to describe the relationships between data. Protocols will also be deposited on protocols.io.

How scientific data will be findable and identifiable

All data on Zenodo, NIH ImmPort, and protocols.io will be identifiable DOIs and have searchable metadata deposited with DataCite and indexed by search engines. In addition, we will mirror data on non-archival sites that are popular and accessible for their features and search optimization: DoltHub for data, GitHub for source code, and R-Universe for installable R software binaries.

When and how long the scientific data will be made available

Data associated with publications will be shared in its processed and cataloged form upon release of associated products (preprints, software, or publications upon acceptance), or the end of the period of performance (inclusive of any approved extensions), whichever comes first. The duration of preservation and sharing of the data will be a minimum of 10 years after the funding period.

V. ACCESS, DISTRIBUTION, OR REUSE CONSIDERATIONS

Factors affecting subsequent access, distribution, or reuse of scientific data

There are no anticipated factors or limitations that will affect the access, distribution or reuse of the scientific data generated by the proposal.

Whether access to scientific data will be controlled

All data generated by this project will be released in full excepting data aggregated from other projects with personally identifiable data.

Protections for privacy, rights, and confidentiality of human research participants

Where data used by this project from other projects is linked with personally identifiable information, only de-identified information will be released. Identifiable data will not be retained. Requests for the identifiable data will be referred to the original projects and institutions.

VI. OVERSIGHT OF DATA MANAGEMENT AND SHARING

PI Ross (ORCiD 0000-0002-2136-0000) will be responsible for oversight of project data management and release of data. The project research assistant at EcoHealth Alliance will be responsible for management of the project database, maintenance of project metadata, aggregation of data from project partners, and releasing data to appropriate repositories under appropriate standards. A data librarian at EcoHealth Alliance will support this role and act as an organizational fallback for data maintenance. Data release will

take place after agreement amongst PIs from all contributing institutions. State of data management and release will be a standing topic at project quarterly and annual meetings.

PROJECT NARRATIVE

This project develops new methods to identify emerging and zoonotic viruses by analyzing the virus-induced antibodies present in wildlife and human populations in contact with wildlife. It will create both new laboratory techniques to test for those antibodies and new mathematical models to analyze data to identify the trace of those viruses. It will test these methods in partnership with international virus surveillance projects in Bangladesh, Thailand, and South Africa.

PROJECT SUMMARY/ABSTRACT

Serosurveillance provides powerful measures of the infectome of wildlife and humans that can shed light on the disease ecology of zoonotic viruses. Yet serological data is plagued with ambiguities that limit our ability to use it to identify zoonotic disease reservoirs or identify populations at risk from exposure to emerging viruses. These issues include cross-reactivity of antigens and antibodies and complex, nonbinary immune responses. The major goal of this proposal is to dramatically improve the utility of antigen-based serosurveillance for new viral discovery and epidemiology of emerging zoonotic diseases by addressing the challenges of cross-reaction and statistical rigor. We will create discovery panels - qualified and optimized panels of multiplexed immunoassays designed to identify a characterize previously unknown viruses only detected through the antibody response of hosts. We will develop new statistical approaches to extract the signals of novel viruses from those of known viruses or mixtures of antibodies from complex histories multiple previous viral exposures, as well as integrate these into models into population-scale epidemiological models. Finally, we will partner with international viral surveillance projects to apply these techniques to identifying and characterizing filoviruses and henipaviruses in bat populations in South Africa and at-risk wildlife workers in Thailand, and to modeling immune-epidemiological dynamics of Nipah virus circulation in bats and people in Bangladesh.

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FOREIGN JUSTIFICATION

This project concerns zoonotic infectious diseases found in wildlife and human populations globally and with the potential to spread and cause outbreaks in the United States. Study populations of interest – high animal-contact workers in Thailand, and known filovirus host bat species in South Africa – were selected because prior results indicated the presence of antibodies to novel viruses that could be better characterized using the methods developed in this proposal. In both cases, the systems are already being studied under U.S. federally-funded disease surveillance projects: The NIH CREID Emerging Infectious Diseases - South East Asia Research Collaboration Hub (U01 AI151797), the DTRA BTRP South Africa Bat Research Network (HDTRA12010025).

In the case of surveillance in Thailand, the lead expert in the disease ecology and epidemiology of the system of interest is Co-I Wacharapluesadee. Archival sera from study of this population are stored at her institution, King Chulalongkorn Memorial Hospital, and she and her team are best equipped to analyze these specimens and interpret the results. Dr. Wacharapluesadee and King Chulalongkorn Memorial Hospital have a history of collaboration with U.S. institutions under USAID and NIH programs.

In the case of surveillance in South Africa, the lead expert in the disease ecology and epidemiology of the system of interest is Co-I Markotter. Archival sera from study of this population are stored at her institution, University of Pretoria, and she and her team are best equipped to analyze these specimens and interpret the results. Dr. Markotter and University of Pretoria under DTRA and NIH programs.

CONSORTIUM/CONTRACTUAL ARRANGEMENTS

This project is a multi-institutional collaboration led by EcoHealth Alliance, New York (PI Ross, Co-I Epstein) that will subcontract funds in accordance to NIH Institutional guidelines to three other institution:

1. **Uniformed Services University of the Health Sciences, Bethesda, MD** (PI Laing)
2. **University of Pretoria** (Co-I Markotter)
3. **King Chulalongkorn Memorial Hospital, Thailand** (Co-I Wacharapluesadee)

The applicant organization (EcoHealth Alliance) is justified in taking the lead on this project because it specializes in understanding the ecological and virological processes underlying zoonotic disease emergence, and has conducted international, multi-disciplinary and multi-partner research around the world for more than 30 years, including joint projects with the listed institutions EHA administers technological and data-management platforms used by partners as part of these project.

PI Ross oversees modeling, statistical design, and data systems fat EcoHealth Alliance. Dr. Ross, a mathematical disease ecologist, is an expert in complex semiparametric modeling approaches and the role of host and pathogen heterogeneity in disease dynamics. He leads multiple multi-institution projects in scientific and statistical software development [80, 81], disease forecasting (Wellcome 226061/Z/22/Z). He has had core roles in quantitative methods for multi-institution projects under USAID, DTRA, and NIAID, including collaborations with all partner institutions on this project.

EcoHealth Alliance will be the lead for administrative components and oversight of this project, and also be the center of development and testing of quantitative methods, software development, data integration, and overall synthesis. Co-I Epstein at EcoHealth Alliance will provide expertise in the virology, disease ecology, and epidemiology of the populations of interest in Bangladesh.

The subcontract institutions will work on specific issues and areas in which they have proven expertise. These areas are:

- Wildlife virology and immunology, Novel serological and assay development; generation of reagents for novel assays; training and technology for serological and molecular protocol development, of Thailand and South Africa laboratory staff for technology transfer for serological and molecular protocol development (**Uniformed Services University of the Health Sciences,** PI Laing).
- Wildlife and human community surveillance and specimen collection, human clinical or hospital syndromic surveillance, screening of serum specimens using serological assays, disease ecology and epidemiology of zoonotic diseases specific to populations in Southern Africa (**University of Pretoria,** Markotter), and Thailand (**King Chulalongkorn Memorial Hospital,** Wacharapluesadee)

PROJECT LEADERSHIP PLAN

I. BACKGROUND AND MULTI-PI RATIONALE

This research program, *Characterizing unknown emerging viruses through targeted serological profiling*, is inherently interdisciplinary, jointly developing *in vitro* and *in silico* methods for serological viral discovery, drawing from the expertise, teams, networks, and resources of both PIs. This project will be co-led by PIs Dr. Noam Ross (EcoHealth Alliance, New York, NY), and Dr. Eric Laing (Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences (USU), Bethesda, MD). The laboratory and computational components, as represented in Aim 1 and Aim 2, are complementary and co-equal. Each PI brings methodological expertise and experience to their component. Dr. Ross, a mathematical disease ecologist, is an expert in complex semiparametric modeling approaches and the role of host and pathogen heterogeneity in disease dynamics. He leads multiple projects in scientific and statistical software development [80, 81]. Dr. Laing, a virologist, is an experienced researcher in the field of infectious diseases, serological application and profiling, and cellular immunology. He is a co-investigator and associate investigator on multiple virus surveillance projects including Epidemiology, Immunology, and Clinical Characteristics of Emerging Infectious Diseases with Pandemic Potential (EpICC-EID)~~EpICC~~, Prospective Assessment of SARS-CoV-Seroconversion~~PASS~~, EID-SEARCH, SABRENET, and along with OSC Munster is a co-investigator on a pending project, Establishment of a Bat Resource for Infectious Disease Research. -The PIs have a history of collaboration as part of multiple projects in disease surveillance, including serological surveillance of wildlife under DTRA-funded surveillance projects in Malaysia (HDTRA11710037) and South Africa (SABRENET, HDTRA12010025), and NIH-funded research on Nipah virus circulation in Bangladesh (U01 AI153420-01). In each they work(ed) jointly on interpretation of serological data and epidemiological models, serving as the methodological experts within those teams.

Co-Investigators Epstein, Markotter, and Wacharapluesadee all lead active federally funded projects in zoonotic viral surveillance and discovery that make use of serological methods developed by PI Laing. Each provide system-specific expertise in the ecology, epidemiology, and virology of the regions and species in those projects, which are the source of samples and data for our applications for Aim 3.

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II. ROLES AND RESPONSIBILITIES

PI Ross will be the lead of the project, responsible for funder communication, budgeting, and reporting, and oversight of sub-awardees. He will be responsible for project data management, statistical methods and development under Aim 2, and analyses under Aim 3. Dr. Ross will be supported by EHA's administrative staff, including organizational-level finance and human resources directors, a federal grants administrator, a data librarian and computing cluster systems administrator. PI Laing will be the lead laboratory processes and development and site lead for USU. He will be responsible for protein and panel design, challenge experiments, and other laboratory experiments under Aim 1. Both PIs will participate equally in interpretation of analyses and manuscript writing.

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Co-Is Markotter and Wacharapluesadee will be oversee the testing of samples from South Africa and Thailand at their institutions, respectively, and act as consortium leads for University of Pretoria and will liaise with PI Laing for logistics and implementation of protocols. SPs Epstein, Markotter, and Wacharapluesadee will all participate in the (re-)analyses and interpretation of results in Aim 3.

III. PROGRAM MANAGEMENT

COMMUNICATION

PIs will coordinate via monthly video calls. These calls will include other Co-Is quarterly, and more frequently as needed starting with Aim 3 activities in Year 3. At the discretion of PI and Co-Is, other laboratory staff from each institution and OSCs will participate in these meetings. The purpose of these meetings will be to keep all parties informed of the status, progress, and plans. Quarterly meetings will be extended to include presentation of preliminary results. Calls will be coordinated by PIs Ross and Laing with monthly rotation of leadership and responsibility of making agendas for and disseminating notes from each meeting.

PIs will use AirTable and GitHub project management boards, administered by PI Ross and EHA administrative staff, to track task status, including laboratory and computational experiments, testing, administrative tasks, manuscript preparation and publication. EHA's internal content sharing hub (secure.eha.io), will be used for sharing interim reports and preliminary results across project participants.

IN-PERSON MEETINGS

Project PIs will meet in-person at least annually at EHA, USU, or at jointly attended conferences in the last quarter of each project year. Following annual meetings PIs will generate annual reports, using this period to communicate and discuss program status and any possible changes to research directions should information indicate failure of certain directions or the possibility of new opportunities. Decision making will be made by all program's PIs. If issues/topics cannot be resolved at this level then PIs (unlikely) will consult with NIH program staff on future allocation of research funds pending any replacement or revision of the program's goals.

We have budgeted additional funds for laboratory exchanges of staff between EHA and USU. The modeling Postdoc at EHA will travel to USU to train in laboratory methods in Year 1 inform mathematical work. A USU graduate student will travel to EHA to in Year 2-3 to study modeling and statistical methods.

In addition, we will take advantage of existing collaborations to liaise with Co-Is. A USU project staff member is visiting scientist at the CKMH and PI Laing travels to CKMH under EID-SEARCH. PI Ross, PI Laing, and co-I Epstein travel regularly to UP for collaboration under other projects (HDTRA12010025, Wellcome 226061/Z/22/Z).

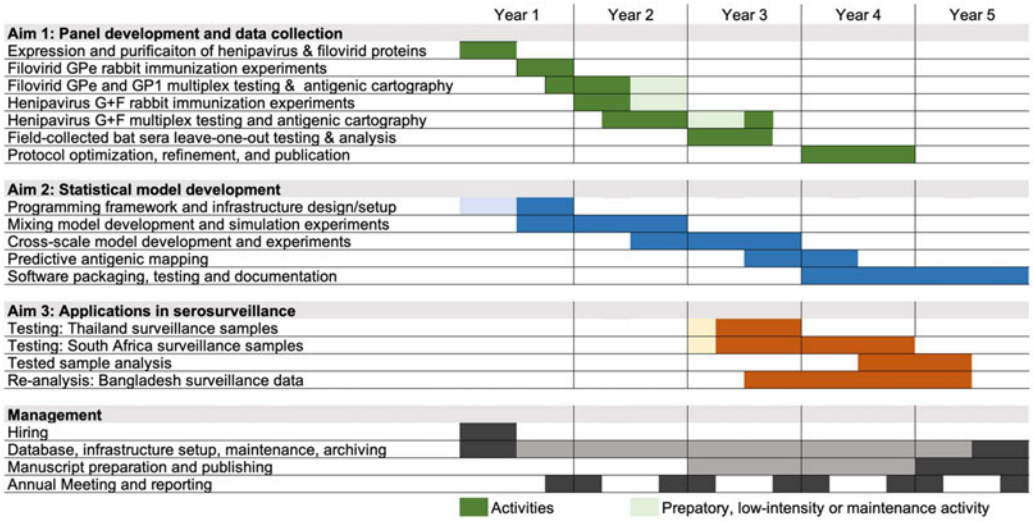
DATA MANAGEMENT

All data and code will be managed in a project database overseen by PI Ross and administered by the project research assistant. All other consortium members will upload data to this database and also maintain local copies in their local information management systems. See DATA MANAGEMENT AND SHARING PLAN for additional details on dat dissemination.

COMPUTATIONAL RIGOR AND REPRODUCIBILITY

Development of model code will follow our framework of *deployment-ready research,* an approach to developing scientific code that can be integrated into reusable software with little additional effort. Code will be maintained in a version control system (git), with versions of models linked to the versions of data they were run against in a versioned database (dolt). Changes to model design or implementation will require code review by a team member. Review will include diagnostics of code quality and comparing output and performance of each version of models, implemented automatically by continuous integration (CI) on our code-sharing platform (GitHub).

PROJECT TIMELINE



COVER LETTER

National Institutes of Health (NIH)
9000 Rockville Pike
Bethesda, Maryland 20892

February 5, 2021

Application for the NIH Research Grant Program Announcement number PA-20-185, entitled NIH Research Project Grant (Parent R01 Clinical Trial Not Allowed), dated May 5, 2020

To whom it may concern:

We are pleased to submit our grant proposal titled "**Characterizing unknown emerging viruses through targeted serological profiling**" for consideration.

Please assign our proposal to the following:

Institutes/Centers: National Institute of Allergy and Infectious Diseases - NIAID

Scientific Review Groups: Transmission of Vector-Borne and Zoonotic Diseases (TVZ)

We know of no individuals (e.g. competitors) who should not review the application.

Our project is aligned with the mission of NIAID because it focuses on detection and characterization of pathogens with potential for emergence, including near neighbors of Ebola virus and Nipah virus, which are priority pathogens. This project addresses NIH's priorities of Immunology and Assay Development, Reagents and Resources, and Epidemiology and Pathogen Discovery under the NIAID Pandemic Preparedness Plan (https://www.niaid.nih.gov/sites/default/files/pandemic-preparedness-plan.pdf).

Two of our subawards, for University of Pretoria (UP) and King Chulalongkorn Memorial Hospital (KCMH), are not active for the first two budget periods of the project. This grant concerns the development, testing, and validation of new methods, with initial development occurring at the main site, EcoHealth Alliance, and consortium partner Uniformed Services University. Testing and validation will involve all consortium partners and begin in Year 3 of the project.

Please note that the proposed work is NOT a Select Agent study because we work only with synthesized antigens and archived sera.

Thank you very much for your consideration.

Sincerely,

(b) (6)

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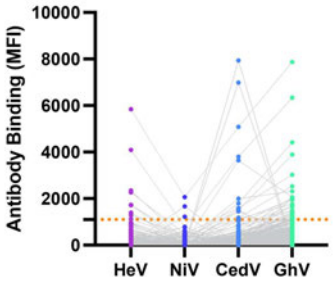
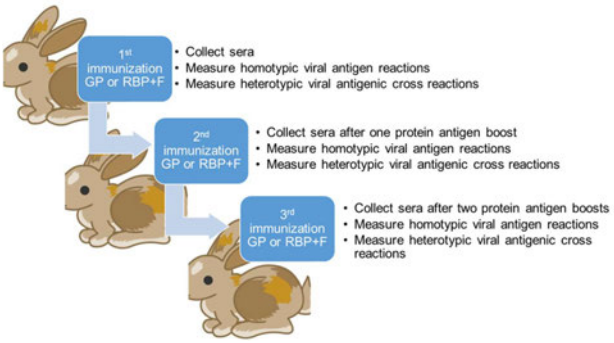
NOTES AND STUFF FROM OLD PROPOSALS

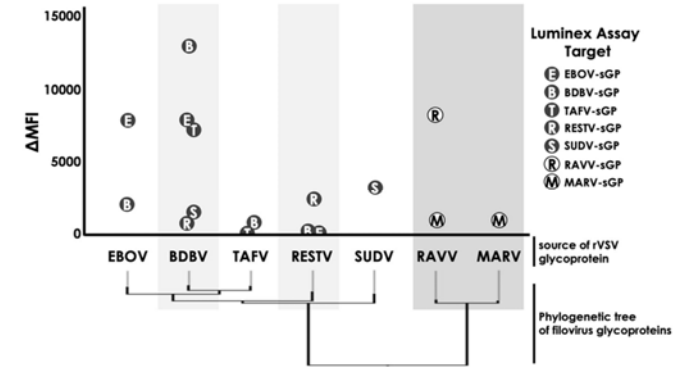
The majority of antibodies induced by virus infections are specific to conformational dependent epitopes found on virus proteins. Antibodies raised against envelope glycoprotein of henipaviruses and ebolaviruses undergo affinity maturation and are the targets of vaccine design and development. However, the confounding effects of cross-reactivity against native-like proteins expressed to retain quaternary structures, and a lack of true controls in understudied wildlife species or for yet-uncharacterized viruses stymies use of rich qualitative data that they produce.

Old World frugivorous bats have been largely considered the wildlife reservoirs of henipaviruses, the discovery of four Asiatic henipaviruses in rodents has come as a suprise [cite]. implies that serosurveillance measures thus far have been unable to appropriately detect the diverse serological profile of extant henipaviruses amongst widlife, livestock, and human hosts. In fact, PI Laing's research team detected a unique serological profile in an archived sera bank of acutely febrile patients in Cambodia that were XX% (44/1400) seropositive for a henipavirus that is seemingly antigenically-distinct and -ancestral to Ghana, Hendra, and Cedar viruses (**Figure JULIET**).



The shape of this serological profile if protein antigens from LayV and the other rodent associated henipaviruses had been included will remain unknown, but the serologic data suggests that there are other serologic footprints left by unknown zoontoic henipaviruses in southeast Asia, the shadows of which can be measured through serological approaches.





IV. SIGNIFICANCE

Serological tests frequently viruses outside their known host range or geographic range (Filovirus, Singapore, etc.).

Serological surveys can capture usable info PCR cannot [11]. Serological screening can identify novel strains that would otherwise be missed, such as this Hendra virus variant [82].

Bayesian clustering approaches [83] are useful but have largely been applied to single-assay surveillance. At higher dimensions, the clustering in high/low expected values confounds with clustering of different viruses.

Outlier-based detection is inherently problematic for rare conditions. Rare values can occur at a rate less than statistically expected outliers.

V. INNOVATION

This project is innovative in in its multi-scale approach, integrating laboratory method development and population-scale surveillance data through modeling to develop.

Estimate the probability of a multiplex panel response to represent exposure to novel coronaviruses. This approach has several statistical antecedents: spectral mixture analysis in remote imaging, antigenic cartography.

Paper on Nipah Panel: [84]

These measures will produce novel insights via *antigenic mapping*. The mixture of high- and low-specificity (RBD and IgG) will allow us to better understand the degree to which phylogenetic similarity between viral species is reflected in antigenic properties.

We will extend techniques of antigenic mapping,[66] 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.

Model 1: mixed effects for unknown responses in wildlife species. Account for variation in wildlife, use phylogenetic information to inform immune response.

Model 2: “spectral mixing” / antigenic mapping approach. Determine the probability that an individual has an exposure to a single virus.

Experiments: test sera exposed to multiple viruses rather than single viruses only, determine additivity. Additive allow for straight spectral mixing, non-additivity requires models with covariance components.

Model 3: Multiple outcome (multivariate probit) models for joint seroprevalence.

In wildlife serological surveillance, we generally lack important controls, including PCR-based or symptom-based confirmation. We lack negative controls because we don’t know the wild-caught status. In the few cases where there are captive-raised animals, these negative controls aren’t representative of wild types that may have had other exposures. In any case the number of species of interests for serological monitoring far exceeds those it would be possible to establish captive models.

Many multi-species surveillance studies.

Analyses need to distinguish between *correlation in assay response*, which may be driven by cross reactions, and *correlation in exposure*, driven by epidemiological and ecological processes.

Pooling data from multiple studies can establish standardized responses for some species, while simultaneously giving a base of evidence for predicting to other species.

Pooling factors allow us to evaluate the level of species- or geography-level information incoroporated into estimates

Making studies comparable by extracting raw data on machine calibration, sample dilution, etc.

Mixed models will allow us to estimate both intra- and interspecific variation in wild-type responses, and develop predictors of this using both phylogenetic and trait-based species data.

Phylogenetic models – assessing phylo-immuno relationship across the panels, allowing for prediction in phylogenetic space

Incorporate measures of variation by test

Benefits – quantitative and scalable interpretation, researchers don’t all have access to the statistical or virological expertise which is required to interpret beyond specific recipes

Nonlinear relationship of cross-reactivity https://link.springer.com/article/10.1007/s10875-021-00997-6

We will screen serum from all subjects for IgG antibodies against Nipah and all other known henipaviruses (Hendra, Cedar, Ghanavirus & Mojiang) using a Luminex multiplexed sphere-based assay developed by the Broder lab and the CSIRO Australian Animal Health Laboratory in Geelong (10).

are acquiring new laboratory experimental data on titer-response curves and cross-reaction against non-target antibodies for Luminex assays.

The core of this proposal is the development of statistical models and the ability to test these models against field data from a variety of systems that has been collected, curated, and interpreted by our team.

Our team has been involved in 17+ years of research in host-pathogen dynamics and spillover processes for zoonotic viruses across continents including Hendra, Nipah, SARS-CoV, SARS-CoV-2, MERS-CoV, and Ebola viruses, as well as development of state-of the-art multiplex assays for serological testing for these viruses. Our data from multiple NIH R01s, as well as other federally-funded work, includes paired individual data on both antibody levels and PCR detection of viral RNA, multiplex data from multiple systems, and serochip data. We will enable broader use of these models in epidemiology through the development of software for both model-fitting and visual diagnostics.

Our team has experience in turning research code into generalizable and sustainably maintainined. scientific software, including designing for user needs, recruiting open-source communities.

Our 3 specific aims are to:

1. Create procedures to detect signals of new viruses from multiplex serological assays.

2. Discovering real signals in high-dimensional serochip data.

and .

Creation of a library of priors: While there are missing baselines for a wide array of serological assays, an accumulating body of evidence can provide input to future routines. We will use data accumulated from a series of projects to build a database of priors. This data will help us answer question such as: how applicable are thresholds and models across species? How consistent are immune responses within and across families of viruses? How do distributions of responses, when normalized, compare across quantitative methods that are related but not linearly proportional with antibody titers?

We will use this database to identify laboratory calibrations that will provide the greatest ability to improve statistical discrimination among responses.

Cross calibration of methods: We have both Luminex data, serochip data, and a spectrum of VNT or pseudo-neutralization assays. All are related to antibody titer but have different mechanisms, and levels of specificity and nonlinearity in response. We will perform cross-calibrations. We will integrate these into our model designs so that mechanisms can be swapped out and serology can be made comparable across populations.

1. We will validate these models with a “leave-one-out” strategy – testing whether a novel virus is identified in sera from a population with a known exposures to a virus, with protiens specific to that protien removed. We will repeat this via both *in silica* and *in vitro* approaches. *In silica*, we will simply remove data from the target viral protiens from multiplex results from a full panel. *In vitro*, we will omit beads for the target viruses from the panel. Comparison of these two approaches will quantify the extent that competitive binding affects the validation technique.

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From: Laing, Eric on behalf of Laing, Eric <eric.laing@usuhs.edu>
To: Noam Ross
Subject: Re: Noam/Eric"s Serology NIH R01 grant submission status
Date: Friday, February 3, 2023 5:08:57 PM
Attachments: Vertebrate Animals Laing.doc
serostats-research-strategy-v2_CURRENT-EDL.docx

Hi Noam,

Attached are very likely my final edits. Feel free to reach out. I added the vertebrate section language (it kicked down 2 blank pages) and also uploaded a doc to the folder "Other Documents."

I think it's there.

- Eric

Eric D. Laing, Ph.D.
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On Fri, Feb 3, 2023 at 11:15 AM Laing, Eric <eric.laing@usuhs.edu> wrote:

> Sorry - I sent the wrong .doc
>
> Eric D. Laing, Ph.D.
> Assistant Professor
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>
> eric.laing@usuhs.edu
>
> On Fri, Feb 3, 2023 at 11:07 AM Noam Ross <ross@ecohealthalliance.org> wrote:
>
> > Thanks! Did we have a biosketch or are we expecting it?
> > --
> >
> > Dr. Noam Ross
> > *Principal Scientist, Computational Research*
> >
> > EcoHealth Alliance
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EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.

On Fri, Feb 3, 2023 at 10:47 AM Laing, Eric <eric.laing@usuhs.edu> wrote:

> Hey - here are Cara's docs
>
> Eric D. Laing, Ph.D.
> Assistant Professor
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> eric.laing@usuhs.edu
>
> On Fri, Feb 3, 2023 at 10:34 AM Noam Ross <ross@ecohealthalliance.org> wrote:
>
> > Great, working on admin stuff and other meetings this morning, but I've put this one up in the shared folder. Will let you know when I'm back on it addressing the things you flagged for me.
> > --
> >
> > Dr. Noam Ross
> > *Principal Scientist, Computational Research*
> >
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> >
> > *EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.*
> >
> > On Fri, Feb 3, 2023 at 12:26 AM Laing, Eric <eric.laing@usuhs.edu> wrote:
> >
> > > Revisions attached.
> > >
> > > Need to tweak Summary Abstract.

Student seminar from 1-2pm tomorrow but I'm free besides that. Just need to add in Vertebrate section.

Let's touch base about any figures tomorrow?

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On Thu, Feb 2, 2023 at 7:25 PM Noam Ross <ross@ecohealthalliance.org> wrote:

> Hi all,
>
> Just sending a state-of-the submission of the serology grant with USU as we barrel to the end of the week and the Monday 5PM deadline.
>
> Grant materials are in this folder: https://ecohealthalliance-my.sharepoint.com/personal/ross_ecohealthalliance_org/_layouts/15/onedrive.aspx?id=%2Fpersonal%2Fross%5Fecohealthalliance%5Forg%2FDocuments%2Fnih%2Dserostats
>
> This includes a document tracker of development and upload status in the base folder. Note that several documents are all consolidated together in the research folder.
>
> About 1/3 of documents and forms are filled out and uploaded in ASSIST (Application 1344425) . The plan is to spend Friday finishing the rest, including filling in the budget and writing the justification, pending any last-minute input from Joe. There is one outstanding administrative item Eric is tracking down for our vertebrate animal testing justification document, and a letter of support we are expecting. These should be done tomorrow, too.
>
> The core research strategy is near-done, but Eric and I have decided to give ourselves a bit of breathing room to revise, taking a break to re-read then uploading and submitting from our end on Sunday. If all goes according to plan, Joe can give organizational approval Monday morning.
>
> I am on a flight to Calfornia 6:30 AM on Monday morning and will pay for internet, but I will assume I'm incapable of doing anything. Should anything not go right with final submission, or an administrative document not arrive until Monday. Collin on my team has full edit capabilities for the proposal ASSIST. Collin please be on standby to liaise with Eric and Joe if something needs to be fixed.
>
> Thank you all for your help!
>
> Noam
> --
>
> Dr. Noam Ross

Principal Scientist, Computational Research

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

SPECIFIC AIMS

Emerging infectious diseases—predominantly caused by zoonotic viruses—have become a major threat to individual and public health as well as the functioning of a global society. Even prior to the SARS-CoV-2 pandemic, outbreaks of consequential emerging pathogens including SARS-CoV, Pandemic influenza 2009, Zika virus, and Ebola virus disease tragically killed, disabled, and caused massive disruptions of regional and global economies and health care systems[2]. The emergence of consequential zoonotic infections is accelerating due to anthropogenic drivers such as climate change, land-use change, intensification of livestock production, and wildlife trade, all of which increase opportunities for people and domestic animals to come into contact with wildlife reservoirs[3-6]. **To reduce the risk of infectious disease epidemics, there is a critical need to detect and *prevent* spillover of zoonotic pathogens from wildlife reservoirs[3,7]**.

Illustrative of this need is the repeated spillover from wildlife reservoirs to human populations of groups of zoonotic viruses such as filoviruses (e.g., Ebola virus [EBOV]) and henipaviruses (e.g., Nipah virus [NIV]). Large-scale outbreaks or clusters of cases of these high-mortality zoonotic viruses have led to their **designation as high priority pathogens by the World Health Organization (WHO)[5]**. However, at present, the detection of outbreaks caused by these pathogens occurs only after the first human infections; a deadly, costly, and remediable failure in prevention[2]. Identifying natural reservoirs of and measuring rates of human exposure to these zoonotic viruses is critical to assessing their potential threat and developing interventions that reduce spillover risk[2,3].

For the proposed study, we have assembled a team of leading experts in the ecology, epidemiology, virology, and immunology of bat-borne viral zoonoses such as filoviruses and henipaviruses, as well as experts in filovirus clinical care and research who are actively working in West Africa. Our study will provide much-needed empirical evidence to help answer the questions: **In a region that is an emerging disease hotspot, which has suffered the world's largest filovirus outbreak, and where there has been evidence of other consequential zoonotic viruses in animals and people, what is the natural reservoir for Ebola virus and how often does spillover of this and other high-consequence viruses happen**? To address these questions, we propose the following specific aims:

Aim 1. To determine the rates of filovirus and henipavirus exposure in people in rural Liberia. We have shown that certain bat species in Liberia may carry EBOV-Zaire, while others carry Marburg virus (MARV) and henipaviruses. Hunting bats is a common activity and we have detected high seroprevalence of Marburg and EBOV antibodies in residents of rural Liberia. Exposure to henipaviruses remains unknown. To test the hypothesis that spillover of both groups of viruses has occurred in this region, we will use an antigen-based multiplex serological assay to screen more than 1,900 archived blood specimens, collected in 2021 and again in 2023 from over 900 adults and children in rural central Liberia as part of a longitudinal study of Lassa fever seroprevalence and seroincidence, for antibodies against all known filoviruses and henipaviruses.

Aim 2. To identify reservoirs and characterize the infection dynamics of filoviruses and henipaviruses in key bat species in Liberia. Our group detected EBOV-Zaire RNA and antibodies in *Miniopterus nimbae*, a cave- and mine-dwelling bat in northern Liberia found near the origin of the 2014 Ebola outbreak in Guinea. We also detected antibodies against EBOV *and* henipaviruses in *Hipposideros* bats that co-roost with *Miniopterus* in multiple locations in Liberia. *Rousettus aegyptiacus*, a common frugivorous bat in Liberia, carries MARV and henipaviruses. We will conduct a 3-year longitudinal study of populations of these bat species near the human cohort studied in **Aim 1**, to identify the reservoirs for Zaire ebolavirus and characterize the temporal dynamics of filovirus and henipavirus circulation in bats using multiplexed serological assays that test for specific IgG antibodies against all known filoviruses and henipaviruses, as well as molecular techniques to screen for Ebola, Marburg, and other filoviruses and henipaviruses.

Aim 3. To characterize factors associated with human exposure to filoviruses and henipaviruses. Combining the data collected in Aim 1 (humans) and Aim 2 (bats), we will identify the demographic, behavioral, and environmental factors associated with an individual's prior exposure to filoviruses and henipaviruses. Detailed animal exposure data collected from the adults and children (some born after the Ebola outbreak) who provided the Aim 1 blood specimens will be used to develop profiles of risk and inform public health and risk reduction communication tools in partnership with the Ministry of Health, Liberia.

RESEARCH STRATEGY

A. SIGNIFICANCE

Zoonotic viruses are responsible for the majority of emerging infectious disease outbreaks, which are occurring at an accelerating rate in global hotspots[1,4,8]. Every outbreak begins with spillover—transmission of the virus from its animal reservoir either directly into human populations or into other animal hosts *en route* to humans (**Figure 1**). **Exactly how often spillover occurs is virtually unknown,** as surveillance systems are primarily designed to detect clusters or outbreaks, and often miss initial individual infections that result from animal contact. To reduce the risk of future epidemics, it will be necessary to limit opportunities for zoonotic virus spillover and to detect spillover at the earliest stages.[2,9] Measuring the efficacy of any interventions designed to reduce spillover and spread of zoonotic pathogens will rely on measuring rates of exposure in at-risk human populations.

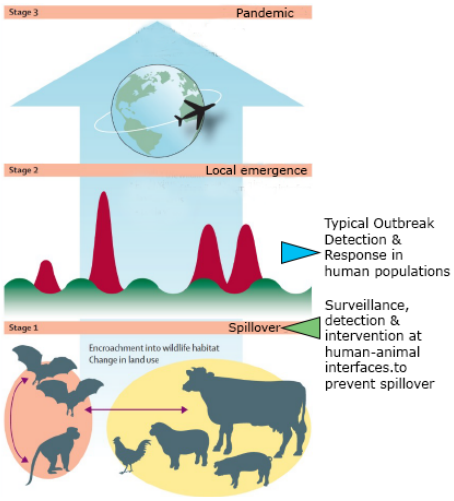


Figure 1. Most pandemics are caused by zoonotic viruses from animal reservoirs. Interventions that prevent spillover, rather than try to contain outbreaks, are needed. Adapted from [1].

Filoviruses and henipaviruses are among the highest consequence zoonotic viruses and have greatly impacted public health. Each group contains zoonotic pathogens that cause significant disease in both people and animals, especially livestock, that have been associated with extremely high rates of morbidity and mortality. Ebola (EBOV) and related viruses, as well as Nipah virus (NIV), a henipavirus, and its related viruses have been listed by the World Health Organization (WHO) among the highest priority pathogens for the development of vaccines and therapeutics, as they represent zoonotic pathogens with high potential to threaten human health, with extremely high mortality rates and few or no therapeutics or vaccines currently available[10-12]. These viruses share other important features which make them high priority: they are all associated with bat hosts that are abundant and widely distributed throughout Africa and Asia, living in close association with people and livestock in some of the most densely populated regions on Earth.

Spillover of these viruses has repeatedly occurred. Beyond the massive 2013-2016 outbreak in West Africa, EBOV outbreaks have occurred repeatedly in Central and East Africa, including a recent Sudan ebolavirus outbreak in Uganda in late 2022[12-14]. Marburg virus (MARV) and Ravn virus (RAVV) comprise the closely related genus *Marburgvirus* and have caused outbreaks in Central and East Africa and Europe with mortality and clinical presentation comparable to Ebola[15,16]. MARV outbreaks were declared in Guinea in 2021, just across the border from the UNC-Liberia research site, and again in Ghana in 2022, marking the first time this virus was reported in either country[17,18]. NIV is an emerging zoonotic paramyxovirus that causes nearly annual outbreaks in Bangladesh, including multiple in 2023, and sporadic outbreaks in India with mean case fatality rates over 70%[13,19-21]. Despite the public health significance of Nipah and related viruses, little is known about their genetic diversity, natural reservoirs, or the frequency of spillover throughout their range, including Africa. Henipaviruses, and NIV in particular, have several characteristics that make them a global health priority: 1) Their bat reservoirs occur throughout Asia and Africa, overlapping human and livestock populations, giving them geographically broad opportunity to cause outbreaks[22]; 2) henipaviruses can be transmitted to people directly from bats or via domestic animals[22]; 3) NIV can be transmitted from person-to-person[23]; 4) NIV spillover has and continues to occur in highly populous and internationally connected regions; 5) repeated spillovers of NIV strains with varying person-to-person transmission rates indicate the ability to evolve **with increased pandemic potential**[24,25]; and 6) Nipah is associated with a high mortality rate in people and currently has no vaccine or treatment[22,24]. While NIV has not been detected in Africa, closely related henipaviruses have been identified in Ghana and Madagascar, though their public health significance remains unknown[26-28]. In West Africa, where bats and

associated henipaviruses occur but no human cases have been identified, **diagnostic testing and systematic surveillance is essentially non-existent**. The detection of antibodies against a Nipah-related virus in hunting communities in Central Africa suggests that cryptic spillover is occuring[29]. **Repeated spillover of henipaviruses creates opportunity for more transmissible strains to emerge[30].**

While bats have been identified as potential hosts for filoviruses including EBOV, the natural reservoir for EBOV-Zaire Ebola in West Africa remains unknown. There is substantial evidence, both serological and molecular, that bat species host filoviruses; however, a definitive natural reservoir or set of reservoirs for EBOV-Zaire has not been identified[31]. Wildlife, including bats, represent a substantial source of animal protein for communities in Liberia[32]. People may be exposed to bat-borne viruses through hunting and butchering, eating food contaminated by bat excreta, or entering caves or mines inhabited by bats. Our group previously found that people living in rural areas of Liberia conduct activities which may increase their risk of contact with bats or their bodily fluids. In the 2013 Ebola outbreak, the index case was a child who was hypothesized to have had contact with bats, yet the exact species was never definitively identified[33]. The route of spillover for most historical Ebola outbreaks remains a mystery. Despite broad efforts to determine the natural reservoirs of EBOV and related viruses, there continues to be a poor understanding of filovirus ecology, with the exception of MARV, whose host has been identified as Egyptian rousette bats (*Rousettus aegyptiacus*)[34-36]. Communities known to hunt bats in Cameroon and India have been found to have antibodies against henipaviruses and filoviruses, respectively, yet nothing is known about community exposure rates in Liberia, where bat hunting is common[29,37]. Our preliminary data (see below) suggest that there is substantial exposure to EBOV as well as MARV among people in central Liberia. While the EBOV antibodies may indicate prior infection from another person such as during the West Africa outbreak, antibodies specific to other filoviruses may signal a localized exposure from animals. Even less is understood about henipaviruses in Africa; identifying antibodies against henipaviruses in people will be critical for understanding where, how, and how often spillover occurs.

The urgent need to develop interventions to prevent spillover of zoonotic high-consequence pathogens can only be met with a better understanding of their natural reservoirs, the rates of exposure in vulnerable human populations, and the ways in which people are exposed. Unrecognized outbreaks provide opportunities for viruses to emerge that may be more transmissible or more pathogenic than previously observed, and limiting these opportunities is an important component of pandemic prevention[3,30]. Typically, by the time an outbreak is recognized in human populations, its containment and control are difficult, if not impossible, to achieve (e.g., HIV, SARS-CoV-2). Understanding the ecology of these viruses, including which species act as reservoirs as well as how and to what extent people are being exposed to filoviruses and henipaviruses, will inform intervention and surveillance strategies. Importantly, it will also provide baseline metrics against which one can measure the efficacy of interventions designed to reduce exposure, such as public health campaigns to promote avoidance of behaviors associated with infection among those most at risk (**Figure 1**).

This proposed study will be one of the most rigorous One Health investigations of spillover of high-consequence zoonotic viruses from bats to humans in an emerging disease hotspot. West Africa is known to be the source of prior outbreaks of emerging infectious diseases and future spillover events there are highly likely. Liberia is typical of this region and contains some of the largest tracts of pristine forest and wildlife biodiversity remaining in West Africa and has a population that depends on wildlife, including bats. Decades ago, a spillover event from a non-human primate to a human triggered an ongoing HIV pandemic that has led to the infection of over 60 million people and 25 million deaths. Only months ago, the orthopoxvirus mpox, which is found in rodents, spread to and within 110 countries across the globe and caused over 85,000 cases. Both originated from African wildlife. The proposed study will leverage the unique infrastructure and expertise we have developed in Liberia to undertake a comprehensive examination of filovirus and henipavirus spillover from bats to humans and provide evidence of previous and possibly on-going transmission events. Our findings will provide essential evidence of the presence and transmission dynamics of these bat-borne viruses and of the risks for infection in people living alongside them.

B. INNOVATION

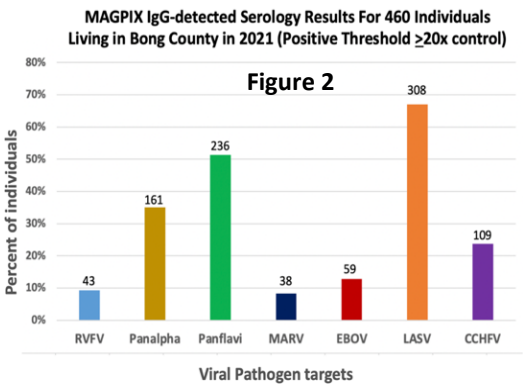
The proposed research is highly innovative in multiple ways, including: 1) Our use of a One Health approach to study the ecology and epidemiology of two groups of high-priority, high-consequence zoonotic viruses in both bats and people in West Africa – an emerging disease hotspot where spillover events have occurred (e.g. Ebola, Marburg, mpox, Lassa fever) and are highly likely to re-occur; 2) Our use of a novel and unique multiplexed serological platform, developed by our group, that will allow us to screen bat and human serum for IgG antibodies against each of the known filoviruses and henipaviruses, simultaneously in a single assay. Longitudinal comprehensive serological studies in bats and people **will provide unprecedented insight into the changes in rates of exposure to known as well as unknown, antigenically related viruses over time in both bats and people (e.g., what is the baseline rate of exposure to henipaviruses and filoviruses and is there evidence of recent exposure?);** 3) We will couple serosurveillance with molecular testing to identify specific filoviruses and henipaviruses carried by bats; and 4) We will connect the bat and human epidemiological studies by analyzing questionnaire data from the human cohort to identify high-risk behaviors and other factors associated with viral exposure. **Results from this analysis will inform the development of public outreach and risk reduction campaigns by the Government of Liberia.**

C. APPROACH

C.1. Overview: Aim 1 will test the hypothesis that spillover of filoviruses and henipaviruses, present in local bat populations, occurs regularly in rural Liberia by testing for IgG antibodies to all known filoviruses and henipaviruses (see **Table 1**) in archived serum samples from more than 900 people living in Bong County (central Liberia). These samples were collected between 2021-2023 at two time points 24 months apart as part of the Coalition for Epidemic Preparedness Innovations (CEPI)-sponsored **ENABLE Study**, an on-going longitudinal study of Lassa fever seroprevalence and seroincidence. Seroprevalence and seroincidence of filoviruses and henipaviruses will be assessed. In **Aim 2,** we will conduct a 36-month longitudinal study of three bat species which are putative reservoirs for EBOV and undescribed henipaviruses. Using a combination of longitudinal serology and molecular testing (PCR and whole genome sequencing) **we will determine the prevalence of filoviruses and henipaviruses in these key bats species associated with the rural human populations studied in Aims 1 and 3**. For **Aim 3,** we will analyze detailed questionnaire data developed collaboratively by our group (EHA and UNC) and collected in 2023 along with the blood samples to be screened in Aim 1. These data will allow us to identify factors associated with exposure to henipaviruses and filoviruses. We will use results from our exposure risk assessment to develop public risk reduction communication strategies with the National Public Health Institute, Liberia, using the results of this study to inform public health strategies. This application is a natural progression of our research activities, and our preliminary data speak to the feasibility of the proposed study.

C.2. Aim 1: To determine the rates of filovirus and henipavirus exposure in people in rural central Liberia.

C.2.1. Rationale and Preliminary Data. The primary goal of this aim is to be able to determine whether populations within Liberia who live in association with bats have evidence of exposure against one or multiple filoviruses and/or henipaviruses and to compare rates of exposure over time. Studies of human populations in Central and West Africa have found evidence of exposure to EBOV prior to the 2013 outbreak and in places not known to have had Ebola outbreaks[38,,39]. UNC Project-Liberia has previously assessed seropositivity to consequential viral pathogens including EBOV and MARV in a subset of ENABLE Study participants. Over 8% of participants had antibodies against MARV, using a conservative seropositivity threshold (**Figure 2**), suggesting



Rift Valley Fever Virus (RVFV), Panalphavirus (Panalpha E), Panflavivirus (Panflavi E), Marburg Virus (MARV), Ebola Virus (EBOV), Lassa Fever Virus (LASV), Congo Crimean Hemorrhagic Fever Virus (CCHFV)

that unreported spillover events have previously occurred. Acute encephalitis is common in western sub-Sharan Africa, yet outbreaks of neurological disease are rarely reported and more than half of all cases are undiagnosed[40]. Among the countries in western Africa reporting cases of meningitis or encephalitis, Liberia has reported the fewest, likely due to a lack of surveillance and capacity to diagnose neurological diseases[40]. NIV encephalitis has never been reported in Africa; however, as described above, two Nipah-like henipaviruses have been described in bat hosts, including one in Ghana[26,28]. Spillover of henipaviruses was reported in bat-hunting communities in Cameroon[29]. In South Africa, we detected henipavirus sequences in *Rousettus aegyptiacus,* bats which also commonly occur in Liberia[27].

In Aim 1, we will test the hypothesis that spillover of both filoviruses and henipaviruses have occurred in rural populations in Liberia by measuring seropositivity to both groups of viruses within the ENABLE Study community cohort. We will also measure changes in exposure rates over a two-year period. While prior studies have provided cross-sectional snapshots of exposure, our longitudinal approach will provide data from the same cohort at two timepoints, 24 months apart, to determine both seroprevalence and seroincidence using a multiplexed serological assay to screen for IgG antibodies against all known filoviruses and henipaviruses.

Settings and Participants. To achieve this aim we will leverage a well characterized cohort of individuals in three rural communities located in Bong County, Liberia (**Figure 3**), which has a population of over 333,400. UNC Project-Liberia has been operating in Liberia since 2014 and established a research site at Phebe Hospital in Bong County, which includes a molecular laboratory that conducts daily qPCR testing for LASV, MARV, and EBOV as well as LASV serological assays using a MAGPIX (see RESOURCES AND FACILITIES). There, following extensive community engagement including meetings with key stakeholders, town hall meetings, and radio messaging, UNC launched the ENABLE Study, the largest passive and active surveillance program for Lassa fever in Liberia. Starting in 2021, 5,005 randomly selected participants 2 years of age and older in Phebe Airstrip, Suakoko, and Rubber Factory were enrolled over a period of 6 months, following informed consent/assent (mean age at enrollment is 22.2 years (range 2-97) and 54.2% are female). **Importantly, 7.7% of the cohort is under the age of 5 years, and therefore, were born after the 2013-14 Ebola outbreak.** Households were selected for recruitment following identification of rooftops by satellite photos and use of a program developed by Médecins Sans Frontières' (MSF) Epicenter to randomly select structures to approach sequentially for participation. The ENABLE Study includes baseline questionnaires and blood specimen collection from all participants with active follow-up by a study community health worker every 2 weeks to determine if a febrile event has occurred, which triggers drawing of blood for LASV, MARV, and EBOV PCR. Baseline blood is tested for Lassa fever virus (LASV) serology. A subset of 1,024 individuals (every 5th approached household is eligible) is also asked to provide blood samples routinely every 6 months for up to 24 months for repeat LASV serology. The blood samples from 0 and 24 months and the associated surveys will be used in **Aims 1 and 3** of this proposal. Informed consent and assent documents include provisions for the use of collected specimens and data for infectious diseases research including for emerging pathogens (see PROTECTION OF HUMAN SUBJECTS).

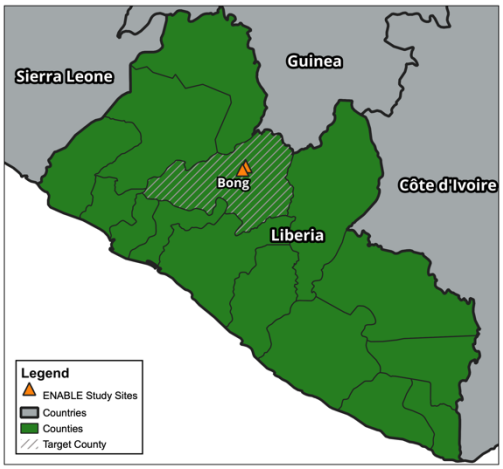


Figure 3. Locations of UNC CEPI ENABLE study sites and proposed target county.

C.2.2. Research Design. We will screen approximately **1,900 archived serum specimens** from participants in the ENABLE Study cohort at two time points ("baseline" at month 0 and again at month 24) for IgG antibodies against specific filoviruses and henipaviruses using the MAGPIX at the UNC molecular lab at Phebe Hospital to run the multiplexed *henipavirus / filovirus* platform to be provided by Co-I Laing in year 1 of the study. The multiplex platform will allow us to determine specifically which filoviruses and henipaviruses people have been exposed to and compare that to the filoviruses and antibodies we observe circulating in local bat populations in **Aim 2**.

The bead-based multiplexed assay has tremendous advantage over traditional ELISAs in that it will identify up to 25 different antibodies against multiple viral agents in a single, 2uL serum sample and uses a high through-put format. The ability to look at reactivity across all serogroups simultaneously will allow us to differentiate among the viruses._Proteins A/G non-specifically bind IgG immunoglobulins, and weakly bind IgM, an immunoglobulin that is the first to be produced during the initial stages of infection. We will conduct a 1-week training at the UNC-Phebe lab for using the assay to detect filovirus and henipavirus antibodies as well as data management, bioinformatics, and analysis. **Table 1** shows the viral species and antigens that will be employed in this assay. The platform has been previously transferred to collaborating labs in the UK, South Africa, India, Bangladesh, Singapore, Thailand, and Malaysia. We have previously detected serological profiles of Asiatic ebolaviruses in fruit bats collected in Singapore[41], and in fruits bats as well as humans actively engaged in bat hunting in northeast India[37].

Table 1. Multiplexed microsphere immune assay including soluble glycoproteins from each known filovirus and henipavirus

Virus (Name/Isolate Host/Country/Year/Strain)	Abbreviation	Accession No.	Soluble Glycoprotein	Mag Bead No.
Ebolaviruses				
Ebola virus/H. sapiens/COD/1976/Yambuku-Mayina	EBOV	NC_002549.1	$GP_{(1,2)}$	34
Bundibugyo virus/H. sapiens/UGA/2007	BDBV	FJ217161.1	$GP_{(1,2)}$	64
Bombali ebolavirus/Mops condylurus/SLE/201	BOMV	NC_039345	$GP_{(1,2)}$	55
Taï Forest virus/H. sapiens/COV/1994/Pauleoula-Cl	TAFV	NC_014372	$GP_{(1,2)}$	57
Sudan virus/H. sapiens/UGA/2000/Gulu-808892	SUDV	NC_006432.1	$GP_{(1,2)}$	54
Reston virus/M. fascicularis/USA/1989/Pennsylvania	RESTVm	AF522874.1	$GP_{(1,2)}$	65
Marburgviruses				
Marburg virus/H. sapiens/KEN/1980/Musoke	MARV	Z12132 S55429	$GP_{(1,2)}$	37
Ravn virus/H. sapiens/AGO/2005/Ang0126	RAVV	NC_024781.1	$GP_{(1,2)}$	62
Dianlovirus				
Mengla virus/Rousettus-WT/CHN/2015/Sharen	MLAV	KX371887.2	$sGP_{(1,2)}$	22
Cuevavirus				
Lloviu virus/M. schreibersii-wt/ESP/2003/Asturias-Bat86	LLOV	NC_016144.1	$sGP_{(1,2)}$	66
Henipaviruses				
Hendra virus/E. caballus/AUS/1994	HeV	NC_001906.3	sG	43
Hendra virus-2G	HeV-2g		sG	48
Nipah virus/H. sapiens/MYS/2000	NiVm	NC_002728.1	sG	46
Nipah virus/H. sapiens/BGD/2004	NiVb	AAY43916.1	sG	56
Cedar virus/Pteropus sp./AUS/2012/CG1a	CedV	NC_025351.1	sG	53
Mojiang virus/R. sladeni/CHN/2014/Tongguan1	MojV	NC_025352.1	sG	29
Ghanaian bat virus/E. helvum/GHA/2009/GH-M47a	GhV	NC_025256.1	sG	35
Angavokely virus	AngV		sG	47

C.2.3. Data Analysis. We will create a project database using Airtable, which will be hosted at EHA and will house all data from Aims 1 and 2 and will link to the REDCap server which stores questionnaire data from Aim 3. Database access will be secure and limited to key personnel (see DATA MANAGEMENT AND SHARING PLAN). Raw MFI values from the MAGPIX will be used to calculate reactivity to each viral antigen and, based on relative strength of reactivity and determinations of negative cutoffs based on reference sera, we will calculate prevalence rates for each time point and compare rates between the two timepoints. Serological profiling can be used to detect reactive antibodies against all specific filoviruses and henipaviruses in a sample and compare strength of reactivity and cross-reactivity against multiple antigens using a single sample[42]. We will compare the antibodies across all specific viral antigens to assess which virus or groups of viruses are responsible for the immunological response[43-45]. We will assess exposure to non-Zaire ebolavirus species, which may indicate spillover of filoviruses has occurred separate to the Ebola outbreak of 2013. We will also look at evidence for exposure to Nipah-like henipaviruses and compare exposure rates to both viral groups within and between our two sample collection time points.

C.2.4. Expected outcomes. We anticipate obtaining serological profiles of exposure to filoviruses and henipaviruses from each individual in our cohort. In previous studies we found antibodies against both EBOV and MARV. While the EBOV antibodies are attributable to the epidemic, MARV antibodies, in patients EBOV IgG negative, suggest possible exposure from an animal (e.g., bat) reservoir. We also anticipate seeing reactivity to

Bombali virus (BOMV), given the presence of the reservoir species in Liberia (*Mops condylurus*) and reactivity to the African henipaviruses (GhV and AngV), although this may represent reactivity to an antigenically related virus. Serological results from this and the bat study will inform our strategy for prioritizing which bat samples we test by PCR.

*C.2.5. Potential problems and solutions***.** While our preliminary data suggest there will be detection of immune responses to filoviruses including EBOV-Zaire and, significantly, MARV, it is possible that evidence of exposure to other pathogens, including henipaviruses, will not be detected. This would be an important finding in this large cohort, especially if these viruses are detected in local bat species, that would warrant further investigation. It is also possible that there will be few or no seroconversion events among the cohort during the 24-month period of follow-up. This too would be reassuring but would suggest a need for longer follow-up of this cohort. To address the possibility that reactivity to EBOV-Zaire virus may be explained by infection during the 2013 Ebola epidemic, we will create a polyclonal sera reference standard utilizing WHO convalescent sera collected from human survivors of Ebola virus disease (EVD). This internal reference standard will be calibrated to the WHO EBOV human serology standard (**Figure 4A**) permitting direct interpolation of anti-EBOV IgG as an MFI to a standard antibody concentration (binding antibody units/mL). The internal reference standard will then be included in our human serology testing strategy allowing semi- or quantitative measurement of anti-EBOV IgG. These EBOV human serology standards will permit us to establish positive and negative predictive performance values for the multiplex assay; furthermore, we will be able to investigate immunoreactivity of EVD survivors against heterotypic ebolaviruses. Our preliminary data indicates that EBOV+ sera IgG is highly cross-reactive with soluble native-like trimeric envelope glycoprotein (GP) antigens from both EBOV and BDBV (Figure 5A). EBOV GP, matrix protein (VP40), and nucleocapsid protein (NP) antigens have all been used in a variety of serological tests; however, the frequency of seroconversion against each antigen has not been fully described. In a longitudinal study of EVD survivors, high seroconversion against GP and VP40 was observed, with affinity maturation occurring overtime against GP[46]. Antigens based on the GP are likely to provide an optimal intrinsic balance of sensitivity and specificity. To limit the degree of heterotypic ebolavirus cross reactions, we will test any anti-ebolavirus GP trimer antigen-seropositive samples with a pan-ebolavirus GP1 antigen-based panel. In preliminary studies, GP1 antigens improve specificity and differentiation of antibodies reactive with homotypic ebolaviruses in polyclonal antisera from rabbits immunized with GP trimer immunogens (**Figure 4B**).

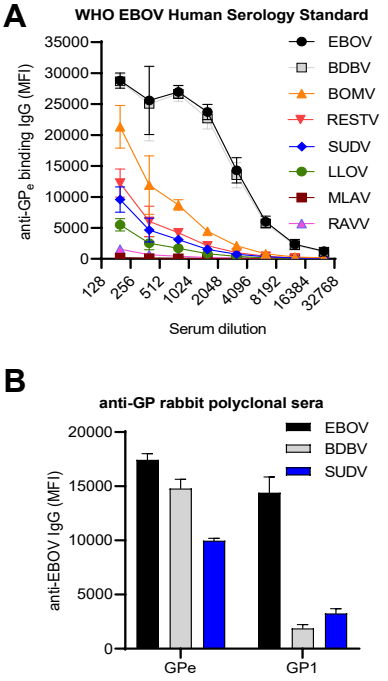
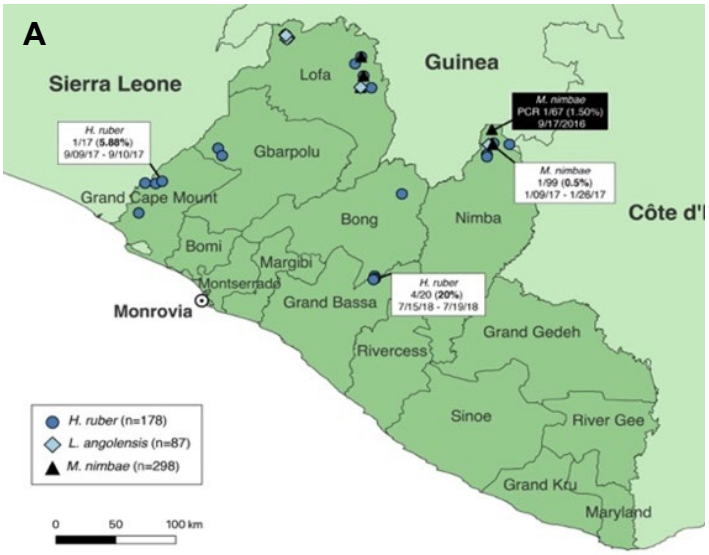


Figure 4. **A)** The WHO EBOV human serology standard was tested for immunoreactivity in a filovirid GP-trimer based multiplex assay. Filovirids are indicated along with sera dilution factors; data is the mean of three independent experiments. **B)** Antisera from EBOV GP-trimer immunized rabbits were tested in multiplex panels comprised of GP-trimer and GP1 subunit proteins. Anti-EBOV polyclonal IgG was less reactive with GP1 from BDBV and SUDV.

C.3. Aim 2. To identify reservoirs and characterize the infection dynamics of filoviruses and henipaviruses in key bat species in Liberia. C.3.1 *Rationale and Preliminary Data.* Bats have been established as important hosts for filoviruses and henipaviruses in Africa and Asia, with combinations of viral isolation, molecular detection or serology providing substantial evidence[31,47-50]. MARV was isolated from Egyptian rousette bats in Uganda, and RNA has since been detected in these bats in Sierra Leone, which borders Liberia[51]. To date, EBOV has never been isolated from bats, although molecular and serological evidence suggest that multiple frugivorous bats may carry EBOV in Central Africa[51,52]. Prior to the 2014 epidemic, Zaire ebolavirus had not been reported in West Africa, and the animal reservoir remains unknown[33]. From 2016-2019, our group (PI Epstein & KP Desmond)

implemented a large-scale effort under the USAID PREDICT Project to identify reservoirs for EBOV in West Africa[53]. This led to the discovery of a new species of Ebola (Bombali virus)[54], the detection of MARV in Egyptian rousette bats in Sierra Leone[34], and **in 2018, we detected, for the first time in West Africa, EBOV RNA and anti-EBOV IgG antibodies in bats (Figure 5A-D)**.



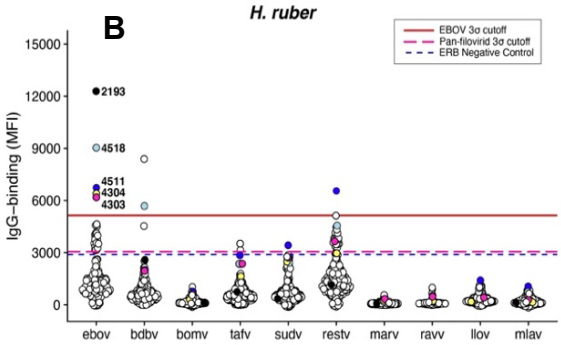
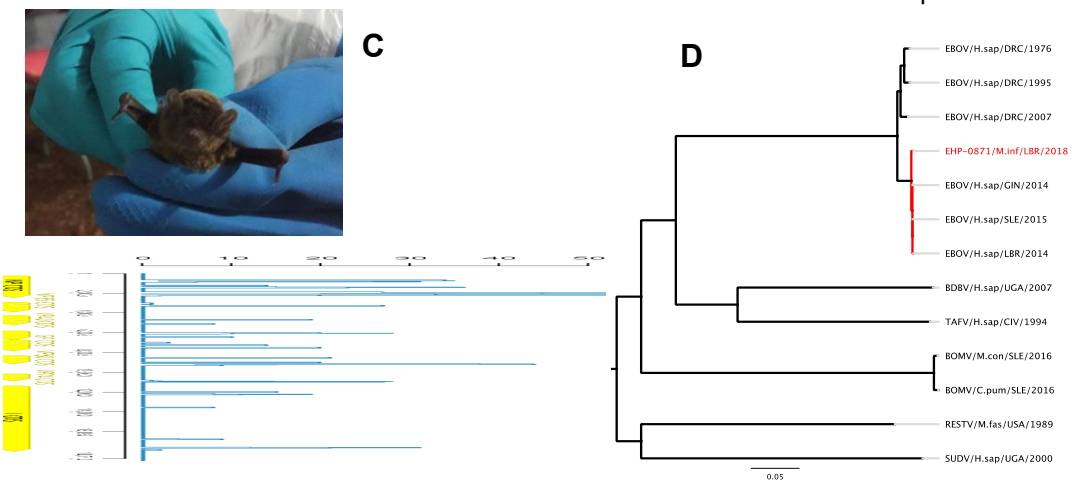


Figure 5. A) Map showing location of EBOV PCR +ive bat (black box) Zaire ebolavirus and MARV in Liberia in 2018. **B)** Serological evidence for EBOV in *H. ruber* from our 2018 study. **C)** RNA genomic sequences from across the *Zaire ebolavirus* genome detected in *Miniopterus nimbae*, Liberia in 2018. **D)** Phylogenetic analysis supports relatedness to 2013 EBOV epidemic virus.



We also detected antibodies reactive against multiple henipaviruses (NiV, Mojiang and GhV) in the same bats (Figure 6). While this finding generated the first evidence of which bats may be reservoirs for Zaire ebolavirus and unknown henipaviruses, longitudinal studies are required to more completely understand the role these species

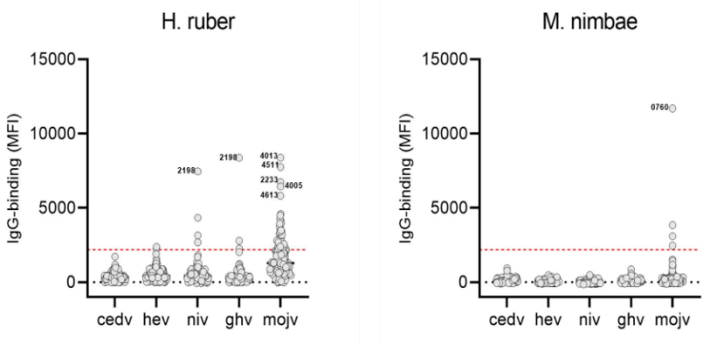


Figure 6. Detection of IgG antibodies reactive to multiple henipaviruses in the two bat species that may carry EBOV in Liberia.

play in EBOV and henipavirus circulation – either as reservoirs that maintain these viruses within a local population, or as incidental hosts, infected by an unknown bat species.

One-off, cross-sectional studies have a low probability of detecting any specific filovirus or henipavirus given their low prevalence and short viremic periods [35,55], and thus provide a poor characterization of viral diversity. The lack of understanding of **filovirus and henipavirus diversity and their natural reservoirs in West Africa significantly impedes risk-based public health interventions to prevent human outbreaks.** Longitudinal sampling is also needed to separate reservoir hosts from spillover hosts. For example, MARV has been isolated repeatedly from *R. aegyptiacus* in Uganda, which, when paired with experimental infection studies showing infection and shedding of MARV by *R. aegyptiacus,* strongly suggests that *R. aegyptiacus* is indeed a MARV reservoir[36,56,57]. Similarly, our longitudinal studies of NIV in *Pteropus medius* bats in Bangladesh, which included the same multiplexed serological assay proposed here, confirmed them as a reservoir for NIV and identified important mechanisms driving viral circulation[30]. **Understanding which species persistently carry filoviruses and henipaviruses will provide important insights into how they are maintained in nature and which species should be targeted for follow-up studies on bat habitat use and human-bat contact patterns.**

C.3.2. Sampling design. We will repeatedly sample three bat species associated with filoviruses or henipaviruses, based on our preliminary data: *M. nimbae* (EBOV), *H. ruber* (EBOV), and *R. aegyptiacus* (MARV & Henipaviruses). We will sample bats at roost sites in Bong County, as close as possible to the human study sites in Aim 1. In Y2-Y4, we will sample 40 individuals of each species every two months for 36 months and collect excreta using plastic tarps placed underneath bat roosts (details below and in C.3.3).

Bat capture and sampling. Briefly, we will use either harp traps or mist nets to capture bats, depending on the capture site. Personnel will wear appropriate personal protective equipment (e.g. Tyvek suits or dedicated long clothing; double nitrile gloves; an N95 respirator and safety glasses or a PAPR)[58]. Traps and nets will be attended continuously during capture periods. Bats will be removed from mist nets immediately upon entanglement to avoid injury. Bats will be removed from harp traps after approximately 1 hour and placed in individual cloth bags until processed. Body measurements, blood, oropharyngeal swabs, and rectal swabs (or fecal pellets) will be collected from each bat. Swabs will be collected in duplicate; one swab will be placed in a cryovial containing Trizol lysis buffer and the other in viral transport medium (VTM). For bats <50g, blood will be collected in a microhematocrit tube and spun down using a portable centrifuge. A bulb will be used to expel serum from the tube into a cryovial. For larger bats >50g, a tuberculin syringe or 1ml syringe and 27g needle will be used to draw blood from the radial artery or vein[58]. Blood will be placed into a conical vial and centrifuged. Serum will be aliquoted into a cryovial using a pipette. During site visits we will also lay out plastic tarps to collect pooled environmental excreta in Trizol and VTM to improve opportunity to detect viral RNA. All samples will be placed in a vapor phase liquid nitrogen dry shipper or portable battery-powered ultracold freezer immediately upon collection and then transferred to a -80C freezer at the Phebe lab until testing.

C.3.3. Testing plan, Data analysis, sample size, and power analysis.

Serology: At the end of Y3, we will screen all serum samples collected to date, at the UNC-Phebe lab, using the MAGPIX multiplex assay for IgG against filoviruses and henipaviruses. We will analyze temporal dynamics in seroprevalence using general additive models (GAMs), which are analyses that we have used successfully in many previous studies of similar design[30]. These methods are well adapted for capturing long-term temporal fluctuations in prevalence and moderately sensitive at capturing short-term changes in seroprevalence which can be used to identify population-level disease outbreaks. For example, we estimated that, with 40 bats sampled every two months, we would be able to statistically detect an increase in seroprevalence (at a 95% CI limit) of greater than 25% between consecutive sampling events > 80% of the time, assuming a bat population seroprevalence of at least 10% in advance of a bat colony infection (estimated by, in brief, by simulating a fluctuating true seroprevalence value, simulating samples given this underlying seroprevalence, fitting a GAM to these samples, and then calculating if CI on true prevalence between the two sampling occasions over which the change in seroprevalence occurred were non-overlapping). While this is a large change, it is within a range to be

expected if infection were to sweep through a bat community[30]. MAGPIX-positive bat sera will be sent to RML for serum neutralization assays using VSV pseudoviruses or viral isolates under BSL4 conditions.

VSV-pseudotype neutralization assays: Serological results will be confirmed using VSV-pseudotype neutralization assays for the following filoviruses EBOV, SUDV, BDBV, RESTV, BOMV, MARV and RAVN. If novel henipavirus sequences are detected, additional assays will be developed to test positive sera. Serum samples will be inactivated using ɣ-irradiation (4 MRad). Neutralization of irradiated and heat-inactivated serum samples will be assessed in Vero 352 E6 cells. Briefly, cells were seeded in 96-well round-bottom plates for 24 hours, serial dilutions of heat-inactivated serum samples will be performed in DMEM supplemented with 2% FBS, penicillin/streptomycin, and L-glutamine. Each plate will contain negative serum control, cell-only control, and virus-only control. VSV EBOV-GFP will be added to each well of the serum dilution plate and the serum-virus mix 357 was incubated at 37˚C for 1 hour. The mix was added to the cells and incubated at 37˚C for 24 hours. The cells will then be fixed with 4% paraformaldehyde at room temperature for 15 minutes and centrifuged at 600 x g for 5 minutes at room temperature. The supernatant will be discarded and FACS+EDTA buffer was added. Samples will be run on the FACSymphony A5 Cell Analyzer (BD Biosciences, Mississauga, ON, Canada) and FITC MFI 362 was measured. Data will be analyzed using FlowJo.

Molecular testing: We will use specific filovirus taqman real-time PCR assays to screen oropharyngeal, fecal, and environmental samples from bats for EBOV and MARV RNA at the UNC-Phebe lab[54,56]. Testing bat samples by PCR may allow us to detect viral RNA during active infection, providing important information about the prevalence and timing of infection within target species. **Because we expect viral prevalence to be significantly lower than seroprevalence, estimated at <5%**[26,28,50], we developed a sampling scheme focused on maximizing our probability of detecting viral RNA with qPCR. To compare power under alternative sampling designs, we built simulation-based power analyses that estimated power to detect: 1) viral EBOV or MARV RNA in at least one bat; and 2) the expected number of positive cases, in each bat species. In brief, we estimated power for total sample sizes between 1500-3000 (500-1000 bats sampled per species) spread across a variable number of visits per year (2-6), while allowing for uncertainty in true infection dynamics in the bat populations, specifically: 1) prevalence during one three-week period (with unknown timing) per year of high prevalence ranging from 1-5%); and 2) an otherwise low background prevalence (0-1%). Given these estimates of yearly infection dynamics, these analyses showed that with 672 samples per species (minimum 32 individual bats per sampling event), spread across 6 sampling occasions per year, we would obtain a per-species probability of acquiring 0 positive samples of <4% (<1% at 40 bats per event – our target number) and an expectation of ~3-6 RNA positive results (~5-10 positives at 40 bats per event). Because henipaviruses have not been previously described in Liberia, we will focus our resources on filovirus testing by PCR. However, if henipavirus serology suggests active circulation, we may send a set of samples to RML for pathogen discovery (see RML LETTER OF SUPPORT).

Viral isolation: PCR-positive cDNA samples and their aliquot in VTM will be sent to RML for further sequencing and attempts at viral isolation under BSL4 conditions. If attempts to culture do not work, we will use next generation sequencing techniques to get whole genome sequence and further characterize the virus at RML. Novel viruses will be characterized and rescued (reverse engineered using a minigenome system to synthetically create infectious viral particles[59]) at RML under secure, biosafety level 4 conditions, then used for serum neutralization assays in bat and human sera to determine more precise exposure rates. Sequences from the glycoproteins of any novel viruses discovered will be used by USU (Co-I Laing) to generate purified proteins and added to the Luminex assay, which will improve its specificity.

VirCapSeq-VERT and myBaits probe-based enrichment for recovery of full-length genome sequences of filoviruses: Although shotgun sequencing provides opportunities to analyze all host and virus genetic material, the larger average genome size of bacteria and hosts compared to viral genomes complicates a detailed analysis of the virome, particularly vertebrate viruses. We have established a positive selection probe capture-based system to enrich for viral sequences. Here, we will take advantage of these probe capture-based methods (VirCapSeq-VERT and myBaits probe library) to enrich for vertebrate viral sequences of interest. Sample libraries will be normalized and combined in 4- to 12-plex reactions for solution capture hybridization using either the

HyperExplore custom bait library version of VirCapSeq-VERT probe set or our custom myBaits probe library. For HyperExplore custom bait library probe set, libraires will be enriched for virus following the SeqCap EZ HyperCap Workflow User's Guide, version 2.3, while for custom myBaits probe library, the myBaits Hybridization Capture for Targeted NGS protocol, Version 4.01 will be used. Sequencing libraries will be normalized and sequenced as 2 X 150 bp fragments on Illumina's MiSeq or NextSeq sequencing platforms, following Illumina's standard procedure (Illumina, San Diego, CA). NGS data will be analyzed using metavirs (https://openomics.github.io/metavirs/), a comprehensive viral metagenomics pipeline to assemble, annotate, and classify viruses. It relies on technologies like Singularity1 to maintain the highest-level of reproducibility. The pipeline consists of a series of data processing and quality-control steps orchestrated by Snakemake2, a flexible and scalable workflow management system, to submit jobs to a cluster. The pipeline is compatible with data generated from Illumina short-read sequencing technologies. As input, it accepts a set of FastQ files and will be run on-premises using the NIAID RML Big Sky supercomputing cluster.

C.3.4. Expected outcomes. Based on our prior work, our multifaceted analysis approach, and calculated power given our proposed temporal sampling strategy, we expect to be able to use temporal serological data to describe filovirus and henipavirus viral dynamics over time. Our molecular screening will primarily focus on filoviruses because we have known targets, and we expect to detect EBOV or MARV RNA, despite low prevalence and short-lived infections. Sequence data from positive samples will 1) provide substantial evidence for the identification of EBOV reservoirs and help explain which virus is responsible for the observed IgG response in bats (and possibly people); and 2) provide valuable information about viral diversity, about which little is known. Henipavirus serology, in the absence of sequence data, will allow us to identify potential bat reservoirs, analyze temporal trends, and generate hypotheses for future targeted studies.

C.3.5. Potential problems and solutions. Serological tests are greatly hindered by the inherent antibody cross-reactivity between antigens from known ebolaviruses, challenging data interpretations that antibodies are EBOV-specific or EBOV, BDBV, and SUDV cross-reactive [41]. This is also true for the henipaviruses in the panel. The advantage of the multiplexed platform is that we can simultaneously see relative MFI values for all the filoviruses and henipaviruses. Glycoproteins from the homologous virus should cause the most intense reaction, particularly at higher dilutions. Co-Is Laing and Munster have demonstrated that homotypic and heterotypic ebolavirus-antibody reactions can be simultaneously measured and accounted for in ERBs challenged with replication-competent VSV-filoGP (**Figure 7**). Still, there is some cross reactivity, and we will use additional tools such as viral neutralization assays as a secondary test, and our statistical approach can correct for a portion of cross reactivity. Past studies have relied on individual ELISAs which had varying degrees of specificity depending on which test was used, but often couldn't rule out cross-reactivity. The Luminex filovirus multiplex assay has predictable patterns of cross-reactivity, but we will still be able to discern to which virus the serum is most reactive.

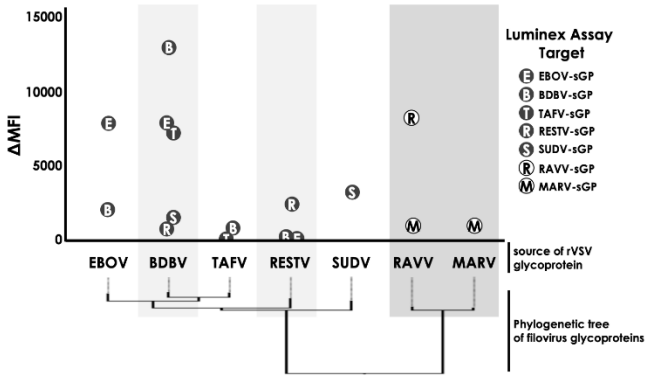


Figure 7. Captive-bred Egyptian rousette bats were experimentally challenged with VSV-filoGPs, the GP source is indicated on the x-axis. In a GP-trimer multiplex test the homotypic ebolavirus bat antisera were most reactive with the homotypic ebolavirus GP-trimer antigen.

C.4. Aim 3: To characterize factors associated with human exposure to filoviruses and henipaviruses.

C.4.1 Background and rationale. As described above, there is substantial interaction between wildlife, domestic animals, and people living in rural Liberia. Our group collected questionnaire data from 585 people across Liberia as part of the USAID-funded Ebola Host Project between 2016 and 2019. When asked about animal exposures during the 2013-2016 Ebola outbreak, participants frequently reported contact with both domestic and wild

animals. Common types of animal contact included animals coming inside the dwelling (58%), cooking or handling animal meat (44%), and eating raw or undercooked meat (31%). Additionally, 26% reported slaughtering and 12% reported hunting or trapping an animal. Elsewhere in West Africa, EHA participated in the response to Ghana's 2022 cluster of MARV cases, working with local partners to administer a behavioral risk questionnaire characterizing bat exposures in three affected communities[17]. Many participants lived in close proximity to bats, with one fifth reporting exposure to bats inside a dwelling or other building in the previous four months. When asked about exposures more relevant to Egyptian rousette bats, a known MARV reservoir and a target species in this proposed study, participants commonly reported bats feeding on fruit trees on their home compound, eating fruit bearing bite marks, and exposure to bats inside a cave or mine. In the ENABLE Study of 5,005 residents of Bong County, which will be the source of preexisting survey data for our proposed study, baseline survey questions mainly focused on rodent exposures relevant to Lassa fever. 14% of participants reported hunting or preparing wild animal meat, but little is currently known about the prevalence and type of other potential bat exposures in this region of Liberia. However, EHA has collaborated with UNC to incorporate additional bat-specific exposure questions into the ENABLE Study's month 24 survey (to be conducted in 2023). These additional questions are based on EHA's experience in Ghana and with the Liberia Ebola Host Project, and the resulting data will provide a clearer picture of human-bat interactions in these communities.

C.4.2. Research design. As described in Aim 1, serum collected at baseline (in 2021) and at month 24 (in 2023) during the ENABLE Study will be screened for filoviruses and henipaviruses (analyses not included in the ENABLE Study protocol), enabling us to calculate seroprevalence at baseline and seroconversion over a two-year period. In addition to providing blood specimens, the 1,024 participants who initially enrolled responded to baseline questionnaires on demographics, healthcare-seeking behaviors, health status, and animal exposures. At month 24, the 921 participants still actively enrolled will be administered repeat questionnaires. This final survey includes additional items based on the Ghanaian questionnaire and asks specifically about bats including the respondents' activities in their habitats (entering forests, caves, and mines), direct contact (hunting, eating handling), indirect contact (presence in home or other used structure), and observed characteristics of bats encountered (fruit tree dwelling). By pairing this existing survey data with the new serology results generated from archived samples under Aim 1, as well as new data on nearby bat infection from Aim 2, we will be able to develop comprehensive risk profiles for exposure to filoviruses and henipaviruses in these communities.

C.4.3 Data analysis. The primary outcome for this analysis will be testing positive for IgG antibodies against any known filoviruses or henipaviruses. For both families of viruses, and for each species of virus within those families, we will use the serology results generated under Aim 1 to examine risk factors for seropositivity at baseline and for seroconversion between baseline and month 24. We plan to conduct a multivariate logistic regression, with appropriate weights applied to account for the single-stage cluster sampling design of the ENABLE Study, in which members of randomly selected households were surveyed and tested. We will use data from both the baseline and month 24 questionnaires to examine the effects of sampling site, participant demographics (e.g., age, gender, occupation), environmental exposures (e.g., animals present in and around the dwelling), and behavioral risks (e.g., direct contact with bats through hunting, preparing, or eating them, entering caves or mines where bats live).

Power analysis: To assess our power to identify risk metrics for seroconversion (or overall seropositivity), we built simulation-based power analyses that consisted of, in brief: 1) simulating survey responses for N (400-1000) individuals, while allowing for moderate unevenness in representation across five binary categories (as large as 80%-20%, for example); 2) examining a range of effect sizes (odds ratios ranging from 1-2.5) for these five binary categorical predictors (e.g., sex, encountered a bat, handled a bat, etc.). Summarizing across 1,200 simulations of surveys of 400-1000 individuals given joint uncertainty in the representation within these surveys and effect sizes, we estimated that we would have at least 80% power to detect significant effects for each predictor given an odds ratio of 1.65 or greater with 1,000 survey responses or ~1.80 (for example, with a rate just under 5% for Group1 = 0, just over 8% for Group1 = 1) with 750 responses.

C.4.4 Expected outcomes. By combining serology results with detailed questionnaire data on participant demographics and animal exposures, we will be able to use odds ratios to identify behaviors significantly associated with seropositivity (e.g., exposure to filoviruses or henipaviruses). We expect to be able to also identify age- and gender-based risk factors given our sample size. Based on previous experience conducting behavioral risk surveys, including a recent study in Ghana following a Marburg outbreak, we expect to be able to develop risk mitigation messaging that uses evidence from Aims 1 and 2 as well as results from this aim.

C.4.5 Potential problems and solutions. We anticipate that most IgG positive individuals for either set of viruses will be adults, with the likelihood of exposure and having antibodies increasing with age. Because we don't know how long IgG antibodies persist at detectable levels, it will not be possible to accurately determine when exposure occurred, with the exception being EBOV antibodies that most likely occurred as a result of contact with another infected person during the 2014-2016 outbreak. We will address this in two ways: by looking at respondents 2-6 years of age, we will know that any filovirus antibodies they have are most likely not due to the epidemic, and we will be able to determine whether there are any significant associations with particular behavior or other factor (e.g., household construction, domestic animal exposure) that promotes direct animal contact, consumption of contaminated fruit, or exposure to bat habitats (e.g., caves or mines). For adult respondents, we will focus on exposures (based on relative MFI values) specific to non-EBOV filoviruses and henipaviruses and see whether there are associations with specific behaviors.

C.5. Project timeline

Project timeline	Y1				Y2				Y3				Y4				Y5			
Activity	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Ethical approval (local IRB / IACUC)																				
Database development																				
Aim 1: Human serum testing																				
Aim 2: Bat sampling																				
Aim 2: Bat serum testing																				
Aim 2: Bat sample PCR																				
Aim 1&2: Confirmatory assays + sequencing (RML)																				
Aim 3: Behavioral risk analysis																				
Aim 3: Risk communication development																				
Publications																				
Data publication																				

D. Summary and Conclusions.

West Africa is a hotspot for emerging zoonotic viruses such as Ebola, Marburg, and potentially Nipah-related henipaviruses—groups that have been identified by WHO as among the most significant pathogens that threaten global health. Liberia is also a biodiversity hotspot, with some of the largest tracts of primary forest remaining in the region; its population relies on wildlife as a primary source of protein, creating continued risk of spillover of high consequence zoonotic pathogens. This project aims to comprehensively characterize the risk of filo- and henipavirus spillover events by measuring seroprevalence and seroincidence among a well characterized cohort of individuals in rural Liberia; identify bat reservoirs for Zaire ebolavirus, other filoviruses, and henipaviruses; and identify host, behavior, and environmental risk factors associated with exposure to these viruses. The results of this work will inform upstream interventions designed to prevent spillover and downstream interventions to facilitate early detection of human infections through implementation of pathogen-specific diagnostics. Our group comprises an unparalleled multidisciplinary group of leading experts in epidemiology, clinical medicine, virology, serology, and disease ecology all with deep experience working in Liberia and implementing zoonotic virus research, as well as strong partnerships with the Liberian public health institutions, giving this project a high likelihood of success in generating impactful results that will help prevent future epidemics in West Africa.

From:	Jon Epstein on behalf of Jon Epstein <epstein@ecohealthalliance.org>
To:	William A. Fischer, II; Wohl, David A; Eric Laing; Munster, Vincent (NIH/NIAID) [E]; Morgan Kain; Shannon Ball; Jim Desmond
Cc:	Madeline Salino
Subject:	Grant submitted!
Date:	Friday, February 3, 2023 4:55:00 PM
Attachments:	NIH R01_Liberia_Research_Final_Feb 2023.pdf

We did it!
Great job everyone! Thanks for all of your input, I think it came together nicely. Special thanks to Shannon and Madeline for all the hard work you did to keep us on track and make sure the proposal came together on time. Attached is the final version, as submitted.

I'll be interested to see what the reviewers think. I requested that it go to the TVZ study section which handles One Health and disease ecology type proposals. I believe it's a new study section, but it's really a good fit and I know several of the members.

Have a great weekend, everyone!

Cheers,
Jon

--

Jonathan H. Epstein DVM, MPH, PhD

Vice President for Science and Outreach

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

Specific Aims

Emerging infectious diseases - predominantly caused by zoonotic viruses - have become a major threat to individual and public health as well as the functioning of a global society. Even prior to the SARS-CoV-2 pandemic, outbreaks of ~~consequential~~ high consequence emerging pathogens including SARS-CoV, Pandemic influenza 2009, Zika virus, and Ebola virus disease have not only caused significant morbidity and mortality but have also ~~tragically killed and disabled and caused~~led to massive disruptions of regional and global economies and health care systems[1]. The ~~emergence~~ size and frequency of ~~consequential~~ high consequence zoonotic infections are increasing~~is accelerating~~ due to anthropogenic drivers such as climate change, land-use change, intensification of livestock production, and wildlife trade, all of which increase opportunity for people and domestic animals to come into contact with wildlife reservoirs[2-5]. **To reduce the risk of infectious disease epidemics, there is a critical need to detect and *prevent* spillover of zoonotic pathogens from wildlife reservoirs[2,6]**.

Illustrative of this need is the repeated spillover from wildlife reservoirs to human populations of groups of zoonotic viruses such as filoviruses (e.g., Ebola virus [EBOV]) and henipaviruses (e.g., Nipah virus [NIV]). Large-scale outbreaks or clusters of cases with these high-mortality zoonotic viruses have led to their being **designated as high priority pathogens by the World Health Organization (WHO)[5]**. However, at present, the detection of outbreaks caused by these pathogens occurs only after clusters of ~~the first~~ human infections and deaths occur; a failure in prevention that is deadly, costly, and remediable[1]. Identifying natural reservoirs of zoonotic viruses and measuring rates of exposure to these viruses in humans can inform both up and down-stream interventions to both~~are critical to assessing their potential threat and developing interventions that~~ reduce the risk of spillover and accelerate early detection.[1,2]

In the proposed study we have assembled a diverse team of experts in ecology, epidemiology, virology and immune response to bat-borne viral zoonoses such as filoviruses and henipaviruses; and filovirus clinical care and research who are actively working in West Africa to provide much needed empirical evidence to help answer the questions: **What is the natural reservoir for Ebola virus and how often does spillover of this and related viruses happen in a region that is an emerging disease hotspot, suffered the world's largest filovirus outbreak, and where there has been evidence of other consequential zoonotic viruses in animals and people**? To address these questions, we propose the following specific aims:

Aim 1. To determine the rates of filovirus and henipavirus exposure in people in rural Liberia. We have shown that certain bat species in Liberia carry EBOV-Zaire, while others carry Marburg virus and henipaviruses. Further, hunting bats is a common activity and we have detected high seroprevalence of Marburg and EBOV antibodies in residents of rural Liberia. Exposure to henipaviruses remains unknown. To test the hypothesis that spillover of both groups of viruses has occurred in this region, we will use an antigen-based multiplex serological assay to screen more than 1,9800 archived blood specimens, collected in 2021 and again in 2023 from over 900 adults and children in rural central Liberia as part of a longitudinal study of Lassa fever seroprevalence and seroincidence, for antibodies against all known filoviruses and henipaviruses.

Aim 2. To identify reservoirs and characterize the infection dynamics of filoviruses and henipaviruses in key bat species in Liberia. Our group detected EBOV-Zaire RNA and antibodies in *Miniopterus nimbae*, a cave- and mine-dwelling bat in northern Liberia found near the origin of the 2014 Ebola outbreak in Guinea. We also detected antibodies against EBOV in *Hipposideros* bats that co-roost with *Miniopterus* in multiple locations in Liberia. *Rousettus aegyptiacus*, a common frugivorous bat in Liberia, carries Marburg virus and henipaviruses. We will conduct a 3-year longitudinal study of populations of these bat species near the human cohort studied in **Aim 1**, to determine whether they are reservoirs for Ebola or other filoviruses and henipaviruses and characterize the temporal dynamics of filoviruses and henipavirus circulation using multiplexed serological assays that test for IgG antibodies against all known filoviruses and henipaviruses, as well as molecular techniques to screen for Ebola, Marburg, Nipah virus, and related viruses.

Aim 3. To characterize behaviors associated with exposure to filoviruses and henipaviruses. Combining the data collected in Aim 1 (humans) and Aim 2 (bats) we will identify the demographic, behavioral, and environmental factors associated with an individual's prior exposure to filoviruses and henipaviruses. Detailed

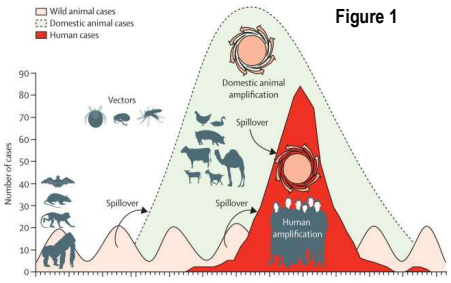
animal exposure data collected from the adults and children (many born after the Ebola outbreak) who provided the Aim 1 blood specimens will be used to develop profiles of risk and inform public health & risk reduction communication tools in partnership with the Ministry of Health, Liberia.

RESEARCH STRATEGY

A. SIGNIFICANCE

Zoonotic viruses are responsible for the majority of emerging infectious disease outbreaks, which are occurring at an accelerating rate in global hotspots[3,7]. Every outbreak begins with spillover – transmission of the virus from its animal reservoir either directly into human populations or into other animal hosts *en route* to humans (**Figure 1**). **Exactly how often spillover occurs is virtually unknown,** as surveillance systems are primarily designed to detect clusters or outbreaks, and often miss initial individual infections that result from animal contact. To reduce the risk of future epidemics, it will be necessary to limit opportunities for zoonotic viruses ~~to~~ spillover and to detect spillover at the earliest stages.[1,8] Measuring the efficacy of any interventions designed to reduce spillover and spread of zoonotic pathogens will rely on measuring rates of exposure in at-risk human populations.

Figure 1



Filoviruses and henipaviruses are among the highest consequence zoonotic viruses and have greatly impacted public health. Each group contains zoonotic pathogens that cause significant disease in both people and animals, especially livestock, that have been associated with extremely high rates of morbidity and mortality. Ebola (EBOV) and related viruses, as well as Nipah virus (NIV), a henipavirus, and its related viruses have been listed by the World Health Organization (WHO) among the highest priority pathogens for the development of vaccines and therapeutics, as they represent zoonotic pathogens with high potential to threaten human health, with extremely high mortality rates and few or no therapeutics or vaccines currently available.[9-11] These viruses share other important features which make them high priority: they are all associated with bat hosts that are abundant and widely distributed throughout Africa and Asia, living in close association with people and livestock in some of the most densely populated regions on Earth.

Spillover of these viruses has repeatedly occurred. Beyond the massive outbreak in West Africa in 2013-16, EBOV outbreaks have occurred repeatedly in Central and East Africa; most recently Sudan ebolavirus caused an outbreak in Uganda in late 2022[11-13]. Marburg virus (MARV) and Ravn virus (RAVV) comprise the closely related genus *Marburgvirus* and have caused outbreaks in Central and East Africa and Europe with mortality and clinical presentation comparable to Ebola [14,15]. Marburg virus outbreaks were declared in Guinea, just across the border from the UNC-Liberia research site in 2021 and then again in Ghana in 2022 marking the first time this virus has been reported in either country[16,17]. ~~Nipah~~ NIV ~~virus~~ is an emerging zoonotic paramyxovirus that has caused nearly annual outbreaks in Bangladesh, including multiple outbreaks in 2023, and sporadically in India with mean case fatality rates greater than 70% [12,18-20]. Despite the public health significance of Nipah and related viruses, little is known about its genetic diversity, natural reservoirs, or frequency of spillover throughout their range, including Africa. Henipaviruses, and ~~Nipah virus~~NIV in particular - have several characteristics that make them a global health priority: 1) Their bat reservoirs occur throughout Asia and Africa, overlapping human and livestock populations, giving them geographically broad opportunity to cause outbreaks [21]; 2) henipaviruses can be transmitted to people directly from bats or via domestic animals [21]; 3) ~~Nipah virus~~NIV can be transmitted from person to person [22]; 4) ~~Nipah virus~~NIV spillover has occurred and continues to occur in highly populous and internationally connected regions; 5) repeated spillovers of NIV strains with varying person-to-person transmission rates indicate the ability to evolve **with increased pandemic potential** [23,24]; and 6) ~~Nipah~~ NIV is associated with a high mortality rate in people and currently has no vaccine or treatment [21,23]. While ~~Nipah virus~~NIV has not been detected in Africa, closely related henipaviruses have been identified in Ghana and Madagascar, though their public health significance remains unknown[25-27]. In West Africa, where bats and associated henipaviruses occur but where no human cases have been identified, **diagnostic testing and systematic surveillance is essentially non-existent**. The detection of antibodies against a Nipah-related virus in hunting communities in Central Africa suggests that cryptic spillover is occuring[28]. **Repeated spillover of henipaviruses creates opportunity for more transmissible strains to emerge[29].**

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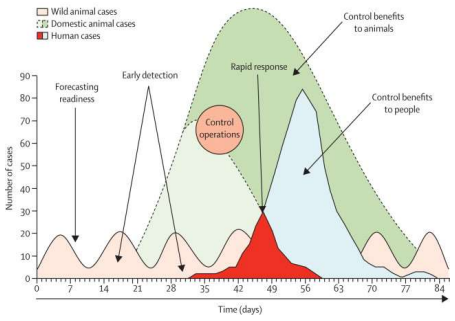
While bats have been identified as potential hosts for filoviruses including EBOV, the natural reservoir for EBOV-Zaire Ebola in West Africa remains unknown. There is substantial evidence, both serological and molecular, that bat species host filoviruses; however, a definitive natural reservoir or set of reservoirs for EBOV-Zaire has not been identified[30]. Wildlife, including bats, represent a substantial source of animal protein for communities in Liberia[31]. People may be exposed to bat-borne viruses through hunting and butchering, eating food contaminated by bat excreta, or by entering caves or mines inhabited by bats. Our group previously found that people living in rural areas of Liberia ~~conduct~~ engage in activities ~~which may~~that increase their risk of contact with bats or their bodily fluids. The index case~~, a child,~~ in the 2013 Ebola outbreak, a child, was hypothesized to have had contact with bats, yet the exact species was never definitively identified[32]. The route of spillover for most historical Ebola outbreaks remains a mystery. Despite broad efforts to determine the natural reservoirs of EBOV and related viruses, there continues to be a poor understanding of filovirus ecology, with the exception of ~~Marburg virus~~MARV, whose host has been identified as Egyptian rousette bats (*Rousettus aegyptiacus*)[33-35]. Communities known to hunt bats in Cameroon and India have been found to have antibodies against henipaviruses and filoviruses, respectively, yet nothing is known about community exposure rates in Liberia, where bat hunting is common[28,36]. Our preliminary data (see below) suggest that there is substantial exposure to EBOV as well as MARV among people in central Liberia. While the EBOV antibodies may indicate prior infection from another person such as during the West Africa outbreak, antibodies specific to other filoviruses may signal a localized exposure from animals. Even less is understood about henipaviruses in ~~Africa, and~~Africa and identifying antibodies against henipaviruses in people will be critical for understanding where, how and how often spillover occurs.

The urgent need to develop interventions to prevent spillover of zoonotic high consequence pathogens can only be met with a better understanding of their natural reservoirs, the rates of exposure in vulnerable human populations, and the ways in which people are exposed. Unrecognized outbreaks provide opportunities for viruses to emerge that may be more transmissible or more pathogenic than previously observed, and limiting these opportunities is an important component of pandemic prevention[2,29]. Typically, by the time an outbreak is recognized in human populations its containment and control are difficult, if not impossible to achieve (e.g., HIV, SARS-CoV-2). Understanding the ecology of these viruses, including which species act as reservoirs as well as how and to what extent people are being exposed to filoviruses and henipaviruses will inform intervention and surveillance strategies and provide baseline metrics against which one can measure the efficacy of interventions designed to reduce exposure, such as public health campaigns to promote avoidance of behaviors associated with infection among those most at risk (**Figure 2**).

This proposed study will be the largest and most rigorous One Health investigation of high-consequence zoonotic viruses in bats and humans in an emerging disease hotspot. West Africa is known to be the source of prior outbreaks of emerging infectious diseases and future spillover events there are highly likely. Liberia is typical of this region and contains some of the largest tracts of pristine forest and wildlife biodiversity remaining in West Africa and has a population that depends on wildlife, including bats. Decades ago, a spillover event from a non-human primate to a human triggered an on-going HIV pandemic that has led to the infection of over 60 million people and 25 million deaths. Only months ago, the orthopoxvirus MPOX found in rodents spread to and within 110 countries across the globe causing over 85,000 cases. Both originated from African wildlife. The proposed study will leverage the unique infrastructure and expertise we have developed in Liberia to undertake a comprehensive examination of filovirus and henipavirus spillover from bats to humans and provide evidence of previous and possibly on-going transmission events. Our findings will provide essential

Figure 2



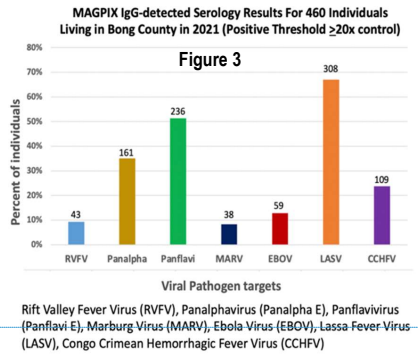
evidence of the presence and transmission dynamics of these bat-borne viruses and identify~~as~~ the risks for infection in people living alongside them.

B. INNOVATION

The proposed research is highly innovative in multiple ways, including: 1) Our use of a one Health approach to study the ecology and epidemiology of two groups of high-priority, high-consequence zoonotic viruses in both bats and people in West Africa – an emerging disease hotspot where spillover events have occurred (e.g. Ebola, Marburg, MPOX, Lassa fever) and are highly likely to re-occur; 2) Our use of a novel and unique multiplexed serological platform, developed by our group, that will allow us to screen bat and human serum for IgG antibodies against each of the known filoviruses and henipaviruses, simultaneously in a single assay. Longitudinal comprehensive serological studies in bats and people **will provide unprecedented insight into the changes in rates of exposure over time to ~~known~~ high consequence viruses in bats; and in people (e.g. what is the baseline rate of exposure to henipaviruses and filoviruses and is there evidence of recent exposure?); and exposure rates to unknown, antigenically related viruses;** 3) We will couple serosurveillance with molecular testing to identify specific filoviruses and henipaviruses carried by bats; and 4) We will connect the bat and human epidemiological studies by analyzing questionnaire data from the human cohort to identify~~ing~~ high risk behaviors associated with viral exposure. **Results from this analysis will inform the development of public outreach and risk reduction campaigns by the Government of Liberia.**

C. APPROACH

C.1. Overview: **Aim 1** will test the hypothesis that spillover of filoviruses and henipaviruses occur regularly in rural Liberia by testing for IgG antibodies to all known filoviruses and (~~see~~ Ttable 1~~3~~) in ~~archived~~ serum samples from more than 900 people living in Bong county (central Liberia), collected between 2021-23 at two time points 24 months apart as part of the Coalition for Epidemic Preparedness Innovations (CEPI)-sponsored **ENABLE Study**, an on-going longitudinal study of Lassa fever seroprevalence and seroincidence. ~~-~~Seroprevalence of filoviruses and henipaviruses will be assessed as will ~~changes~~ seroincidence~~between the two collection timepoints~~. In **Aim 2,** we will conduct a 36-month longitudinal study of three bat species which are putative reservoirs for EBOV and undescribed henipaviruses. Using a combination of longitudinal serology and molecular testing (PCR and whole genome sequencing) **we will determine the prevalence of filoviruses and henipaviruses in ~~three~~ these key bats species associated with the rural human populations studied in Aims 1 and 3**. For **Aim 3,** we will analyze detailed questionnaire data developed collaboratively by our group (EHA and UNC) and collected in 2023 along with blood samples to be screened in Aim 1. These data will allow us to identify ~~behaviors~~ factors associated with exposure to henipaviruses and filoviruses. We will use results from our behavioral and exposure risk assessment to develop public risk reduction communication strategies with the National Public Health Institute, Liberia, using the results of this study to inform public health strategies. This application is a natural progression of our research activities, and our preliminary data speak to the feasibility of the proposed study.



Rift Valley Fever Virus (RVFV), Panalphavirus (Panalpha E), Panflavivirus (Panflavi E), Marburg Virus (MARV), Ebola Virus (EBOV), Lassa Fever Virus (LASV), Congo Crimean Hemorrhagic Fever Virus (CCHFV)

C.2. Aim 1: To determine the rates of filovirus and henipavirus exposure in people in central rural Liberia.

C.2.1. *Rationale and Preliminary Data.* The primary goal of this aim is to be able to determine whether populations within Liberia who live in association with bats have evidence of exposure against one or multiple filoviruses and/or henipaviruses and to compare rates of exposure over time. Studies of human populations in Central and West Africa have found evidence of exposure to EBOV prior to the 2013 outbreak and in places not known to have had Ebola outbreaks[37,,38]. UNC Project-Liberia has previously assessed seropositivity to

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~~consequential~~ high consequence viral pathogens including EBOV and MARV in a subset of ~~CEPI~~ ENABLE Study participants. ~~About~~ Over 8~~1~~0% of participants had antibodies against MARV using a conservative threshold for seropositivity (**Figure 3**), suggesting that unreported spillover events have previously occurred. Acute encephalitis is common in western sub-Sharan Africa, yet outbreaks of neurological disease are rarely reported and more than half of all cases are undiagnosed[39]. Among the countries in western Africa reporting cases of meningitis or encephalitis, Liberia has reported the fewest, likely due to a lack of surveillance and capacity to diagnose neurological diseases[39]. ~~Nipah virus~~NIV encephalitis has never been reported in Africa, however, as described above, two Nipah-like henipaviruses have been described in bat hosts, including one in Ghana[25,27]. Spillover of henipaviruses was reported in bat-hunting communities in Cameroon[28]. In South Africa, we detected henipavirus sequences in *Rousettus aegyptiacus,* bats which also commonly occur in Liberia[26].

In Aim 1, we will test the hypothesis that spillover of both filoviruses and henipaviruses have occurred in rural populations in Liberia by measuring seropositivity to both groups of viruses within the CEPI ENABLE Study community cohort. We will also measure changes in exposure rates over a two-year period. While prior studies have provided cross-sectional snapshots of exposure, our longitudinal approach will provide data from the same cohort at two timepoints, 24 months apart, to determine both seroprevalence and sero-incidence using a multiplexed serological assay to screen for IgG antibodies against all known filoviruses and henipaviruses.

Settings and Participants. To achieve this aim, we will leverage a well characterized cohort of individuals in three rural communities located in Bong County, Liberia (**Figure 4**), which has a population of over 333,400. UNC Project-Liberia has been operating in Liberia since 2014 and established a research site at Phebe Hospital in Bong County that includes a molecular laboratory that conducts daily qPCR testing for LASV, MARV, and EBOV as well as LASV serological assays using a MAGPIX (SEE RESOURCES AND FACILITIES). There, following extensive community engagement including meetings with key stakeholders, town hall meetings, and radio messaging, UNC launched the CEPI **ENABLE Study**, the largest passive and active surveillance program for Lassa fever in Liberia. Started in 2021~~0~~, 5,005 randomly selected participants 2 years of age and older in Phebe Airstrip, Suakoko, and Rubber Factory were enrolled over 6 months following informed consent/assent (mean age at enrollment is 22.2 years (range 2-97) and 54.2% are female). **Importantly, 7.7% of the cohort is under the age of 5 years, and therefore, were born after the 2013-14 Ebola outbreak (Table 1).** Households were selected for recruitment following identification of roof tops by satellite photos and use of a program developed by Médecins Sans Frontières' (MSF) Epicentre to randomly select~~ed~~ structures to ~~consecutively~~ approach sequentially for participation. The ENABLE Study includes baseline questionnaires and blood specimen collection from all participants with active follow-up by a study community health worker every 2 weeks to determine i~~f~~s a febrile event has occurred, which triggers drawing of blood for LASV, MARV, and EBOV PCR. Baseline blood is tested for Lassa fever virus (LASV) serology. A subset of 1,024 individuals (every 5th approached household is eligible) is also asked to provide blood samples routinely every 6 months for up to 24 months for repeat LASV serology. The blood samples from 0 and 24 months and the associated surveys will be used in **Aims 1 and 3** of this proposal. Informed consent and assent documents include provisions for the use of collected specimens and data for infectious diseases research including for emerging pathogens (see PROTECTION OF HUMAN SUBJECTS).

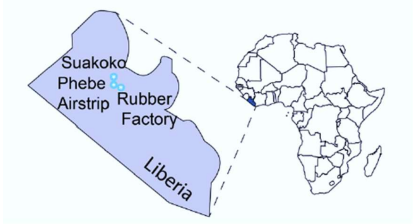

Figure 4. Map of Liberia with Bong County sites shown in inset.

C.2.2. Research Design. We will screen approximately **1,900 archived serum specimens** from participants in the ENABLE Study cohort at two time points ("baseline" (Month 0) and (Month 24)) for IgG antibodies against specific filoviruses and henipaviruses using the MAGPIX at the UNC molecular lab at Phebe H~~h~~ospital to run the multiplexed *henipavirus / filovirus* platform to be provided by Co-I Laing in year 1. The multiplex platform will allow us to determine specifically which filoviruses and henipaviruses people have been exposed to and compare that to the filoviruses and antibodies we observe circulating in local bat populations in

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Aim 2. The bead-based multiplexed assay has tremendous advantage over traditional ELISAs in that it will identify up to 25 different antibodies against multiple viral agents in a single, 2uL serum sample and uses a high through-put format. The ability to look at reactivity across all serogroups simultaneously will allow us to differentiate among the viruses. Proteins A/G non-specifically bind IgG immunoglobulins, and weakly bind IgM, an immunoglobulin that is the first to be produced during the initial stages of infection. We will conduct a 1-week training at the UNC-Phebe lab for using the assay to detect filovirus and henipavirus antibodies as well as data management, bioinformatics and analysis. **Table 13** shows the viral species and antigens that will be employed in this assay. The platform has been previously transferred to collaborating labs in the UK, South Africa, India, Bangladesh, Singapore, Thailand and Malaysia. We have previously detected serological profiles of Asiatic ebolaviruses in fruit bats collected in Singapore[40] and in fruits bats and humans actively engaged in bat hunting in northeast India[36].

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Table 13. Multiplexed microsphere immune assay including soluble glycoproteins from each known filovirus and henipavirus

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Virus (Name/Isolate Host/Country/Year/Strain)	Abbreviation	Accession No.	Soluble Glycoprotein	Mag Bead No.
Ebolaviruses				
Ebola virus/H. sapiens/COD/1976/Yambuku-Mayina	EBOV	NC_002549.1	$GP_{(1,2)}$	34
Bundibugyo virus/H. sapiens/UGA/2007	BDBV	FJ217161.1	$GP_{(1,2)}$	64
Bombali ebolavirus/Mops condylurus/SLE/201	BOMV	NC_039345	$GP_{(1,2)}$	55
Taï Forest virus/H. sapiens/COV/1994/Pauleoula-Cl	TAFV	NC_014372	$GP_{(1,2)}$	57
Sudan virus/H. sapiens/UGA/2000/Gulu-808892	SUDV	NC_006432.1	$GP_{(1,2)}$	54
Reston virus/M. fascicularis/USA/1989/Pennsylvania	RESTVm	AF522874.1	$GP_{(1,2)}$	65
Marburgviruses				
Marburg virus/H. sapiens/KEN/1980/Musoke	MARV	Z12132 S55429	$GP_{(1,2)}$	37
Ravn virus/H. sapiens/AGO/2005/Ang0126	RAVV	NC_024781.1	$GP_{(1,2)}$	62
Dianlovirus				
Mengla virus/Rousettus-WT/CHN/2015/Sharen	MLAV	KX371887.2	$sGP_{(1,2)}$	22
Cuevavirus				
Lloviu virus/M. schreibersii-wt/ESP/2003/Asturias-Bat86	LLOV	NC_016144.1	$sGP_{(1,2)}$	66
Henipaviruses				
Hendra virus/E. caballus/AUS/1994	HeV	NC_001906.3	sG	43
Hendra virus-2G	HeV-2g		sG	48
Nipah virus/H. sapiens/MYS/2000	NiVm	NC_002728.1	sG	46
Nipah virus/H. sapiens/BGD/2004	NiVb	AAY43916.1	sG	56
Cedar virus/Pteropus sp./AUS/2012/CG1a	CedV	NC_025351.1	sG	53
Mojiang virus/R. sladeni/CHN/2014/Tongguan1	MojV	NC_025352.1	sG	29
Ghanaian bat virus/E. helvum/GHA/2009/GH-M47a	GhV	NC_025256.1	sG	35
Angavokely virus	AngV		sG	47

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C.2.3. Data Analysis. We will create a project database using Airtable, which will be hosted at EHA and will house all data from Aims 1 and 2 and will link to the RedCap server which stores questionnaire data from Aim 3. Database access will be secure and limited to key personnel (see data management plan). Raw MFI values from the MAGPIX will be used to calculate reactivity to each viral antigen and based on relative strength of reactivity (**Fig 5**) and determinations of negative cutoffs based on reference sera, we will calculate prevalence rates for each time point and compare rates between the two timepoints. Serological profiling can be used to detect reactive antibodies against all specific filoviruses and henipaviruses in a samples and compare strength of reactivity and cross-reactivity against multiple antigens

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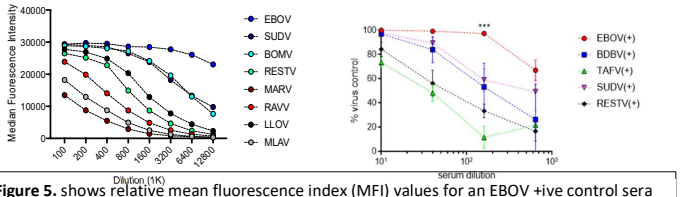

Figure 5. shows relative mean fluorescence index (MFI) values for an EBOV +ive control sera across serial dilutions. While there is cross reactivity, the EBOV MFI values are consistently greater than the other viral GPs and at higher dilutions the separation becomes significant.

using a single sample[41]. We will compare the antibodies across all specific viral antigens to assess which virus or groups of viruses are responsible for the immunological response[42-44]. We will assess exposure to non-Zaire ebolavirus species, which may indicate spillover of filoviruses has occurred separate to the Ebola outbreak of 2013. We will also look at evidence for exposure to Nipah-like henipaviruses and compare exposure rates to both viral groups within and between our two sample collection time points.

C.2.4. Expected outcomes. We anticipate obtaining serological profiles of exposure to filoviruses and henipaviruses from each individual in our cohort. In previous studies we found antibodies against both ~~Ebola~~ EBOV and ~~Marburg~~ MARV virus. While the ~~Ebola~~ EBOV antibodies are attributable to the 2013-2016 epidemic, ~~Marburg~~ MARV antibodies, in ~~patients~~ EBOV IgG negative patients, suggest possible exposure from an animal (e.g. bat) reservoir. We also anticipate seeing reactivity to ~~to~~ Bombali virus (BOMV), given the presence of the reservoir species in Liberia (*Mops condylurus*) and reactivity to the African henipaviruses (GhV and AngV), although this may represent reactivity to an antigenically related virus. Serological results from this and the bat study will inform our strategy for prioritizing which bat samples we test by PCR.

C.2.5. Potential problems and solutions. While our preliminary data suggest there will be detection of immune responses to filoviruses including EBOV-Zaire and, significantly, MARV, it is possible that evidence of exposure to other pathogens, including henipaviruses will not be detected. This would be an important finding in this large cohort, especially if these viruses are detected in local bat species, that would warrant further investigation. It is also possible that there will be few or no seroconversion events among the cohort during the 24-month period of follow-up. This too would be reassuring but would suggest a need for longer follow-up of this cohort. To address the possibility that reactivity to EBOV-Zaire virus may be explained by infection during the 2013 Ebola epidemic, we will create a polyclonal sera reference standard utilizing ~~convalescent~~ sera collected from human survivors of EVD. This internal reference standard will be calibrated to the WHO EBOV human serology standard (**Fig 6A**) permitting direct interpolation of anti-EBOV IgG as ~~a~~ MFI to a standard antibody concentration (binding antibody units/mL). The internal reference standard will then be included in our human serology testing strategy allowing semi- or quantitative measurement of anti-EBOV IgG. These EBOV human serology standards will permit us to establish positive and negative predictive performance values for the multiplex assay; furthermore, we will be able to investigate immunoreactivity of EVD survivors against heterotypic ebolaviruses. Our preliminary data indicates that EBOV+ sera IgG is highly cross-reactive with soluble native-like trimeric envelope glycoprotein (GP) antigens from both EBOV and BDBV (Figure 6A). EBOV GP, matrix protein (VP40), and nucleocapsid protein (NP) antigens have all been used in a variety of serological tests, however, the frequency of seroconversion against each antigen has not been fully described. In a longitudinal study of EVD survivors, high seroconversion against GP and VP40 was observed, with affinity maturation occurring overtime against GP[45]. Antigens based on the GP are likely to provide an optimal intrinsic balance of sensitivity and specificity. To limit the degree of heterotypic ebolavirus cross reactions we will test any anti-ebolavirus GP trimer antigen-seropositive samples with a pan-ebolavirus GP1 antigen-based panel. In preliminary studies, GP1 antigens improve specificity and differentiation of antibodies reactive with homotypic ebolaviruses in polyclonal antisera from rabbits immunized with GP trimer immunogens (**Figure 6B**).

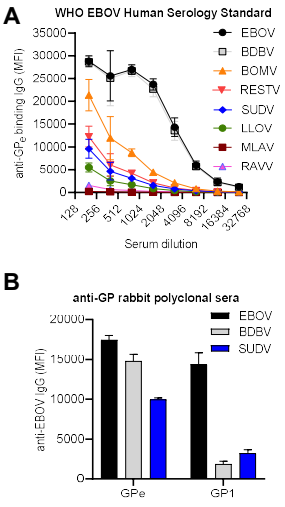

FIGURE 6A & B. A) The WHO EBOV human serology standard was testeding for immunoreactivity in a filovirid GP-trimer based multiplex assay. Filovirids are indicated along with sera dilution factors; data is the mean of three independent experiments. B) Antisera from EBOV GP-trimer immunized rabbits were tested in multiplex panels comprised of GP-trimer and GP1 subunit proteins. Anti-EBOV polyclonal IgG was less reactive with GP1 from BDBV and SUDV.

C.3. Aim 2. To identify reservoirs and

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characterize the infection dynamics of filoviruses and henipaviruses in key bat species in Liberia.

C.3.1 *Rationale and Pp*reliminary data. Bats have been established as important hosts for filoviruses and henipaviruses in Africa and Asia, with combinations of viral isolation, molecular detection or serology providing substantial evidence [30,46-49]. MARV was isolated from Egyptian rousette bats in Uganda, and RNA has since been detected in these bats in Sierra Leone, which borders Liberia [50]. To date, infectious EBOV has never been isolated from bats, although molecular and serological evidence suggest that multiple frugivorous bats may carry EBOV in Central Africa[50,51]. Prior to the 2014 epidemic, Zaire ebolavirus had not been reported in West Africa, and the animal reservoir remains unknown[32]. From 2016-2019, our group (PI Epstein & KP Desmond) implemented a large-scale project to identify reservoirs for EBOV in West Africa, under the USAID PREDICT Project[52]. This effort led to the discovery of a new species of Ebola (Bombali virus)[53], the detection of MARV in E Egyptian rousette

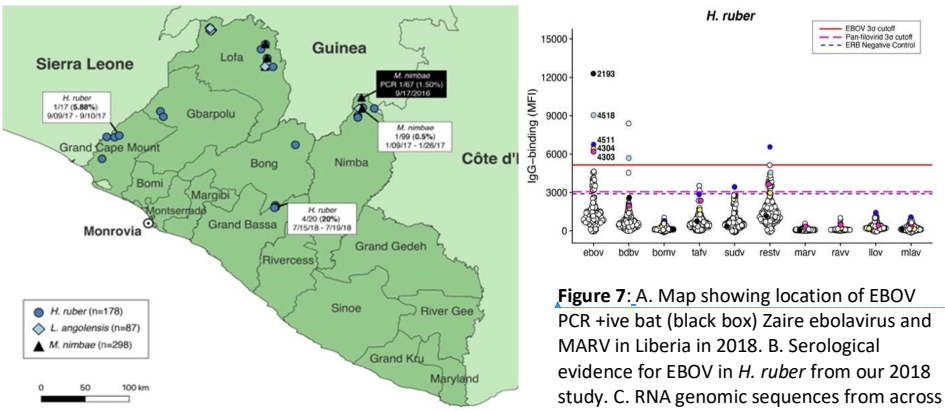
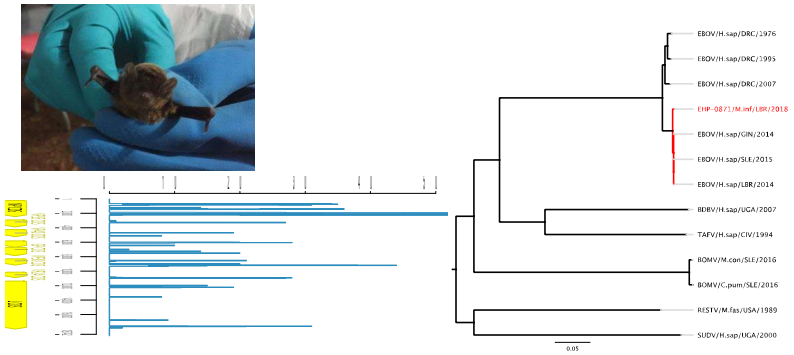


Figure 7: A. Map showing location of EBOV PCR +ive bat (black box) Zaire ebolavirus and MARV in Liberia in 2018. B. Serological evidence for EBOV in *H. ruber* from our 2018 study. C. RNA genomic sequences from across the *Zaire ebolavirus* genome detected in *Miniopterus nimbae*, Liberia in 2018. D. Phylogenetic analysis supports relatedness to 2013 EBOV epidemic virus.



batsRBs in Sierra Leone[33], and **in 2018, we detected, for the first time in West Africa, EBOV RNA and anti-EBOV IgG antibodies in bats (Figure 7A-D)**.

While this finding generated the first evidence of which bats

may be reservoirs for Zaire ebolavirus, longitudinal studies are required to more completely understand the role

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these species play in EBOV circulation – either as reservoirs that maintain the virus within a local population, or as incidental hosts, infected by an unknown bat species. One-off, cross-sectional studies have a low probability of detecting any specific filovirus or henipavirus given their low prevalence and short viremic periods [34,54], and thus provide a poor characterization of viral diversity. The lack of understanding of **filovirus and henipavirus diversity and their natural reservoirs in West Africa significantly impedes risk-based public health interventions to prevent human outbreaks.** Longitudinal sampling is needed to separate reservoir hosts from spillover hosts. For example, MARV has been isolated repeatedly from *R. aegyptiacus* in Uganda, which, when paired with experimental infection studies showing infection and shedding of MARV by *R. aegyptiacus,* strongly suggests that *R. aegyptiacus* is indeed a ~~Marburg virus~~MARV reservoir[35,55,56]. Similarly, our longitudinal studies of ~~Nipah virus~~NIV in *Pteropus medius* bats in Bangladesh, which included the same multiplexed serological assay proposed here, confirmed them as a reservoir for NIV and identified important mechanisms driving viral circulation[29]. **Understanding which species persistently carry filoviruses and henipaviruses will provide important insights into how ~~they~~ these groups of viruses are maintained in nature and what species should be targeted for follow-up studies on bat habitat use and human-bat contact patterns.**

C.3.2. *Sampling design*. We will repeatedly sample three bat species associated with filoviruses or henipaviruses, based on our preliminary data: *M. nimbae* (EBOV), *H. ruber* (EBOV), and *R. aegyptiacus* (MARV & Henipaviruses). We will sample bats at roost sites in Bong county, as close as possible to the human study sites in Aim 1. In Y2-Y4, we will sample 40 individuals of each species every two months for 36 months and collect excreta using plastic tarps placed underneath bat roosts (details below and in C.3.3).

Bat capture and sampling. Briefly, we will use either harp traps or mist nets to capture bats, depending on the capture site. Personnel will wear appropriate personal protective equipment (e.g. Tyvek suits or dedicated long clothing; double nitrile gloves; an N95 respirator and safety glasses or a PAPR [57]. Traps and nets will be attended continuously during capture periods. Bats will be removed from mist nets immediately upon entanglement to avoid injury. Bats will be removed from harp traps after approximately 1 hour and bats will be placed in individual cloth bags until processed. Body measurements, blood, oropharyngeal swabs, and rectal swabs (or fecal pellets) will be collected from each bat. Swabs will be collected in duplicate; one swab will be placed in a cryovial containing Trizol lysis buffer and the other in viral transport medium (VTM). For bats <50g, blood will be collected in a microhematocrit tube and spun down using a portable centrifuge. A bulb will be used to expel serum from the tube into a cryovial. For larger bats >50g, a tuberculin syringe or 1ml syringe and 27g needle will be used to draw blood from the radial artery or vein[57]. Blood will be placed into a conical vial and centrifuged. Serum will be aliquoted into a cryovial using a pipette. During site visits we will also lay out plastic tarps to collect pooled environmental excreta in Trizol and VTM to improve opportunity to detect viral RNA. All samples will be placed in a vapor phase liquid nitrogen dry shipper or portable battery-powered ultracold freezer, immediately upon collection and then transferred to a -80C freezer at the Phebe lab until testing.

C.3.3. *Testing plan, ~~d~~Data analysis, sample size, and power analysis. Serology:* At the end of Y3, we will screen all serum samples collected to date using the MAGPIX multiplex assay for IgG against filoviruses and henipaviruses. We will analyze temporal dynamics in seroprevalence using general additive models (GAMs), which are analyses that we have used successfully in many previous studies of similar design[29]. These methods are well adapted for capturing long-term temporal fluctuations in prevalence and moderately sensitive at capturing short-term changes in seroprevalence which can be used to identify population-level disease outbreaks. For example, we estimated that with 40 bats sampled every two months, we would be able to statistically detect an increase in seroprevalence (at a 95% CI limit) of greater than 25% between consecutive sampling events > 80% of the time, assuming a bat population seroprevalence of at least 10% in advance of a bat colony infection (estimated by, in brief, by simulating a fluctuating true seroprevalence value, simulating samples given this underlying seroprevalence, fitting a GAM to these samples, and then calculating if CI on true prevalence between the two sampling occasions over which the change in seroprevalence occurred were non-overlapping). While this is a large change, it is within a range to be expected if infection were to sweep through a bat community[29]. MAGPIX-positive bat sera will be sent to RML for serum neutralization assays using viral isolates or VSP pseudoviruses under BSL4 conditions.

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VSV-pseudotype neutralization assays: Serological results will be confirmed using VSV-pseudotype neutralization assays for the following filoviruses EBOV, SUDV, BDBV, RETV, BOMV, MARV and RAVN. If novel henipavirus sequences are detected, additional assays will be developed to test positive sera. Serum samples will be inactivated using ɣ-irradiation (4 MRad). Neutralization of irradiated and heat-inactivated serum samples will be assessed in Vero 352 E6 cells. Briefly, cells were seeded in 96-well round-bottom plates for 24 hours, serial dilutions of heat-inactivated serum samples will be performed in DMEM supplemented with 2% FBS, penicillin/streptomycin, and L-glutamine. Each plate will contain negative serum control, cell-only control, and virus-only control. VSV EBOV-GFP will be added to each well of the serum dilution plate and the serum-virus mix 357 was incubated at 37˚C for 1-hour. The mix was added to the cells and incubated at 37˚C for 24 hours. The cells will then fixed with 4% paraformaldehyde at room temperature 359 for 15-minutes and centrifuged at 600 x g for 5-minutes at room temperature. The supernatant will be discarded and FACS+EDTA buffer was added. Samples will be run on the FACSymphony A5 Cell Analyzer (BD Biosciences, Mississauga, ON, Canada) and FITC MFI 362 was measured. Data will be analyzed using FlowJo.

Molecular testing: We will use specific filovirus taqman real-time PCR assays to screen oropharyngeal, fecal and environmental samples from bats for EBOV and MARV RNA at the UNC-Phebe lab[55,58]. Testing bat samples by PCR may allow us to detect viral RNA during active infection, providing important information about the prevalence and timing of infection within target species. **Because we expect viral prevalence to be significantly lower than seroprevalence, estimated at <5%[25,27,49],** we developed a sampling scheme focused on maximizing our probability of detecting viral RNA with qPCR. To compare power under alternative sampling designs, we built simulation-based power analyses that estimated power to detect: 1) viral EBOV or MARV RNA in at least one bat; and 2) the expected number of positive cases, in each bat species. In brief, we estimated power for total sample sizes between 1500-3000 (between 500 and 1000 bats sampled per species) spread across a variable number of visits per year (2-6), while allowing for uncertainty in true infection dynamics in the bat populations, specifically: 1) prevalence during one three-week period (with unknown timing) per year of high prevalence ranging from 1-5%); and 2) an otherwise low background prevalence (0-1%). Given these estimates of yearly infection dynamics, these analyses showed with 672 samples per species (minimum 32 individual bats per sampling event), spread across 6 sampling occasions per year, we would obtain a per-species probability of acquiring 0 positive samples of <4% (<1% at 40 bats per event – our target number) and an expectation of ~3-6 RNA positive results (~5-10 positives at 40 bats per event). Because henipaviruses have not been previously described in Liberia, we will focus our resources on filovirus testing by PCR. However, if henipavirus serology suggests active circulation, we may send a set of samples to RML for pathogen discovery (SEE RML LETTER OF SUPPORT).

Viral isolation: PCR-positive cDNA samples and their aliquot in VTM will be sent to RML for further sequencing and attempts at viral isolation under BSL4 conditions. If attempts to culture do not work, we will use next generation sequencing techniques to get whole genome sequence and further characterize the virus at RML. Novel viruses will be characterized and rescued (reverse engineered using a minigenome system to synthetically create infectious viral particles [59]) at RML under secure, biosafety level 4 conditions, then used for serum neutralization assays in bat and human sera to determine more precise exposure rates. Sequences from the glycoproteins of any novel viruses discovered will be used by USU (Co-I Laing) to generate purified proteins and added to the Luminex assay, which will improve its specificity.

VirCapSeq-VERT and myBaits probe-based enrichment for recovery of full-length genome sequences of filoviruses: Although shotgun sequencing provides opportunities to analyze all host and virus genetic material, the larger average genome size of bacteria and hosts compared to viral genomes complicates a detailed analysis of the virome, particularly vertebrate viruses. We have established a positive selection probe capture-based

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system to enrich for viral sequences. Here, we will take advantage of these probe capture-based methods (VirCapSeq-VERT and myBaits probe library) to enrich for vertebrate viral sequences of interest. Sample libraries will be normalized and combined in 4- to 12-plex reactions for solution capture hybridization using either the HyperExplore custom bait library version of VirCapSeq-VERT probe set or our custom myBaits probe library. For HyperExplore custom bait library probe set, libraires will be enriched for virus following the SeqCap EZ HyperCap Workflow User's Guide, version 2.3, while for custom myBaits probe library, the myBaits Hybridization Capture for Targeted NGS protocol, Version 4.01 will be used. Sequencing libraries will be normalized and sequenced as 2 X 150 bp fragments on Illumina's MiSeq or NextSeq sequencing platforms, following Illumina's standard procedure (Illumina, San Diego, CA). NGS data will be analyzed using metavirs (https://openomics.github.io/metavirs/), a comprehensive viral metagenomics pipeline to assemble, annotate, and classify viruses. It relies on technologies like Singularity1 to maintain the highest-level of reproducibility. The pipeline consists of a series of data processing and quality-control steps orchestrated by Snakemake2, a flexible and scalable workflow management system, to submit jobs to a cluster. The pipeline is compatible with data generated from Illumina short-read sequencing technologies. As input, it accepts a set of FastQ files and will be run on-premises using the NIAID RML Big Sky supercomputing cluster.

C.3.45. *Expected outcomes.* Based on our prior work, our multifaceted analysis approach, and calculated power given our proposed temporal sampling strategy, we expect to be able to use temporal serological data to describe filovirus and henipavirus viral dynamics over time. Our molecular screening will primarily focus on filoviruses because we have known targets, and we expect to detect EBOV or MARV RNA, despite low prevalence and short-lived infections. Sequence data from positive samples will: 1) provide substantial evidence for the identification of EBOV reservoirs and help explain which virus is responsible for the observed IgG response in bats (and possibly people); and 2) provide valuable information about viral diversity, about which little is known. Henipavirus serology, in the absence of sequence data, will allow us to identify potential bat reservoirs, analyze temporal trends, and generate hypotheses for future targeted studies.

C.3.56. *Potential problems and solutions*

Serological tests are greatly hindered by the inherent antibody cross-reactivity between antigens from known ebolaviruses, challenging data interpretations that antibodies are EBOV-specific or EBOV, BDBV, and SUDV cross-reactive [40]. This is also true for the henipaviruses in the panel. The advantage of the multiplexed platform is that we can simultaneously see relative MFI values for all the filoviruses and henipaviruses. Glycoproteins from the homologous virus should cause the most intense reaction, particularly at higher dilutions. Co-Is Laing and Munster have demonstrated that homotypic and heterotypic ebolavirus-antibody reactions can be simultaneously measured and accounted for in ERBs challenged with replication-competent VSV-filoGP (**Figure 8**). Still, there is some cross reactivity, and we will use additional tools such as viral neutralization assays as a secondary test, and our statistical approach can correct for a portion of cross reactivity. Past studies have relied on individual ELISAs which had varying degrees of specificity depending on which test was used, but often couldn't rule out cross-reactivity. The Luminex filovirus multiplex assay has predictable patterns of cross-reactivity, but we will still be able to discern to which virus the serum is most reactive.

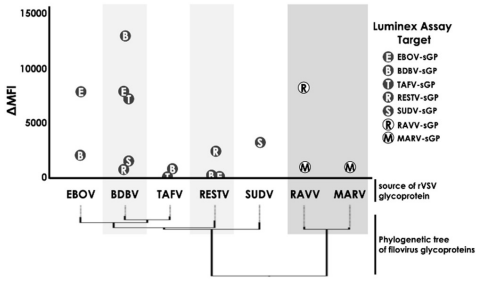

Figure 8. Captive-bred Egyptian rousette bats ~~ERBs~~ were experimentally challenged with VSV-filoGPs, the GP source is indicated on the x-axis. In a GP-trimer multiplex test the homotypic ebolavirus bat antisera were most reactive with the homotypic ebolavirus GP-trimer antigen.

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C4. Aim 3: To characterize behaviors associated with exposure to filoviruses and henipaviruses.

C.4.1 Background and rationale. As described above, there is substantial interaction between wildlife, domestic animals, and people living in rural Liberia. Our group collected questionnaire data from 585 people across Liberia as part of the USAID-funded Ebola Host Project between 2016 and 2019. When asked about animal exposures during the 2013-2016 Ebola outbreak, participants frequently reported contact with both domestic and wild animals. Common types of animal contact included animals coming inside the dwelling (58%), cooking or handling animal meat (44%), and eating raw or undercooked meat (31%). Additionally, 26% reported slaughtering and 12% reported hunting or trapping an animal. In the ENABLE Study of 5,005 residents of Bong County, survey questions mainly focused on rodent exposures relevant to Lassa fever, but 12% of participants reported hunting or preparing wild animal meat. Little is known about the prevalence and type of other potential bat exposures in this region of Liberia. Elsewhere in West Africa, EcoHealth Alliance participated in the response to Ghana's 2022 cluster of MARV cases, working with local partners to administer a behavioral risk questionnaire characterizing bat exposures in three affected communities[16]. Many participants lived in close proximity to bats, with one fifth reported exposure to bats inside a dwelling or other building in the previous four months. When asked about exposures more relevant to Egyptian Rousette bats, a known MARV reservoir and a target species in this proposed study, participants commonly reported bats feeding on fruit trees on their home compound, eating fruit bearing bite marks, and exposure to bats inside a cave or mine.

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C.4.2. *Research design*. As described in Aim 1, serum collected at baseline (in 2021) and at month 24 (in 2023) during the ENABLE Study will be screened for filoviruses and henipaviruses antibodies (analyses not included in the ENABLE Study protocol), enabling us to ~~calculate~~ determine both seroprevalence at baseline and seroconversion over a two-year period. In addition to providing blood specimens, the 1,024 participants who initially enrolled responded to baseline questionnaires on demographics, healthcare-seeking behaviors, health status, and animal exposures. At month 24, the 921 participants (90%) still actively enrolled are administered repeat questionnaires. This final survey includes additional items based on the Ghanaian questionnaire and asks specifically about bats including the respondents' activities in their habitats (entering forests, caves, and mines), direct contact (hunting, eating handling), indirect contact (presence in home or other used structure), and observed characteristics of bats encountered (fruit tree dwelling). By pairing this existing survey data with the new serology results generated from archived samples under Aim 1, as well as new data on nearby bat infection from Aim 2, we will be able to develop comprehensive risk profiles for exposure to filoviruses and henipaviruses in this community.

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C.4.3 *Data analysis.* The primary outcome for this analysis will be testing positive for IgG antibodies against any known filoviruses or henipaviruses. For both families of viruses, and for each species of virus within those families, we will use the serology results generated under Aim 1 to examine risk factors for seropositivity at baseline and for seroconversion between baseline and month 24. We plan to conduct a multivariate logistic regression, with appropriate weights applied to account for the single-stage cluster sampling design of the ENABLE Study, in which members of randomly selected households were surveyed and tested. We will use data from both the baseline and month 24 questionnaires to examine the effects of sampling site, participant demographics (e.g., age, gender, occupation), environmental exposures (~~e.g.~~e.g., animals present in and around the dwelling), and behavioral risks (e.g., direct contact with bats through hunting, preparing, or eating them, entering caves or mines where bats live).

Commented [FWAI18]: the primary outcome for Aim 3 is really risk behavior associated with IgG positivity but NOT IgG positivity.

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Power analysis: To assess our power to identify risk metrics for seroconversion (or overall seropositivity), we built simulation-based power analyses that consisted of, in brief: 1) simulating survey responses for N (400-1,000) individuals, while allowing for moderate unevenness in representation across five binary categories (as

great as 80%-20% for each); 2) examining a range of effect sizes (odds ratios ranging from 1-2.5) for these five binary categorical predictors (e.g., sex, encountered a bat, handled a bat, etc.). Summarizing across 1,200 simulations of surveys of 400-1,000 individuals given joint uncertainty in the representation within these surveys and effect sizes, we estimated that we would have at least 80% power to detect significant effects for each predictor given an odds ratio of 1.65 or greater with 1,000 survey responses or ~1.80 (for example, with a rate just under 5% for Group1 = 0, just over 8% for Group1 = 1) with 750 responses.

C.4.4 *Expected outcomes.* By combining serology results with detailed questionnaire data on participant demographics and animal exposures, we will be able to use odds ratios to identify behaviors significantly associated with seropositivity (e.g., exposure to filoviruses or henipaviruses). We expect to be able to also identify age and gender-based risk factors given our sample size. Based on previous experience conducting behavioral risk surveys, including a recent study in Ghana following a Marburg outbreak, we expect to be able to develop risk mitigation messaging that uses evidence from Aims 1 and 2 as well as results from this aim.

C.4.5 *Potential problems and solutions*. We anticipate that most IgG positive individuals for either set of viruses will be adults, with the likelihood of exposure and having antibodies increasing with age. Because we don't know how long IgG antibodies persist at detectable levels, it will not be possible to accurately determine when exposure occurred, with the exception being EBOV antibodies that most likely occurred as a result of contact with another infected person during the 2014-2016 outbreak. We will address this in two ways: by looking at respondents 21-6 ~~yrs~~ years ~~old~~of age, we will know that any filovirus antibodies they have are most likely not due to the epidemic, and we will be able to determine whether there are any significant associations with particular behavior or other factor (household construction, domestic animal exposure) that promotes direct animal contact, consumption of contaminated fruit, or exposure to bat habitats (e.g., caves or mines). For adult respondents, we will focus on exposures (based on relative MFI values) specific to non-EBOV filoviruses and henipaviruses and see whether there are associations with specific behaviors.

C.5. Project timeline

Project timeline	Y1				Y2				Y3				Y4				Y5			
Activity	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Ethical approval (local IRB / IACUC)																				
Database development																				
Aim 1: Human serum testing																				
Aim 2: Bat sampling																				
Aim 2: Bat serum testing																				
Aim 2: Bat sample PCR																				
Aim 1&2: Confirmatory assays + sequencing (RML)																				
Aim 3: Behavioral risk analysis																				
Aim 3: Risk communication development																				
Publications																				
Data publication																				

D. Summary and Conclusions.

West Africa is a hotspot for emerging zoonotic viruses such as Ebola, Marburg and potentially Nipah-related henipaviruses – groups that have been identified by WHO as among the most significant pathogens that threaten global health. Liberia is also a biodiversity hotspot, with some of the largest tracts of primary forest remaining in the region, and its population relies on wildlife as a primary source of protein, creating continued risk of spillover of high consequence zoonotic pathogens. This study aims to comprehensively characterize the risk of filo- and Henipavirus cross over events by measuring seroprevelaence and seroincidence among a well characterized cohort of individuals in rural Liberia, identify potential bat reservoirs for filoviruses and

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Henipaviruses, and identify host, behavior, and environmental risk factors associated with seroprevalence. The results of this work will inform upstreatm interventions designed to prevent spill over events from occurring and downstream interventions to facilitate early detection of human infections through implementation of pathogen-specific diagnostics~~This project has the potential to determine the origins of Zaire ebolavirus in West Africa, characterize the circulation of Ebola and other filoviruses and henipaviruses in common bat species, identify background rates of spillover in rural human populations, and identify high risk behaviors associated with exposure to these bat borne zoonoses~~. Our group comprises an unparalleled multidisciplinary group of leading experts in epidemiology, clinical medicine, virology, serology, and disease ecology all with deep experience working in Liberia, strong partnerships with the Liberian public health institutions, and implementing zoonotic virus research, giving this project a high chance of success in generating impactful results that will help prevent future epidemics in West Africa.

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Specific Aims

Emerging infectious diseases - predominantly caused by zoonotic viruses - have become a major threat to individual and public health as well as the functioning of a global society. Even prior to the SARS-CoV-2 pandemic, outbreaks of consequential emerging pathogens including SARS-CoV, Pandemic influenza 2009, Zika virus, and Ebola virus disease tragically killed and disabled and caused massive disruptions of regional and global economies and health care systems[1]. The emergence of consequential zoonotic infections is accelerating due to anthropogenic drivers such as climate change, land-use change, intensification of livestock production, and wildlife trade, all of which increase opportunity for people and domestic animals to come into contact with wildlife reservoirs[2-5]. **To reduce the risk of infectious disease epidemics, there is a critical need to detect and *prevent* spillover of zoonotic pathogens from wildlife reservoirs[2,6]**.

Illustrative of this need is the repeated spillover from wildlife reservoirs to human populations of groups of zoonotic viruses such as filoviruses (e.g., Ebola virus [EBOV]) and henipaviruses (e.g., Nipah virus [NIV]). Large-scale outbreaks or clusters of cases with these high-mortality zoonotic viruses have led to their being **designated as high priority pathogens by the World Health Organization (WHO)[5]**. However, at present, the detection of outbreaks caused by these pathogens occurs only after the first human infections; a failure in prevention that is deadly, costly, and remediable[1]. Identifying natural reservoirs of zoonotic viruses and measuring rates of exposure to these viruses in humans are critical to assessing their potential threat and developing interventions that reduce the risk of spillover.[1,2]

In the proposed study we have assembled a diverse team of experts in ecology, epidemiology, virology and immune response to bat-borne viral zoonoses such as filoviruses and henipaviruses; and filovirus clinical care and research who are actively working in West Africa to provide much needed empirical evidence to help answer the questions: **What is the natural reservoir for Ebola virus and how often does spillover of this and related viruses happen in a region that is an emerging disease hotspot, suffered the world's largest filovirus outbreak, and where there has been evidence of other consequential zoonotic viruses in animals and people**? To address these questions, we propose the following specific aims:

Aim 1. To determine the rates of filovirus and henipavirus exposure in people in rural Liberia. We have shown that certain bat species in Liberia carry EBOV-Zaire, while others carry Marburg virus and henipaviruses. Further, hunting bats is a common activity and we have detected high seroprevalence of Marburg and EBOV antibodies in residents of rural Liberia. Exposure to henipaviruses remains unknown. To test the hypothesis that spillover of both groups of viruses has occurred in this region, we will use an antigen-based multiplex serological assay to screen more than 1,9800 archived blood specimens, collected in 2021 and again in 2023 from over 900 adults and children in rural central Liberia as part of a longitudinal study of Lassa fever seroprevalence and seroincidence, for antibodies against all known filoviruses and henipaviruses.

Aim 2. To identify reservoirs and characterize the infection dynamics of filoviruses and henipaviruses in key bat species in Liberia. Our group detected EBOV-Zaire RNA and antibodies in *Miniopterus nimbae*, a cave- and mine-dwelling bat in northern Liberia found near the origin of the 2014 Ebola outbreak in Guinea. We also detected antibodies against EBOV in *Hipposideros* bats that co-roost with *Miniopterus* in multiple locations in Liberia. *Rousettus aegyptiacus*, a common frugivorous bat in Liberia, carries Marburg virus and henipaviruses. We will conduct a 3-year longitudinal study of populations of these bat species near the human cohort studied in **Aim 1**, to determine whether they are reservoirs for Ebola or other filoviruses and henipaviruses and characterize the temporal dynamics of filoviruses and henipavirus circulation using multiplexed serological assays that test for IgG antibodies against all known filoviruses and henipaviruses, as well as molecular techniques to screen for Ebola, Marburg, Nipah virus, and related viruses.

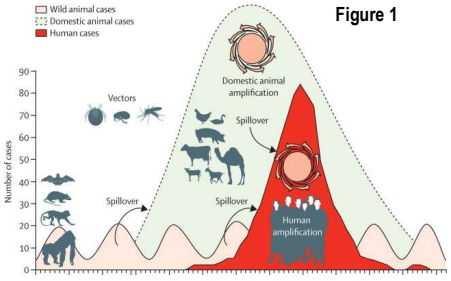
Aim 3. To characterize behaviors associated with exposure to filoviruses and henipaviruses. Combining the data collected in Aim 1 (humans) and Aim 2 (bats) we will identify the demographic, behavioral, and environmental factors associated with an individual's prior exposure to filoviruses and henipaviruses. Detailed animal exposure data collected from the adults and children (many born after the Ebola outbreak) who provided the Aim 1 blood specimens will be used to develop profiles of risk and inform public health & risk reduction communication tools in partnership with the Ministry of Health, Liberia.

RESEARCH STRATEGY

A. SIGNIFICANCE

Zoonotic viruses are responsible for the majority of emerging infectious disease outbreaks, which are occurring at an accelerating rate in global hotspots[3,7]. Every outbreak begins with spillover – transmission of the virus from its animal reservoir either directly into human populations or into other animal hosts *en route* to humans (**Figure 1**). **Exactly how often spillover occurs is virtually unknown,** as surveillance systems are primarily designed to detect clusters or outbreaks, and often miss initial individual infections that result from animal contact. To reduce the risk of future epidemics, it will be necessary to limit opportunities for zoonotic virus~~es to~~ spillover and to detect spillover at the earliest stages.[1,8] Measuring the efficacy of any interventions designed to reduce spillover and spread of zoonotic pathogens will rely on measuring rates of exposure in at-risk human populations.

Figure 1



Filoviruses and henipaviruses are among the highest consequence zoonotic viruses and have greatly impacted public health. Each group contains zoonotic pathogens that cause significant disease in both people and animals, especially livestock, that have been associated with extremely high rates of morbidity and mortality. Ebola (EBOV) and related viruses, as well as Nipah virus (NIV), a henipavirus, and its related viruses have been listed by the World Health Organization (WHO) among the highest priority pathogens for the development of vaccines and therapeutics, as they represent zoonotic pathogens with high potential to threaten human health, with extremely high mortality rates and few or no therapeutics or vaccines currently available.[9-11] These viruses share other important features which make them high priority: they are all associated with bat hosts that are abundant and widely distributed throughout Africa and Asia, living in close association with people and livestock in some of the most densely populated regions on Earth.

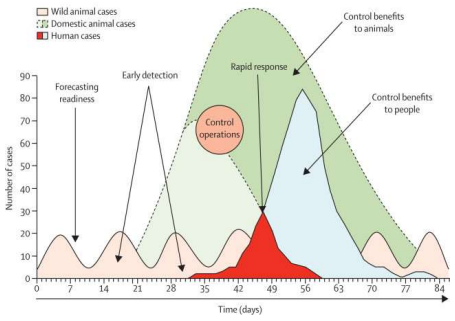
Spillover of these viruses has repeatedly occurred. Beyond the massive outbreak in West Africa in 2013-16, EBOV outbreaks have occurred repeatedly in Central and East Africa; most recently Sudan ebolavirus caused an outbreak in Uganda in late 2022[11-13]. Marburg virus (MARV) and Ravn virus (RAVV) comprise the closely related genus *Marburgvirus* and have caused outbreaks in Central and East Africa and Europe with mortality and clinical presentation comparable to Ebola [14,15]. Marburg virus outbreaks were declared in Guinea, just across the border from the UNC-Liberia research site in 2021 and then again in Ghana in 2022 marking the first time this virus has been reported in either country[16,17]. ~~Nipah~~ NIV ~~virus~~ is an emerging zoonotic paramyxovirus that has caused nearly annual outbreaks in Bangladesh, including multiple outbreaks in 2023, and sporadically in India with mean case fatality rates greater than 70% [12,18-20]. Despite the public health significance of Nipah and related viruses, little is known about its genetic diversity, natural reservoirs, or frequency of spillover throughout their range, including Africa. Henipaviruses, and ~~Nipah virus~~NIV in particular - have several characteristics that make them a global health priority: 1) Their bat reservoirs occur throughout Asia and Africa, overlapping human and livestock populations, giving them geographically broad opportunity to cause outbreaks [21]; 2) henipaviruses can be transmitted to people directly from bats or via domestic animals [21]; 3) ~~Nipah virus~~NIV can be transmitted from person to person [22]; 4) ~~Nipah virus~~NIV spillover has occurred and continues to occur in highly populous and internationally connected regions; 5) repeated spillovers of NIV strains with varying person-to-person transmission rates indicate the ability to evolve **with increased pandemic potential** [23,24]; and 6) Nipah is associated with a high mortality rate in people and currently has no vaccine or treatment [21,23]. While ~~Nipah virus~~NIV has not been detected in Africa, closely related henipaviruses have been identified in Ghana and Madagascar, though their public health significance remains unknown[25-27]. In West Africa, where bats and associated henipaviruses occur but where no human cases have been identified, **diagnostic testing and systematic surveillance is essentially non-existent**. The detection of antibodies against a Nipah-related virus in hunting communities in Central Africa suggests that cryptic spillover is occuring[28]. **Repeated spillover of henipaviruses creates opportunity for more transmissible strains to emerge[29].**

While bats have been identified as potential hosts for filoviruses including EBOV, the natural reservoir for EBOV-Zaire Ebola in West Africa remains unknown. There is substantial evidence, both serological and molecular, that bat species host filoviruses; however, a definitive natural reservoir or set of reservoirs for EBOV-Zaire has not been identified[30]. Wildlife, including bats, represent a substantial source of animal protein for communities in Liberia[31]. People may be exposed to bat-borne viruses through hunting and butchering, eating food contaminated by bat excreta, or by entering caves or mines inhabited by bats. Our group previously found that people living in rural areas of Liberia conduct activities which may increase their risk of contact with bats or their bodily fluids. The index case, a child, in the 2013 Ebola outbreak was hypothesized to have had contact with bats, yet the exact species was never definitely identified[32]. The route of spillover for most historical Ebola outbreaks remains a mystery. Despite broad efforts to determine the natural reservoirs of EBOV and related viruses, there continues to be a poor understanding of filovirus ecology, with the exception of ~~Marburg virus~~MARV, whose host has been identified as Egyptian rousette bats (*Rousettus aegyptiacus*)[33-35]. Communities known to hunt bats in Cameroon and India have been found to have antibodies against henipaviruses and filoviruses, respectively, yet nothing is known about community exposure rates in Liberia, where bat hunting is common[28,36]. Our preliminary data (see below) suggest that there is substantial exposure to EBOV as well as MARV among people in central Liberia. While the EBOV antibodies may indicate prior infection from another person such as during the West Africa outbreak, antibodies specific to other filoviruses may signal a localized exposure from animals. Even less is understood about henipaviruses in ~~Africa, and~~Africa and identifying antibodies against henipaviruses in people will be critical for understanding where, how and how often spillover occurs.

The urgent need to develop interventions to prevent spillover of zoonotic high consequence pathogens can only be met with a better understanding of their natural reservoirs, the rates of exposure in vulnerable human populations, and the ways in which people are exposed. Unrecognized outbreaks provide opportunities for viruses to emerge that may be more transmissible or more pathogenic than previously observed, and limiting these opportunities is an important component of pandemic prevention[2,29]. Typically, by the time an outbreak is recognized in human populations its containment and control are difficult, if not impossible to achieve (e.g., HIV, SARS-CoV-2). Understanding the ecology of these viruses, including which species act as reservoirs as well as how and to what extent people are being exposed to filoviruses and henipaviruses will inform intervention and surveillance strategies and provide baseline metrics against which one can measure the efficacy of interventions designed to reduce exposure, such as public health campaigns to promote avoidance of behaviors associated with infection among those most at risk (**Figure 2**).

This proposed study will be the largest and most rigorous One Health investigation of high-consequence zoonotic viruses in bats and humans in an emerging disease hotspot. West Africa is known to be the source of prior outbreaks of emerging infectious diseases and future spillover events there are highly likely. Liberia is typical of this region and contains some of the largest tracts of pristine forest and wildlife biodiversity remaining in West Africa and has a population that depends on wildlife, including bats. Decades ago, a spillover event from a non-human primate to a human triggered an on-going HIV pandemic that has led to the infection of over 60 million people and 25 million deaths. Only months ago, the orthopoxvirus MPOX found in rodents spread to and within 110 countries across the globe causing over 85,000 cases. Both originated from African wildlife. The proposed study will leverage the unique infrastructure and expertise we have developed in Liberia to undertake a comprehensive examination of filovirus and henipavirus spillover from bats to humans and provide evidence of previous and possibly on-going transmission events. Our findings will provide essential

Figure 2



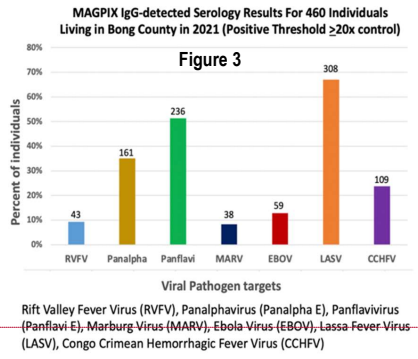
evidence of the presence and transmission dynamics of these bat-borne viruses and as the risks for infection in people living alongside them.

B. INNOVATION

The proposed research is highly innovative in multiple ways, including: 1) Our use of a one Health approach to study the ecology and epidemiology of two groups of high-priority, high-consequence zoonotic viruses in both bats and people in West Africa – an emerging disease hotspot where spillover events have occurred (e.g. Ebola, Marburg, MPOX, Lassa fever) and are highly likely to re-occur; 2) Our use of a novel and unique multiplexed serological platform, developed by our group, that will allow us to screen bat and human serum for IgG antibodies against each of the known filoviruses and henipaviruses, simultaneously in a single assay. Longitudinal comprehensive serological studies in bats and people **will provide unprecedented insight into the changes in rates of exposure over time to known viruses in bats; and in people (e.g. what is the baseline rate of exposure to henipaviruses and filoviruses and is there evidence of recent exposure?); and exposure rates to unknown, antigenically related viruses;** 3) We will couple serosurveillance with molecular testing to identify specific filoviruses and henipaviruses carried by bats; and 4) We will connect the bat and human epidemiological studies by analyzing questionnaire data from the human cohort to identifying high risk behaviors associated with viral exposure. **Results from this analysis will inform the development of public outreach and risk reduction campaigns by the Government of Liberia.**

C. APPROACH

C.1. Overview: **Aim 1** will test the hypothesis that spillover of filoviruses and henipaviruses occur regularly in rural Liberia by testing for IgG antibodies to all known filoviruses and (see Ttable 13) in ~~archived~~ serum samples from more than 900 people living in Bong county (central Liberia), collected between 2021-23 at two time points 24 months apart as part of the Coalition for Epidemic Preparedness Innovations (CEPI)-sponsored **ENABLE Study**, an on-going longitudinal study of Lassa fever seroprevalence and seroincidence. ~~-~~Seroprevalence of filoviruses and henipaviruses will be assessed as will ~~changes~~ seroincidence~~between the two collection timepoints~~. In **Aim 2,** we will conduct a 36-month longitudinal study of three bat species which are putative reservoirs for EBOV and undescribed henipaviruses. Using a combination of longitudinal serology and molecular testing (PCR and whole genome sequencing) **we will determine the prevalence of filoviruses and henipaviruses in ~~three~~ these key bats species associated with the rural human populations studied in Aims 1 and 3**. For **Aim 3,** we will analyze detailed questionnaire data developed collaboratively by our group (EHA and UNC) and collected in 2023 along with blood samples to be screened in Aim 1. These data will allow us to identify ~~behaviors~~ factors associated with exposure to henipaviruses and filoviruses. We will use results from our behavioral and exposure risk assessment to develop public risk reduction communication strategies with the National Public Health Institute, Liberia, using the results of this study to inform public health strategies. This application is a natural progression of our research activities, and our preliminary data speak to the feasibility of the proposed study.



~~Rift Valley Fever Virus (RVFV), Panalphavirus (Panalpha E), Panflavivirus (Panflavi E), Marburg Virus (MARV), Ebola Virus (EBOV), Lassa Fever Virus (LASV), Congo Crimean Hemorrhagic Fever Virus (CCHFV)~~

C.2. Aim 1: To determine the rates of filovirus and henipavirus exposure in people in central rural Liberia.

C.2.1. *Rationale and Preliminary Data.* The primary goal of this aim is to be able to determine whether populations within Liberia who live in association with bats have evidence of exposure against one or multiple filoviruses and/or henipaviruses and to compare rates of exposure over time. Studies of human populations in Central and West Africa have found evidence of exposure to EBOV prior to the 2013 outbreak and in places not known to have had Ebola outbreaks[37,,38]. UNC Project-Liberia has previously assessed seropositivity to

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consequential viral pathogens including EBOV and MARV in a subset of ~~CEPI~~ ENABLE Study participants. ~~About~~ Over 8~~1~~0% of participants had antibodies against MARV using a conservative threshold for seropositivity (**Figure 3**), suggesting that unreported spillover events have previously occurred. Acute encephalitis is common in western sub-Sharan Africa, yet outbreaks of neurological disease are rarely reported and more than half of all cases are undiagnosed[39]. Among the countries in western Africa reporting cases of meningitis or encephalitis, Liberia has reported the fewest, likely due to a lack of surveillance and capacity to diagnose neurological diseases[39]. ~~Nipah virus~~NIV encephalitis has never been reported in Africa, however, as described above, two Nipah-like henipaviruses have been described in bat hosts, including one in Ghana[25,27]. Spillover of henipaviruses was reported in bat-hunting communities in Cameroon[28]. In South Africa, we detected henipavirus sequences in *Rousettus aegyptiacus,* bats which also commonly occur in Liberia[26].

In Aim 1, we will test the hypothesis that spillover of both filoviruses and henipaviruses have occurred in rural populations in Liberia by measuring seropositivity to both groups of viruses within the CEPI ENABLE Study community cohort. We will also measure changes in exposure rates over a two-year period. While prior studies have provided cross-sectional snapshots of exposure, our longitudinal approach will provide data from the same cohort at two timepoints, 24 months apart, to determine both seroprevalence and sero-incidence using a multiplexed serological assay to screen for IgG antibodies against all known filoviruses and henipaviruses.

Settings and Participants. To achieve this aim we will leverage a well characterized cohort of individuals in three rural communities located in Bong County, Liberia (**Figure 4**), which has a population of over 333,400. UNC Project-Liberia has been operating in Liberia since 2014 and established a research site at Phebe Hospital in Bong County that includes a molecular laboratory that conducts daily qPCR testing for LASV, MARV, and EBOV as well as LASV serological assays using a MAGPIX (SEE RESOURCES AND FACILITIES). There, following extensive community engagement including meetings with key stakeholders, town hall meetings, and radio messaging, UNC launched the CEPI **ENABLE Study**, the largest passive and active surveillance program for Lassa fever in Liberia. Started in 2021~~0~~, 5,005 randomly selected participants 2 years of age and older in Phebe Airstrip, Suakoko, and Rubber Factory were enrolled over 6 months following informed consent/assent (mean age at enrollment is 22.2 years (range 2-97) and 54.2% are female). **Importantly, 7.7% of the cohort is under the age of 5 years, and therefore, were born after the 2013-14 Ebola outbreak (Table 1).** Households were selected for recruitment following identification of roof tops by satellite photos and use of a program developed by Médecins Sans Frontières' (MSF) Epicentre to randomly select~~ed~~ structures to ~~consecutively~~ approach sequentially for participation. The ENABLE Study includes baseline questionnaires and blood specimen collection from all participants with active follow-up by a study community health worker every 2 weeks to determine if~~s~~ a febrile event has occurred, which triggers drawing of blood for LASV, MARV, and EBOV PCR. Baseline blood is tested for Lassa fever virus (LASV) serology. A subset of 1,024 individuals (every 5th approached household is eligible) is also asked to provide blood samples routinely every 6 months for up to 24 months for repeat LASV serology. The blood samples from 0 and 24 months and the associated surveys will be used in **Aims 1 and 3** of this proposal. Informed consent and assent documents include provisions for the use of collected specimens and data for infectious diseases research including for emerging pathogens (see PROTECTION OF HUMAN SUBJECTS).

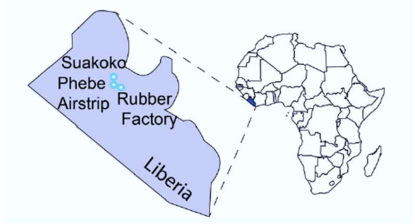

Figure 4. Map of Liberia with Bong County sites shown in inset.

C.2.2. Research Design. We will screen approximately **1,900 archived serum specimens** from participants in the ENABLE Study cohort at two time points ("baseline" (Month 0) and (Month 24)) for IgG antibodies against specific filoviruses and henipaviruses using the MAGPIX at the UNC molecular lab at Phebe H~~h~~ospital to run the multiplexed *henipavirus / filovirus* platform to be provided by Co-I Laing in year 1. The multiplex platform will allow us to determine specifically which filoviruses and henipaviruses people have been exposed to and compare that to the filoviruses and antibodies we observe circulating in local bat populations in **Aim 2**. The bead-based multiplexed assay has tremendous advantage over traditional ELISAs in that it will

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identify up to 25 different antibodies against multiple viral agents in a single, 2uL serum sample and uses a high through-put format. The ability to look at reactivity across all serogroups simultaneously will allow us to differentiate among the viruses. Proteins A/G non-specifically bind IgG immunoglobulins, and weakly bind IgM, an immunoglobulin that is the first to be produced during the initial stages of infection. We will conduct a 1-week training at the UNC-Phebe lab for using the assay to detect filovirus and henipavirus antibodies as well as data management, bioinformatics and analysis. **Table 13** shows the viral species and antigens that will be employed in this assay. The platform has been previously transferred to collaborating labs in the UK, South Africa, India, Bangladesh, Singapore, Thailand and Malaysia. We have previously detected serological profiles of Asiatic ebolaviruses in fruit bats collected in Singapore[40] and in fruits bats and humans actively engaged in bat hunting in northeast India[36].

Table 13. Multiplexed microsphere immune assay including soluble glycoproteins from each known filovirus and henipavirus

Virus (Name/Isolate Host/Country/Year/Strain)	Abbreviation	Accession No.	Soluble Glycoprotein	Mag Bead No.
Ebolaviruses				
Ebola virus/H. sapiens/COD/1976/Yambuku-Mayina	EBOV	NC_002549.1	$GP_{(1,2)}$	34
Bundibugyo virus/H. sapiens/UGA/2007	BDBV	FJ217161.1	$GP_{(1,2)}$	64
Bombali ebolavirus/Mops condylurus/SLE/201	BOMV	NC_039345	$GP_{(1,2)}$	55
Taï Forest virus/H. sapiens/COV/1994/Pauleoula-Cl	TAFV	NC_014372	$GP_{(1,2)}$	57
Sudan virus/H. sapiens/UGA/2000/Gulu-808892	SUDV	NC_006432.1	$GP_{(1,2)}$	54
Reston virus/M. fascicularis/USA/1989/Pennsylvania	RESTVm	AF522874.1	$GP_{(1,2)}$	65
Marburgviruses				
Marburg virus/H. sapiens/KEN/1980/Musoke	MARV	Z12132 S55429	$GP_{(1,2)}$	37
Ravn virus/H. sapiens/AGO/2005/Ang0126	RAVV	NC_024781.1	$GP_{(1,2)}$	62
Dianlovirus				
Mengla virus/Rousettus-WT/CHN/2015/Sharen	MLAV	KX371887.2	$sGP_{(1,2)}$	22
Cuevavirus				
Lloviu virus/M. schreibersii-wt/ESP/2003/Asturias-Bat86	LLOV	NC_016144.1	$sGP_{(1,2)}$	66
Henipaviruses				
Hendra virus/E. caballus/AUS/1994	HeV	NC_001906.3	sG	43
Hendra virus-2G	HeV-2g		sG	48
Nipah virus/H. sapiens/MYS/2000	NiVm	NC_002728.1	sG	46
Nipah virus/H. sapiens/BGD/2004	NiVb	AAY43916.1	sG	56
Cedar virus/Pteropus sp./AUS/2012/CG1a	CedV	NC_025351.1	sG	53
Mojiang virus/R. sladeni/CHN/2014/Tongguan1	MojV	NC_025352.1	sG	29
Ghanaian bat virus/E. helvum/GHA/2009/GH-M47a	GhV	NC_025256.1	sG	35
Angavokely virus	AngV		sG	47

C.2.3. Data Analysis. We will create a project database using Airtable, which will be hosted at EHA and will house all data from Aims 1 and 2 and will link to the RedCap server which stores questionnaire data from Aim 3. Database access will be secure and limited to key personnel (see data management plan). Raw MFI values from the MAGPIX will be used to calculate reactivity to each viral antigen and based on relative strength of reactivity (**Fig 5**) and determinations of negative cutoffs based on reference sera, we will calculate prevalence rates for each time point and compare rates between the two timepoints. Serological profiling can be used to detect reactive antibodies against all specific filoviruses and henipaviruses in a samples and compare strength of reactivity and cross-reactivity against multiple antigens using a single sample[41]. We will compare the antibodies across all specific viral antigens to assess which virus or

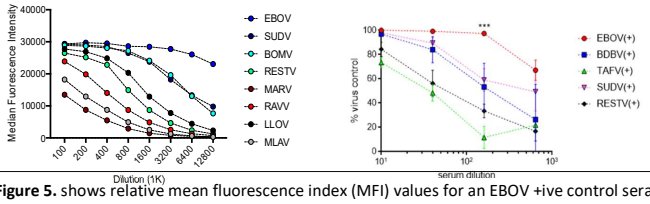

Figure 5. shows relative mean fluorescence index (MFI) values for an EBOV +ive control sera across serial dilutions. While there is cross reactivity, the EBOV MFI values are consistently greater than the other viral GPs and at higher dilutions the separation becomes significant.

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groups of viruses are responsible for the immunological response[42-44]. We will assess exposure to non-Zaire ebolavirus species, which may indicate spillover of filoviruses has occurred separate to the Ebola outbreak of 2013. We will also look at evidence for exposure to Nipah-like henipaviruses and compare exposure rates to both viral groups within and between our two sample collection time points.

C.2.4. Expected outcomes. We anticipate obtaining serological profiles of exposure to filoviruses and henipaviruses from each individual in our cohort. In previous studies we found antibodies against both ~~Ebola~~ EBOV and ~~Marburg~~ MARV virus. While the ~~Ebola~~ EBOV antibodies are attributable to the epidemic, ~~Marburg~~ MARV antibodies, in patients EBOV IgG negative, suggest possible exposure from an animal (e.g. bat) reservoir. We also anticipate seeing reactivity to ~~to~~ Bombali virus (BOMV), given the presence of the reservoir species in Liberia (*Mops condylurus*) and reactivity to the African henipaviruses (GhV and AngV), although this may represent reactivity to an antigenically related virus. Serological results from this and the bat study will inform our strategy for prioritizing which bat samples we test by PCR.

C.2.5. Potential problems and solutions. While our preliminary data suggest there will be detection of immune responses to filoviruses including EBOV-Zaire and, significantly, MARV, it is possible that evidence of exposure to other pathogens, including henipaviruses will not be detected. This would be an important finding in this large cohort, especially if these viruses are detected in local bat species, that would warrant further investigation. It is also possible that there will be few or no seroconversion events among the cohort during the 24-month period of follow-up. This too would be reassuring but would suggest a need for longer follow-up of this cohort. To address the possibility that reactivity to EBOV-Zaire virus may be explained by infection during the 2013 Ebola epidemic, we will create a polyclonal sera reference standard utilizing convalescent sera collected from human survivors of EVD. This internal reference standard will be calibrated to the WHO EBOV human serology standard (**Fig 6A**) permitting direct interpolation of anti-EBOV IgG as a MFI to a standard antibody concentration (binding antibody units/mL). The internal reference standard will then be included in our human serology testing strategy allowing semi- or quantitative measurement of anti-EBOV IgG. These EBOV human serology standards will permit us to establish positive and negative predictive performance values for the multiplex assay; furthermore, we will be able to investigate immunoreactivity of EVD survivors against heterotypic ebolaviruses. Our preliminary data indicates that EBOV+ sera IgG is highly cross-reactive with soluble native-like trimeric envelope glycoprotein (GP) antigens from both EBOV and BDBV (Figure 6A). EBOV GP, matrix protein (VP40), and nucleocapsid protein (NP) antigens have all been used in a variety of serological tests, however, the frequency of seroconversion against each antigen has not been fully described. In a longitudinal study of EVD survivors, high seroconversion against GP and VP40 was observed, with affinity maturation occurring overtime against GP[45]. Antigens based on the GP are likely to provide an optimal intrinsic balance of sensitivity and specificity. To limit the degree of heterotypic ebolavirus cross reactions we will test any anti-ebolavirus GP trimer antigen-seropositive samples with a pan-ebolavirus GP1 antigen-based panel. In preliminary studies, GP1 antigens improve specificity and differentiation of antibodies reactive with homotypic ebolaviruses in polyclonal antisera from rabbits immunized with GP trimer immunogens (**Figure 6B**).

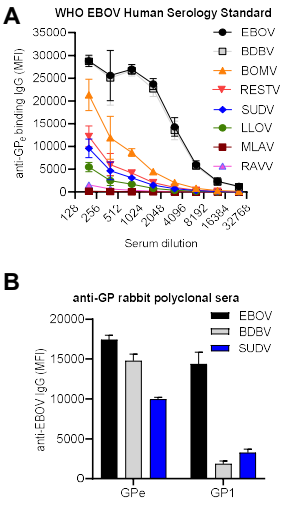

FIGURE 6A & B. A) The WHO EBOV human serology standard was testeding for immunoreactivity in a filovirid GP-trimer based multiplex assay. Filovirids are indicated along with sera dilution factors; data is the mean of three independent experiments. B) Antisera from EBOV GP-trimer immunized rabbits were tested in multiplex panels comprised of GP-trimer and GP1 subunit proteins. Anti-EBOV polyclonal IgG was less reactive with GP1 from BDBV and SUDV.

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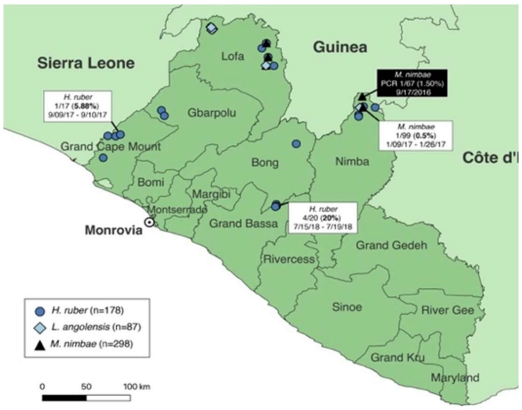
C.3. Aim 2. To identify reservoirs and characterize the infection dynamics of filoviruses and henipaviruses in key bat species in Liberia.

C.3.1 *Rationale and ~~P~~preliminary data.* Bats have been established as important hosts for filoviruses and henipaviruses in Africa and Asia, with combinations of viral isolation, molecular detection or serology providing substantial evidence [30,46-49]. MARV was isolated from Egyptian rousette bats in Uganda, and RNA has since been detected in these bats in Sierra Leone, which borders Liberia [50]. To date, EBOV has never been isolated from bats, although molecular and serological evidence suggest that multiple frugivorous bats may carry EBOV in Central Africa[50,51]. Prior to the 2014 epidemic, Zaire ebolavirus had not been reported in West Africa, and the animal reservoir remains unknown[32]. From 2016-2019, our group (PI Epstein & KP Desmond) implemented a large-scale project to identify reservoirs for EBOV in West Africa, under the USAID PREDICT Project[52]. This effort led to the discovery of a new species of Ebola (Bombali virus)[53], the detection of MARV in E Egyptian rousette bats~~RBs~~ in Sierra Leone[33], and **in 2018, we detected, for the first time in West Africa, EBOV RNA and anti-EBOV**



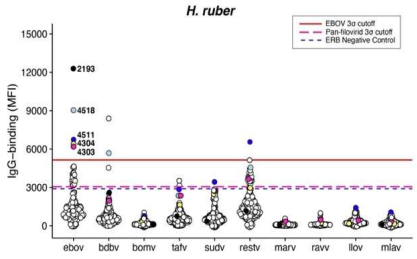
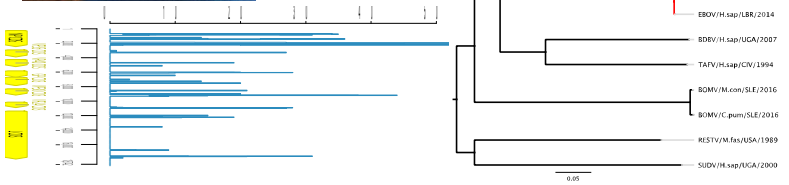


Figure 7: A. Map showing location of EBOV PCR +ive bat (black box) Zaire ebolavirus and MARV in Liberia in 2018. B. Serological evidence for EBOV in *H. ruber* from our 2018 study. C. RNA genomic sequences from across the *Zaire ebolavirus* genome detected in *Miniopterus nimbae*, Liberia in 2018. D. Phylogenetic analysis supports relatedness to 2013 EBOV epidemic virus.



IgG antibodies in bats (Figure 7A-D).

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may be reservoirs for Zaire ebolavirus, longitudinal studies are required to more completely understand the role these species play in EBOV circulation – either as reservoirs that maintain the virus within a local population, or as incidental hosts, infected by an unknown bat species. One-off, cross-sectional studies have a low probability of detecting any specific filovirus or henipavirus given their low prevalence and short viremic periods [34,54], and thus provide a poor characterization of viral diversity. The lack of understanding of **filovirus and henipavirus diversity and their natural reservoirs in West Africa significantly impedes risk-based public health interventions to prevent human outbreaks.** Longitudinal sampling is needed to separate reservoir hosts from spillover hosts. For example, MARV has been isolated repeatedly from *R. aegyptiacus* in Uganda, which, when paired with experimental infection studies showing infection and shedding of MARV by *R. aegyptiacus,* strongly suggests that *R. aegyptiacus* is indeed a ~~Marburg virus~~MARV reservoir[35,55,56]. Similarly, our longitudinal studies of ~~Nipah virus~~NIV in *Pteropus medius* bats in Bangladesh, which included the same multiplexed serological assay proposed here, confirmed them as a reservoir for NIV and identified important mechanisms driving viral circulation[29]. **Understanding which species persistently carry filoviruses and henipaviruses will provide important insights into how they are maintained in nature and what species should be targeted for follow-up studies on bat habitat use and human-bat contact patterns.**

C.3.2. *Sampling design*. We will repeatedly sample three bat species associated with filoviruses or henipaviruses, based on our preliminary data: *M. nimbae* (EBOV), *H. ruber* (EBOV), and *R. aegyptiacus* (MARV & Henipaviruses). We will sample bats at roost sites in Bong county, as close as possible to the human study sites in Aim 1. In Y2-Y4, we will sample 40 individuals of each species every two months for 36 months and collect excreta using plastic tarps placed underneath bat roosts (details below and in C.3.3).

Bat capture and sampling. Briefly, we will use either harp traps or mist nets to capture bats, depending on the capture site. Personnel will wear appropriate personal protective equipment (e.g. Tyvek suits or dedicated long clothing; double nitrile gloves; an N95 respirator and safety glasses or a PAPR [57]. Traps and nets will be attended continuously during capture periods. Bats will be removed from mist nets immediately upon entanglement to avoid injury. Bats will be removed from harp traps after approximately 1 hour and bats will be placed in individual cloth bags until processed. Body measurements, blood, oropharyngeal swabs, and rectal swabs (or fecal pellets) will be collected from each bat. Swabs will be collected in duplicate; one swab will be placed in a cryovial containing Trizol lysis buffer and the other in viral transport medium (VTM). For bats <50g, blood will be collected in a microhematocrit tube and spun down using a portable centrifuge. A bulb will be used to expel serum from the tube into a cryovial. For larger bats >50g, a tuberculin syringe or 1ml syringe and 27g needle will be used to draw blood from the radial artery or vein[57]. Blood will be placed into a conical vial and centrifuged. Serum will be aliquoted into a cryovial using a pipette. During site visits we will also lay out plastic tarps to collect pooled environmental excreta in Trizol and VTM to improve opportunity to detect viral RNA. All samples will be placed in a vapor phase liquid nitrogen dry shipper or portable battery-powered ultracold freezer, immediately upon collection and then transferred to a -80C freezer at the Phebe lab until testing.

C.3.3. *Testing plan, ~~d~~Data analysis, sample size, and power analysis*. *Serology:* At the end of Y3, we will screen all serum samples collected to date using the MAGPIX multiplex assay for IgG against filoviruses and henipaviruses. We will analyze temporal dynamics in seroprevalence using general additive models (GAMs), which are analyses that we have used successfully in many previous studies of similar design[29]. These methods are well adapted for capturing long-term temporal fluctuations in prevalence and moderately sensitive at capturing short-term changes in seroprevalence which can be used to identify population-level disease outbreaks. For example, we estimated that with 40 bats sampled every two months, we would be able to statistically detect an increase in seroprevalence (at a 95% CI limit) of greater than 25% between consecutive sampling events > 80% of the time, assuming a bat population seroprevalence of at least 10% in advance of a bat colony infection (estimated by, in brief, by simulating a fluctuating true seroprevalence value, simulating samples given this underlying seroprevalence, fitting a GAM to these samples, and then calculating if CI on true prevalence between the two sampling occasions over which the change in seroprevalence occurred were non-overlapping). While this is a large change, it is within a range to be expected if infection were to sweep through a bat community[29]. MAGPIX-positive bat sera will be sent to RML for serum neutralization assays using viral isolates or VSV pseudoviruses under BSL4 conditions.

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VSV-pseudotype neutralization assays: Serological results will be confirmed using VSV-pseudotype neutralization assays for the following filoviruses EBOV, SUDV, BDBV, RESTV, BOMV, MARV and RAVN. If novel henipavirus sequences are detected, additional assays will be developed to test positive sera. Serum samples will be inactivated using ɣ-irradiation (4 MRad). Neutralization of irradiated and heat-inactivated serum samples will be assessed in Vero 352 E6 cells. Briefly, cells were seeded in 96-well round-bottom plates for 24 hours, serial dilutions of heat-inactivated serum samples will be performed in DMEM supplemented with 2% FBS, penicillin/streptomycin, and L-glutamine. Each plate will contain negative serum control, cell-only control, and virus-only control. VSV EBOV-GFP will be added to each well of the serum dilution plate and the serum-virus mix 357 was incubated at 37˚C for 1-hour. The mix was added to the cells and incubated at 37˚C for 24 hours. The cells will then fixed with 4% paraformaldehyde at room temperature 359 for 15-minutes and centrifuged at 600 x g for 5-minutes at room temperature. The supernatant will be discarded and FACS+EDTA buffer was added. Samples will be run on the FACSymphony A5 Cell Analyzer (BD Biosciences, Mississauga, ON, Canada) and FITC MFI 362 was measured. Data will be analyzed using FlowJo.

Molecular testing: We will use specific filovirus taqman real-time PCR assays to screen oropharyngeal, fecal and environmental samples from bats for EBOV and MARV RNA at the UNC-Phebe lab[55,58]. Testing bat samples by PCR may allow us to detect viral RNA during active infection, providing important information about the prevalence and timing of infection within target species. **Because we expect viral prevalence to be significantly lower than seroprevalence, estimated at <5%[25,27,49],** we developed a sampling scheme focused on maximizing our probability of detecting viral RNA with qPCR. To compare power under alternative sampling designs, we built simulation-based power analyses that estimated power to detect: 1) viral EBOV or MARV RNA in at least one bat; and 2) the expected number of positive cases, in each bat species. In brief, we estimated power for total sample sizes between 1500-3000 (between 500 and 1000 bats sampled per species) spread across a variable number of visits per year (2-6), while allowing for uncertainty in true infection dynamics in the bat populations, specifically: 1) prevalence during one three-week period (with unknown timing) per year of high prevalence ranging from 1-5%); and 2) an otherwise low background prevalence (0-1%). Given these estimates of yearly infection dynamics, these analyses showed with 672 samples per species (minimum 32 individual bats per sampling event), spread across 6 sampling occasions per year, we would obtain a per-species probability of acquiring 0 positive samples of <4% (<1% at 40 bats per event – our target number) and an expectation of ~3-6 RNA positive results (~5-10 positives at 40 bats per event). Because henipaviruses have not been previously described in Liberia, we will focus our resources on filovirus testing by PCR. However, if henipavirus serology suggests active circulation, we may send a set of samples to RML for pathogen discovery (SEE RML LETTER OF SUPPORT).

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Viral isolation: PCR-positive cDNA samples and their aliquot in VTM will be sent to RML for further sequencing and attempts at viral isolation under BSL4 conditions. If attempts to culture do not work, we will use next generation sequencing techniques to get whole genome sequence and further characterize the virus at RML. Novel viruses will be characterized and rescued (reverse engineered using a minigenome system to synthetically create infectious viral particles [59]) at RML under secure, biosafety level 4 conditions, then used for serum neutralization assays in bat and human sera to determine more precise exposure rates. Sequences from the glycoproteins of any novel viruses discovered will be used by USU (Co-I Laing) to generate purified proteins and added to the Luminex assay, which will improve its specificity.

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VirCapSeq-VERT and myBaits probe-based enrichment for recovery of full-length genome sequences of filoviruses: Although shotgun sequencing provides opportunities to analyze all host and virus genetic material, the larger average genome size of bacteria and hosts compared to viral genomes complicates a detailed analysis of the virome, particularly vertebrate viruses. We have established a positive selection probe capture-based

system to enrich for viral sequences. Here, we will take advantage of these probe capture-based methods (VirCapSeq-VERT and myBaits probe library) to enrich for vertebrate viral sequences of interest. Sample libraries will be normalized and combined in 4- to 12-plex reactions for solution capture hybridization using either the HyperExplore custom bait library version of VirCapSeq-VERT probe set or our custom myBaits probe library. For HyperExplore custom bait library probe set, libraires will be enriched for virus following the SeqCap EZ HyperCap Workflow User's Guide, version 2.3, while for custom myBaits probe library, the myBaits Hybridization Capture for Targeted NGS protocol, Version 4.01 will be used. Sequencing libraries will be normalized and sequenced as 2 X 150 bp fragments on Illumina's MiSeq or NextSeq sequencing platforms, following Illumina's standard procedure (Illumina, San Diego, CA). NGS data will be analyzed using metavirs (https://openomics.github.io/metavirs/), a comprehensive viral metagenomics pipeline to assemble, annotate, and classify viruses. It relies on technologies like Singularity1 to maintain the highest-level of reproducibility. The pipeline consists of a series of data processing and quality-control steps orchestrated by Snakemake2, a flexible and scalable workflow management system, to submit jobs to a cluster. The pipeline is compatible with data generated from Illumina short-read sequencing technologies. As input, it accepts a set of FastQ files and will be run on-premises using the NIAID RML Big Sky supercomputing cluster.

C.3.~~4~~5. *Expected outcomes.* Based on our prior work, our multifaceted analysis approach, and calculated power given our proposed temporal sampling strategy, we expect to be able to use temporal serological data to describe filovirus and henipavirus viral dynamics over time. Our molecular screening will primarily focus on filoviruses because we have known targets, and we expect to detect EBOV or MARV RNA, despite low prevalence and short-lived infections. Sequence data from positive samples will: 1) provide substantial evidence for the identification of EBOV reservoirs and help explain which virus is responsible for the observed IgG response in bats (and possibly people); and 2) provide valuable information about viral diversity, about which little is known. Henipavirus serology, in the absence of sequence data, will allow us to identify potential bat reservoirs, analyze temporal trends, and generate hypotheses for future targeted studies.

C.3.~~5~~6. *Potential problems and solutions*

Serological tests are greatly hindered by the inherent antibody cross-reactivity between antigens from known ebolaviruses, challenging data interpretations that antibodies are EBOV-specific or EBOV, BDBV, and SUDV cross-reactive [40]. This is also true for the henipaviruses in the panel. The advantage of the multiplexed platform is that we can simultaneously see relative MFI values for all the filoviruses and henipaviruses. Glycoproteins from the homologous virus should cause the most intense reaction, particularly at higher dilutions. Co-Is Laing and Munster have demonstrated that homotypic and heterotypic ebolavirus-antibody reactions can be simultaneously measured and accounted for in ERBs challenged with replication-competent VSV-filoGP (**Figure 8**). Still, there is some cross reactivity, and we will use additional tools such as viral neutralization assays as a secondary test, and our statistical approach can correct for a portion of cross reactivity. Past studies have relied on individual ELISAs which had varying degrees of specificity depending on which test was used, but often couldn't rule out cross-reactivity. The Luminex filovirus multiplex assay has predictable patterns of cross-reactivity, but we will still be able to discern to which virus the serum is most reactive.

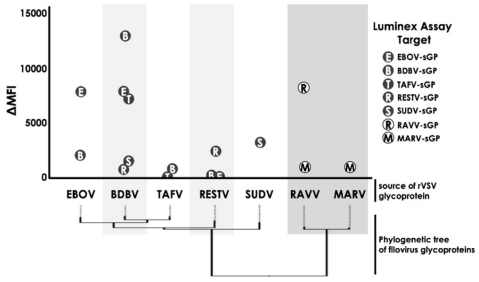

Figure 8. Captive-bred Egyptian rousette bats ~~ERBs~~ were experimentally challenged with VSV-filoGPs, the GP source is indicated on the x-axis. In a GP-trimer multiplex test the homotypic ebolavirus bat antisera were most reactive with the homotypic ebolavirus GP-trimer antigen.

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C4. Aim 3: To characterize behaviors associated with exposure to filoviruses and henipaviruses.

C.4.1 Background and rationale. As described above, there is substantial interaction between wildlife, domestic animals, and people living in rural Liberia. Our group collected questionnaire data from 585 people across Liberia as part of the USAID-funded Ebola Host Project between 2016 and 2019. When asked about animal exposures during the 2013-2016 Ebola outbreak, participants frequently reported contact with both domestic and wild animals. Common types of animal contact included animals coming inside the dwelling (58%), cooking or handling animal meat (44%), and eating raw or undercooked meat (31%). Additionally, 26% reported slaughtering and 12% reported hunting or trapping an animal. In the ENABLE Study of 5,005 residents of Bong County, survey questions mainly focused on rodent exposures relevant to Lassa fever, but 12% of participants reported hunting or preparing wild animal meat. Little is known about the prevalence and type of other potential bat exposures in this region of Liberia. Elsewhere in West Africa, EcoHealth Alliance participated in the response to Ghana's 2022 cluster of MARV cases, working with local partners to administer a behavioral risk questionnaire characterizing bat exposures in three affected communities[16]. Many participants lived in close proximity to bats, with one fifth reported exposure to bats inside a dwelling or other building in the previous four months. When asked about exposures more relevant to Egyptian Rousette bats, a known MARV reservoir and a target species in this proposed study, participants commonly reported bats feeding on fruit trees on their home compound, eating fruit bearing bite marks, and exposure to bats inside a cave or mine.

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C.4.2. *Research design*. As described in Aim 1, serum collected at baseline (in 2021) and at month 24 (in 2023) during the ENABLE Study will be screened for filoviruses and henipaviruses (analyses not included in the ENABLE Study protocol), enabling us to calculate seroprevalence at baseline and seroconversion over a two-year period. In addition to providing blood specimens, the 1,024 participants who initially enrolled responded to baseline questionnaires on demographics, healthcare-seeking behaviors, health status, and animal exposures. At month 24, the 921 participants still actively enrolled are administered repeat questionnaires. This final survey includes additional items based on the Ghanaian questionnaire and asks specifically about bats including the respondents' activities in their habitats (entering forests, caves, and mines), direct contact (hunting, eating handling), indirect contact (presence in home or other used structure), and observed characteristics of bats encountered (fruit tree dwelling). By pairing this existing survey data with the new serology results generated from archived samples under Aim 1, as well as new data on nearby bat infection from Aim 2, we will be able to develop comprehensive risk profiles for exposure to filoviruses and henipaviruses in this community.

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C.4.3 *Data analysis.* The primary outcome for this analysis will be testing positive for IgG antibodies against any known filoviruses or henipaviruses. For both families of viruses, and for each species of virus within those families, we will use the serology results generated under Aim 1 to examine risk factors for seropositivity at baseline and for seroconversion between baseline and month 24. We plan to conduct a multivariate logistic regression, with appropriate weights applied to account for the single-stage cluster sampling design of the ENABLE Study, in which members of randomly selected households were surveyed and tested. We will use data from both the baseline and month 24 questionnaires to examine the effects of sampling site, participant demographics (e.g., age, gender, occupation), environmental exposures (~~e.g.~~e.g., animals present in and around the dwelling), and behavioral risks (e.g., direct contact with bats through hunting, preparing, or eating them, entering caves or mines where bats live).

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Power analysis: To assess our power to identify risk metrics for seroconversion (or overall seropositivity), we built simulation-based power analyses that consisted of, in brief: 1) simulating survey responses for N (400-1,000) individuals, while allowing for moderate unevenness in representation across five binary categories (as great as 80%-20% for each); 2) examining a range of effect sizes (odds ratios ranging from 1-2.5) for these five

binary categorical predictors (e.g., sex, encountered a bat, handled a bat, etc.). Summarizing across 1,200 simulations of surveys of 400-1,000 individuals given joint uncertainty in the representation within these surveys and effect sizes, we estimated that we would have at least 80% power to detect significant effects for each predictor given an odds ratio of 1.65 or greater with 1,000 survey responses or ~1.80 (for example, with a rate just under 5% for Group1 = 0, just over 8% for Group1 = 1) with 750 responses.

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C.4.4 *Expected outcomes.* By combining serology results with detailed questionnaire data on participant demographics and animal exposures, we will be able to use odds ratios to identify behaviors significantly associated with seropositivity (e.g., exposure to filoviruses or henipaviruses). We expect to be able to also identify age and gender-based risk factors given our sample size. Based on previous experience conducting behavioral risk surveys, including a recent study in Ghana following a Marburg outbreak, we expect to be able to develop risk mitigation messaging that uses evidence from Aims 1 and 2 as well as results from this aim.

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C.4.5 *Potential problems and solutions*. We anticipate that most IgG positive individuals for either set of viruses will be adults, with the likelihood of exposure and having antibodies increasing with age. Because we don't know how long IgG antibodies persist at detectable levels, it will not be possible to accurately determine when exposure occurred, with the exception being EBOV antibodies that most likely occurred as a result of contact with another infected person during the 2014-2016 outbreak. We will address this in two ways: by looking at respondents 21-6 yrs years oldof age, we will know that any filovirus antibodies they have are most likely not due to the epidemic, and we will be able to determine whether there are any significant associations with particular behavior or other factor (household construction, domestic animal exposure) that promotes direct animal contact, consumption of contaminated fruit, or exposure to bat habitats (e.g., caves or mines). For adult respondents, we will focus on exposures (based on relative MFI values) specific to non-EBOV filoviruses and henipaviruses and see whether there are associations with specific behaviors.

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C.5. Project timeline

Project timeline	Y1				Y2				Y3				Y4				Y5			
Activity	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Ethical approval (local IRB / IACUC)																				
Database development																				
Aim 1: Human serum testing																				
Aim 2: Bat sampling																				
Aim 2: Bat serum testing																				
Aim 2: Bat sample PCR																				
Aim 1&2: Confirmatory assays + sequencing (RML)																				
Aim 3: Behavioral risk analysis																				
Aim 3: Risk communication development																				
Publications																				
Data publication																				

D. Summary and Conclusions.

West Africa is a hotspot for emerging zoonotic viruses such as Ebola, Marburg and potentially Nipah-related henipaviruses – groups that have been identified by WHO as among the most significant pathogens that threaten global health. Liberia is also a biodiversity hotspot, with some of the largest tracts of primary forest remaining in the region; , and its population relies on wildlife as a primary source of protein, creating continued risk of spillover of high consequence zoonotic pathogens. This project has the potential to determine the origins of Zaire ebolavirus in West Africa, characterize the circulation of Ebola and other filoviruses and henipaviruses in common bat species, identify background rates of spillover in rural human populations, and identify high-risk behaviors associated with exposure to these bat-borne zoonoses. Our group comprises an unparalleled

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multidisciplinary group of leading experts in epidemiology, clinical medicine, virology, serology, and disease ecology all with deep experience working in Liberia, strong partnerships with the Liberian public health institutions, and implementing zoonotic virus research, giving this project a high chance of success in generating impactful results that will help prevent future epidemics in West Africa.

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Specific Aims

Emerging infectious diseases - predominantly caused by zoonotic viruses - have become a major threat to individual and public health as well as the functioning of a global society. Even prior to the SARS-CoV-2 pandemic, outbreaks of consequential emerging pathogens including SARS-CoV, Pandemic influenza 2009, Zika virus, and Ebola virus disease tragically killed and disabled and caused massive disruptions of regional and global economies and health care systems[1]. The emergence of consequential zoonotic infections is accelerating due to anthropogenic drivers such as climate change, land-use change, intensification of livestock production, and wildlife trade, all of which increase opportunity for people and domestic animals to come into contact with wildlife reservoirs[2-5]. **To reduce the risk of infectious disease epidemics, there is a critical need to detect and *prevent* spillover of zoonotic pathogens from wildlife reservoirs[2,6]**.

Illustrative of this need is the repeated spillover from wildlife reservoirs to human populations of groups of zoonotic viruses such as filoviruses (e.g., Ebola virus [EBOV]) and henipaviruses (e.g., Nipah virus [NIV]). Large-scale outbreaks or clusters of cases with these high-mortality zoonotic viruses have led to their being **designated as high priority pathogens by the World Health Organization (WHO)[5]**. However, at present, the detection of outbreaks caused by these pathogens occurs only after the first human infections; a failure in prevention that is deadly, costly, and remediable[1]. Identifying natural reservoirs of zoonotic viruses and measuring rates of exposure to these viruses in humans are critical to assessing their potential threat and developing interventions that reduce the risk of spillover.[1,2]

In the proposed study we have assembled a diverse team of experts in ecology, epidemiology, virology and immune response to bat-borne viral zoonoses such as filoviruses and henipaviruses; and filovirus clinical care and research who are actively working in West Africa to provide much needed empirical evidence to help answer the questions: **What is the natural reservoir for Ebola virus and how often does spillover of this and related viruses happen in a region that is an emerging disease hotspot, suffered the world's largest filovirus outbreak, and where there has been evidence of other consequential zoonotic viruses in animals and people**? To address these questions, we propose the following specific aims:

Aim 1. To determine the rates of filovirus and henipavirus exposure in people in rural Liberia. We have shown that certain bat species in Liberia carry EBOV-Zaire, while others carry Marburg virus and henipaviruses. Further, hunting bats is a common activity and we have detected high seroprevalence of Marburg and EBOV antibodies in residents of rural Liberia. Exposure to henipaviruses remains unknown. To test the hypothesis that spillover of both groups of viruses has occurred in this region, we will use an antigen-based multiplex serological assay to screen more than 1,9800 archived blood specimens, collected in 2021 and again in 2023 from over 900 adults and children in rural central Liberia as part of a longitudinal study of Lassa fever seroprevalence and seroincidence, for antibodies against all known filoviruses and henipaviruses.

Aim 2. To identify reservoirs and characterize the infection dynamics of filoviruses and henipaviruses in key bat species in Liberia. Our group detected EBOV-Zaire RNA and antibodies in *Miniopterus nimbae*, a cave- and mine-dwelling bat in northern Liberia found near the origin of the 2014 Ebola outbreak in Guinea. We also detected antibodies against EBOV in *Hipposideros* bats that co-roost with *Miniopterus* in multiple locations in Liberia. *Rousettus aegyptiacus*, a common frugivorous bat in Liberia, carries Marburg virus and henipaviruses. We will conduct a 3-year longitudinal study of populations of these bat species near the human cohort studied in **Aim 1**, to determine whether they are reservoirs for Ebola or other filoviruses and henipaviruses and characterize the temporal dynamics of filoviruses and henipavirus circulation using multiplexed serological assays that test for IgG antibodies against all known filoviruses and henipaviruses, as well as molecular techniques to screen for Ebola, Marburg, Nipah virus, and related viruses.

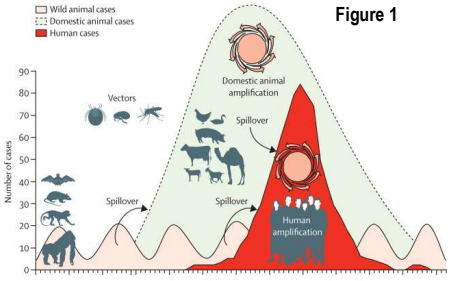
Aim 3. To characterize behaviors associated with exposure to filoviruses and henipaviruses. Combining the data collected in Aim 1 (humans) and Aim 2 (bats) we will identify the demographic, behavioral, and environmental factors associated with an individual's prior exposure to filoviruses and henipaviruses. Detailed animal exposure data collected from the adults and children (many born after the Ebola outbreak) who provided the Aim 1 blood specimens will be used to develop profiles of risk and inform public health & risk reduction communication tools in partnership with the Ministry of Health, Liberia.

RESEARCH STRATEGY

A. SIGNIFICANCE

Zoonotic viruses are responsible for the majority of emerging infectious disease outbreaks, which are occurring at an accelerating rate in global hotspots[3,7]. Every outbreak begins with spillover – transmission of the virus from its animal reservoir either directly into human populations or into other animal hosts *en route* to humans (**Figure 1**). **Exactly how often spillover occurs is virtually unknown,** as surveillance systems are primarily designed to detect clusters or outbreaks, and often miss initial individual infections that result from animal contact. To reduce the risk of future epidemics, it will be necessary to limit opportunities for zoonotic virus~~es to~~ spillover and to detect spillover at the earliest stages.[1,8] Measuring the efficacy of any interventions designed to reduce spillover and spread of zoonotic pathogens will rely on measuring rates of exposure in at-risk human populations.

Figure 1



Filoviruses and henipaviruses are among the highest consequence zoonotic viruses and have greatly impacted public health. Each group contains zoonotic pathogens that cause significant disease in both people and animals, especially livestock, that have been associated with extremely high rates of morbidity and mortality. Ebola (EBOV) and related viruses, as well as Nipah virus (NIV), a henipavirus, and its related viruses have been listed by the World Health Organization (WHO) among the highest priority pathogens for the development of vaccines and therapeutics, as they represent zoonotic pathogens with high potential to threaten human health, with extremely high mortality rates and few or no therapeutics or vaccines currently available.[9-11] These viruses share other important features which make them high priority: they are all associated with bat hosts that are abundant and widely distributed throughout Africa and Asia, living in close association with people and livestock in some of the most densely populated regions on Earth.

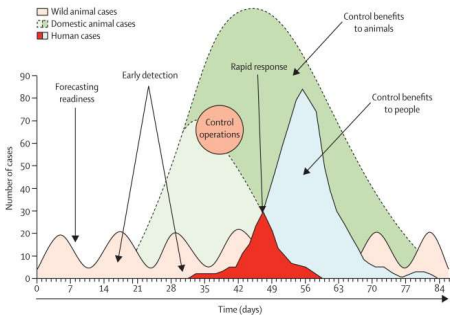
Spillover of these viruses has repeatedly occurred. Beyond the massive outbreak in West Africa in 2013-16, EBOV outbreaks have occurred repeatedly in Central and East Africa; most recently Sudan ebolavirus caused an outbreak in Uganda in late 2022[11-13]. Marburg virus (MARV) and Ravn virus (RAVV) comprise the closely related genus *Marburgvirus* and have caused outbreaks in Central and East Africa and Europe with mortality and clinical presentation comparable to Ebola [14,15]. Marburg virus outbreaks were declared in Guinea, just across the border from the UNC-Liberia research site in 2021 and then again in Ghana in 2022 marking the first time this virus has been reported in either country[16,17]. ~~Nipah~~ NIV ~~virus~~ is an emerging zoonotic paramyxovirus that has caused nearly annual outbreaks in Bangladesh, including multiple outbreaks in 2023, and sporadically in India with mean case fatality rates greater than 70% [12,18-20]. Despite the public health significance of Nipah and related viruses, little is known about its genetic diversity, natural reservoirs, or frequency of spillover throughout their range, including Africa. Henipaviruses, and ~~Nipah virus~~NIV in particular - have several characteristics that make them a global health priority: 1) Their bat reservoirs occur throughout Asia and Africa, overlapping human and livestock populations, giving them geographically broad opportunity to cause outbreaks [21]; 2) henipaviruses can be transmitted to people directly from bats or via domestic animals [21]; 3) ~~Nipah virus~~NIV can be transmitted from person to person [22]; 4) ~~Nipah virus~~NIV spillover has occurred and continues to occur in highly populous and internationally connected regions; 5) repeated spillovers of NIV strains with varying person-to-person transmission rates indicate the ability to evolve **with increased pandemic potential** [23,24]; and 6) Nipah is associated with a high mortality rate in people and currently has no vaccine or treatment [21,23]. While ~~Nipah virus~~NIV has not been detected in Africa, closely related henipaviruses have been identified in Ghana and Madagascar, though their public health significance remains unknown[25-27]. In West Africa, where bats and associated henipaviruses occur but where no human cases have been identified, **diagnostic testing and systematic surveillance is essentially non-existent**. The detection of antibodies against a Nipah-related virus in hunting communities in Central Africa suggests that cryptic spillover is occuring[28]. **Repeated spillover of henipaviruses creates opportunity for more transmissible strains to emerge[29].**

While bats have been identified as potential hosts for filoviruses including EBOV, the natural reservoir for EBOV-Zaire Ebola in West Africa remains unknown. There is substantial evidence, both serological and molecular, that bat species host filoviruses; however, a definitive natural reservoir or set of reservoirs for EBOV-Zaire has not been identified[30]. Wildlife, including bats, represent a substantial source of animal protein for communities in Liberia[31]. People may be exposed to bat-borne viruses through hunting and butchering, eating food contaminated by bat excreta, or by entering caves or mines inhabited by bats. Our group previously found that people living in rural areas of Liberia conduct activities which may increase their risk of contact with bats or their bodily fluids. The index case, a child, in the 2013 Ebola outbreak was hypothesized to have had contact with bats, yet the exact species was never definitely identified[32]. The route of spillover for most historical Ebola outbreaks remains a mystery. Despite broad efforts to determine the natural reservoirs of EBOV and related viruses, there continues to be a poor understanding of filovirus ecology, with the exception of ~~Marburg virus~~MARV, whose host has been identified as Egyptian rousette bats (*Rousettus aegyptiacus*)[33-35]. Communities known to hunt bats in Cameroon and India have been found to have antibodies against henipaviruses and filoviruses, respectively, yet nothing is known about community exposure rates in Liberia, where bat hunting is common[28,36]. Our preliminary data (see below) suggest that there is substantial exposure to EBOV as well as MARV among people in central Liberia. While the EBOV antibodies may indicate prior infection from another person such as during the West Africa outbreak, antibodies specific to other filoviruses may signal a localized exposure from animals. Even less is understood about henipaviruses in ~~Africa, and~~Africa and identifying antibodies against henipaviruses in people will be critical for understanding where, how and how often spillover occurs.

The urgent need to develop interventions to prevent spillover of zoonotic high consequence pathogens can only be met with a better understanding of their natural reservoirs, the rates of exposure in vulnerable human populations, and the ways in which people are exposed. Unrecognized outbreaks provide opportunities for viruses to emerge that may be more transmissible or more pathogenic than previously observed, and limiting these opportunities is an important component of pandemic prevention[2,29]. Typically, by the time an outbreak is recognized in human populations its containment and control are difficult, if not impossible to achieve (e.g., HIV, SARS-CoV-2). Understanding the ecology of these viruses, including which species act as reservoirs as well as how and to what extent people are being exposed to filoviruses and henipaviruses will inform intervention and surveillance strategies and provide baseline metrics against which one can measure the efficacy of interventions designed to reduce exposure, such as public health campaigns to promote avoidance of behaviors associated with infection among those most at risk (**Figure 2**).

This proposed study will be the largest and most rigorous One Health investigation of high-consequence zoonotic viruses in bats and humans in an emerging disease hotspot. West Africa is known to be the source of prior outbreaks of emerging infectious diseases and future spillover events there are highly likely. Liberia is typical of this region and contains some of the largest tracts of pristine forest and wildlife biodiversity remaining in West Africa and has a population that depends on wildlife, including bats. Decades ago, a spillover event from a non-human primate to a human triggered an on-going HIV pandemic that has led to the infection of over 60 million people and 25 million deaths. Only months ago, the orthopoxvirus MPOX found in rodents spread to and within 110 countries across the globe causing over 85,000 cases. Both originated from African wildlife. The proposed study will leverage the unique infrastructure and expertise we have developed in Liberia to undertake a comprehensive examination of filovirus and henipavirus spillover from bats to humans and provide evidence of previous and possibly on-going transmission events. Our findings will provide essential

Figure 2



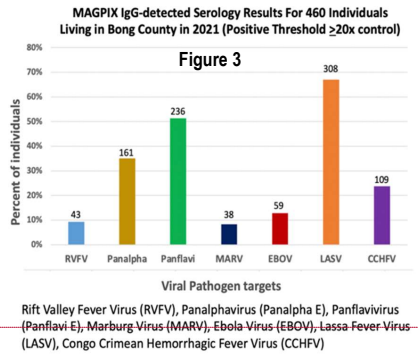
evidence of the presence and transmission dynamics of these bat-borne viruses and as the risks for infection in people living alongside them.

B. INNOVATION

The proposed research is highly innovative in multiple ways, including: 1) Our use of a one Health approach to study the ecology and epidemiology of two groups of high-priority, high-consequence zoonotic viruses in both bats and people in West Africa – an emerging disease hotspot where spillover events have occurred (e.g. Ebola, Marburg, MPOX, Lassa fever) and are highly likely to re-occur; 2) Our use of a novel and unique multiplexed serological platform, developed by our group, that will allow us to screen bat and human serum for IgG antibodies against each of the known filoviruses and henipaviruses, simultaneously in a single assay. Longitudinal comprehensive serological studies in bats and people **will provide unprecedented insight into the changes in rates of exposure over time to known viruses in bats; and in people (e.g. what is the baseline rate of exposure to henipaviruses and filoviruses and is there evidence of recent exposure?); and exposure rates to unknown, antigenically related viruses;** 3) We will couple serosurveillance with molecular testing to identify specific filoviruses and henipaviruses carried by bats; and 4) We will connect the bat and human epidemiological studies by analyzing questionnaire data from the human cohort to identifying high risk behaviors associated with viral exposure. **Results from this analysis will inform the development of public outreach and risk reduction campaigns by the Government of Liberia.**

C. APPROACH

C.1. Overview: **Aim 1** will test the hypothesis that spillover of filoviruses and henipaviruses occur regularly in rural Liberia by testing for IgG antibodies to all known filoviruses and (see Ttable 13) in ~~archived~~ serum samples from more than 900 people living in Bong county (central Liberia), collected between 2021-23 at two time points 24 months apart as part of the Coalition for Epidemic Preparedness Innovations (CEPI)-sponsored **ENABLE Study**, an on-going longitudinal study of Lassa fever seroprevalence and seroincidence. ~~-~~Seroprevalence of filoviruses and henipaviruses will be assessed as will ~~changes~~ seroincidence~~between the two collection timepoints~~. In **Aim 2,** we will conduct a 36-month longitudinal study of three bat species which are putative reservoirs for EBOV and undescribed henipaviruses. Using a combination of longitudinal serology and molecular testing (PCR and whole genome sequencing) **we will determine the prevalence of filoviruses and henipaviruses in ~~three~~ these key bats species associated with the rural human populations studied in Aims 1 and 3**. For **Aim 3,** we will analyze detailed questionnaire data developed collaboratively by our group (EHA and UNC) and collected in 2023 along with blood samples to be screened in Aim 1. These data will allow us to identify ~~behaviors~~ factors associated with exposure to henipaviruses and filoviruses. We will use results from our behavioral and exposure risk assessment to develop public risk reduction communication strategies with the National Public Health Institute, Liberia, using the results of this study to inform public health strategies. This application is a natural progression of our research activities, and our preliminary data speak to the feasibility of the proposed study.



~~Rift Valley Fever Virus (RVFV), Panalphavirus (Panalpha E), Panflavivirus (Panflavi E), Marburg Virus (MARV), Ebola Virus (EBOV), Lassa Fever Virus (LASV), Congo Crimean Hemorrhagic Fever Virus (CCHFV)~~

C.2. Aim 1: To determine the rates of filovirus and henipavirus exposure in people in central rural Liberia.

C.2.1. *Rationale and Preliminary Data.* The primary goal of this aim is to be able to determine whether populations within Liberia who live in association with bats have evidence of exposure against one or multiple filoviruses and/or henipaviruses and to compare rates of exposure over time. Studies of human populations in Central and West Africa have found evidence of exposure to EBOV prior to the 2013 outbreak and in places not known to have had Ebola outbreaks[37,,38]. UNC Project-Liberia has previously assessed seropositivity to

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consequential viral pathogens including EBOV and MARV in a subset of ~~CEPI~~ ENABLE Study participants. ~~About~~ Over 8~~1~~0% of participants had antibodies against MARV using a conservative threshold for seropositivity (**Figure 3**), suggesting that unreported spillover events have previously occurred. Acute encephalitis is common in western sub-Sharan Africa, yet outbreaks of neurological disease are rarely reported and more than half of all cases are undiagnosed[39]. Among the countries in western Africa reporting cases of meningitis or encephalitis, Liberia has reported the fewest, likely due to a lack of surveillance and capacity to diagnose neurological diseases[39]. ~~Nipah virus~~NIV encephalitis has never been reported in Africa, however, as described above, two Nipah-like henipaviruses have been described in bat hosts, including one in Ghana[25,27]. Spillover of henipaviruses was reported in bat-hunting communities in Cameroon[28]. In South Africa, we detected henipavirus sequences in *Rousettus aegyptiacus,* bats which also commonly occur in Liberia[26].

In Aim 1, we will test the hypothesis that spillover of both filoviruses and henipaviruses have occurred in rural populations in Liberia by measuring seropositivity to both groups of viruses within the CEPI ENABLE Study community cohort. We will also measure changes in exposure rates over a two-year period. While prior studies have provided cross-sectional snapshots of exposure, our longitudinal approach will provide data from the same cohort at two timepoints, 24 months apart, to determine both seroprevalence and sero-incidence using a multiplexed serological assay to screen for IgG antibodies against all known filoviruses and henipaviruses.

Settings and Participants. To achieve this aim we will leverage a well characterized cohort of individuals in three rural communities located in Bong County, Liberia (**Figure 4**), which has a population of over 333,400. UNC Project-Liberia has been operating in Liberia since 2014 and established a research site at Phebe Hospital in Bong County that includes a molecular laboratory that conducts daily qPCR testing for LASV, MARV, and EBOV as well as LASV serological assays using a MAGPIX (SEE RESOURCES AND FACILITIES). There, following extensive community engagement including meetings with key stakeholders, town hall meetings, and radio messaging, UNC launched the CEPI **ENABLE Study**, the largest passive and active surveillance program for Lassa fever in Liberia. Started in 2021~~0~~, 5,005 randomly selected participants 2 years of age and older in Phebe Airstrip, Suakoko, and Rubber Factory were enrolled over 6 months following informed consent/assent (mean age at enrollment is 22.2 years (range 2-97) and 54.2% are female). **Importantly, 7.7% of the cohort is under the age of 5 years, and therefore, were born after the 2013-14 Ebola outbreak (Table 1).** Households were selected for recruitment following identification of roof tops by satellite photos and use of a program developed by Médecins Sans Frontières' (MSF) Epicentre to randomly select~~ed~~ structures to ~~consecutively~~ approach sequentially for participation. The ENABLE Study includes baseline questionnaires and blood specimen collection from all participants with active follow-up by a study community health worker every 2 weeks to determine if~~s~~ a febrile event has occurred, which triggers drawing of blood for LASV, MARV, and EBOV PCR. Baseline blood is tested for Lassa fever virus (LASV) serology. A subset of 1,024 individuals (every 5th approached household is eligible) is also asked to provide blood samples routinely every 6 months for up to 24 months for repeat LASV serology. The blood samples from 0 and 24 months and the associated surveys will be used in **Aims 1 and 3** of this proposal. Informed consent and assent documents include provisions for the use of collected specimens and data for infectious diseases research including for emerging pathogens (see PROTECTION OF HUMAN SUBJECTS).

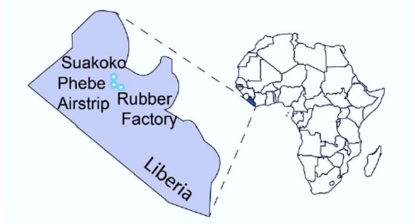

Figure 4. Map of Liberia with Bong County sites shown in inset.

C.2.2. Research Design. We will screen approximately **1,900 archived serum specimens** from participants in the ENABLE Study cohort at two time points ("baseline" (Month 0) and (Month 24)) for IgG antibodies against specific filoviruses and henipaviruses using the MAGPIX at the UNC molecular lab at Phebe H~~h~~ospital to run the multiplexed *henipavirus / filovirus* platform to be provided by Co-I Laing in year 1. The multiplex platform will allow us to determine specifically which filoviruses and henipaviruses people have been exposed to and compare that to the filoviruses and antibodies we observe circulating in local bat populations in **Aim 2**. The bead-based multiplexed assay has tremendous advantage over traditional ELISAs in that it will

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identify up to 25 different antibodies against multiple viral agents in a single, 2uL serum sample and uses a high through-put format. The ability to look at reactivity across all serogroups simultaneously will allow us to differentiate among the viruses. Proteins A/G non-specifically bind IgG immunoglobulins, and weakly bind IgM, an immunoglobulin that is the first to be produced during the initial stages of infection. We will conduct a 1-week training at the UNC-Phebe lab for using the assay to detect filovirus and henipavirus antibodies as well as data management, bioinformatics and analysis. **Table 13** shows the viral species and antigens that will be employed in this assay. The platform has been previously transferred to collaborating labs in the UK, South Africa, India, Bangladesh, Singapore, Thailand and Malaysia. We have previously detected serological profiles of Asiatic ebolaviruses in fruit bats collected in Singapore[40] and in fruits bats and humans actively engaged in bat hunting in northeast India[36].

Table 13. Multiplexed microsphere immune assay including soluble glycoproteins from each known filovirus and henipavirus

Virus (Name/Isolate Host/Country/Year/Strain)	Abbreviation	Accession No.	Soluble Glycoprotein	Mag Bead No.
Ebolaviruses				
Ebola virus/H. sapiens/COD/1976/Yambuku-Mayina	EBOV	NC_002549.1	$GP_{(1,2)}$	34
Bundibugyo virus/H. sapiens/UGA/2007	BDBV	FJ217161.1	$GP_{(1,2)}$	64
Bombali ebolavirus/Mops condylurus/SLE/201	BOMV	NC_039345	$GP_{(1,2)}$	55
Taï Forest virus/H. sapiens/COV/1994/Pauleoula-Cl	TAFV	NC_014372	$GP_{(1,2)}$	57
Sudan virus/H. sapiens/UGA/2000/Gulu-808892	SUDV	NC_006432.1	$GP_{(1,2)}$	54
Reston virus/M. fascicularis/USA/1989/Pennsylvania	RESTVm	AF522874.1	$GP_{(1,2)}$	65
Marburgviruses				
Marburg virus/H. sapiens/KEN/1980/Musoke	MARV	Z12132 S55429	$GP_{(1,2)}$	37
Ravn virus/H. sapiens/AGO/2005/Ang0126	RAVV	NC_024781.1	$GP_{(1,2)}$	62
Dianlovirus				
Mengla virus/Rousettus-WT/CHN/2015/Sharen	MLAV	KX371887.2	$sGP_{(1,2)}$	22
Cuevavirus				
Lloviu virus/M. schreibersii-wt/ESP/2003/Asturias-Bat86	LLOV	NC_016144.1	$sGP_{(1,2)}$	66
Henipaviruses				
Hendra virus/E. caballus/AUS/1994	HeV	NC_001906.3	sG	43
Hendra virus-2G	HeV-2g		sG	48
Nipah virus/H. sapiens/MYS/2000	NiVm	NC_002728.1	sG	46
Nipah virus/H. sapiens/BGD/2004	NiVb	AAY43916.1	sG	56
Cedar virus/Pteropus sp./AUS/2012/CG1a	CedV	NC_025351.1	sG	53
Mojiang virus/R. sladeni/CHN/2014/Tongguan1	MojV	NC_025352.1	sG	29
Ghanaian bat virus/E. helvum/GHA/2009/GH-M47a	GhV	NC_025256.1	sG	35
Angavokely virus	AngV		sG	47

C.2.3. Data Analysis. We will create a project database using Airtable, which will be hosted at EHA and will house all data from Aims 1 and 2 and will link to the RedCap server which stores questionnaire data from Aim 3. Database access will be secure and limited to key personnel (see data management plan). Raw MFI values from the MAGPIX will be used to calculate reactivity to each viral antigen and based on relative strength of reactivity (**Fig 5**) and determinations of negative cutoffs based on reference sera, we will calculate prevalence rates for each time point and compare rates between the two timepoints. Serological profiling can be used to detect reactive antibodies against all specific filoviruses and henipaviruses in a samples and compare strength of reactivity and cross-reactivity against multiple antigens using a single sample[41]. We will compare the antibodies across all specific viral antigens to assess which virus or

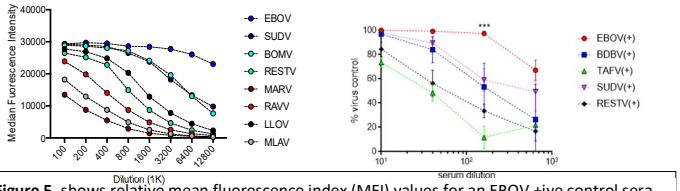

Figure 5. shows relative mean fluorescence index (MFI) values for an EBOV +ive control sera across serial dilutions. While there is cross reactivity, the EBOV MFI values are consistently greater than the other viral GPs and at higher dilutions the separation becomes significant.

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groups of viruses are responsible for the immunological response[42-44]. We will assess exposure to non-Zaire ebolavirus species, which may indicate spillover of filoviruses has occurred separate to the Ebola outbreak of 2013. We will also look at evidence for exposure to Nipah-like henipaviruses and compare exposure rates to both viral groups within and between our two sample collection time points.

C.2.4. Expected outcomes. We anticipate obtaining serological profiles of exposure to filoviruses and henipaviruses from each individual in our cohort. In previous studies we found antibodies against both ~~Ebola~~ EBOV and ~~Marburg~~ MARV virus. While the ~~Ebola~~ EBOV antibodies are attributable to the epidemic, ~~Marburg~~ MARV antibodies, in patients EBOV IgG negative, suggest possible exposure from an animal (e.g. bat) reservoir. We also anticipate seeing reactivity to ~~to~~ Bombali virus (BOMV), given the presence of the reservoir species in Liberia (*Mops condylurus*) and reactivity to the African henipaviruses (GhV and AngV), although this may represent reactivity to an antigenically related virus. Serological results from this and the bat study will inform our strategy for prioritizing which bat samples we test by PCR.

C.2.5. Potential problems and solutions. While our preliminary data suggest there will be detection of immune responses to filoviruses including EBOV-Zaire and, significantly, MARV, it is possible that evidence of exposure to other pathogens, including henipaviruses will not be detected. This would be an important finding in this large cohort, especially if these viruses are detected in local bat species, that would warrant further investigation. It is also possible that there will be few or no seroconversion events among the cohort during the 24-month period of follow-up. This too would be reassuring but would suggest a need for longer follow-up of this cohort. To address the possibility that reactivity to EBOV-Zaire virus may be explained by infection during the 2013 Ebola epidemic, we will create a polyclonal sera reference standard utilizing convalescent sera collected from human survivors of EVD. This internal reference standard will be calibrated to the WHO EBOV human serology standard (**Fig 6A**) permitting direct interpolation of anti-EBOV IgG as a MFI to a standard antibody concentration (binding antibody units/mL). The internal reference standard will then be included in our human serology testing strategy allowing semi- or quantitative measurement of anti-EBOV IgG. These EBOV human serology standards will permit us to establish positive and negative predictive performance values for the multiplex assay; furthermore, we will be able to investigate immunoreactivity of EVD survivors against heterotypic ebolaviruses. Our preliminary data indicates that EBOV+ sera IgG is highly cross-reactive with soluble native-like trimeric envelope glycoprotein (GP) antigens from both EBOV and BDBV (Figure 6A). EBOV GP, matrix protein (VP40), and nucleocapsid protein (NP) antigens have all been used in a variety of serological tests, however, the frequency of seroconversion against each antigen has not been fully described. In a longitudinal study of EVD survivors, high seroconversion against GP and VP40 was observed, with affinity maturation occurring overtime against GP[45]. Antigens based on the GP are likely to provide an optimal intrinsic balance of sensitivity and specificity. To limit the degree of heterotypic ebolavirus cross reactions we will test any anti-ebolavirus GP trimer antigen-seropositive samples with a pan-ebolavirus GP1 antigen-based panel. In preliminary studies, GP1 antigens improve specificity and differentiation of antibodies reactive with homotypic ebolaviruses in polyclonal antisera from rabbits immunized with GP trimer immunogens (**Figure 6B**).

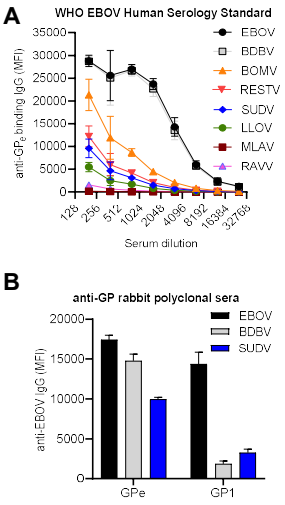

FIGURE 6A & B. A) The WHO EBOV human serology standard was testeding for immunoreactivity in a filovirid GP-trimer based multiplex assay. Filovirids are indicated along with sera dilution factors; data is the mean of three independent experiments. B) Antisera from EBOV GP-trimer immunized rabbits were tested in multiplex panels comprised of GP-trimer and GP1 subunit proteins. Anti-EBOV polyclonal IgG was less reactive with GP1 from BDBV and SUDV.

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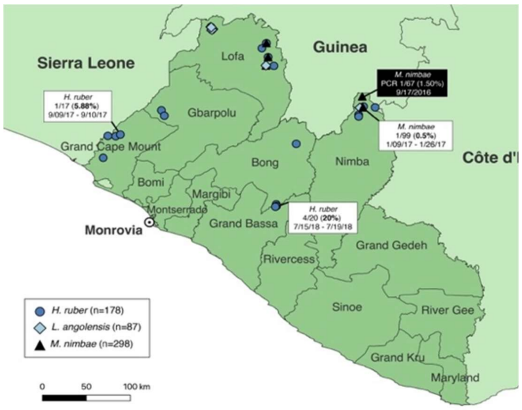
C.3. Aim 2. To identify reservoirs and characterize the infection dynamics of filoviruses and henipaviruses in key bat species in Liberia.

C.3.1 *Rationale and ~~P~~preliminary data.* Bats have been established as important hosts for filoviruses and henipaviruses in Africa and Asia, with combinations of viral isolation, molecular detection or serology providing substantial evidence [30,46-49]. MARV was isolated from Egyptian rousette bats in Uganda, and RNA has since been detected in these bats in Sierra Leone, which borders Liberia [50]. To date, EBOV has never been isolated from bats, although molecular and serological evidence suggest that multiple frugivorous bats may carry EBOV in Central Africa[50,51]. Prior to the 2014 epidemic, Zaire ebolavirus had not been reported in West Africa, and the animal reservoir remains unknown[32]. From 2016-2019, our group (PI Epstein & KP Desmond) implemented a large-scale project to identify reservoirs for EBOV in West Africa, under the USAID PREDICT Project[52]. This effort led to the discovery of a new species of Ebola (Bombali virus)[53], the detection of MARV in E Egyptian rousette bats~~RBs~~ in Sierra Leone[33], and **in 2018, we detected, for the first time in West Africa, EBOV RNA and anti-EBOV**



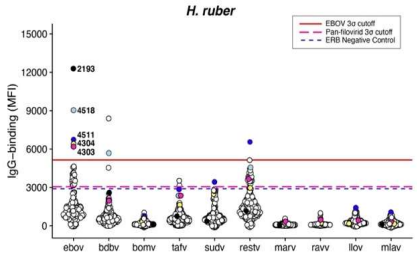
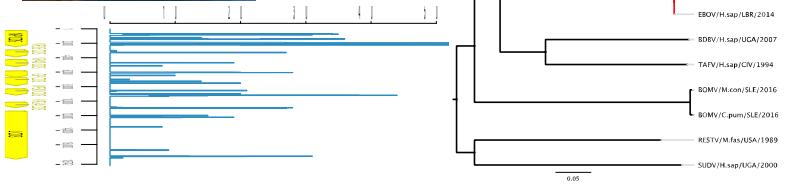


Figure 7: A. Map showing location of EBOV PCR +ive bat (black box) Zaire ebolavirus and MARV in Liberia in 2018. B. Serological evidence for EBOV in *H. ruber* from our 2018 study. C. RNA genomic sequences from across the *Zaire ebolavirus* genome detected in *Miniopterus nimbae*, Liberia in 2018. D. Phylogenetic analysis supports relatedness to 2013 EBOV epidemic virus.



IgG antibodies in bats (Figure 7A-D).

While this finding generated the first evidence of which bats

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may be reservoirs for Zaire ebolavirus, longitudinal studies are required to more completely understand the role these species play in EBOV circulation – either as reservoirs that maintain the virus within a local population, or as incidental hosts, infected by an unknown bat species. One-off, cross-sectional studies have a low probability of detecting any specific filovirus or henipavirus given their low prevalence and short viremic periods [34,54], and thus provide a poor characterization of viral diversity. The lack of understanding of **filovirus and henipavirus diversity and their natural reservoirs in West Africa significantly impedes risk-based public health interventions to prevent human outbreaks.** Longitudinal sampling is needed to separate reservoir hosts from spillover hosts. For example, MARV has been isolated repeatedly from *R. aegyptiacus* in Uganda, which, when paired with experimental infection studies showing infection and shedding of MARV by *R. aegyptiacus,* strongly suggests that *R. aegyptiacus* is indeed a ~~Marburg virus~~MARV reservoir[35,55,56]. Similarly, our longitudinal studies of ~~Nipah virus~~NIV in *Pteropus medius* bats in Bangladesh, which included the same multiplexed serological assay proposed here, confirmed them as a reservoir for NIV and identified important mechanisms driving viral circulation[29]. **Understanding which species persistently carry filoviruses and henipaviruses will provide important insights into how they are maintained in nature and what species should be targeted for follow-up studies on bat habitat use and human-bat contact patterns.**

C.3.2. *Sampling design*. We will repeatedly sample three bat species associated with filoviruses or henipaviruses, based on our preliminary data: *M. nimbae* (EBOV), *H. ruber* (EBOV), and *R. aegyptiacus* (MARV & Henipaviruses). We will sample bats at roost sites in Bong county, as close as possible to the human study sites in Aim 1. In Y2-Y4, we will sample 40 individuals of each species every two months for 36 months and collect excreta using plastic tarps placed underneath bat roosts (details below and in C.3.3).

Bat capture and sampling. Briefly, we will use either harp traps or mist nets to capture bats, depending on the capture site. Personnel will wear appropriate personal protective equipment (e.g. Tyvek suits or dedicated long clothing; double nitrile gloves; an N95 respirator and safety glasses or a PAPR [57]. Traps and nets will be attended continuously during capture periods. Bats will be removed from mist nets immediately upon entanglement to avoid injury. Bats will be removed from harp traps after approximately 1 hour and bats will be placed in individual cloth bags until processed. Body measurements, blood, oropharyngeal swabs, and rectal swabs (or fecal pellets) will be collected from each bat. Swabs will be collected in duplicate; one swab will be placed in a cryovial containing Trizol lysis buffer and the other in viral transport medium (VTM). For bats <50g, blood will be collected in a microhematocrit tube and spun down using a portable centrifuge. A bulb will be used to expel serum from the tube into a cryovial. For larger bats >50g, a tuberculin syringe or 1ml syringe and 27g needle will be used to draw blood from the radial artery or vein[57]. Blood will be placed into a conical vial and centrifuged. Serum will be aliquoted into a cryovial using a pipette. During site visits we will also lay out plastic tarps to collect pooled environmental excreta in Trizol and VTM to improve opportunity to detect viral RNA. All samples will be placed in a vapor phase liquid nitrogen dry shipper or portable battery-powered ultracold freezer, immediately upon collection and then transferred to a -80C freezer at the Phebe lab until testing.

C.3.3. *Testing plan, ~~d~~Data analysis, sample size, and power analysis. Serology:* At the end of Y3, we will screen all serum samples collected to date using the MAGPIX multiplex assay for IgG against filoviruses and henipaviruses. We will analyze temporal dynamics in seroprevalence using general additive models (GAMs), which are analyses that we have used successfully in many previous studies of similar design[29]. These methods are well adapted for capturing long-term temporal fluctuations in prevalence and moderately sensitive at capturing short-term changes in seroprevalence which can be used to identify population-level disease outbreaks. For example, we estimated that with 40 bats sampled every two months, we would be able to statistically detect an increase in seroprevalence (at a 95% CI limit) of greater than 25% between consecutive sampling events > 80% of the time, assuming a bat population seroprevalence of at least 10% in advance of a bat colony infection (estimated by, in brief, by simulating a fluctuating true seroprevalence value, simulating samples given this underlying seroprevalence, fitting a GAM to these samples, and then calculating if CI on true prevalence between the two sampling occasions over which the change in seroprevalence occurred were non-overlapping). While this is a large change, it is within a range to be expected if infection were to sweep through a bat community[29]. MAGPIX-positive bat sera will be sent to RML for serum neutralization assays using viral isolates or VSV pseudoviruses under BSL4 conditions.

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VSV-pseudotype neutralization assays: Serological results will be confirmed using VSV-pseudotype neutralization assays for the following filoviruses EBOV, SUDV, BDBV, RETV, BOMV, MARV and RAVN. If novel henipavirus sequences are detected, additional assays will be developed to test positive sera. Serum samples will be inactivated using ɣ-irradiation (4 MRad). Neutralization of irradiated and heat-inactivated serum samples will be assessed in Vero 352 E6 cells. Briefly, cells were seeded in 96-well round-bottom plates for 24 hours, serial dilutions of heat-inactivated serum samples will be performed in DMEM supplemented with 2% FBS, penicillin/streptomycin, and L-glutamine. Each plate will contain negative serum control, cell-only control, and virus-only control. VSV EBOV-GFP will be added to each well of the serum dilution plate and the serum-virus mix 357 was incubated at 37˚C for 1-hour. The mix was added to the cells and incubated at 37˚C for 24 hours. The cells will then fixed with 4% paraformaldehyde at room temperature 359 for 15-minutes and centrifuged at 600 x g for 5-minutes at room temperature. The supernatant will be discarded and FACS+EDTA buffer was added. Samples will be run on the FACSymphony A5 Cell Analyzer (BD Biosciences, Mississauga, ON, Canada) and FITC MFI 362 was measured. Data will be analyzed using FlowJo.

Molecular testing: We will use specific filovirus taqman real-time PCR assays to screen oropharyngeal, fecal and environmental samples from bats for EBOV and MARV RNA at the UNC-Phebe lab[55,58]. Testing bat samples by PCR may allow us to detect viral RNA during active infection, providing important information about the prevalence and timing of infection within target species. **Because we expect viral prevalence to be significantly lower than seroprevalence, estimated at <5%[25,27,49],** we developed a sampling scheme focused on maximizing our probability of detecting viral RNA with qPCR. To compare power under alternative sampling designs, we built simulation-based power analyses that estimated power to detect: 1) viral EBOV or MARV RNA in at least one bat; and 2) the expected number of positive cases, in each bat species. In brief, we estimated power for total sample sizes between 1500-3000 (between 500 and 1000 bats sampled per species) spread across a variable number of visits per year (2-6), while allowing for uncertainty in true infection dynamics in the bat populations, specifically: 1) prevalence during one three-week period (with unknown timing) per year of high prevalence ranging from 1-5%); and 2) an otherwise low background prevalence (0-1%). Given these estimates of yearly infection dynamics, these analyses showed with 672 samples per species (minimum 32 individual bats per sampling event), spread across 6 sampling occasions per year, we would obtain a per-species probability of acquiring 0 positive samples of <4% (<1% at 40 bats per event – our target number) and an expectation of ~3-6 RNA positive results (~5-10 positives at 40 bats per event). Because henipaviruses have not been previously described in Liberia, we will focus our resources on filovirus testing by PCR. However, if henipavirus serology suggests active circulation, we may send a set of samples to RML for pathogen discovery (SEE RML LETTER OF SUPPORT).

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Viral isolation: PCR-positive cDNA samples and their aliquot in VTM will be sent to RML for further sequencing and attempts at viral isolation under BSL4 conditions. If attempts to culture do not work, we will use next generation sequencing techniques to get whole genome sequence and further characterize the virus at RML. Novel viruses will be characterized and rescued (reverse engineered using a minigenome system to synthetically create infectious viral particles [59]) at RML under secure, biosafety level 4 conditions, then used for serum neutralization assays in bat and human sera to determine more precise exposure rates. Sequences from the glycoproteins of any novel viruses discovered will be used by USU (Co-I Laing) to generate purified proteins and added to the Luminex assay, which will improve its specificity.

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VirCapSeq-VERT and myBaits probe-based enrichment for recovery of full-length genome sequences of filoviruses: Although shotgun sequencing provides opportunities to analyze all host and virus genetic material, the larger average genome size of bacteria and hosts compared to viral genomes complicates a detailed analysis of the virome, particularly vertebrate viruses. We have established a positive selection probe capture-based

system to enrich for viral sequences. Here, we will take advantage of these probe capture-based methods (VirCapSeq-VERT and myBaits probe library) to enrich for vertebrate viral sequences of interest. Sample libraries will be normalized and combined in 4- to 12-plex reactions for solution capture hybridization using either the HyperExplore custom bait library version of VirCapSeq-VERT probe set or our custom myBaits probe library. For HyperExplore custom bait library probe set, libraires will be enriched for virus following the SeqCap EZ HyperCap Workflow User's Guide, version 2.3, while for custom myBaits probe library, the myBaits Hybridization Capture for Targeted NGS protocol, Version 4.01 will be used. Sequencing libraries will be normalized and sequenced as 2 X 150 bp fragments on Illumina's MiSeq or NextSeq sequencing platforms, following Illumina's standard procedure (Illumina, San Diego, CA). NGS data will be analyzed using metavirs (https://openomics.github.io/metavirs/), a comprehensive viral metagenomics pipeline to assemble, annotate, and classify viruses. It relies on technologies like Singularity1 to maintain the highest-level of reproducibility. The pipeline consists of a series of data processing and quality-control steps orchestrated by Snakemake2, a flexible and scalable workflow management system, to submit jobs to a cluster. The pipeline is compatible with data generated from Illumina short-read sequencing technologies. As input, it accepts a set of FastQ files and will be run on-premises using the NIAID RML Big Sky supercomputing cluster.

C.3.~~4~~5. *Expected outcomes.* Based on our prior work, our multifaceted analysis approach, and calculated power given our proposed temporal sampling strategy, we expect to be able to use temporal serological data to describe filovirus and henipavirus viral dynamics over time. Our molecular screening will primarily focus on filoviruses because we have known targets, and we expect to detect EBOV or MARV RNA, despite low prevalence and short-lived infections. Sequence data from positive samples will: 1) provide substantial evidence for the identification of EBOV reservoirs and help explain which virus is responsible for the observed IgG response in bats (and possibly people); and 2) provide valuable information about viral diversity, about which little is known. Henipavirus serology, in the absence of sequence data, will allow us to identify potential bat reservoirs, analyze temporal trends, and generate hypotheses for future targeted studies.

C.3.~~5~~6. *Potential problems and solutions*

Serological tests are greatly hindered by the inherent antibody cross-reactivity between antigens from known ebolaviruses, challenging data interpretations that antibodies are EBOV-specific or EBOV, BDBV, and SUDV cross-reactive [40]. This is also true for the henipaviruses in the panel. The advantage of the multiplexed platform is that we can simultaneously see relative MFI values for all the filoviruses and henipaviruses. Glycoproteins from the homologous virus should cause the most intense reaction, particularly at higher dilutions. Co-Is Laing and Munster have demonstrated that homotypic and heterotypic ebolavirus-antibody reactions can be simultaneously measured and accounted for in ERBs challenged with replication-competent VSV-filoGP (**Figure 8**). Still, there is some cross reactivity, and we will use additional tools such as viral neutralization assays as a secondary test, and our statistical approach can correct for a portion of cross reactivity. Past studies have relied on individual ELISAs which had varying degrees of specificity depending on which test was used, but often couldn't rule out cross-reactivity. The Luminex filovirus multiplex assay has predictable patterns of cross-reactivity, but we will still be able to discern to which virus the serum is most reactive.

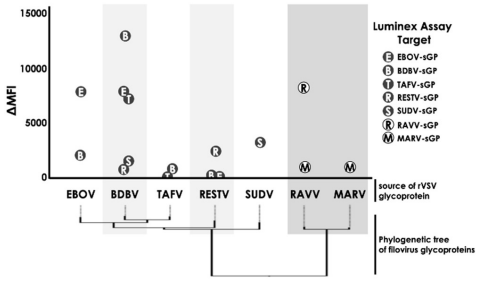

Figure 8. Captive-bred Egyptian rousette bats ~~ERBs~~ were experimentally challenged with VSV-filoGPs, the GP source is indicated on the x-axis. In a GP-trimer multiplex test the homotypic ebolavirus bat antisera were most reactive with the homotypic ebolavirus GP-trimer antigen.

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C4. Aim 3: To characterize behaviors associated with exposure to filoviruses and henipaviruses.

C.4.1 Background and rationale. As described above, there is substantial interaction between wildlife, domestic animals, and people living in rural Liberia. Our group collected questionnaire data from 585 people across Liberia as part of the USAID-funded Ebola Host Project between 2016 and 2019. When asked about animal exposures during the 2013-2016 Ebola outbreak, participants frequently reported contact with both domestic and wild animals. Common types of animal contact included animals coming inside the dwelling (58%), cooking or handling animal meat (44%), and eating raw or undercooked meat (31%). Additionally, 26% reported slaughtering and 12% reported hunting or trapping an animal. In the ENABLE Study of 5,005 residents of Bong County, survey questions mainly focused on rodent exposures relevant to Lassa fever, but 12% of participants reported hunting or preparing wild animal meat. Little is known about the prevalence and type of other potential bat exposures in this region of Liberia. Elsewhere in West Africa, EcoHealth Alliance participated in the response to Ghana's 2022 cluster of MARV cases, working with local partners to administer a behavioral risk questionnaire characterizing bat exposures in three affected communities[16]. Many participants lived in close proximity to bats, with one fifth reported exposure to bats inside a dwelling or other building in the previous four months. When asked about exposures more relevant to Egyptian Rousette bats, a known MARV reservoir and a target species in this proposed study, participants commonly reported bats feeding on fruit trees on their home compound, eating fruit bearing bite marks, and exposure to bats inside a cave or mine.

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C.4.2. *Research design*. As described in Aim 1, serum collected at baseline (in 2021) and at month 24 (in 2023) during the ENABLE Study will be screened for filoviruses and henipaviruses (analyses not included in the ENABLE Study protocol), enabling us to calculate seroprevalence at baseline and seroconversion over a two-year period. In addition to providing blood specimens, the 1,024 participants who initially enrolled responded to baseline questionnaires on demographics, healthcare-seeking behaviors, health status, and animal exposures. At month 24, the 921 participants still actively enrolled are administered repeat questionnaires. This final survey includes additional items based on the Ghanaian questionnaire and asks specifically about bats including the respondents' activities in their habitats (entering forests, caves, and mines), direct contact (hunting, eating handling), indirect contact (presence in home or other used structure), and observed characteristics of bats encountered (fruit tree dwelling). By pairing this existing survey data with the new serology results generated from archived samples under Aim 1, as well as new data on nearby bat infection from Aim 2, we will be able to develop comprehensive risk profiles for exposure to filoviruses and henipaviruses in this community.

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C.4.3 *Data analysis.* The primary outcome for this analysis will be testing positive for IgG antibodies against any known filoviruses or henipaviruses. For both families of viruses, and for each species of virus within those families, we will use the serology results generated under Aim 1 to examine risk factors for seropositivity at baseline and for seroconversion between baseline and month 24. We plan to conduct a multivariate logistic regression, with appropriate weights applied to account for the single-stage cluster sampling design of the ENABLE Study, in which members of randomly selected households were surveyed and tested. We will use data from both the baseline and month 24 questionnaires to examine the effects of sampling site, participant demographics (e.g., age, gender, occupation), environmental exposures (~~e.g.~~e.g., animals present in and around the dwelling), and behavioral risks (e.g., direct contact with bats through hunting, preparing, or eating them, entering caves or mines where bats live).

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Power analysis: To assess our power to identify risk metrics for seroconversion (or overall seropositivity), we built simulation-based power analyses that consisted of, in brief: 1) simulating survey responses for N (400-1,000) individuals, while allowing for moderate unevenness in representation across five binary categories (as great as 80%-20% for each); 2) examining a range of effect sizes (odds ratios ranging from 1-2.5) for these five

binary categorical predictors (e.g., sex, encountered a bat, handled a bat, etc.). Summarizing across 1,200 simulations of surveys of 400-1,000 individuals given joint uncertainty in the representation within these surveys and effect sizes, we estimated that we would have at least 80% power to detect significant effects for each predictor given an odds ratio of 1.65 or greater with 1,000 survey responses or ~1.80 (for example, with a rate just under 5% for Group1 = 0, just over 8% for Group1 = 1) with 750 responses.

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C.4.4 *Expected outcomes.* By combining serology results with detailed questionnaire data on participant demographics and animal exposures, we will be able to use odds ratios to identify behaviors significantly associated with seropositivity (e.g., exposure to filoviruses or henipaviruses). We expect to be able to also identify age and gender-based risk factors given our sample size. Based on previous experience conducting behavioral risk surveys, including a recent study in Ghana following a Marburg outbreak, we expect to be able to develop risk mitigation messaging that uses evidence from Aims 1 and 2 as well as results from this aim.

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C.4.5 *Potential problems and solutions*. We anticipate that most IgG positive individuals for either set of viruses will be adults, with the likelihood of exposure and having antibodies increasing with age. Because we don't know how long IgG antibodies persist at detectable levels, it will not be possible to accurately determine when exposure occurred, with the exception being EBOV antibodies that most likely occurred as a result of contact with another infected person during the 2014-2016 outbreak. We will address this in two ways: by looking at respondents 21-6 yrs years oldof age, we will know that any filovirus antibodies they have are most likely not due to the epidemic, and we will be able to determine whether there are any significant associations with particular behavior or other factor (household construction, domestic animal exposure) that promotes direct animal contact, consumption of contaminated fruit, or exposure to bat habitats (e.g., caves or mines). For adult respondents, we will focus on exposures (based on relative MFI values) specific to non-EBOV filoviruses and henipaviruses and see whether there are associations with specific behaviors.

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C.5. Project timeline

Project timeline	Y1				Y2				Y3				Y4				Y5			
Activity	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Ethical approval (local IRB / IACUC)																				
Database development																				
Aim 1: Human serum testing																				
Aim 2: Bat sampling																				
Aim 2: Bat serum testing																				
Aim 2: Bat sample PCR																				
Aim 1&2: Confirmatory assays + sequencing (RML)																				
Aim 3: Behavioral risk analysis																				
Aim 3: Risk communication development																				
Publications																				
Data publication																				

D. Summary and Conclusions.

West Africa is a hotspot for emerging zoonotic viruses such as Ebola, Marburg and potentially Nipah-related henipaviruses – groups that have been identified by WHO as among the most significant pathogens that threaten global health. Liberia is also a biodiversity hotspot, with some of the largest tracts of primary forest remaining in the region, and its population relies on wildlife as a primary source of protein, creating continued risk of spillover of high consequence zoonotic pathogens. This project has the potential to determine the origins of Zaire ebolavirus in West Africa, characterize the circulation of Ebola and other filoviruses and henipaviruses in common bat species, identify background rates of spillover in rural human populations, and identify high-risk behaviors associated with exposure to these bat-borne zoonoses. Our group comprises an unparalleled

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multidisciplinary group of leading experts in epidemiology, clinical medicine, virology, serology, and disease ecology all with deep experience working in Liberia, strong partnerships with the Liberian public health institutions, and implementing zoonotic virus research, giving this project a high chance of success in generating impactful results that will help prevent future epidemics in West Africa.

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Specific Aims

Emerging infectious diseases - predominantly caused by zoonotic viruses - have become a major threat to individual and public health as well as the functioning of a global society. Even prior to the SARS-CoV-2 pandemic, outbreaks of consequential emerging pathogens including SARS-CoV, Pandemic influenza 2009, Zika virus, and Ebola virus disease tragically killed and disabled and caused massive disruptions of regional and global economies and health care systems[1]. The emergence of consequential zoonotic infections is accelerating due to anthropogenic drivers such as climate change, land-use change, intensification of livestock production, and wildlife trade, all of which increase opportunity for people and domestic animals to come into contact with wildlife reservoirs[2-5]. **To reduce the risk of infectious disease epidemics, there is a critical need to detect and *prevent* spillover of zoonotic pathogens from wildlife reservoirs[2,6]**.

Illustrative of this need is the repeated spillover from wildlife reservoirs to human populations of groups of zoonotic viruses such as filoviruses (e.g., Ebola virus [EBOV]) and henipaviruses (e.g., Nipah virus [NIV]). Large-scale outbreaks or clusters of cases with these high-mortality zoonotic viruses have led to their being **designated as high priority pathogens by the World Health Organization (WHO)[5]**. However, at present, the detection of outbreaks caused by these pathogens occurs only after the first human infections; a failure in prevention that is deadly, costly, and remediable[1]. Identifying natural reservoirs of zoonotic viruses and measuring rates of exposure to these viruses in humans are critical to assessing their potential threat and developing interventions that reduce the risk of spillover.[1,2]

In the proposed study we have assembled a diverse team of experts in ecology, epidemiology, virology and immune response to bat-borne viral zoonoses such as filoviruses and henipaviruses; and filovirus clinical care and research who are actively working in West Africa to provide much needed empirical evidence to help answer the questions: **What is the natural reservoir for Ebola virus and how often does spillover of this and related viruses happen in a region that is an emerging disease hotspot, suffered the world's largest filovirus outbreak, and where there has been evidence of other consequential zoonotic viruses in animals and people**? To address these questions, we propose the following specific aims:

Aim 1. To determine the rates of filovirus and henipavirus exposure in people in rural Liberia. We have shown that certain bat species in Liberia carry EBOV-Zaire, while others carry Marburg virus and henipaviruses. Further, hunting bats is a common activity and we have detected high seroprevalence of Marburg and EBOV antibodies in rural Liberia. Exposure to henipaviruses remains unknown. To test the hypothesis that spillover of both groups of viruses has occurred in this region, we will use an antigen-based multiplex serological assay to screen more than 1,800 archived blood specimens, collected in 2021 and again in 2023 from over 900 adults and children in rural central Liberia, for antibodies against all known filoviruses and henipaviruses.

Aim 2. To identify reservoirs and characterize the infection dynamics of filoviruses and henipaviruses in key bat species in Liberia. Our group detected EBOV-Zaire RNA and antibodies in *Miniopterus nimbae*, a cave- and mine-dwelling bat in northern Liberia found near the origin of the 2014 Ebola outbreak in Guinea. We also detected antibodies against EBOV in *Hipposideros* bats that co-roost with *Miniopterus* in multiple locations in Liberia. *Rousettus aegyptiacus*, a common frugivorous bat in Liberia, carries Marburg virus and henipaviruses. We will conduct a 3-year longitudinal study of populations of these bat species near the human cohort studied in **Aim 1**, to determine whether they are reservoirs for Ebola or other filoviruses and henipaviruses and characterize the temporal dynamics of filoviruses and henipavirus circulation using multiplexed serological assays that test for IgG antibodies against all known filoviruses and henipaviruses, as well as molecular techniques to screen for Ebola, Marburg, Nipah virus, and related viruses.

Aim 3. To characterize behaviors associated with exposure to filoviruses and henipaviruses. Combining the data collected in Aim 1 (humans) and Aim 2 (bats) we will identify the demographic, behavioral, and environmental factors associated with an individual's prior exposure to filoviruses and henipaviruses. Detailed animal exposure data collected from the adults and children (many born after the Ebola outbreak) who provided the Aim 1 blood specimens will be used to develop profiles of risk and inform public health & risk reduction communication tools in partnership with the Ministry of Health, Liberia.

RESEARCH STRATEGY

A. SIGNIFICANCE

Zoonotic viruses are responsible for the majority of emerging infectious disease outbreaks, which are occurring at an accelerating rate in global hotspots[3,7]. Every outbreak begins with spillover – transmission of the virus from its animal reservoir either directly into human populations or into other animal hosts *en route* to humans (**Figure 1**). **Exactly how often spillover occurs is virtually unknown,** as surveillance systems are primarily designed to detect clusters or outbreaks, and often miss initial individual infections that result from animal contact. To reduce the risk of future epidemics, it will be necessary to limit opportunities for zoonotic viruses to spillover and to detect spillover at the earliest stages.[1,8] Measuring the efficacy of any interventions designed to reduce spillover and spread of zoonotic pathogens will rely on measuring rates of exposure in at-risk human populations.

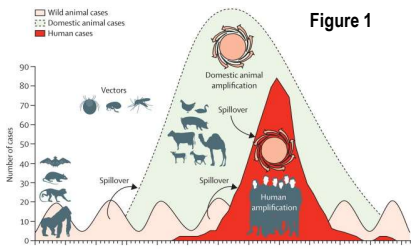


Figure 1

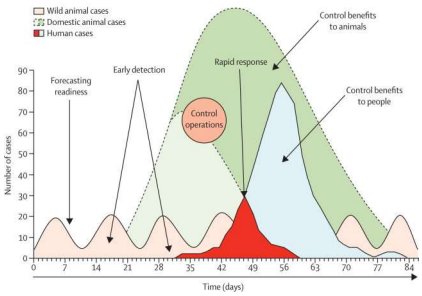
Filoviruses and henipaviruses are among the highest consequence zoonotic viruses and have greatly impacted public health. Each group contains zoonotic pathogens that cause significant disease in both people and animals, especially livestock, that have been associated with extremely high rates of morbidity and mortality. Ebola (EBOV) and related viruses, as well as Nipah virus (NIV), a henipavirus, and its related viruses have been listed by the World Health Organization (WHO) among the highest priority pathogens for the development of vaccines and therapeutics, as they represent zoonotic pathogens with high potential to threaten human health, with extremely high mortality rates and few or no therapeutics or vaccines currently available.[9-11] These viruses share other important features which make them high priority: they are all associated with bat hosts that are abundant and widely distributed throughout Africa and Asia, living in close association with people and livestock in some of the most densely populated regions on Earth.

Spillover of these viruses has repeatedly occurred. Beyond the massive outbreak in West Africa in 2013-16, EBOV outbreaks have occurred repeatedly in Central and East Africa; most recently Sudan ebolavirus caused an outbreak in Uganda in late 2022[11-13]. Marburg virus (MARV) and Ravn virus (RAVV) comprise the closely related genus *Marburgvirus* and have caused outbreaks in Central and East Africa and Europe with mortality and clinical presentation comparable to Ebola [14,15]. Marburg virus outbreaks were declared in Guinea, just across the border from the UNC-Liberia research site in 2021 and then again in Ghana in 2022 marking the first time this virus has been reported in either country[16,17]. Nipah virus is an emerging zoonotic paramyxovirus that has caused nearly annual outbreaks in Bangladesh, including multiple outbreaks in 2023, and sporadically in India with mean case fatality rates greater than 70% [12,18-20]. Despite the public health significance of Nipah and related viruses, little is known about its genetic diversity, natural reservoirs, or frequency of spillover throughout their range, including Africa. Henipaviruses, and Nipah virus in particular - have several characteristics that make them a global health priority: 1) Their bat reservoirs occur throughout Asia and Africa, overlapping human and livestock populations, giving them geographically broad opportunity to cause outbreaks [21]; 2) henipaviruses can be transmitted to people directly from bats or via domestic animals [21]; 3) Nipah virus can be transmitted from person to person [22]; 4) Nipah virus spillover has occurred and continues to occur in highly populous and internationally connected regions; 5) repeated spillovers of NIV strains with varying person-to-person transmission rates indicate the ability to evolve **with increased pandemic potential** [23,24]; and 6) Nipah is associated with a high mortality rate in people and currently has no vaccine or treatment [21,23]. While Nipah virus has not been detected in Africa, closely related henipaviruses have been identified in Ghana and Madagascar, though their public health significance remains unknown[25-27]. In West Africa, where bats and associated henipaviruses occur but where no human cases have been identified, **diagnostic testing and systematic surveillance is essentially non-existent**. The detection of antibodies against a Nipah-related virus in hunting communities in Central Africa suggests that cryptic spillover is occuring[28]. **Repeated spillover of henipaviruses creates opportunity for more transmissible strains to emerge[29].**

While bats have been identified as potential hosts for filoviruses including EBOV, the natural reservoir for EBOV-Zaire Ebola in West Africa remains unknown. There is substantial evidence, both serological and molecular, that bat species host filoviruses; however, a definitive natural reservoir or set of reservoirs for EBOV-Zaire has not been identified[30]. Wildlife, including bats, represent a substantial source of animal protein for communities in Liberia[31]. People may be exposed to bat-borne viruses through hunting and butchering, eating food contaminated by bat excreta, or by entering caves or mines inhabited by bats. Our group previously found that people living in rural areas of Liberia conduct activities which may increase their risk of contact with bats or their bodily fluids. The index case, a child, in the 2013 Ebola outbreak was hypothesized to have had contact with bats, yet the exact species was never definitely identified[32]. The route of spillover for most historical Ebola outbreaks remains a mystery. Despite broad efforts to determine the natural reservoirs of EBOV and related viruses, there continues to be a poor understanding of filovirus ecology, with the exception of Marburg virus, whose host has been identified as Egyptian rousette bats (*Rousettus aegyptiacus*)[33-35]. Communities known to hunt bats in Cameroon and India have been found to have antibodies against henipaviruses and filoviruses, respectively, yet nothing is known about community exposure rates in Liberia, where bat hunting is common[28,36]. Our preliminary data (see below) suggest that there is substantial exposure to EBOV as well as MARV among people in central Liberia. While the EBOV antibodies may indicate prior infection from another person such as during the West Africa outbreak, antibodies specific to other filoviruses may signal a localized exposure from animals. Even less is understood about henipaviruses in Africa, and identifying antibodies against henipaviruses in people will be critical for understanding where, how and how often spillover occurs.

The urgent need to develop interventions to prevent spillover of zoonotic high consequence pathogens can only be met with a better understanding of their natural reservoirs, the rates of exposure in vulnerable human populations, and the ways in which people are exposed. Unrecognized outbreaks provide opportunities for viruses to emerge that may be more transmissible or more pathogenic than previously observed, and limiting these opportunities is an important component of pandemic prevention[2,29]. Typically, by the time an outbreak is recognized in human populations its containment and control are difficult, if not impossible to achieve (e.g., HIV, SARS-CoV-2). Understanding the ecology of these viruses, including which species act as reservoirs as well as how and to what extent people are being exposed to filoviruses and henipaviruses will inform intervention and surveillance strategies and provide baseline metrics against which one can measure the efficacy of interventions designed to reduce exposure, such as public health campaigns to promote avoidance of behaviors associated with infection among those most at risk (**Figure 2**).

Figure 2



This proposed study will be the largest and most rigorous One Health investigation of high-consequence zoonotic viruses in bats and humans in an emerging disease hotspot. West Africa is known to be the source of prior outbreaks of emerging infectious diseases and future spillover events there are highly likely. Liberia is typical of this region and contains some of the largest tracts of pristine forest and wildlife biodiversity remaining in West Africa and has a population that depends on wildlife, including bats. Decades ago, a spillover event from a non-human primate to a human triggered an on-going HIV pandemic that has led to the infection of over 60 million people and 25 million deaths. Only months ago, the orthopoxvirus MPOX found in rodents spread to and within 110 countries across the globe causing over 85,000 cases. Both originated from African wildlife. The proposed study will leverage the unique infrastructure and expertise we have developed in Liberia to undertake a comprehensive examination of filovirus and henipavirus spillover from bats to humans and provide evidence of previous and possibly on-going transmission events. Our findings will provide essential evidence of the presence and transmission dynamics of these bat-borne viruses and as the risks for infection in people living alongside them.

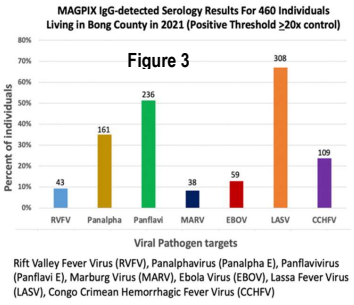
B. INNOVATION

The proposed research is highly innovative in multiple ways, including: 1) Our use of a one Health approach to study the ecology and epidemiology of two groups of high-priority, high-consequence zoonotic viruses in both bats and people in West Africa – an emerging disease hotspot where spillover events have occurred (e.g. Ebola, Marburg, MPOX, Lassa fever) and are highly likely to re-occur; 2) Our use of a novel and unique multiplexed serological platform, developed by our group, that will allow us to screen bat and human serum for IgG antibodies against each of the known filoviruses and henipaviruses, simultaneously in a single assay. Longitudinal comprehensive serological studies in bats and people **will provide unprecedented insight into the changes in rates of exposure over time to known viruses in bats; and in people (e.g. what is the baseline rate of exposure to henipaviruses and filoviruses and is there evidence of recent exposure?); and exposure rates to unknown, antigenically related viruses;** 3) We will couple serosurveillance with molecular testing to identify specific filoviruses and henipaviruses carried by bats; and 4) We will connect the bat and human epidemiological studies by analyzing questionnaire data from the human cohort to identifying high risk behaviors associated with viral exposure. **Results from this analysis will inform the development of public outreach and risk reduction campaigns by the Government of Liberia.**

C. APPROACH

C.1. Overview: **Aim 1** will test the hypothesis that spillover of filoviruses and henipaviruses occur regularly in rural Liberia by testing for IgG antibodies to all known filoviruses and (see table 3) in archived serum samples from 900 people living in Bong county (central Liberia), collected at two time points 24 months apart. Seroprevalence of filoviruses and henipaviruses will be assessed as will changes between the two collection timepoints. In **Aim 2,** we will conduct a 36-month longitudinal study of three bat species which are putative reservoirs for EBOV and undescribed henipaviruses. Using a combination of longitudinal serology and molecular testing (PCR and whole genome sequencing) **we will determine the prevalence of filoviruses and henipaviruses in three key bats species associated with the rural human populations studied in Aims 1 and 3**. For **Aim 3,** we will analyze detailed questionnaire data developed collaboratively by our group (EHA and UNC) and collected in 2023 along with blood samples to be screened in Aim 1. These data will allow us to identify behaviors associated with exposure to henipaviruses and filoviruses. We will use results from our behavioral risk assessment to develop public risk reduction communication strategies with the National Public Health Institute, Liberia, using the results of this study to inform public health strategies. This application is a natural progression of our research activities, and our preliminary data speak to the feasibility of the proposed study.

C.2. Aim 1: To determine the rates of filovirus and henipavirus exposure in people in central rural Liberia. C.2.1. *Rationale and Preliminary Data.* The primary goal of this aim is to be able to determine whether populations within Liberia who live in association with bats have evidence of exposure against one or multiple filoviruses and/or henipaviruses and to compare rates of exposure over time. Studies of human populations in Central and West Africa have found evidence of exposure to EBOV prior to the 2013 outbreak and in places not known to have had Ebola outbreaks[37,,38]. UNC Project-Liberia has previously assessed seropositivity to consequential viral pathogens including EBOV and MARV in a subset of CEPI ENABLE Study participants. About 10% of participants had antibodies against MARV (**Figure 3**), suggesting that unreported spillover events have previously occurred. Acute encephalitis is common in western sub-Sharan Africa, yet outbreaks of neurological disease are rarely reported and more than half of all cases are undiagnosed[39]. Among the countries in western Africa reporting cases of meningitis or encephalitis, Liberia has reported the fewest, likely due to a lack of surveillance



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and capacity to diagnose neurological diseases[39]. Nipah virus encephalitis has never been reported in Africa, however, as described above, two Nipah-like henipaviruses have been described in bat hosts, including one in Ghana[25,27]. Spillover of henipaviruses was reported in bat-hunting communities in Cameroon[28]. In South Africa, we detected henipavirus sequences in *Rousettus aegyptiacus,* bats which also commonly occur in Liberia[26].

In Aim 1, we will test the hypothesis that spillover of both filoviruses and henipaviruses have occurred in rural populations in Liberia by measuring seropositivity to both groups of viruses within the CEPI ENABLE Study community cohort. We will also measure changes in exposure rates over a two-year period. While prior studies have provided cross-sectional snapshots of exposure, our longitudinal approach will provide data from the same cohort at two timepoints, 24 months apart, to determine both seroprevalence and sero-incidence using a multiplexed serological assay to screen for IgG antibodies against all known filoviruses and henipaviruses.

Settings and Participants. To achieve this aim we will leverage a well characterized cohort of individuals in three rural communities located in Bong County, Liberia (**Figure 4**), which has a population of over 333,400. UNC Project-Liberia has been operating in Liberia since 2014 and established a research site at Phebe Hospital in Bong County that includes a molecular laboratory that conducts daily qPCR testing for LASV, MARV, and EBOV as well as LASV serological assays using a MAGPIX (SEE RESOURCES AND FACILITIES). There, following extensive community engagement including meetings with key stakeholders, town hall meetings, and radio messaging, UNC launched the CEPI ENABLE Study, the largest passive and active surveillance program for Lassa fever in Liberia. Started in 2020, 5,005 randomly selected participants 2 years of age and older in Phebe Airstrip, Suakoko, and Rubber Factory were enrolled over 6 months following informed consent/assent (mean age at enrollment is 22.2 years (range 2-97) and 54.2% are female). **Importantly, 7.7% of the cohort is under the age of 5 years, and therefore, were born after the 2013-14 Ebola outbreak (Table 1).**

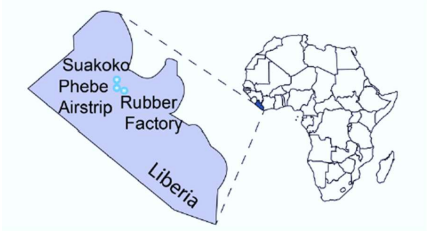

Figure 4. Map of Liberia with Bong County sites shown in inset.

Households were selected for recruitment following identification of roof tops by satellite photos and use of a program developed by Médecins Sans Frontières' (MSF) Epicentre to randomly selected structures to consecutively approach. The ENABLE Study includes baseline questionnaires and blood specimen collection from all participants with active follow-up by a study community health worker every 2 weeks to determine is a febrile event has occurred, which triggers drawing of blood for LASV, MARV, and EBOV PCR. A subset of 1,024 individuals is also asked to provide blood samples routinely every 6 months for up to 24 months. The blood samples from 0 and 24 months and the associated surveys will be used in **Aims 1 and 3** of this proposal. Informed consent and assent documents include provisions for the use of collected specimens and data for infectious diseases research including for emerging pathogens (see PROTECTION OF HUMAN SUBJECTS).

C.2.2. Research Design. We will screen approximately **1,900 archived serum specimens** from participants in the ENABLE Study cohort at two time points ("baseline" (Month 0) and (Month 24)) for IgG antibodies against specific filoviruses and henipaviruses using the MAGPIX at the UNC molecular lab at Phebe hospital to run the multiplexed *henipavirus / filovirus* platform to be provided by Co-I Laing in year 1. The multiplex platform will allow us to determine specifically which filoviruses and henipaviruses people have been exposed to and compare that to the filoviruses and antibodies we observe circulating in local bat populations in **Aim 2**. The bead-based multiplexed assay has tremendous advantage over traditional ELISAs in that it will identify up to 25 different antibodies against multiple viral agents in a single, 2uL serum sample and uses a high through-put format. The ability to look at reactivity across all serogroups simultaneously will allow us to differentiate among the viruses. Proteins A/G non-specifically bind IgG immunoglobulins, and weakly bind IgM, an immunoglobulin that is the first to be produced during the initial stages of infection. We will conduct a 1-week training at the UNC-Phebe lab for using the assay to detect filovirus and henipavirus antibodies as well as data management, bioinformatics and analysis. **Table 3** shows the viral species and antigens that will be employed in this assay. The platform has been previously transferred to collaborating labs in the UK, South

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Africa, India, Bangladesh, Singapore, Thailand and Malaysia. We have previously detected serological profiles of Asiatic ebolaviruses in fruit bats collected in Singapore[40] and in fruits bats and humans actively engaged in bat hunting in northeast India[36].

Table 3. Multiplexed microsphere immune assay including soluble glycoproteins from each known filovirus and henipavirus

Virus (Name/Isolate Host/Country/Year/Strain)	Abbreviation	Accession No.	Soluble Glycoprotein	Mag Bead No.
Ebolaviruses				
Ebola virus/H. sapiens/COD/1976/Yambuku-Mayina	EBOV	NC_002549.1	$GP_{(1,2)}$	34
Bundibugyo virus/H. sapiens/UGA/2007	BDBV	FJ217161.1	$GP_{(1,2)}$	64
Bombali ebolavirus/Mops condylurus/SLE/201	BOMV	NC_039345	$GP_{(1,2)}$	55
Taï Forest virus/H. sapiens/COV/1994/Pauleoula-CI	TAFV	NC_014372	$GP_{(1,2)}$	57
Sudan virus/H. sapiens/UGA/2000/Gulu-808892	SUDV	NC_006432.1	$GP_{(1,2)}$	54
Reston virus/M. fascicularis/USA/1989/Pennsylvania	RESTVm	AF522874.1	$GP_{(1,2)}$	65
Marburgviruses				
Marburg virus/H. sapiens/KEN/1980/Musoke	MARV	Z12132 S55429	$GP_{(1,2)}$	37
Ravn virus/H. sapiens/AGO/2005/Ang0126	RAVV	NC_024781.1	$GP_{(1,2)}$	62
Dianlovirus				
Mengla virus/Rousettus-WT/CHN/2015/Sharen	MLAV	KX371887.2	$sGP_{(1,2)}$	22
Cuevavirus				
86	LLOV	NC_016144.1	$sGP_{(1,2)}$	66
Henipaviruses				
Hendra virus/E. caballus/AUS/1994	HeV	NC_001906.3	sG	43
Hendra virus-2G	HeV-2g		sG	48
Nipah virus/H. sapiens/MYS/2000	NiVm	NC_002728.1	sG	46
Nipah virus/H. sapiens/BGD/2004	NiVb	AAY43916.1	sG	56
Cedar virus/Pteropus sp./AUS/2012/CG1a	CedV	NC_025351.1	sG	53
Mojiang virus/R. sladeni/CHN/2014/Tongguan1	MojV	NC_025352.1	sG	29
Ghanaian bat virus/E. helvum/GHA/2009/GH-M47a	GhV	NC_025256.1	sG	35
Angavokely virus	AngV		sG	47

C.2.3. Data Analysis. We will create a project database using Airtable, which will be hosted at EHA and will house all data from Aims 1 and 2 and will link to the RedCap server which stores questionnaire data from Aim 3. Database access will be secure and limited to key personnel (see data management plan). Raw MFI values from the MAGPIX will be used to calculate reactivity to each viral antigen and based on relative strength of reactivity (**Fig 5**) and determinations of negative cutoffs based on reference sera, we will calculate prevalence rates for each time point and compare rates between the two timepoints. Serological profiling can be used to detect reactive antibodies against all specific filoviruses and henipaviruses in a samples and compare strength of reactivity and cross-reactivity against multiple antigens using a single sample[41]. We will compare the antibodies across all specific viral antigens to assess which virus or groups of viruses are responsible for the immunological response[42-44]. We will assess exposure to non-Zaire ebolavirus species, which may indicate spillover of filoviruses has occurred separate to the Ebola outbreak of 2013. We will also look at evidence for exposure to Nipah-like henipaviruses and compare exposure rates to both viral groups within and between our two sample collection time points.

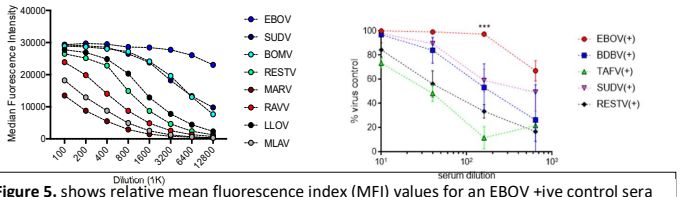


Figure 5. shows relative mean fluorescence index (MFI) values for an EBOV +ive control sera across serial dilutions. While there is cross reactivity, the EBOV MFI values are consistently greater than the other viral GPs and at higher dilutions the separation becomes significant.

C.2.4. Expected outcomes. We anticipate obtaining serological profiles of exposure to filoviruses and henipaviruses from each individual in our cohort. In previous studies we found antibodies against both Ebola and Marburg virus. While the Ebola antibodies are attributable to the epidemic, Marburg antibodies, in

patients EBOV IgG negative, suggest possible exposure from an animal (e.g. bat) reservoir. We also anticipate seeing reactivity to to Bombali virus (BOMV), given the presence of the reservoir species in Liberia (*Mops condylurus*) and reactivity to the African henipaviruses (GhV and AngV), although this may represent reactivity to an antigenically related virus. Serological results from this and the bat study will inform our strategy for prioritizing which bat samples we test by PCR.

*C.2.5. Potential problems and solutions***.** While our preliminary data suggest there will be detection of immune responses to filoviruses including EBOV-Zaire and, significantly, MARV, it is possible that evidence of exposure to other pathogens, including henipaviruses will not be detected. This would be an important finding in this large cohort, especially if these viruses are detected in local bat species, that would warrant further investigation. It is also possible that there will be few or no seroconversion events among the cohort during the 24-month period of follow-up. This too would be reassuring but would suggest a need for longer follow-up of this cohort. To address the possibility that reactivity to EBOV-Zaire virus may be explained by infection during the 2013 Ebola epidemic, we will create a polyclonal sera reference standard utilizing convalescent sera collected from human survivors of EVD. This internal reference standard will be calibrated to the WHO EBOV human serology standard (**Fig 6A**) permitting direct interpolation of anti-EBOV IgG as a MFI to a standard antibody concentration (binding antibody units/mL). The internal reference standard will then be included in our human serology testing strategy allowing semi- or quantitative measurement of anti-EBOV IgG. These EBOV human serology standards will permit us to establish positive and negative predictive performance values for the multiplex assay, furthermore, we will be able to investigate immunoreactivity of EVD survivors against heterotypic ebolaviruses. Our preliminary data indicates that EBOV+ sera IgG is highly cross-reactive with soluble native-like trimeric envelope glycoprotein (GP) antigens from both EBOV and BDBV (Figure 6A). EBOV GP, matrix protein (VP40), and nucleocapsid protein (NP) antigens have all been used in a variety of serological tests, however, the frequency of seroconversion against each antigen has not been fully described. In a longitudinal study of EVD survivors, high seroconversion against GP and VP40 was observed, with affinity maturation occurring overtime against GP[45]. Antigens based on the GP are likely to provide an optimal intrinsic balance of sensitivity and specificity. To limit the degree of heterotypic ebolavirus cross reactions we will test any anti-ebolavirus GP trimer antigen-seropositive samples with a pan-ebolavirus GP1 antigen-based panel. In preliminary studies, GP1 antigens improve specificity and differentiation of antibodies reactive with homotypic ebolaviruses in polyclonal antisera from rabbits immunized with GP trimer immunogens (**Figure 6B**).

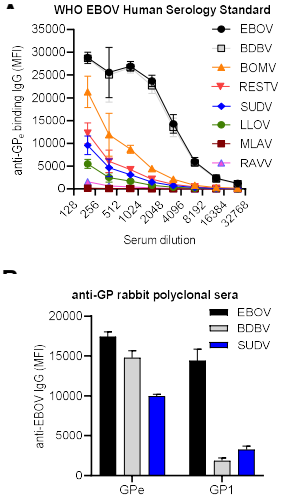


FIGURE 6A & B. A) The WHO EBOV human serology standard was testing for immunoreactivity in a filovirid GP-trimer based multiplex assay. Filovirids are indicated along with sera dilution factors; data is the mean of three independent experiments. B) Antisera from EBOV GP-trimer immunized rabbits were tested in multiplex panels comprised of GP-trimer and GP1 subunit proteins. Anti-EBOV polyclonal IgG was less reactive with GP1 from BDBV and SUDV.

C.3. Aim 2. To identify reservoirs and characterize the infection dynamics of filoviruses and henipaviruses in key bat species in Liberia. C3.1 *Rationale and preliminary data.* Bats have been established as important hosts for filoviruses and henipaviruses in Africa and Asia, with combinations of viral isolation, molecular detection or serology providing substantial evidence [30,46-49]. MARV was isolated from Egyptian rousette bats in Uganda, and RNA has since been detected in these bats in Sierra Leone, which borders Liberia [50]. To date, EBOV has never been isolated from bats, although molecular and serological evidence suggest that multiple frugivorous bats may carry EBOV in Central Africa[50,51]. Prior to the 2014 epidemic, Zaire ebolavirus had not been reported in West Africa, and the animal reservoir remains unknown[32]. From 2016-2019, our group (PI Epstein & KP Desmond) implemented a large-scale project to identify reservoirs for EBOV in West Africa, under the USAID PREDICT Project[52]. This effort led to the discovery of a new species of Ebola (Bombali virus)[53], the detection of MARV in ERBs in Sierra Leone[33], and **in 2018, we detected, for the first time in West Africa, EBOV RNA and anti-EBOV IgG antibodies in bats (Figure 7A-D)**. While this finding generated the first evidence of which bats

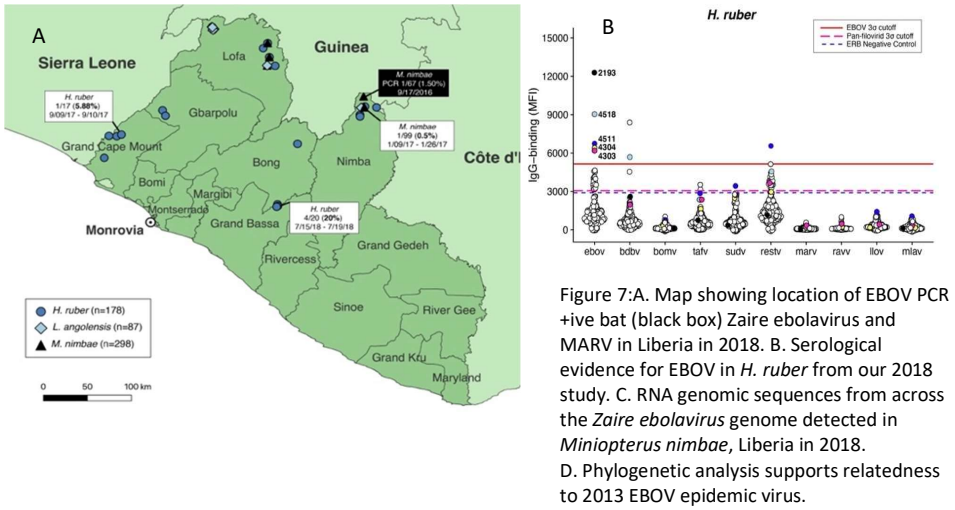
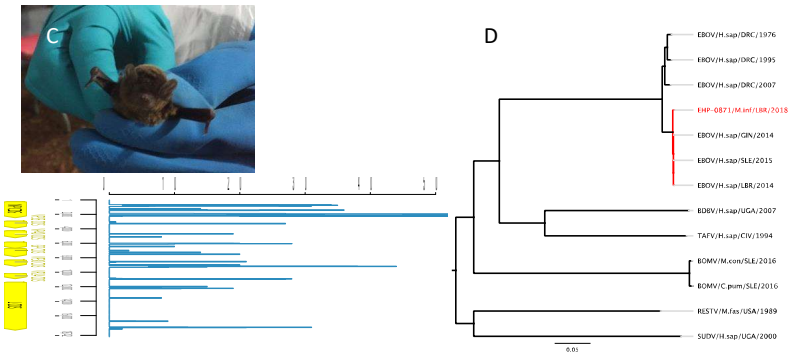


Figure 7:A. Map showing location of EBOV PCR +ive bat (black box) Zaire ebolavirus and MARV in Liberia in 2018. B. Serological evidence for EBOV in *H. ruber* from our 2018 study. C. RNA genomic sequences from across the *Zaire ebolavirus* genome detected in *Miniopterus nimbae*, Liberia in 2018. D. Phylogenetic analysis supports relatedness to 2013 EBOV epidemic virus.



may be reservoirs for Zaire ebolavirus, longitudinal studies are required to more completely understand the role these species play in EBOV circulation – either as reservoirs that maintain the virus within a local population, or as incidental hosts, infected by an unknown bat species. One -off, cross-sectional studies have a

low probability of detecting any specific filovirus or henipavirus given their low prevalence and short viremic periods [34,54], and thus provide a poor characterization of viral diversity. The lack of understanding of **filovirus and henipavirus diversity and their natural reservoirs in West Africa significantly impedes risk-based public health interventions to prevent human outbreaks.** Longitudinal sampling is needed to separate reservoir hosts from spillover hosts. For example, MARV has been isolated repeatedly from *R. aegyptiacus* in Uganda, which, when paired with experimental infection studies showing infe ction and shedding of MARV by *R. aegyptiacus,* strongly suggests that *R. aegyptiacus* is indeed a Marburg virus reservoir[35,55,56]. Similarly, our longitudinal studies of Nipah virus in *Pteropus medius* bats in Bangladesh, which included the same multiplexed serological assay proposed here, confirmed them as a reservoir for NIV and identified important mechanisms driving viral circulation[29]. **Understanding which species persistently carry filoviruses and henipaviruses will provide important insights into how they are maintained in nature and what species should be targeted for follow-up studies on bat habitat use and human-bat contact patterns.**

3.2. *Sampling design*. We will repeatedly sample three bat species associated with filoviruses or henipaviruses, based on our preliminary data: *M. nimbae* (EBOV), *H. ruber* (EBOV), and *R. aegyptiacus* (MARV & Henipaviruses). We will sample bats at roost sites in Bong county, as close as possible to the human study sites in Aim 1. In Y2-Y4, we will sample 40 individuals of each species every two months for 36 months and collect excreta using plastic tarps placed underneath bat roosts (details below and in C3.3).

Bat capture and sampling. Briefly, we will use either harp traps or mist nets to capture bats, depending on the capture site. Personnel will wear appropriate personal protective equipment (e.g. Tyvek suits or dedicated long clothing; double nitrile gloves; an N95 respirator and safety glasses or a PAPR [57]. Traps and nets will be attended continuously during capture periods. Bats will be removed from mist nets immediately upon entanglement to avoid injury. Bats will be removed from harp traps after approximately 1 hour and bats will be placed in individual cloth bags until processed. Body measurements, blood, oropharyngeal swabs, and rectal swabs (or fecal pellets) will be collected from each bat. Swabs will be collected in duplicate; one swab will be placed in a cryovial containing Trizol lysis buffer and the other in viral transport medium (VTM). For bats <50g, blood will be collected in a microhematocrit tube and spun down using a portable centrifuge. A bulb will be used to expel serum from the tube into a cryovial. For larger bats >50g, a tuberculin syringe or 1ml syringe and 27g needle will be used to draw blood from the radial artery or vein[57]. Blood will be placed into a conical vial and centrifuged. Serum will be aliquoted into a cryovial using a pipette. During site visits we will also lay out plastic tarps to collect pooled environmental excreta in Trizol and VTM to improve opportunity to detect viral RNA. All samples will be placed in a vapor phase liquid nitrogen dry shipper or portable battery-powered ultracold freezer, immediately upon collection and then transferred to a -80C freezer at the Phebe lab until testing.

C3.3. *Testing plan, Data analysis, sample size, and power analysis*. *Serology: At the end of Y3,* we will screen all serum samples collected to date using the MAGPIX multiplex assay for IgG against filoviruses and henipaviruses. We will analyze temporal dynamics in seroprevalence using general additive models (GAMs), which are analyses that we have used successfully in many previous studies of similar design[29]. These methods are well adapted for capturing long-term temporal fluctuations in prevalence and moderately sensitive at capturing short-term changes in seroprevalence which can be used to identify population-level disease outbreaks. For example, we estimated that with 40 bats sampled every two months, we would be able to statistically detect an increase in seroprevalence (at a 95% CI limit) of greater than 25% between consecutive sampling events > 80% of the time, assuming a bat population seroprevalence of at least 10% in advance of a bat colony infection (estimated by, in brief, by simulating a fluctuating true seroprevalence value, simulating samples given this underlying seroprevalence, fitting a GAM to these samples, and then calculating if CI on true prevalence between the two sampling occasions over which the change in seroprevalence occurred were non-overlapping). While this is a large change, it is within a range to be expected if infection were to sweep through a bat community[29]. MAGPIX-positive bat sera will be sent to RML for serum neutralization assays using viral isolates or VSV pseudoviruses under BSL4 conditions.

VSV-pseudotype neutralization assays: Serological results will be confirmed using VSV-pseudotype neutralization assays for the following filoviruses EBOV, SUDV, BDBV, RETV, BOMV, MARV and RAVN. If novel

henipavirus sequences are detected, additional assays will be developed to test positive sera. Serum samples will be inactivated using γ-irradiation (4 MRad). Neutralization of irradiated and heat-inactivated serum samples will be assessed in Vero 352 E6 cells. Briefly, cells were seeded in 96-well round-bottom plates for 24 hours, serial dilutions of heat-inactivated serum samples will be performed in DMEM supplemented with 2% FBS, penicillin/streptomycin, and L-glutamine. Each plate will contain negative serum control, cell-only control, and virus-only control. VSV EBOV-GFP will be added to each well of the serum dilution plate and the serum-virus mix 357 was incubated at 37˚C for 1-hour. The mix was added to the cells and incubated at 37˚C for 24 hours. The cells will then fixed with 4% paraformaldehyde at room temperature 359 for 15-minutes and centrifuged at 600 x g for 5-minutes at room temperature. The supernatant will be discarded and FACS+EDTA buffer was added. Samples will be run on the FACSymphony A5 Cell Analyzer (BD Biosciences, Mississauga, ON, Canada) and FITC MFI 362 was measured. Data will be analyzed using FlowJo.

Molecular testing: We will use specific filovirus taqman real-time PCR assays to screen oropharyngeal, fecal and environmental samples from bats for EBOV and MARV RNA at the UNC-Phebe lab[55,58]. Testing bat samples by PCR may allow us to detect viral RNA during active infection, providing important information about the prevalence and timing of infection within target species. **Because we expect viral prevalence to be significantly lower than seroprevalence, estimated at <5%[25,27,49],** we developed a sampling scheme focused on maximizing our probability of detecting viral RNA with qPCR. To compare power under alternative sampling designs, we built simulation-based power analyses that estimated power to detect: 1) viral EBOV or MARV RNA in at least one bat; and 2) the expected number of positive cases, in each bat species. In brief, we estimated power for total sample sizes between 1500-3000 (between 500 and 1000 bats sampled per species) spread across a variable number of visits per year (2-6), while allowing for uncertainty in true infection dynamics in the bat populations, specifically: 1) prevalence during one three-week period (with unknown timing) per year of high prevalence ranging from 1-5%); and 2) an otherwise low background prevalence (0-1%). Given these estimates of yearly infection dynamics, these analyses showed with 672 samples per species (minimum 32 individual bats per sampling event), spread across 6 sampling occasions per year, we would obtain a per-species probability of acquiring 0 positive samples of <4% (<1% at 40 bats per event – our target number) and an expectation of ~3-6 RNA positive results (~5-10 positives at 40 bats per event). Because henipaviruses have not been previously described in Liberia, we will focus our resources on filovirus testing by PCR. However, if henipavirus serology suggests active circulation, we may send a set of samples to RML for pathogen discovery (see RML letter of support).

Viral isolation: PCR-positive cDNA samples and their aliquot in VTM will be sent to RML for further sequencing and attempts at viral isolation under BSL4 conditions. If attempts to culture do not work, we will use next generation sequencing techniques to get whole genome sequence and further characterize the virus at RML. Novel viruses will be characterized and rescued (reverse engineered using a minigenome system to synthetically create infectious viral particles [59]) at RML under secure, biosafety level 4 conditions, then used for serum neutralization assays in bat and human sera to determine more precise exposure rates. Sequences from the glycoproteins of any novel viruses discovered will be used by USU (Co-I Laing) to generate purified proteins and added to the Luminex assay, which will improve its specificity.

VirCapSeq-VERT and myBaits probe-based enrichment for recovery of full-length genome sequences of filoviruses: Although shotgun sequencing provides opportunities to analyze all host and virus genetic material, the larger average genome size of bacteria and hosts compared to viral genomes complicates a detailed analysis of the virome, particularly vertebrate viruses. We have established a positive selection probe capture-based system to enrich for viral sequences. Here, we will take advantage of these probe capture-based methods (VirCapSeq-VERT and myBaits probe library) to enrich for vertebrate viral sequences of interest. Sample libraries will be normalized and combined in 4- to 12-plex reactions for solution capture hybridization using either the HyperExplore custom bait library version of VirCapSeq-VERT probe set or our custom myBaits probe library. For HyperExplore custom bait library probe set, libraires will be enriched for virus following the

SeqCap EZ HyperCap Workflow User's Guide, version 2.3, while for custom myBaits probe library, the myBaits Hybridization Capture for Targeted NGS protocol, Version 4.01 will be used. Sequencing libraries will be normalized and sequenced as 2 X 150 bp fragments on Illumina's MiSeq or NextSeq sequencing platforms, following Illumina's standard procedure (Illumina, San Diego, CA). NGS data will be analyzed using metavirs (https://openomics.github.io/metavirs/), a comprehensive viral metagenomics pipeline to assemble, annotate, and classify viruses. It relies on technologies like Singularity1 to maintain the highest-level of reproducibility. The pipeline consists of a series of data processing and quality-control steps orchestrated by Snakemake2, a flexible and scalable workflow management system, to submit jobs to a cluster. The pipeline is compatible with data generated from Illumina short-read sequencing technologies. As input, it accepts a set of FastQ files and will be run on-premises using the NIAID RML Big Sky supercomputing cluster.

C3.5. *Expected outcomes.* Based on our prior work, our multifaceted analysis approach, and calculated power given our proposed temporal sampling strategy, we expect to be able to use temporal serological data to describe filovirus and henipavirus viral dynamics over time. Our molecular screening will primarily focus on filoviruses because we have known targets, and we expect to detect EBOV or MARV RNA, despite low prevalence and short-lived infections. Sequence data from positive samples will 1) provide substantial evidence for the identification of EBOV reservoirs and help explain which virus is responsible for the observed IgG response in bats (and possibly people); and 2) provide valuable information about viral diversity, about which little is known. Henipavirus serology, in the absence of sequence data, will allow us to identify potential bat reservoirs, analyze temporal trends, and generate hypotheses for future targeted studies.

C3.6. *Potential problems and solutions*

Serological tests are greatly hindered by the inherent antibody cross-reactivity between antigens from known ebolaviruses, challenging data interpretations that antibodies are EBOV-specific or EBOV, BDBV, and SUDV cross-reactive [40]. This is also true for the henipaviruses in the panel. The advantage of the multiplexed platform is that we can simultaneously see relative MFI values for all the filoviruses and henipaviruses. Glycoproteins from the homologous virus should cause the most intense reaction, particularly at higher dilutions. Co-Is Laing and Munster have demonstrated that homotypic and heterotypic ebolavirus-antibody reactions can be simultaneously measured and accounted for in ERBs challenged with replication-competent VSV-filoGP (**Figure 8**). Still, there is some cross reactivity, and we will use additional tools such as viral neutralization assays as a secondary test, and our statistical approach can correct for a portion of cross reactivity. Past studies have relied on individual ELISAs which had varying degrees of specificity depending on which test was used, but often couldn't rule out cross-reactivity. The Luminex filovirus multiplex assay has predictable patterns of cross-reactivity, but we will still be able to discern to which virus the serum is most reactive.

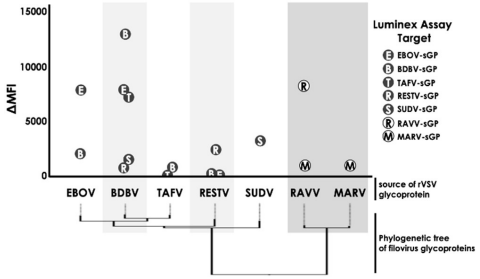


Figure 8. Captive-bred ERBs were experimentally challenged with VSV-filoGPs, the GP source is indicated on the x-axis. In a GP-trimer multiplex test the homotypic ebolavirus bat antisera were most reactive with the homotypic ebolavirus GP-trimer antigen.

C4. Aim 3: To characterize behaviors associated with exposure to filoviruses and henipaviruses. *C4.1 Background and rationale.* As described above, there is substantial interaction between wildlife, domestic animals, and people living in rural Liberia. Our group collected questionnaire data from 585 people across Liberia as part of the USAID-funded Ebola Host Project between 2016 and 2019. When asked about animal exposures during the 2013-2016 Ebola outbreak, participants frequently reported contact with both domestic

and wild animals. Common types of animal contact included animals coming inside the dwelling (58%), cooking or handling animal meat (44%), and eating raw or undercooked meat (31%). Additionally, 26% reported slaughtering and 12% reported hunting or trapping an animal. In the ENABLE Study of 5,005 residents of Bong County, survey questions mainly focused on rodent exposures relevant to Lassa fever, but 12% of participants reported hunting or preparing wild animal meat. Little is known about the prevalence and type of other potential bat exposures in this region of Liberia. Elsewhere in West Africa, EcoHealth Alliance participated in the response to Ghana's 2022 cluster of MARV cases, working with local partners to administer a behavioral risk questionnaire characterizing bat exposures in three affected communities[16]. Many participants lived in close proximity to bats, with one fifth reported exposure to bats inside a dwelling or other building in the previous four months. When asked about exposures more relevant to Egyptian Rousette bats, a known MARV reservoir and a target species in this proposed study, participants commonly reported bats feeding on fruit trees on their home compound, eating fruit bearing bite marks, and exposure to bats inside a cave or mine.

C4.2. *Research design*. As described in Aim 1, serum collected at baseline (in 2021) and at month 24 (in 2023) during the ENABLE Study will be screened for filoviruses and henipaviruses, enabling us to calculate seroprevalence at baseline and seroconversion over a two-year period. In addition to providing blood specimens, the 1,024 participants who initially enrolled responded to baseline questionnaires on demographics, healthcare-seeking behaviors, health status, and animal exposures. At month 24, the 921 participants still actively enrolled are administered repeat questionnaires. This final survey includes additional items based on the Ghanaian questionnaire and asks specifically about bats including the respondents' activities in their habitats (entering forests, caves, and mines), direct contact (hunting, eating handling), indirect contact (presence in home or other used structure), and observed characteristics of bats encountered (fruit tree dwelling). By pairing this existing survey data with the new serology results generated from archived samples under Aim 1, as well as new data on nearby bat infection from Aim 2, we will be able to develop comprehensive risk profiles for exposure to filoviruses and henipaviruses in this community.

C4.3 *Data analysis.* The primary outcome for this analysis will be testing positive for IgG antibodies against any known filoviruses or henipaviruses. For both families of viruses, and for each species of virus within those families, we will use the serology results generated under Aim 1 to examine risk factors for seropositivity at baseline and seroconversion between baseline and month 24. We plan to conduct a multivariate logistic regression, with appropriate weights applied to account for the single-stage cluster sampling design of the ENABLE Study, in which members of randomly selected households were surveyed and tested. We will use data from both the baseline and month 24 questionnaires to examine the effects of sampling site, participant demographics (e.g., age, gender, occupation), environmental exposures (e.g. animals present in and around the dwelling), and behavioral risks (e.g. direct contact with bats through hunting, preparing, or eating them, entering caves or mines where bats live).

Power analysis: To assess our power to identify risk metrics for seroconversion (or overall seropositivity), we built simulation-based power analyses that consisted of, in brief: 1) simulating survey responses for N (400-1000) individuals, while allowing for moderate unevenness in representation across five binary categories (as great as 80%-20% for each); 2) examining a range of effect sizes (odds ratios ranging from 1-2.5) for these five binary categorical predictors (e.g., sex, encountered a bat, handled a bat, etc.). Summarizing across 1,200 simulations of surveys of 400-1000 individuals given joint uncertainty in the representation within these surveys and effect sizes, we estimated that we would have at least 80% power to detect significant effects for each predictor given an odds ratio of 1.65 or greater with 1,000 survey responses or ~1.80 (for example, with a rate just under 5% for Group1 = 0, just over 8% for Group1 = 1) with 750 responses.

C4.4 *Expected outcomes.* By combining serology results with detailed questionnaire data on participant demographics and animal exposures, we will be able to use odds ratios to identify behaviors significantly associated with seropositivity (e.g. exposure to filoviruses or henipaviruses). We expect to be able to also

identify age and gender-based risk factors given our sample size. Based on previous experience conducting behavioral risk surveys, including a recent study in Ghana following a Marburg outbreak, we expect to be able to develop risk mitigation messaging that uses evidence from Aims 1 and 2 as well as results from this aim.

C4.5 *Potential problems and solutions*. We anticipate that most IgG positive individuals for either set of viruses will be adults, with the likelihood of exposure and having antibodies increasing with age. Because we don't know how long IgG antibodies persist at detectable levels, it will not be possible to accurately determine when exposure occurred, with the exception being EBOV antibodies that most likely occurred as a result of contact with another infected person during the 2014-2016 outbreak. We will address this in two ways: by looking at respondents 1-6 yrs old, we will know that any filovirus antibodies they have are most likely not due to the epidemic, and we will be able to determine whether there are any significant associations with particular behavior that promotes direct animal contact, consumption of contaminated fruit, or exposure to bat habitats (e.g. caves or mines). For adult respondents, we will focus on exposures (based on relative MFI values) specific to non-EBOV filoviruses and henipaviruses and see whether there are associations with specific behaviors.

C.5. Project timeline

Project timeline	Y1				Y2				Y3				Y4				Y5			
Activity	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Ethical approval (local IRB / IACUC)																				
Database development																				
Aim 1: Human serum testing																				
Aim 2: Bat sampling																				
Aim 2: Bat serum testing																				
Aim 2: Bat sample PCR																				
Aim 1&2: Confirmatory assays + sequencing (RML)																				
Aim 3: Behavioral risk analysis																				
Aim 3: Risk communication development																				
Publications																				
Data publication																				

D. Summary and Conclusions.

West Africa is a hotspot for emerging zoonotic viruses such as Ebola, Marburg and potentially Nipah-related henipaviruses – groups that have been identified by WHO as among the most significant pathogens that threaten global health. Liberia is also a biodiversity hotspot, with some of the largest tracts of primary forest remaining in the region, and its population relies on wildlife as a primary source of protein, creating continued risk of spillover of high consequence zoonotic pathogens. This project has the potential to determine the origins of Zaire ebolavirus in West Africa, characterize the circulation of Ebola and other filoviruses and henipaviruses in common bat species, identify background rates of spillover in rural human populations, and identify high-risk behaviors associated with exposure to these bat-borne zoonoses. Our group comprises an unparalleled multidisciplinary group of leading experts in epidemiology, clinical medicine, virology, serology, and disease ecology all with deep experience working in Liberia and implementing zoonotic virus research, giving this project a high chance of success in generating impactful results that will help prevent future epidemics in West Africa.

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From: Fischer, William A. II on behalf of Fischer, William A. II <william_fischer@med.unc.edu>
To: Wohl, David A; Jon Epstein
Cc: Laing, Eric; Munster, Vincent (NIH/NIAID) [E]; Shannon Ball; Morgan Kain; Madeline Salino
Subject: Re: Complete draft 7 for rapid review
Date: Thursday, February 2, 2023 10:38:25 PM
Attachments: Liberia R01 Technical proposal d7_clean_DW_WF.docx

Well done – see attached for minor edits and suggestions.

I know we're tight on space but a figure triangulating seroprevalence in humans, PCR positivity in bats, and host/behavior/environmental risk factors might be helpful to tie the three aims together.

Thanks for everyones' work on this!!

B

William A. Fischer II, MD
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From: Wohl, David A <david_wohl@med.unc.edu>
Date: Thursday, February 2, 2023 at 5:48 PM
To: Jon Epstein <epstein@ecohealthalliance.org>
Cc: Laing, Eric <eric.laing@usuhs.edu>, Fischer, William A. II <william_fischer@med.unc.edu>, Munster, Vincent (NIH/NIAID) [E] <vincent.munster@nih.gov>, Shannon Ball <ball@ecohealthalliance.org>, Morgan Kain <kain@ecohealthalliance.org>, Madeline Salino <salino@ecohealthalliance.org>
Subject: Re: Complete draft 7 for rapid review

Still…

That is important. If there is smoke the is fire and agree important to say that it is present and understudied.

Thanks

D

From: Jon Epstein <epstein@ecohealthalliance.org>
Date: Thursday, February 2, 2023 at 5:45 PM
To: Wohl, David A <david_wohl@med.unc.edu>
Cc: Laing, Eric <eric.laing@usuhs.edu>, Fischer, William A. II <william_fischer@med.unc.edu>, Munster, Vincent (NIH/NIAID) [E] <vincent.munster@nih.gov>, Shannon Ball <ball@ecohealthalliance.org>, Morgan Kain <kain@ecohealthalliance.org>, Madeline Salino <salino@ecohealthalliance.org>
Subject: Re: Complete draft 7 for rapid review

Antibodies....

Our work is done :)

On Thu, Feb 2, 2023 at 5:43 PM Wohl, David A <david_wohl@med.unc.edu> wrote:

> You found a henipavirus in bats in west Africa?
>
> If so, what I said:

David Alain Wohl, MD
Institute of Global Health and Infectious Diseases/HIV Prevention & Treatment Clinical Trials Unit/Viral Hemorrhagic Fevers Research Working Group
University of North Carolina - Chapel Hill
+1 919 843 2723

Sent from my iPhone

> On Feb 2, 2023, at 5:07 PM, Laing, Eric <eric.laing@usuhs.edu> wrote:
>
> I think I get why you're sticking with "Nipah-like henipavirus," but it seems odd to me. Is every henipavirus, "Nipah-like." Why not just call out Ghana virus, or shrew-associated Langya virus that causes acute febrile illness in humans.
>
> The manuscript write-up was EBOV focused but when my student rant the multiplex we included henipaviruses. This prelim you should use that would address the henipavirus concern. I'd drop Figure 5 and use this

Eric D. Laing, Ph.D.
Assistant Professor
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On Thu, Feb 2, 2023 at 4:33 PM Wohl, David A <david_wohl@med.unc.edu> wrote:

> Hi Jon,
>
> See attached. Looks solid.
>
> Main issues to consider:
>
> The conflating of filoviruses and henipaviruses makes me nervous (as Jean DeMarco often says). We have good rationale for expecting filoviruses in bats and humans. For henipaviruses, not so much. This could be shifted to a strength if acknowledged not just at the end but earlier and make clear that this is an opportunity to look for these viruses given the bat species and limited ability to detect encephalitis in Liberia – we could detect the tinderbox for a future outbreak.
>
> I do think in the limitations section we need to address more explicitly that not finding henipavirus exposure in bats or people is possible and is ok in that this is an important negative finding given the potential. I am concerned reviewers will be attracted to henipavirus and unclear if their reaction will be sweet or sour.
>
> Less of an issue is the seroincidence. I am of two minds about this. We can double down and take our chances that reviewers won't ding us for placing bets on a 24 month follow-up period by going the route selected. Alternatively, we could acknowledge that incidence is less certain but that we hypothesize is not uncommon. Given hours left to submit, doubling down makes sense.

For Aim 3, I agree could be clearer about putative factors for infection/exposure. It is not just behavior but also structural (literally and figuratively). Environment plays a role too as does super poverty among the impoverished. Could list hypotheses regarding risks including men>women, hunters vs non, having domestic animals vs not, less sturdy housing vs more, etc.

Let us know if you need anything else.

Thanks for leading the charge.

D

From: Jon Epstein <epstein@ecohealthalliance.org>
Date: Thursday, February 2, 2023 at 2:31 PM
To: Fischer, William A. II <william_fischer@med.unc.edu>, Wohl, David A <david_wohl@med.unc.edu>, Eric Laing <eric.laing@usuhs.edu>, Munster, Vincent (NIH/NIAID) [E] <vincent.munster@nih.gov>, Shannon Ball <ball@ecohealthalliance.org>, Morgan Kain <kain@ecohealthalliance.org>
Cc: Madeline Salino <salino@ecohealthalliance.org>
Subject: Complete draft 7 for rapid review

Hi all,
I've compiled a near final draft, incorporating the text you each provided and then cutting it down to fit within the 13 page (1 page specific aims and 12 page research strategy) limit. Thank you all for excellent contributions to this. I'm sure it's not perfect, but I think it's solid.

If you're able to go through it today and check it for overall readability and accuracy where you have preliminary data, that would be fantastic. Please do use track changes and change the file name to add your initials when you edit, and please do not add too much text, as there's not much space at the end.

I do think Aim 3 might be a little thin, so if Shannon, Dave and Billy could look through that and see if you think it's missing anything, there's space for a few extra lines there.

Please return your edits to me by 7pm, if possible, so I can finalize the draft. Our plan is to submit at noon tomorrow and deal with any errors that may pop up.

While you're reviewing this, I'll work on the summary and narrative, and other peripheral pieces.

Thanks again. We're in the home stretch!

> Cheers,
> Jon
>
> --
> Jonathan H. Epstein DVM, MPH, PhD
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<Liberia R01 Technical proposal d7_clean_DW-EDL.docx>

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Specific Aims

Emerging infectious diseases - predominantly caused by zoonotic viruses - have become a major threat to individual and public health as well as the functioning of a global society. Even prior to the SARS-CoV-2 pandemic, outbreaks of consequential emerging pathogens including SARS-CoV, Pandemic influenza 2009, Zika virus, and Ebola virus disease tragically killed, ~~ and~~ disabled, and caused massive disruptions of regional and global economies and health care systems[1]. The emergence of consequential zoonotic infections is accelerating due to anthropogenic drivers such as climate change, land-use change, intensification of livestock production, and wildlife trade, all of which increase opportunity for people and domestic animals to come into contact with wildlife reservoirs[2-5]. **To reduce the risk of infectious disease epidemics, there is a critical need to detect and *prevent* spillover of zoonotic pathogens from wildlife reservoirs[2,6]**.

Illustrative of this need is the repeated spillover from wildlife reservoirs to human populations of groups of zoonotic viruses such as filoviruses (e.g., Ebola virus [EBOV]) and henipaviruses (e.g., Nipah virus [NIV]). Large-scale outbreaks or clusters of cases with these high-mortality zoonotic viruses have led to their being **designated as high priority pathogens by the World Health Organization (WHO)[5]**. However, at present, the detection of outbreaks caused by these pathogens occurs only after the first human infections; a failure in prevention that is deadly, costly, and remediable[1]. Identifying natural reservoirs of zoonotic viruses and measuring rates of human exposure to these viruses ~~in humans~~ are critical to assessing their potential threat and developing interventions that reduce ~~the risk of~~ spillover risk.[1,2]

In the proposed study we have assembled a diverse team of experts in the ecology, epidemiology, virology, and ~~immune response~~immunology of~~to~~ bat-borne viral zoonoses such as filoviruses and henipaviruses, as well as; ~~experts in~~and filovirus clinical care and research who are actively working in West Africa. With this team, our study will ~~to~~ provide much needed empirical evidence to help answer the questions: **What is the natural reservoir for Ebola virus and how often does spillover of this and related viruses happen in a region that is an emerging disease hotspot, suffered the world's largest filovirus outbreak, and where there has been evidence of other consequential zoonotic viruses in animals and people**?

To address these questions, we propose the following specific aims:

Aim 1. To determine the rates of filovirus and henipavirus exposure in people in rural Liberia. We have shown that certain bat species in Liberia carry EBOV-Zaire, while others carry Marburg virus and henipaviruses. Further, hunting bats is a common activity and we have detected high seroprevalence of Marburg and EBOV antibodies in rural Liberia. Exposure to henipaviruses remains unknown. To test the hypothesis that spillover of both groups of viruses has occurred in this region, we will use an antigen-based multiplex serological assay to screen more than 1,800 archived blood specimens, collected in 2021 and again in 2023 from over 900 adults and children in rural central Liberia, for antibodies against all known filoviruses and henipaviruses.

Aim 2. To identify reservoirs and characterize the infection dynamics of filoviruses and henipaviruses in key bat species in Liberia. Our group detected EBOV-Zaire RNA and antibodies in *Miniopterus nimbae*, a cave- and mine-dwelling bat in northern Liberia found near the origin of the 2014 Ebola outbreak in Guinea. We also detected antibodies against EBOV in *Hipposideros* bats that co-roost with *Miniopterus* in multiple locations in Liberia. *Rousettus aegyptiacus*, a common frugivorous bat in Liberia, carries Marburg virus and henipaviruses. We will conduct a 3-year longitudinal study of populations of these bat species near the human cohort studied in **Aim 1**, to determine whether they are reservoirs for Ebola or other filoviruses and henipaviruses and characterize the temporal dynamics of filoviruses and henipavirus circulation using multiplexed serological assays that test for IgG antibodies against all known filoviruses and henipaviruses, as well as molecular techniques to screen for Ebola, Marburg, Nipah virus, and related viruses.

Aim 3. To characterize behaviors associated with exposure to filoviruses and henipaviruses. Combining the data collected in Aim 1 (humans) and Aim 2 (bats) we will identify the demographic, behavioral, and environmental factors associated with an individual's prior exposure to filoviruses and henipaviruses. Detailed animal exposure data collected from the adults and children (many born after the Ebola outbreak) who provided the Aim 1 blood specimens will be used to develop profiles of risk and inform public health & risk reduction communication tools in partnership with the Ministry of Health, Liberia.

Formatted: Indent: First line: 0"

Commented [MK1]: Reads slightly odd to me given the bolded aim of "people". Maybe flip this paragraph around?

Commented [MV([2]: Is this correct? Which henipavirus comes from Rousettus?

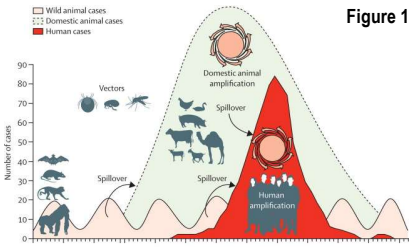
Commented [MV([3]: Wouldn't you say this stronger, to link it o our initial work detecting EBOV? More like identification of the exact reservoir?

RESEARCH STRATEGY

A. SIGNIFICANCE

Zoonotic viruses are responsible for the majority of emerging infectious disease outbreaks, which are occurring at an accelerating rate in global hotspots[3,7]. Every outbreak begins with spillover – transmission of the virus from its animal reservoir either directly into human populations or into other animal hosts *en route* to humans (**Figure 1**). **Exactly how often spillover occurs is virtually unknown,** as surveillance systems are primarily designed to detect clusters or outbreaks; thus current surveillance , ~~and~~ often misses initial individual infections that result from animal contact. To reduce the risk of future epidemics, it will be necessary to limit opportunities for zoonotic viruses to spillover and to detect spillover at the earliest stages.[1,8] Measuring the efficacy of any interventions designed to reduce spillover and spread of zoonotic pathogens will rely on measuring rates of exposure in at-risk human populations.

Figure 1



Commented [MK4]: This figure seems overly complicated to me for the sentence in which it is referenced, especially without any caption. Do you expect a reviewer to stop and parse this figure and continue reading with a stronger understanding of what is going on? I feel a reviewer could just get annoyed…

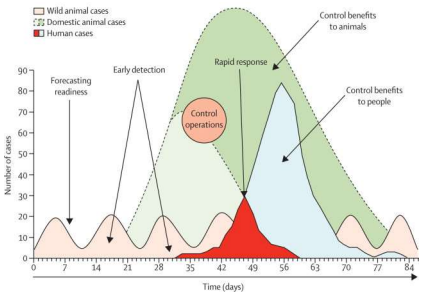
Filoviruses and henipaviruses are among the highest consequence zoonotic viruses and have greatly impacted public health. Each group contains zoonotic pathogens that cause significant disease in both people and animals, especially livestock, that have been associated with extremely high rates of morbidity and mortality. Ebola (EBOV) and related viruses, as well as Nipah virus (NIV), a henipavirus, and its related viruses have been listed by the World Health Organization (WHO) among the highest priority pathogens for the development of vaccines and therapeutics, as they represent zoonotic pathogens with high potential to threaten human health, with extremely high mortality rates and few or no therapeutics or vaccines currently available.[9-11] These viruses share other important features which make them high priority: they are all associated with bat hosts that are abundant and widely distributed throughout Africa and Asia, living in close association with people and livestock in some of the most densely populated regions on Earth.

Spillover of these viruses has repeatedly occurred. Beyond the massive outbreak in West Africa in 2013-16, EBOV outbreaks have occurred repeatedly in Central and East Africa; most recently Sudan ebolavirus caused an outbreak in Uganda in late 2022[11-13]. Marburg virus (MARV) and Ravn virus (RAVV) comprise the closely related genus *Marburgvirus* and have caused outbreaks in Central and East Africa and Europe with mortality and clinical presentation comparable to Ebola [14,15]. Marburg virus outbreaks were declared in Guinea in 2021, just across the border from the UNC-Liberia research site, ~~in 2021~~ and then again in Ghana in 2022 marking the first time this virus has been reported in either country[16,17]. Nipah virus is an emerging zoonotic paramyxovirus that has caused nearly annual outbreaks in Bangladesh, including multiple outbreaks in 2023, and sporadically in India with mean case fatality rates greater than 70% [12,18-20]. Despite the public health significance of Nipah and related viruses, little is known about its genetic diversity, natural reservoirs, or frequency of spillover throughout their range, including Africa. Henipaviruses, and Nipah virus in particular - have several characteristics that make them a global health priority: 1) Their bat reservoirs occur throughout Asia and Africa, overlapping human and livestock populations, giving them geographically broad opportunity to cause outbreaks [21]; 2) henipaviruses can be transmitted to people directly from bats or via domestic animals [21]; 3) Nipah virus can be transmitted from person to person [22]; 4) Nipah virus spillover has occurred and continues to occur in highly populous and internationally connected regions; 5) repeated spillovers of NIV strains with varying person-to-person transmission rates indicate the ability to evolve **with increased pandemic potential** [23,24]; and 6) Nipah is associated with a high mortality rate in people and currently has no vaccine or treatment [21,23]. While Nipah virus has not been detected in Africa, closely related henipaviruses have been identified in Ghana and Madagascar, though their public health significance remains unknown[25-27]. In West Africa, where bats and associated henipaviruses occur but where no human cases have been identified, **diagnostic testing and systematic surveillance is essentially non-existent**. The detection of antibodies against a Nipah-related virus in hunting communities in Central Africa suggests that cryptic spillover is occuring[28]. **Repeated spillover of henipaviruses creates opportunity for more transmissible strains to emerge[29].**

While bats have been identified as potential hosts for filoviruses including EBOV, the natural reservoir for EBOV-Zaire Ebola in West Africa remains unknown. There is substantial evidence, both serological and molecular, that bat species host filoviruses; however, a definitive natural reservoir or set of reservoirs for EBOV-Zaire has not been identified[30]. Wildlife, including bats, represent a substantial source of animal protein for communities in Liberia[31]. People may be exposed to bat-borne viruses through hunting and butchering, eating food contaminated by bat excreta, or by entering caves or mines inhabited by bats. Our group previously found that people living in rural areas of Liberia conduct activities which may increase their risk of contact with bats or their bodily fluids. The index case, a child, in the 2013 Ebola outbreak was hypothesized to have had contact with bats, yet the exact species was never definitely identified[32]. The route of spillover for most historical Ebola outbreaks remains a mystery. Despite broad efforts to determine the natural reservoirs of EBOV and related viruses, there continues to be a poor understanding of filovirus ecology, with the exception of Marburg virus, whose host has been identified as Egyptian rousette bats (*Rousettus aegyptiacus*)[33-35]. Communities known to hunt bats in Cameroon and India have been found to have antibodies against henipaviruses and filoviruses, respectively, yet nothing is known about community exposure rates in Liberia, where bat hunting is common[28,36]. Our preliminary data (see below) suggest that there is substantial exposure to EBOV as well as MARV among people in central Liberia. While the EBOV antibodies may indicate prior infection from another person such as during the West Africa outbreak, antibodies specific to other filoviruses may signal a localized exposure from animals. Even less is understood about henipaviruses in Africa~~, and~~ identifying antibodies against henipaviruses in people will be critical for understanding where, how, and how often spillover occurs.

The urgent need to develop interventions to prevent spillover of zoonotic high consequence pathogens can only be met with a better understanding of their natural reservoirs, the rates of exposure in vulnerable human populations, and the ways in which people are exposed. Unrecognized outbreaks provide opportunities for viruses to emerge that may be more transmissible or more pathogenic than previously observed, and limiting these opportunities is an important component of pandemic prevention[2,29]. Typically, by the time an outbreak is recognized in human populations its containment and control are difficult, if not impossible to achieve (e.g., HIV, SARS-CoV-2). Understanding the ecology of these viruses, including which species act as reservoirs as well as how and to what extent people are being exposed to filoviruses and henipaviruses will inform intervention and surveillance strategies and provide baseline metrics against which one can measure the efficacy of interventions designed to reduce exposure, such as public health campaigns to promote avoidance of behaviors associated with infection among those most at risk (**Figure 2**).

Figure 2



This proposed study will be the largest and most rigorous One Health investigation of high-consequence zoonotic viruses in bats and humans in an emerging disease hotspot. West Africa is known to be the source of prior outbreaks of emerging infectious diseases and future spillover events there are highly likely. Liberia is typical of this region and contains some of the largest tracts of pristine forest and wildlife biodiversity remaining in West Africa and has a population that depends on wildlife, including bats. Decades ago, a spillover event from a non-human primate to a human triggered an on-going HIV pandemic that has led to the infection of over 60 million people and 25 million deaths. Only months ago, the orthopoxvirus MPOX virus~~found in~~originating from rodents spread to and within 110 countries across the globe causing over 85,000 cases. Both originated from African wildlife. The proposed study will leverage the unique infrastructure and expertise we have developed in Liberia to undertake a comprehensive examination of filovirus and henipavirus spillover from bats to humans and provide evidence of previous and possibly on-going transmission events. Our findings will provide essential evidence of the presence and transmission dynamics of these bat-borne viruses and as the risks for infection in people living alongside them.

B. INNOVATION

The proposed research is highly innovative in multiple ways, including: 1) Our use of a Oone Health approach to study the ecology and epidemiology of two groups of high-priority, high-consequence zoonotic viruses in both bats and people in West Africa – an emerging disease hotspot where spillover events have occurred (e.g. Ebola, Marburg, MPOX, Lassa fever) and are highly likely to re-occur; 2) Our use of a novel and unique multiplexed serological platform, developed by our group, that will allow us to screen bat and human serum for IgG antibodies against each of the known filoviruses and henipaviruses, simultaneously in a single assay. Longitudinal comprehensive serological studies in bats and people **will provide unprecedented insight into the changes in rates of exposure over time to known viruses in both bats and people (e.g., what is the baseline rate of exposure to henipaviruses and filoviruses and is there evidence of recent exposure?), as well as exposure rates to unknown, antigenically related viruses;** 3) We will couple serosurveillance with molecular testing to identify specific filoviruses and henipaviruses carried by bats; and 4) We will connect the bat and human epidemiological studies by analyzing questionnaire data from the human cohort to identifying high risk behaviors associated with viral exposure. **Results from this analysis will inform the development of public outreach and risk reduction campaigns by the Government of Liberia.**

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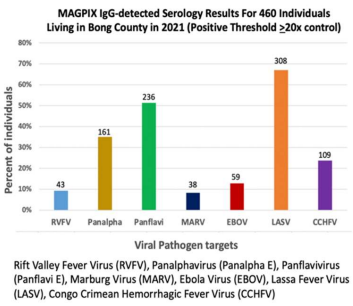
C. APPROACH

C.1. Overview: **Aim 1** will test the hypothesis that spillover of filoviruses and henipaviruses occur regularly in rural Liberia by testing for IgG antibodies to all known filoviruses and (see table 3) in archived serum samples from 900 people living in Bong county (central Liberia), collected at two time points 24 months apart. Seroprevalence of filoviruses and henipaviruses will be assessed as will changes between the two collection timepoints. In **Aim 2,** we will conduct a 36-month longitudinal study of three bat species which are putative reservoirs for EBOV and undescribed henipaviruses. Using a combination of longitudinal serology and molecular testing (PCR and whole genome sequencing) **we will determine the prevalence of filoviruses and henipaviruses in three key bats species associated with the rural human populations studied in Aims 1 and 3**. For **Aim 3,** we will analyze detailed questionnaire data developed collaboratively by our group (EHA and UNC) and collected in 2023 along with blood samples to be screened in Aim 1. These data will allow us to identify behaviors associated with exposure to henipaviruses and filoviruses. We will use results from our behavioral risk assessment to develop public risk reduction communication strategies with the National Public Health Institute, Liberia, using the results of this study to inform public health strategies. This application is a natural progression of our research activities, and our preliminary data speak to the feasibility of the proposed study.

C.2. Aim 1: To determine the rates of filovirus and henipavirus exposure in people in central rural Liberia.

C.2.1. *Rationale and Preliminary Data.* The primary goal of this aim is to be able to determine whether populations within Liberia who live in association with bats have evidence of exposure against one or multiple filoviruses and/or henipaviruses and to compare rates of exposure over time. Studies of human populations in Central and West Africa have found evidence of exposure to EBOV prior to the 2013 outbreak and in places not known to have had Ebola outbreaks[37,,38]. UNC Project-Liberia has previously assessed seropositivity to consequential viral pathogens including EBOV and MARV in a subset of CEPI ENABLE Study participants. About 10% of participants had antibodies against MARV (**Figure 3**), suggesting that unreported spillover events have previously occurred. Acute encephalitis is common in western sub-Sharan Africa, yet outbreaks of neurological disease are rarely reported and more than half of all cases are undiagnosed[39]. Among the countries in western Africa reporting cases of meningitis or encephalitis, Liberia has reported the fewest, likely due to a lack of surveillance and capacity to diagnose neurological diseases[39]. Nipah

Figure 3



Rift Valley Fever Virus (RVFV), Panalphavirus (Panalpha E), Panflavivirus (Panflavi E), Marburg Virus (MARV), Ebola Virus (EBOV), Lassa Fever Virus (LASV), Congo Crimean Hemorrhagic Fever Virus (CCHFV)

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virus encephalitis has never been reported in Africa, however, as described above, two Nipah-like henipaviruses have been described in bat hosts, including one in Ghana[25,27]. Spillover of henipaviruses was reported in bat-hunting communities in Cameroon[28]. In South Africa, we detected henipavirus sequences in *Rousettus aegyptiacus,* bats which also commonly occur in Liberia[26].

In Aim 1, we will test the hypothesis that spillover of both filoviruses and henipaviruses have occurred in rural populations in Liberia by measuring seropositivity to both groups of viruses within the CEPI ENABLE Study community cohort. We will also measure changes in exposure rates over a two-year period. While prior studies have provided cross-sectional snapshots of exposure, our longitudinal approach will provide data from the same cohort at two timepoints, 24 months apart, to determine both seroprevalence and sero-incidence using a multiplexed serological assay to screen for IgG antibodies against all known filoviruses and henipaviruses.

Settings and Participants. To achieve this aim, we will leverage a well characterized cohort of individuals in three rural communities located in Bong County, Liberia (**Figure 4**), which has a population of over 333,400. UNC Project-Liberia has been operating in Liberia since 2014 and established a research site at Phebe Hospital in Bong County that includes a molecular laboratory that conducts daily qPCR testing for LASV, MARV, and EBOV as well as LASV serological assays using a MAGPIX (SEE RESOURCES AND FACILITIES). There, following extensive community engagement including meetings with key stakeholders, town hall meetings, and radio messaging, UNC launched the CEPI ENABLE Study, the largest passive and active surveillance program for Lassa fever in Liberia. Started in 2020, 5,005 randomly selected participants 2 years of age and older in Phebe Airstrip, Suakoko, and Rubber Factory were enrolled over 6 months following informed consent/assent (mean age at enrollment is 22.2 years (range 2-97) and 54.2% are female). **Importantly, 7.7% of the cohort is under the age of 5 years, and therefore, were born after the 2013-14 Ebola outbreak (Table 1).** Households were selected for recruitment following identification of roof tops by satellite photos and use of a program developed by Médecins Sans Frontières' (MSF) Epicentre to randomly selected structures to consecutively approach. The ENABLE Study includes baseline questionnaires and blood specimen collection from all participants with active follow-up by a study community health worker every 2 weeks to determine is a febrile event has occurred, which triggers drawing of blood for LASV, MARV, and EBOV PCR. A subset of 1,024 individuals is also asked to provide blood samples routinely every 6 months for up to 24 months. The blood samples from 0 and 24 months and the associated surveys will be used in **Aims 1 and 3** of this proposal. Informed consent and assent documents include provisions for the use of collected specimens and data for infectious diseases research including for emerging pathogens (see PROTECTION OF HUMAN SUBJECTS).

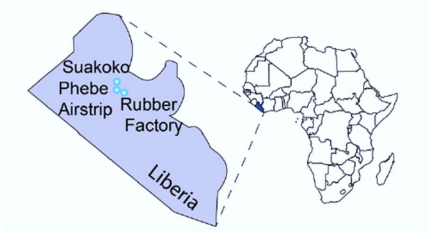

Figure 4. Map of Liberia with Bong County sites shown in inset.

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C.2.2. Research Design. We will screen approximately **1,900 archived serum specimens** from participants in the ENABLE Study cohort at two time points ("baseline" [~~(~~Month 0]~~)~~ and ~~(~~Month 24~~)~~) for IgG antibodies against specific filoviruses and henipaviruses using the MAGPIX at the UNC molecular lab at Phebe hospital to run the multiplexed *henipavirus / filovirus* platform to be provided by Co-I Laing in year 1. The multiplex platform will allow us to determine specifically which filoviruses and henipaviruses people have been exposed to and compare that to the filoviruses and antibodies we observe circulating in local bat populations in **Aim 2**. The bead-based multiplexed assay has tremendous advantage over traditional ELISAs in that it will identify up to 25 different antibodies against multiple viral agents in a single, 2uL serum sample and uses a high through-put format. The ability to look at reactivity across all serogroups simultaneously will allow us to differentiate among the viruses. Proteins A/G non-specifically bind IgG immunoglobulins, and weakly bind IgM, an immunoglobulin that is the first to be produced during the initial stages of infection. We will conduct a 1-week training at the UNC-Phebe lab for using the assay to detect filovirus and henipavirus antibodies as well as data management, bioinformatics, and analysis. **Table 3** shows the viral species and antigens that will be employed in this assay. The platform has

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been previously transferred to collaborating labs in the UK, South Africa, India, Bangladesh, Singapore, Thailand, and Malaysia. We have previously detected serological profiles of Asiatic ebolaviruses in fruit bats collected in Singapore[40] and in fruits bats and humans actively engaged in bat hunting in northeast India[36].

Table 3. Multiplexed microsphere immune assay including soluble glycoproteins from each known filovirus and henipavirus

Virus (Name/Isolate Host/Country/Year/Strain)	Abbreviation	Accession No.	Soluble Glycoprotein	Mag Bead No.
Ebolaviruses				
Ebola virus/H. sapiens/COD/1976/Yambuku-Mayina	EBOV	NC_002549.1	$GP_{(1,2)}$	34
Bundibugyo virus/H. sapiens/UGA/2007	BDBV	FJ217161.1	$GP_{(1,2)}$	64
Bombali ebolavirus/Mops condylurus/SLE/201	BOMV	NC_039345	$GP_{(1,2)}$	55
Taï Forest virus/H. sapiens/COV/1994/Pauleoula-CI	TAFV	NC_014372	$GP_{(1,2)}$	57
Sudan virus/H. sapiens/UGA/2000/Gulu-808892	SUDV	NC_006432.1	$GP_{(1,2)}$	54
Reston virus/M. fascicularis/USA/1989/Pennsylvania	RESTVm	AF522874.1	$GP_{(1,2)}$	65
Marburgviruses				
Marburg virus/H. sapiens/KEN/1980/Musoke	MARV	Z12132 S55429	$GP_{(1,2)}$	37
Ravn virus/H. sapiens/AGO/2005/Ang0126	RAVV	NC_024781.1	$GP_{(1,2)}$	62
Dianlovirus				
Mengla virus/Rousettus-WT/CHN/2015/Sharen	MLAV	KX371887.2	$sGP_{(1,2)}$	22
Cuevavirus				
86	LLOV	NC_016144.1	$sGP_{(1,2)}$	66
Henipaviruses				
Hendra virus/E. caballus/AUS/1994	HeV	NC_001906.3	sG	43
Hendra virus-2G	HeV-2g		sG	48
Nipah virus/H. sapiens/MYS/2000	NiVm	NC_002728.1	sG	46
Nipah virus/H. sapiens/BGD/2004	NiVb	AAY43916.1	sG	56
Cedar virus/Pteropus sp./AUS/2012/CG1a	CedV	NC_025351.1	sG	53
Mojiang virus/R. sladeni/CHN/2014/Tongguan1	MojV	NC_025352.1	sG	29
Ghanaian bat virus/E. helvum/GHA/2009/GH-M47a	GhV	NC_025256.1	sG	35
Angavokely virus	AngV		sG	47

C.2.3. Data Analysis. We will create a project database using Airtable, which will be hosted at EHA and will house all data from Aims 1 and 2 and will link to the RedCap server which stores questionnaire data from Aim 3. Database access will be secure and limited to key personnel (see data management plan). Raw MFI values from the MAGPIX will be used to calculate reactivity to each viral antigen and based on relative strength of reactivity (**Fig 5**) and determinations of negative cutoffs based on reference sera, we will calculate prevalence rates for each time point and compare rates between the two timepoints. Serological profiling can be used to detect reactive antibodies against all specific filoviruses and henipaviruses in a samples and compare strength of reactivity and cross-reactivity against multiple antigens using a single sample[41]. We will compare the antibodies across all specific viral antigens to assess which virus or groups of viruses are responsible for the immunological response[42-44]. We will assess exposure to non-Zaire ebolavirus species, which may indicate spillover of filoviruses has occurred separate to the Ebola outbreak of 2013. We will also look at evidence for exposure to Nipah-like henipaviruses and compare exposure rates to both viral groups within and between our two sample collection time points.

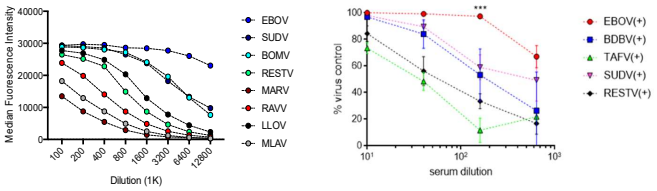

Figure 5. shows relative mean fluorescence index (MFI) values for an EBOV +ive control sera across serial dilutions. While there is cross reactivity, the EBOV MFI values are consistently greater than the other viral GPs and at higher dilutions the separation becomes significant.

C.2.4. Expected outcomes. We anticipate obtaining serological profiles of exposure to filoviruses and henipaviruses from each individual in our cohort. In previous studies we found antibodies against both Ebola and Marburg virus. While the Ebola antibodies are attributable to the epidemic, Marburg antibodies, in

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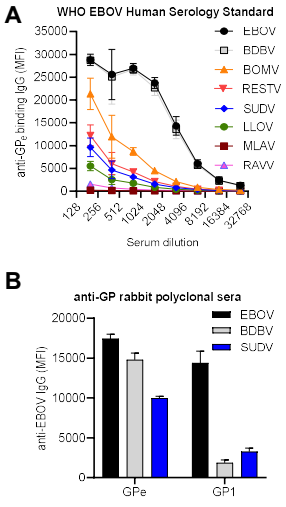
patients EBOV IgG negative, suggest possible exposure from an animal (e.g., bat) reservoir. We also anticipate seeing reactivity ~~to~~ to Bombali virus (BOMV), given the presence of the reservoir species in Liberia (*Mops condylurus*) and reactivity to the African henipaviruses (GhV and AngV), although this may represent reactivity to an antigenically related virus. Serological results from this and the bat study will inform our strategy for prioritizing which bat samples we test by PCR.

C.2.5. Potential problems and solutions. -While our preliminary data suggest there will be detection of immune responses to filoviruses including EBOV-Zaire and, significantly, MARV, it is possible that evidence of exposure to other pathogens, including henipaviruses will not be detected. This would be an important finding in this large cohort, especially if these viruses are detected in local bat species, that would warrant further investigation. It is also possible that there will be few or no seroconversion events among the cohort during the 24-month period of follow-up. This too would be reassuring but would suggest a need for longer follow-up of this cohort. To address the possibility that reactivity to EBOV-Zaire virus may be explained by infection during the 2013 Ebola epidemic, we will create a polyclonal sera reference standard utilizing convalescent sera collected from human survivors of EVD. This internal reference standard will be calibrated to the WHO EBOV human serology standard (**Fig 6A**) permitting direct interpolation of anti-EBOV IgG as an MFI to a standard antibody concentration (binding antibody units/mL). The internal reference standard will then be included in our human serology testing strategy allowing semi- or quantitative measurement of anti-EBOV IgG. These EBOV human serology standards will permit us to establish positive and negative predictive performance values for the multiplex assay, furthermore, we will be able to investigate immunoreactivity of EVD survivors against heterotypic ebolaviruses. Our preliminary data indicates that EBOV+ sera IgG is highly cross-reactive with soluble native-like trimeric envelope glycoprotein (GP) antigens from both EBOV and BDBV (Figure 6A). EBOV GP, matrix protein (VP40), and nucleocapsid protein (NP) antigens have all been used in a variety of serological tests, however, the frequency of seroconversion against each antigen has not been fully described. In a longitudinal study of EVD survivors, high seroconversion against GP and VP40 was observed, with affinity maturation occurring overtime against GP[45]. Antigens based on the GP are likely to provide an optimal intrinsic balance of sensitivity and specificity. To limit the degree of heterotypic ebolavirus cross reactions we will test any anti-ebolavirus GP trimer antigen-seropositive samples with a pan-ebolavirus GP1 antigen-based panel. In preliminary studies, GP1 antigens improve specificity and differentiation of antibodies reactive with homotypic ebolaviruses in polyclonal antisera from rabbits immunized with GP trimer immunogens (**Figure 6B**).



Figure~~IGURE 6A & B~~. A) The WHO EBOV human serology standard was testing for immunoreactivity in a filovirid GP-trimer based multiplex assay. Filovirids are indicated along with sera dilution factors; data is the mean of three independent experiments. **B)** Antisera from EBOV GP-trimer immunized rabbits were tested in multiplex panels comprised of GP-trimer and GP1 subunit proteins. Anti-EBOV polyclonal IgG was less reactive with GP1 from BDBV and SUDV.

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C.3. Aim 2. To identify reservoirs and characterize the infection dynamics of filoviruses and henipaviruses in key bat species in Liberia.

C.3.1 *Rationale and preliminary data.* Bats have been established as important hosts for filoviruses and henipaviruses in Africa and Asia, with combinations of viral isolation, molecular detection or serology providing substantial evidence [30,46-49]. MARV was isolated from Egyptian rousette bats in Uganda, and RNA has since been detected in these bats in Sierra Leone, which borders Liberia [50]. To date, EBOV has never been isolated from bats, although molecular and serological evidence suggest that multiple frugivorous bats may carry EBOV in Central Africa[50,51]. Prior to the 2014 epidemic, Zaire ebolavirus had not been reported in West Africa, and the animal reservoir remains unknown[32]. From 2016-2019, our group (PI Epstein & KP Desmond) implemented a large-scale project to identify reservoirs for EBOV in West Africa, under the USAID PREDICT Project[52]. This effort led to the discovery of a new species of Ebola (Bombali virus)[53], the detection of MARV in ERBs in Sierra Leone[33], and **in 2018, we detected, for the first time in West Africa, EBOV RNA and anti-EBOV IgG antibodies in bats (Figure 7A-D)**.

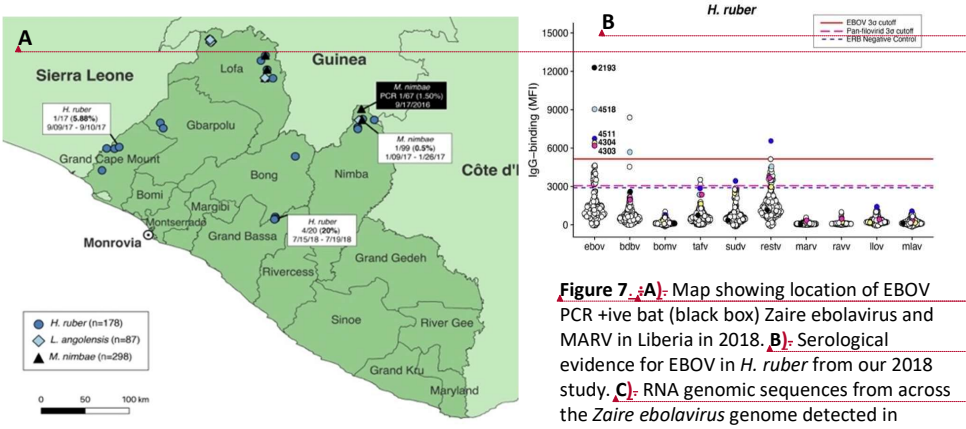
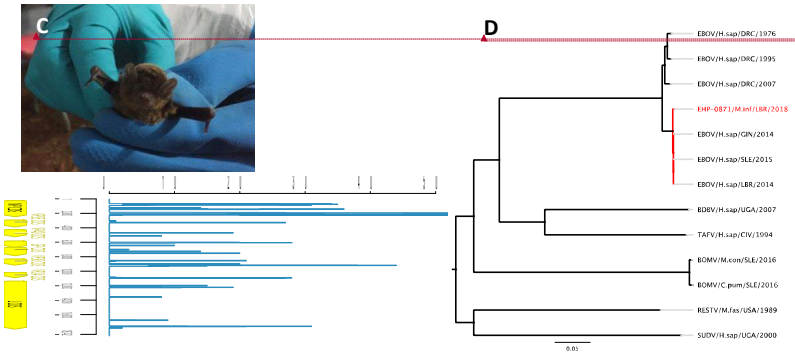


Figure 7. A) Map showing location of EBOV PCR +ive bat (black box) Zaire ebolavirus and MARV in Liberia in 2018. **B)** Serological evidence for EBOV in *H. ruber* from our 2018 study. **C)** RNA genomic sequences from across the *Zaire ebolavirus* genome detected in *Miniopterus nimbae*, Liberia in 2018. **D)** Phylogenetic analysis supports relatedness to 2013 EBOV epidemic virus.



While this finding generated the first evidence of which bats

may be reservoirs for Zaire ebolavirus, longitudinal studies are required to more completely understand the role these species play in EBOV circulation – either as reservoirs that maintain the virus within a local population, or as incidental hosts, infected by an unknown bat species. One-off, cross-sectional studies have a low probability of detecting any specific filovirus or henipavirus given their low prevalence and short viremic periods [34,54], and thus provide a poor characterization of viral diversity. The lack of understanding of **filovirus and henipavirus diversity and their natural reservoirs in West Africa significantly impedes risk-based public health interventions to prevent human outbreaks.** Longitudinal sampling is also needed to separate reservoir hosts from spillover hosts. For example, MARV has been isolated repeatedly from *R. aegyptiacus* in Uganda, which, when paired with experimental infection studies showing infection and shedding of MARV by *R. aegyptiacus,* strongly suggests that *R. aegyptiacus* is indeed a Marburg virus reservoir[35,55,56]. Similarly, our longitudinal studies of Nipah virus in *Pteropus medius* bats in Bangladesh, which included the same multiplexed serological assay proposed here, confirmed them as a reservoir for NIV and identified important mechanisms driving viral circulation[29]. **Understanding which species persistently carry filoviruses and henipaviruses will provide important insights into how they are maintained in nature and what species should be targeted for follow-up studies on bat habitat use and human-bat contact patterns.**

C.3.2. *Sampling design*. We will repeatedly sample three bat species associated with filoviruses or henipaviruses, based on our preliminary data: *M. nimbae* (EBOV), *H. ruber* (EBOV), and *R. aegyptiacus* (MARV & Henipaviruses). We will sample bats at roost sites in Bong county, as close as possible to the human study sites in Aim 1. In Y2-Y4, we will sample 40 individuals of each species every two months for 36 months and collect excreta using plastic tarps placed underneath bat roosts (details below and in C3.3).

Bat capture and sampling. Briefly, we will use either harp traps or mist nets to capture bats, depending on the capture site. Personnel will wear appropriate personal protective equipment (e.g., Tyvek suits or dedicated long clothing; double nitrile gloves; an N95 respirator and safety glasses or a PAPR) [57]. Traps and nets will be attended continuously during capture periods. Bats will be removed from mist nets immediately upon entanglement to avoid injury. Bats will be removed from harp traps after approximately 1 hour and ~~bats will be~~ placed in individual cloth bags until processed. Body measurements, blood, oropharyngeal swabs, and rectal swabs (or fecal pellets) will be collected from each bat. Swabs will be collected in duplicate; one swab will be placed in a cryovial containing Trizol lysis buffer and the other in viral transport medium (VTM). For bats <50g, blood will be collected in a microhematocrit tube and spun down using a portable centrifuge. A bulb will be used to expel serum from the tube into a cryovial. For larger bats >50g, a tuberculin syringe or 1ml syringe and 27g needle will be used to draw blood from the radial artery or vein[57]. Blood will be placed into a conical vial and centrifuged. Serum will be aliquoted into a cryovial using a pipette. During site visits we will also lay out plastic tarps to collect pooled environmental excreta in Trizol and VTM to improve opportunity to detect viral RNA. All samples will be placed in a vapor phase liquid nitrogen dry shipper or portable battery-powered ultracold freezer, immediately upon collection and then transferred to a -80C freezer at the Phebe lab until testing.

C.3.3. *Testing plan, Data analysis, sample size, and power analysis*.

Serology: At the end of Y3, we will screen all serum samples collected to date using the MAGPIX multiplex assay for IgG against filoviruses and henipaviruses. We will analyze temporal dynamics in seroprevalence using general additive models (GAMs), which are analyses that we have used successfully in many previous studies of similar design[29]. These methods are well adapted for capturing long-term temporal fluctuations in prevalence and moderately sensitive at capturing short-term changes in seroprevalence which can be used to identify population-level disease outbreaks. For example, we estimated that with 40 bats sampled every two months, we would be able to statistically detect an increase in seroprevalence (at a 95% CI limit) of greater than 25% between consecutive sampling events > 80% of the time, assuming a bat population seroprevalence of at least 10% in advance of a bat colony infection (estimated by, in brief, by simulating a fluctuating true seroprevalence value, simulating samples given this underlying seroprevalence, fitting a GAM to these samples, and then calculating if CI on true prevalence between the two sampling occasions over which the change in seroprevalence occurred were non-overlapping). While this is a large change, it is within a range to

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be expected if infection were to sweep through a bat community[29]. MAGPIX-positive bat sera will be sent to RML for serum neutralization assays using viral isolates or VSV pseudoviruses under BSL4 conditions.

VSV-pseudotype neutralization assays: Serological results will be confirmed using VSV-pseudotype neutralization assays for the following filoviruses EBOV, SUDV, BDBV, RETV, BOMV, MARV and RAVN. If novel henipavirus sequences are detected, additional assays will be developed to test positive sera. Serum samples will be inactivated using γ-irradiation (4 MRad). Neutralization of irradiated and heat-inactivated serum samples will be assessed in Vero 352 E6 cells. Briefly, cells were seeded in 96-well round-bottom plates for 24 hours, serial dilutions of heat-inactivated serum samples will be performed in DMEM supplemented with 2% FBS, penicillin/streptomycin, and L-glutamine. Each plate will contain negative serum control, cell-only control, and virus-only control. VSV EBOV-GFP will be added to each well of the serum dilution plate and the serum-virus mix 357 was incubated at 37˚C for 1-hour. The mix was added to the cells and incubated at 37˚C for 24 hours. The cells will then fixed with 4% paraformaldehyde at room temperature 359 for 15-minutes and centrifuged at 600 x g for 5-minutes at room temperature. The supernatant will be discarded and FACS+EDTA buffer was added. Samples will be run on the FACSymphony A5 Cell Analyzer (BD Biosciences, Mississauga, ON, Canada) and FITC MFI 362 was measured. Data will be analyzed using FlowJo.

Molecular testing: We will use specific filovirus taqman real-time PCR assays to screen oropharyngeal, fecal and environmental samples from bats for EBOV and MARV RNA at the UNC-Phebe lab[55,58]. Testing bat samples by PCR may allow us to detect viral RNA during active infection, providing important information about the prevalence and timing of infection within target species. **Because we expect viral prevalence to be significantly lower than seroprevalence, estimated at <5%[25,27,49],** we developed a sampling scheme focused on maximizing our probability of detecting viral RNA with qPCR. To compare power under alternative sampling designs, we built simulation-based power analyses that estimated power to detect: 1) viral EBOV or MARV RNA in at least one bat; and 2) the expected number of positive cases, in each bat species. In brief, we estimated power for total sample sizes between 1500-3000 (between 500 and 1000 bats sampled per species) spread across a variable number of visits per year (2-6), while allowing for uncertainty in true infection dynamics in the bat populations, specifically: 1) prevalence during one three-week period (with unknown timing) per year of high prevalence ranging from 1-5%); and 2) an otherwise low background prevalence (0-1%). Given these estimates of yearly infection dynamics, these analyses showed that with 672 samples per species (minimum 32 individual bats per sampling event), spread across 6 sampling occasions per year, we would obtain a per-species probability of acquiring 0 positive samples of <4% (<1% at 40 bats per event – our target number) and an expectation of ~3-6 RNA positive results (~5-10 positives at 40 bats per event). Because henipaviruses have not been previously described in Liberia, we will focus our resources on filovirus testing by PCR. However, if henipavirus serology suggests active circulation, we may send a set of samples to RML for pathogen discovery (see RML letter of support).

Viral isolation: PCR-positive cDNA samples and their aliquot in VTM will be sent to RML for further sequencing and attempts at viral isolation under BSL4 conditions. If attempts to culture do not work, we will use next generation sequencing techniques to get whole genome sequence and further characterize the virus at RML. Novel viruses will be characterized and rescued (reverse engineered using a minigenome system to synthetically create infectious viral particles [59]) at RML under secure, biosafety level 4 conditions, then used for serum neutralization assays in bat and human sera to determine more precise exposure rates. Sequences from the glycoproteins of any novel viruses discovered will be used by USU (Co-I Laing) to generate purified proteins and added to the Luminex assay, which will improve its specificity.

VirCapSeq-VERT and myBaits probe-based enrichment for recovery of full-length genome sequences of filoviruses: Although shotgun sequencing provides opportunities to analyze all host and virus genetic material, the larger average genome size of bacteria and hosts compared to viral genomes complicates a detailed analysis of the virome, particularly vertebrate viruses. We have established a positive selection probe capture-

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based system to enrich for viral sequences. Here, we will take advantage of these probe capture-based methods (VirCapSeq-VERT and myBaits probe library) to enrich for vertebrate viral sequences of interest. Sample libraries will be normalized and combined in 4- to 12-plex reactions for solution capture hybridization using either the HyperExplore custom bait library version of VirCapSeq-VERT probe set or our custom myBaits probe library. For HyperExplore custom bait library probe set, libraires will be enriched for virus following the SeqCap EZ HyperCap Workflow User's Guide, version 2.3, while for custom myBaits probe library, the myBaits Hybridization Capture for Targeted NGS protocol, Version 4.01 will be used. Sequencing libraries will be normalized and sequenced as 2 X 150 bp fragments on Illumina's MiSeq or NextSeq sequencing platforms, following Illumina's standard procedure (Illumina, San Diego, CA). NGS data will be analyzed using metavirs (https://openomics.github.io/metavirs/), a comprehensive viral metagenomics pipeline to assemble, annotate, and classify viruses. It relies on technologies like Singularity1 to maintain the highest-level of reproducibility. The pipeline consists of a series of data processing and quality-control steps orchestrated by Snakemake2, a flexible and scalable workflow management system, to submit jobs to a cluster. The pipeline is compatible with data generated from Illumina short-read sequencing technologies. As input, it accepts a set of FastQ files and will be run on-premises using the NIAID RML Big Sky supercomputing cluster.

C.3.5. *Expected outcomes.* -Based on our prior work, our multifaceted analysis approach, and calculated power given our proposed temporal sampling strategy, we expect to be able to use temporal serological data to describe filovirus and henipavirus viral dynamics over time. Our molecular screening will primarily focus on filoviruses because we have known targets, and we expect to detect EBOV or MARV RNA, despite low prevalence and short-lived infections. Sequence data from positive samples will 1) provide substantial evidence for the identification of EBOV reservoirs and help explain which virus is responsible for the observed IgG response in bats (and possibly people); and 2) provide valuable information about viral diversity, about which little is known. Henipavirus serology, in the absence of sequence data, will allow us to identify potential bat reservoirs, analyze temporal trends, and generate hypotheses for future targeted studies.

C.3.6. *Potential problems and solutions.*
Serological tests are greatly hindered by the inherent antibody cross-reactivity between antigens from known ebolaviruses, challenging data interpretations that antibodies are EBOV-specific or EBOV, BDBV, and SUDV cross-reactive [40]. This is also true for the henipaviruses in the panel. The advantage of the multiplexed platform is that we can simultaneously see relative MFI values for all the filoviruses and henipaviruses. Glycoproteins from the homologous virus should cause the most intense reaction, particularly at higher dilutions. Co-Is Laing and Munster have demonstrated that homotypic and heterotypic ebolavirus-antibody reactions can be simultaneously measured and accounted for in ERBs challenged with replication-competent VSV-filoGP (**Figure 8**). Still, there is some cross reactivity, and we will use additional tools such as viral neutralization assays as a secondary test, and our statistical approach can correct for a portion of cross reactivity. Past studies have relied on individual ELISAs which had varying degrees of specificity depending on which test was used, but often couldn't rule out cross-reactivity. The Luminex filovirus multiplex assay has predictable patterns of cross-reactivity, but we will still be able to discern to which virus the serum is most reactive.

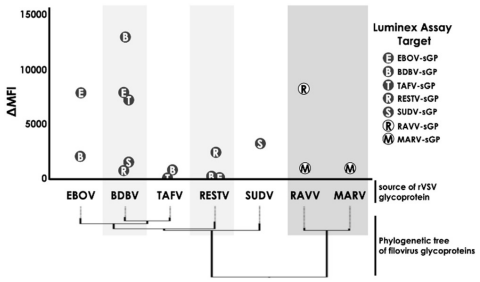

Figure 8. Captive-bred ERBs were experimentally challenged with VSV-filoGPs, the GP source is indicated on the x-axis. In a GP-trimer multiplex test the homotypic ebolavirus bat antisera were most reactive with the homotypic ebolavirus GP-trimer antigen.

C4. Aim 3: To characterize behaviors associated with exposure to filoviruses and henipaviruses.

C.4.1 Background and rationale. As described above, there is substantial interaction between wildlife, domestic animals, and people living in rural Liberia. Our group collected questionnaire data from 585 people across Liberia as part of the USAID-funded Ebola Host Project between 2016 and 2019. When asked about animal exposures during the 2013-2016 Ebola outbreak, participants frequently reported contact with both domestic and wild animals. Common types of animal contact included animals coming inside the dwelling (58%), cooking or handling animal meat (44%), and eating raw or undercooked meat (31%). Additionally, 26% reported slaughtering and 12% reported hunting or trapping an animal. In the ENABLE Study of 5,005 residents of Bong County, survey questions mainly focused on rodent exposures relevant to Lassa fever, but 12% of participants reported hunting or preparing wild animal meat. Little is known about the prevalence and type of other potential bat exposures in this region of Liberia. Elsewhere in West Africa, EcoHealth Alliance participated in the response to Ghana's 2022 cluster of MARV cases, working with local partners to administer a behavioral risk questionnaire characterizing bat exposures in three affected communities[16]. Many participants lived in close proximity to bats, with one fifth reported exposure to bats inside a dwelling or other building in the previous four months. When asked about exposures more relevant to Egyptian Rousette bats, a known MARV reservoir and a target species in this proposed study, participants commonly reported bats feeding on fruit trees on their home compound, eating fruit bearing bite marks, and exposure to bats inside a cave or mine.

C.4.2. *Research design*. As described in Aim 1, serum collected at baseline (in 2021) and at month 24 (in 2023) during the ENABLE Study will be screened for filoviruses and henipaviruses, enabling us to calculate seroprevalence at baseline and seroconversion over a two-year period. In addition to providing blood specimens, the 1,024 participants who initially enrolled responded to baseline questionnaires on demographics, healthcare-seeking behaviors, health status, and animal exposures. At month 24, the 921 participants still actively enrolled are administered repeat questionnaires. This final survey includes additional items based on the Ghanaian questionnaire and asks specifically about bats including the respondents' activities in their habitats (entering forests, caves, and mines), direct contact (hunting, eating handling), indirect contact (presence in home or other used structure), and observed characteristics of bats encountered (fruit tree dwelling). By pairing this existing survey data with the new serology results generated from archived samples under Aim 1, as well as new data on nearby bat infection from Aim 2, we will be able to develop comprehensive risk profiles for exposure to filoviruses and henipaviruses in this community.

C.4.3. *Data analysis.* The primary outcome for this analysis will be testing positive for IgG antibodies against any known filoviruses or henipaviruses. For both families of viruses, and for each species of virus within those families, we will use the serology results generated under Aim 1 to examine risk factors for seropositivity at baseline and seroconversion between baseline and month 24. We plan to conduct a multivariate logistic regression, with appropriate weights applied to account for the single-stage cluster sampling design of the ENABLE Study, in which members of randomly selected households were surveyed and tested. We will use data from both the baseline and month 24 questionnaires to examine the effects of sampling site, participant demographics (e.g., age, gender, occupation), environmental exposures (e.g., animals present in and around the dwelling), and behavioral risks (e.g., direct contact with bats through hunting, preparing, or eating them, entering caves or mines where bats live).

Power analysis: To assess our power to identify risk metrics for seroconversion (or overall seropositivity), we built simulation-based power analyses that consisted of, in brief: 1) simulating survey responses for N (400-1000) individuals, while allowing for moderate unevenness in representation across five binary categories (as largegreat as 80%-20,% for eachexample); 2) examining a range of effect sizes (odds ratios ranging from 1-2.5) for these five binary categorical predictors (e.g., sex, encountered a bat, handled a bat, etc.). Summarizing across 1,200 simulations of surveys of 400-1000 individuals given joint uncertainty in the representation within these surveys and effect sizes, we estimated that we would have at least 80% power

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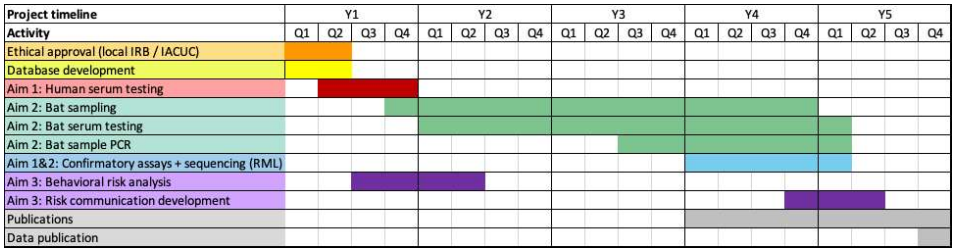
to detect significant effects for each predictor given an odds ratio of 1.65 or greater with 1,000 survey responses or ~1.80 (for example, with a rate just under 5% for Group1 = 0, just over 8% for Group1 = 1) with 750 responses.

C.4.4. *Expected outcomes.* By combining serology results with detailed questionnaire data on participant demographics and animal exposures, we will be able to use odds ratios to identify behaviors significantly associated with seropositivity (e.g., exposure to filoviruses or henipaviruses). We expect to be able to also identify age and gender-based risk factors given our sample size. Based on previous experience conducting behavioral risk surveys, including a recent study in Ghana following a Marburg outbreak, we expect to be able to develop risk mitigation messaging that uses evidence from Aims 1 and 2 as well as results from this aim.

C.4.5. *Potential problems and solutions*. We anticipate that most IgG positive individuals for either set of viruses will be adults, with the likelihood of exposure and having antibodies increasing with age. Because we don't know how long IgG antibodies persist at detectable levels, it will not be possible to accurately determine when exposure occurred, with the exception being EBOV antibodies that most likely occurred as a result of contact with another infected person during the 2014-2016 outbreak. We will address this in two ways: by looking at respondents 1-6 yrs old, we will know that any filovirus antibodies they have are most likely not due to the epidemic, and we will be able to determine whether there are any significant associations with particular behavior that promotes direct animal contact, consumption of contaminated fruit, or exposure to bat habitats (e.g. caves or mines). For adult respondents, we will focus on exposures (based on relative MFI values) specific to non-EBOV filoviruses and henipaviruses and see whether there are associations with specific behaviors.

C.5. Project timeline



D. Summary and Conclusions.

West Africa is a hotspot for emerging zoonotic viruses such as Ebola, Marburg and potentially Nipah-related henipaviruses – groups that have been identified by WHO as among the most significant pathogens that threaten global health. Liberia is also a biodiversity hotspot, with some of the largest tracts of primary forest remaining in the region, and its population relies on wildlife as a primary source of protein, creating continued risk of spillover of high consequence zoonotic pathogens. This project has the potential to determine the origins of Zaire ebolavirus in West Africa, characterize the circulation of Ebola and other filoviruses and henipaviruses in common bat species, identify background rates of spillover in rural human populations, and identify high-risk behaviors associated with exposure to these bat-borne zoonoses. Our group comprises an unparalleled multidisciplinary group of leading experts in epidemiology, clinical medicine, virology, serology, and disease ecology all with deep experience working in Liberia and implementing zoonotic virus research, giving this project a high chance of success in generating impactful results that will help prevent future epidemics in West Africa.

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From: Munster, Vincent (NIH/NIAID) [E] on behalf of Munster, Vincent (NIH/NIAID) [E] <vincent.munster@nih.gov>
To: Wohl, David A; Jon Epstein; Fischer, William A. II; Eric Laing; Shannon Ball; Morgan Kain
Cc: Madeline Salino
Subject: RE: Complete draft 7 for rapid review
Date: Thursday, February 2, 2023 5:04:58 PM
Attachments: Liberia R01 Technical proposal d7_clean MPKVM.docx

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Couple of very minor comments,

I agree with David on the henipa's as you are coupling a clear direct public health threat (Filos's – EBOV and MARV) with a history of large outbreaks, with a group of viruses for which there is no direct evidence of causing any morbidity and mortality in Africa. It makes complete sense to do exactly what we are proposing, but i think it would be stronger to argue that the risk of henipa's is currently unknown. Particularly tying this back to the human data, you know that most filo's will have direct zoonotic potential, for anything you find in the bats that will remain clear unless you can isolate said virus from a human case.

Other than that, cool proposal, great team!

Vincent Munster, PhD
Chief Virus Ecology Section
Rocky Mountain Laboratories
NIAID/NIH

From: Wohl, David A <david_wohl@med.unc.edu>
Sent: Thursday, February 2, 2023 2:33 PM
To: Jon Epstein <epstein@ecohealthalliance.org>; Fischer, William A. II <william_fischer@med.unc.edu>; Eric Laing <eric.laing@usuhs.edu>; Munster, Vincent (NIH/NIAID) [E] <vincent.munster@nih.gov>; Shannon Ball <ball@ecohealthalliance.org>; Morgan Kain <kain@ecohealthalliance.org>
Cc: Madeline Salino <salino@ecohealthalliance.org>
Subject: [EXTERNAL] Re: Complete draft 7 for rapid review

Hi Jon,

See attached. Looks solid.

Main issues to consider:

The conflating of filoviruses and henipaviruses makes me nervous (as Jean DeMarco often says). We have good rationale for expecting filoviruses in bats and humans. For henipaviruses, not so much. This could be shifted to a strength if acknowledged not just at the end but earlier and make clear that this is an opportunity to look for these viruses given the bat species and limited ability to detect encephalitis in Liberia – we could detect the tinderbox for a future outbreak.

I do think in the limitations section we need to address more explicitly that not finding henipavirus exposure in bats or people is possible and is ok in that this is an important negative finding given the potential. I am concerned reviewers will be attracted to henipavirus and unclear if their reaction will be sweet or sour.

Less of an issue is the seroincidence. I am of two minds about this. We can double down and take our chances that reviewers won't ding us for placing bets on a 24 month follow-up period by going the route selected. Alternatively, we could acknowledge that incidence is less certain but that we hypothesize is not uncommon. Given hours left to submit, doubling down makes sense.

For Aim 3, I agree could be clearer about putative factors for infection/exposure. It is not just behavior but also structural (literally and figuratively). Environment plays a role too as does super poverty among the impoverished. Could list hypotheses regarding risks including men>women, hunters vs non, having domestic animals vs not, less sturdy housing vs more, etc.

Let us know if you need anything else.

Thanks for leading the charge.

D

From: Jon Epstein <epstein@ecohealthalliance.org>
Date: Thursday, February 2, 2023 at 2:31 PM
To: Fischer, William A. II <william_fischer@med.unc.edu>, Wohl, David A <david_wohl@med.unc.edu>, Eric Laing <eric.laing@usuhs.edu>, Munster, Vincent (NIH/NIAID) [E] <vincent.munster@nih.gov>, Shannon Ball <ball@ecohealthalliance.org>, Morgan Kain <kain@ecohealthalliance.org>
Cc: Madeline Salino <salino@ecohealthalliance.org>
Subject: Complete draft 7 for rapid review

Hi all,
I've compiled a near final draft, incorporating the text you each provided and then cutting it down to fit within the 13 page (1 page specific aims and 12 page research strategy) limit. Thank you all for excellent contributions to this. I'm sure it's not perfect, but I think it's solid.

If you're able to go through it today and check it for overall readability and accuracy where you have preliminary data, that would be fantastic. Please do use track changes and change the file name to add your initials when you edit, and please do not add too much text, as

there's not much space at the end.

I do think Aim 3 might be a little thin, so if Shannon, Dave and Billy could look through that and see if you think it's missing anything, there's space for a few extra lines there.

Please return your edits to me by 7pm, if possible, so I can finalize the draft. Our plan is to submit at noon tomorrow and deal with any errors that may pop up.

While you're reviewing this, I'll work on the summary and narrative, and other peripheral pieces.

Thanks again. We're in the home stretch!

Cheers,
Jon

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

Specific Aims

Emerging infectious diseases - predominantly caused by zoonotic viruses - have become a major threat to individual and public health as well as the functioning of a global society. Even prior to the SARS-CoV-2 pandemic, outbreaks of consequential emerging pathogens including SARS-CoV, Pandemic influenza 2009, Zika virus, and Ebola virus disease tragically killed, ~~and~~ disabled, and caused massive disruptions of regional and global economies and health care systems[1]. The emergence of consequential zoonotic infections is accelerating due to anthropogenic drivers such as climate change, land-use change, intensification of livestock production, and wildlife trade, all of which increase opportunity for people and domestic animals to come into contact with wildlife reservoirs[2-5]. **To reduce the risk of infectious disease epidemics, there is a critical need to detect and *prevent* spillover of zoonotic pathogens from wildlife reservoirs[2,6]**.

Illustrative of this need is the repeated spillover from wildlife reservoirs to human populations of groups of zoonotic viruses such as filoviruses (e.g., Ebola virus [EBOV]) and henipaviruses (e.g., Nipah virus [NIV]). Large-scale outbreaks or clusters of cases with these high-mortality zoonotic viruses have led to their being **designated as high priority pathogens by the World Health Organization (WHO)[5]**. However, at present, the detection of outbreaks caused by these pathogens occurs only after the first human infections; a failure in prevention that is deadly, costly, and remediable[1]. Identifying natural reservoirs of zoonotic viruses and measuring rates of human exposure to these viruses ~~in humans~~ are critical to assessing their potential threat and developing interventions that reduce ~~the risk of~~ spillover risk.[1,2]

In the proposed study we have assembled a diverse team of experts in the ecology, epidemiology, virology, and ~~immune response~~immunology of~~to~~ bat-borne viral zoonoses such as filoviruses and henipaviruses, as well as; ~~experts in~~and filovirus clinical care and research who are actively working in West Africa. With this team, our study will ~~to~~ provide much needed empirical evidence to help answer the questions: **What is the natural reservoir for Ebola virus and how often does spillover of this and related viruses happen in a region that is an emerging disease hotspot, suffered the world's largest filovirus outbreak, and where there has been evidence of other consequential zoonotic viruses in animals and people**?

To address these questions, we propose the following specific aims:

Aim 1. To determine the rates of filovirus and henipavirus exposure in people in rural Liberia. We have shown that certain bat species in Liberia carry EBOV-Zaire, while others carry Marburg virus and henipaviruses. Further, hunting bats is a common activity and we have detected high seroprevalence of Marburg and EBOV antibodies in rural Liberia. Exposure to henipaviruses remains unknown. To test the hypothesis that spillover of both groups of viruses has occurred in this region, we will use an antigen-based multiplex serological assay to screen more than 1,800 archived blood specimens, collected in 2021 and again in 2023 from over 900 adults and children in rural central Liberia, for antibodies against all known filoviruses and henipaviruses.

Aim 2. To identify reservoirs and characterize the infection dynamics of filoviruses and henipaviruses in key bat species in Liberia. Our group detected EBOV-Zaire RNA and antibodies in *Miniopterus nimbae*, a cave- and mine-dwelling bat in northern Liberia found near the origin of the 2014 Ebola outbreak in Guinea. We also detected antibodies against EBOV in *Hipposideros* bats that co-roost with *Miniopterus* in multiple locations in Liberia. *Rousettus aegyptiacus*, a common frugivorous bat in Liberia, carries Marburg virus and henipaviruses. We will conduct a 3-year longitudinal study of populations of these bat species near the human cohort studied in **Aim 1**, to determine whether they are reservoirs for Ebola or other filoviruses and henipaviruses and characterize the temporal dynamics of filoviruses and henipavirus circulation using multiplexed serological assays that test for IgG antibodies against all known filoviruses and henipaviruses, as well as molecular techniques to screen for Ebola, Marburg, Nipah virus, and related viruses.

Aim 3. To characterize behaviors associated with exposure to filoviruses and henipaviruses. Combining the data collected in Aim 1 (humans) and Aim 2 (bats) we will identify the demographic, behavioral, and environmental factors associated with an individual's prior exposure to filoviruses and henipaviruses. Detailed animal exposure data collected from the adults and children (many born after the Ebola outbreak) who provided the Aim 1 blood specimens will be used to develop profiles of risk and inform public health & risk reduction communication tools in partnership with the Ministry of Health, Liberia.

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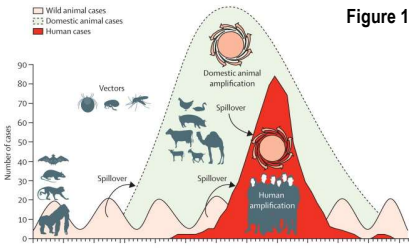
RESEARCH STRATEGY

A. SIGNIFICANCE

Zoonotic viruses are responsible for the majority of emerging infectious disease outbreaks, which are occurring at an accelerating rate in global hotspots[3,7]. Every outbreak begins with spillover – transmission of the virus from its animal reservoir either directly into human populations or into other animal hosts *en route* to humans (**Figure 1**). **Exactly how often spillover occurs is virtually unknown,** as surveillance systems are primarily designed to detect clusters or outbreaks; thus current surveillance , ~~and~~ often misses initial individual infections that result from animal contact. To reduce the risk of future epidemics, it will be necessary to limit opportunities for zoonotic viruses to spillover and to detect spillover at the earliest stages.[1,8] Measuring the efficacy of any interventions designed to reduce spillover and spread of zoonotic pathogens will rely on measuring rates of exposure in at-risk human populations.

Figure 1



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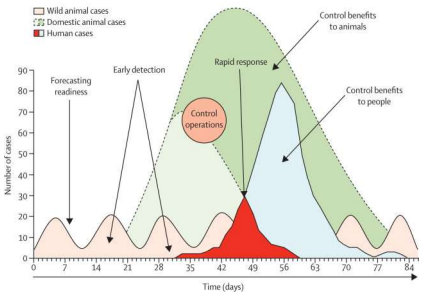
Filoviruses and henipaviruses are among the highest consequence zoonotic viruses and have greatly impacted public health. Each group contains zoonotic pathogens that cause significant disease in both people and animals, especially livestock, that have been associated with extremely high rates of morbidity and mortality. Ebola (EBOV) and related viruses, as well as Nipah virus (NIV), a henipavirus, and its related viruses have been listed by the World Health Organization (WHO) among the highest priority pathogens for the development of vaccines and therapeutics, as they represent zoonotic pathogens with high potential to threaten human health, with extremely high mortality rates and few or no therapeutics or vaccines currently available.[9-11] These viruses share other important features which make them high priority: they are all associated with bat hosts that are abundant and widely distributed throughout Africa and Asia, living in close association with people and livestock in some of the most densely populated regions on Earth.

Spillover of these viruses has repeatedly occurred. Beyond the massive outbreak in West Africa in 2013-16, EBOV outbreaks have occurred repeatedly in Central and East Africa; most recently Sudan ebolavirus caused an outbreak in Uganda in late 2022[11-13]. Marburg virus (MARV) and Ravn virus (RAVV) comprise the closely related genus *Marburgvirus* and have caused outbreaks in Central and East Africa and Europe with mortality and clinical presentation comparable to Ebola [14,15]. Marburg virus outbreaks were declared in Guinea in 2021, just across the border from the UNC-Liberia research site, ~~in 2021~~ and then again in Ghana in 2022 marking the first time this virus has been reported in either country[16,17]. Nipah virus is an emerging zoonotic paramyxovirus that has caused nearly annual outbreaks in Bangladesh, including multiple outbreaks in 2023, and sporadically in India with mean case fatality rates greater than 70% [12,18-20]. Despite the public health significance of Nipah and related viruses, little is known about its genetic diversity, natural reservoirs, or frequency of spillover throughout their range, including Africa. Henipaviruses, and Nipah virus in particular - have several characteristics that make them a global health priority: 1) Their bat reservoirs occur throughout Asia and Africa, overlapping human and livestock populations, giving them geographically broad opportunity to cause outbreaks [21]; 2) henipaviruses can be transmitted to people directly from bats or via domestic animals [21]; 3) Nipah virus can be transmitted from person to person [22]; 4) Nipah virus spillover has occurred and continues to occur in highly populous and internationally connected regions; 5) repeated spillovers of NIV strains with varying person-to-person transmission rates indicate the ability to evolve **with increased pandemic potential** [23,24]; and 6) Nipah is associated with a high mortality rate in people and currently has no vaccine or treatment [21,23]. While Nipah virus has not been detected in Africa, closely related henipaviruses have been identified in Ghana and Madagascar, though their public health significance remains unknown[25-27]. In West Africa, where bats and associated henipaviruses occur but where no human cases have been identified, **diagnostic testing and systematic surveillance is essentially non-existent**. The detection of antibodies against a Nipah-related virus in hunting communities in Central Africa suggests that cryptic spillover is occuring[28]. **Repeated spillover of henipaviruses creates opportunity for more transmissible strains to emerge[29].**

While bats have been identified as potential hosts for filoviruses including EBOV, the natural reservoir for EBOV-Zaire Ebola in West Africa remains unknown. There is substantial evidence, both serological and molecular, that bat species host filoviruses; however, a definitive natural reservoir or set of reservoirs for EBOV-Zaire has not been identified[30]. Wildlife, including bats, represent a substantial source of animal protein for communities in Liberia[31]. People may be exposed to bat-borne viruses through hunting and butchering, eating food contaminated by bat excreta, or by entering caves or mines inhabited by bats. Our group previously found that people living in rural areas of Liberia conduct activities which may increase their risk of contact with bats or their bodily fluids. The index case, a child, in the 2013 Ebola outbreak was hypothesized to have had contact with bats, yet the exact species was never definitely identified[32]. The route of spillover for most historical Ebola outbreaks remains a mystery. Despite broad efforts to determine the natural reservoirs of EBOV and related viruses, there continues to be a poor understanding of filovirus ecology, with the exception of Marburg virus, whose host has been identified as Egyptian rousette bats (*Rousettus aegyptiacus*)[33-35]. Communities known to hunt bats in Cameroon and India have been found to have antibodies against henipaviruses and filoviruses, respectively, yet nothing is known about community exposure rates in Liberia, where bat hunting is common[28,36]. Our preliminary data (see below) suggest that there is substantial exposure to EBOV as well as MARV among people in central Liberia. While the EBOV antibodies may indicate prior infection from another person such as during the West Africa outbreak, antibodies specific to other filoviruses may signal a localized exposure from animals. Even less is understood about henipaviruses in Africa~~, and~~ identifying antibodies against henipaviruses in people will be critical for understanding where, how, and how often spillover occurs.

The urgent need to develop interventions to prevent spillover of zoonotic high consequence pathogens can only be met with a better understanding of their natural reservoirs, the rates of exposure in vulnerable human populations, and the ways in which people are exposed. Unrecognized outbreaks provide opportunities for viruses to emerge that may be more transmissible or more pathogenic than previously observed, and limiting these opportunities is an important component of pandemic prevention[2,29]. Typically, by the time an outbreak is recognized in human populations its containment and control are difficult, if not impossible to achieve (e.g., HIV, SARS-CoV-2). Understanding the ecology of these viruses, including which species act as reservoirs as well as how and to what extent people are being exposed to filoviruses and henipaviruses will inform intervention and surveillance strategies and provide baseline metrics against which one can measure the efficacy of interventions designed to reduce exposure, such as public health campaigns to promote avoidance of behaviors associated with infection among those most at risk (**Figure 2**).

Figure 2



This proposed study will be the largest and most rigorous One Health investigation of high-consequence zoonotic viruses in bats and humans in an emerging disease hotspot. West Africa is known to be the source of prior outbreaks of emerging infectious diseases and future spillover events there are highly likely. Liberia is typical of this region and contains some of the largest tracts of pristine forest and wildlife biodiversity remaining in West Africa and has a population that depends on wildlife, including bats. Decades ago, a spillover event from a non-human primate to a human triggered an on-going HIV pandemic that has led to the infection of over 60 million people and 25 million deaths. Only months ago, the orthopoxvirus MPOX found in rodents spread to and within 110 countries across the globe causing over 85,000 cases. Both originated from African wildlife. The proposed study will leverage the unique infrastructure and expertise we have developed in Liberia to undertake a comprehensive examination of filovirus and henipavirus spillover from bats to humans and provide evidence of previous and possibly on-going transmission events. Our findings will provide essential evidence of the presence and transmission dynamics of these bat-borne viruses and as the risks for infection in people living alongside them.

B. INNOVATION

The proposed research is highly innovative in multiple ways, including: 1) Our use of a Oone Health approach to study the ecology and epidemiology of two groups of high-priority, high-consequence zoonotic viruses in both bats and people in West Africa – an emerging disease hotspot where spillover events have occurred (e.g. Ebola, Marburg, MPOX, Lassa fever) and are highly likely to re-occur; 2) Our use of a novel and unique multiplexed serological platform, developed by our group, that will allow us to screen bat and human serum for IgG antibodies against each of the known filoviruses and henipaviruses, simultaneously in a single assay. Longitudinal comprehensive serological studies in bats and people **will provide unprecedented insight into the changes in rates of exposure over time to known viruses in both bats and people (e.g., what is the baseline rate of exposure to henipaviruses and filoviruses and is there evidence of recent exposure?), as well as exposure rates to unknown, antigenically related viruses;** 3) We will couple serosurveillance with molecular testing to identify specific filoviruses and henipaviruses carried by bats; and 4) We will connect the bat and human epidemiological studies by analyzing questionnaire data from the human cohort to identifying high risk behaviors associated with viral exposure. **Results from this analysis will inform the development of public outreach and risk reduction campaigns by the Government of Liberia.**

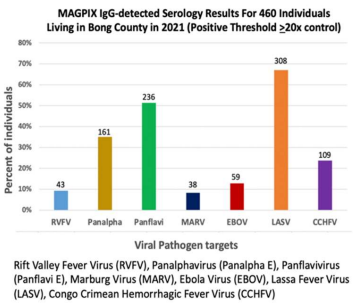
C. APPROACH

C.1. Overview: **Aim 1** will test the hypothesis that spillover of filoviruses and henipaviruses occur regularly in rural Liberia by testing for IgG antibodies to all known filoviruses and (see table 3) in archived serum samples from 900 people living in Bong county (central Liberia), collected at two time points 24 months apart. Seroprevalence of filoviruses and henipaviruses will be assessed as will changes between the two collection timepoints. In **Aim 2,** we will conduct a 36-month longitudinal study of three bat species which are putative reservoirs for EBOV and undescribed henipaviruses. Using a combination of longitudinal serology and molecular testing (PCR and whole genome sequencing) **we will determine the prevalence of filoviruses and henipaviruses in three key bats species associated with the rural human populations studied in Aims 1 and 3**. For **Aim 3,** we will analyze detailed questionnaire data developed collaboratively by our group (EHA and UNC) and collected in 2023 along with blood samples to be screened in Aim 1. These data will allow us to identify behaviors associated with exposure to henipaviruses and filoviruses. We will use results from our behavioral risk assessment to develop public risk reduction communication strategies with the National Public Health Institute, Liberia, using the results of this study to inform public health strategies. This application is a natural progression of our research activities, and our preliminary data speak to the feasibility of the proposed study.

C.2. Aim 1: To determine the rates of filovirus and henipavirus exposure in people in central rural Liberia.

C.2.1. *Rationale and Preliminary Data.* The primary goal of this aim is to be able to determine whether populations within Liberia who live in association with bats have evidence of exposure against one or multiple filoviruses and/or henipaviruses and to compare rates of exposure over time. Studies of human populations in Central and West Africa have found evidence of exposure to EBOV prior to the 2013 outbreak and in places not known to have had Ebola outbreaks[37,,38]. UNC Project-Liberia has previously assessed seropositivity to consequential viral pathogens including EBOV and MARV in a subset of CEPI ENABLE Study participants. About 10% of participants had antibodies against MARV (**Figure 3**), suggesting that unreported spillover events have previously occurred. Acute encephalitis is common in western sub-Sharan Africa, yet outbreaks of neurological disease are rarely reported and more than half of all cases are undiagnosed[39]. Among the countries in western Africa reporting cases of meningitis or encephalitis, Liberia has reported the fewest, likely due to a lack of surveillance and capacity to diagnose neurological diseases[39]. Nipah

Figure 3



Rift Valley Fever Virus (RVFV), Panalphavirus (Panalpha E), Panflavivirus (Panflavi E), Marburg Virus (MARV), Ebola Virus (EBOV), Lassa Fever Virus (LASV), Congo Crimean Hemorrhagic Fever Virus (CCHFV)

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virus encephalitis has never been reported in Africa, however, as described above, two Nipah-like henipaviruses have been described in bat hosts, including one in Ghana[25,27]. Spillover of henipaviruses was reported in bat-hunting communities in Cameroon[28]. In South Africa, we detected henipavirus sequences in *Rousettus aegyptiacus,* bats which also commonly occur in Liberia[26].

In Aim 1, we will test the hypothesis that spillover of both filoviruses and henipaviruses have occurred in rural populations in Liberia by measuring seropositivity to both groups of viruses within the CEPI ENABLE Study community cohort. We will also measure changes in exposure rates over a two-year period. While prior studies have provided cross-sectional snapshots of exposure, our longitudinal approach will provide data from the same cohort at two timepoints, 24 months apart, to determine both seroprevalence and sero-incidence using a multiplexed serological assay to screen for IgG antibodies against all known filoviruses and henipaviruses.

Settings and Participants. To achieve this aim, we will leverage a well characterized cohort of individuals in three rural communities located in Bong County, Liberia (**Figure 4**), which has a population of over 333,400. UNC Project-Liberia has been operating in Liberia since 2014 and established a research site at Phebe Hospital in Bong County that includes a molecular laboratory that conducts daily qPCR testing for LASV, MARV, and EBOV as well as LASV serological assays using a MAGPIX (SEE RESOURCES AND FACILITIES). There, following extensive community engagement including meetings with key stakeholders, town hall meetings, and radio messaging, UNC launched the CEPI ENABLE Study, the largest passive and active surveillance program for Lassa fever in Liberia. Started in 2020, 5,005 randomly selected participants 2 years of age and older in Phebe Airstrip, Suakoko, and Rubber Factory were enrolled over 6 months following informed consent/assent (mean age at enrollment is 22.2 years (range 2-97) and 54.2% are female). **Importantly, 7.7% of the cohort is under the age of 5 years, and therefore, were born after the 2013-14 Ebola outbreak (Table 1).** Households were selected for recruitment following identification of roof tops by satellite photos and use of a program developed by Médecins Sans Frontières' (MSF) Epicentre to randomly selected structures to consecutively approach. The ENABLE Study includes baseline questionnaires and blood specimen collection from all participants with active follow-up by a study community health worker every 2 weeks to determine is a febrile event has occurred, which triggers drawing of blood for LASV, MARV, and EBOV PCR. A subset of 1,024 individuals is also asked to provide blood samples routinely every 6 months for up to 24 months. The blood samples from 0 and 24 months and the associated surveys will be used in **Aims 1 and 3** of this proposal. Informed consent and assent documents include provisions for the use of collected specimens and data for infectious diseases research including for emerging pathogens (see PROTECTION OF HUMAN SUBJECTS).

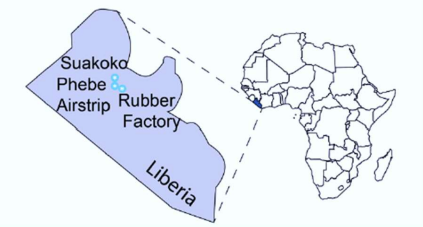

Figure 4. Map of Liberia with Bong County sites shown in inset.

C.2.2. Research Design. We will screen approximately **1,900 archived serum specimens** from participants in the ENABLE Study cohort at two time points ("baseline" [~~(~~Month 0]~~)~~ and ~~(~~Month 24~~)~~) for IgG antibodies against specific filoviruses and henipaviruses using the MAGPIX at the UNC molecular lab at Phebe hospital to run the multiplexed *henipavirus / filovirus* platform to be provided by Co-I Laing in year 1. The multiplex platform will allow us to determine specifically which filoviruses and henipaviruses people have been exposed to and compare that to the filoviruses and antibodies we observe circulating in local bat populations in **Aim 2**. The bead-based multiplexed assay has tremendous advantage over traditional ELISAs in that it will identify up to 25 different antibodies against multiple viral agents in a single, 2uL serum sample and uses a high through-put format. The ability to look at reactivity across all serogroups simultaneously will allow us to differentiate among the viruses. Proteins A/G non-specifically bind IgG immunoglobulins, and weakly bind IgM, an immunoglobulin that is the first to be produced during the initial stages of infection. We will conduct a 1-week training at the UNC-Phebe lab for using the assay to detect filovirus and henipavirus antibodies as well as data management, bioinformatics, and analysis. **Table 3** shows the viral species and antigens that will be employed in this assay. The platform has

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been previously transferred to collaborating labs in the UK, South Africa, India, Bangladesh, Singapore, Thailand, and Malaysia. We have previously detected serological profiles of Asiatic ebolaviruses in fruit bats collected in Singapore[40] and in fruits bats and humans actively engaged in bat hunting in northeast India[36].

Table 3. Multiplexed microsphere immune assay including soluble glycoproteins from each known filovirus and henipavirus

Virus (Name/Isolate Host/Country/Year/Strain)	Abbreviation	Accession No.	Soluble Glycoprotein	Mag Bead No.
Ebolaviruses				
Ebola virus/H. sapiens/COD/1976/Yambuku-Mayina	EBOV	NC_002549.1	$GP_{(1,2)}$	34
Bundibugyo virus/H. sapiens/UGA/2007	BDBV	FJ217161.1	$GP_{(1,2)}$	64
Bombali ebolavirus/Mops condylurus/SLE/201	BOMV	NC_039345	$GP_{(1,2)}$	55
Taï Forest virus/H. sapiens/COV/1994/Pauleoula-CI	TAFV	NC_014372	$GP_{(1,2)}$	57
Sudan virus/H. sapiens/UGA/2000/Gulu-808892	SUDV	NC_006432.1	$GP_{(1,2)}$	54
Reston virus/M. fascicularis/USA/1989/Pennsylvania	RESTVm	AF522874.1	$GP_{(1,2)}$	65
Marburgviruses				
Marburg virus/H. sapiens/KEN/1980/Musoke	MARV	Z12132 S55429	$GP_{(1,2)}$	37
Ravn virus/H. sapiens/AGO/2005/Ang0126	RAVV	NC_024781.1	$GP_{(1,2)}$	62
Dianlovirus				
Mengla virus/Rousettus-WT/CHN/2015/Sharen	MLAV	KX371887.2	$sGP_{(1,2)}$	22
Cuevavirus				
86	LLOV	NC_016144.1	$sGP_{(1,2)}$	66
Henipaviruses				
Hendra virus/E. caballus/AUS/1994	HeV	NC_001906.3	sG	43
Hendra virus-2G	HeV-2g		sG	48
Nipah virus/H. sapiens/MYS/2000	NiVm	NC_002728.1	sG	46
Nipah virus/H. sapiens/BGD/2004	NiVb	AAY43916.1	sG	56
Cedar virus/Pteropus sp./AUS/2012/CG1a	CedV	NC_025351.1	sG	53
Mojiang virus/R. sladeni/CHN/2014/Tongguan1	MojV	NC_025352.1	sG	29
Ghanaian bat virus/E. helvum/GHA/2009/GH-M47a	GhV	NC_025256.1	sG	35
Angavokely virus	AngV		sG	47

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C.2.3. Data Analysis. We will create a project database using Airtable, which will be hosted at EHA and will house all data from Aims 1 and 2 and will link to the RedCap server which stores questionnaire data from Aim 3. Database access will be secure and limited to key personnel (see data management plan). Raw MFI values from the MAGPIX will be used to calculate reactivity to each viral antigen and based on relative strength of reactivity (**Fig 5**) and determinations of negative cutoffs based on reference sera, we will calculate prevalence rates for each time point and compare rates between the two timepoints. Serological profiling can be used to detect reactive antibodies against all specific filoviruses and henipaviruses in a samples and compare strength of reactivity and cross-reactivity against multiple antigens using a single sample[41]. We will compare the antibodies across all specific viral antigens to assess which virus or groups of viruses are responsible for the immunological response[42-44]. We will assess exposure to non-Zaire ebolavirus species, which may indicate spillover of filoviruses has occurred separate to the Ebola outbreak of 2013. We will also look at evidence for exposure to Nipah-like henipaviruses and compare exposure rates to both viral groups within and between our two sample collection time points.

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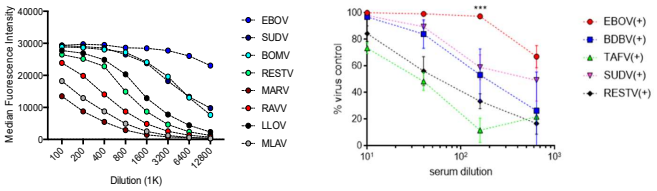

Figure 5. shows relative mean fluorescence index (MFI) values for an EBOV +ive control sera across serial dilutions. While there is cross reactivity, the EBOV MFI values are consistently greater than the other viral GPs and at higher dilutions the separation becomes significant.

C.2.4. Expected outcomes. We anticipate obtaining serological profiles of exposure to filoviruses and henipaviruses from each individual in our cohort. In previous studies we found antibodies against both Ebola and Marburg virus. While the Ebola antibodies are attributable to the epidemic, Marburg antibodies, in

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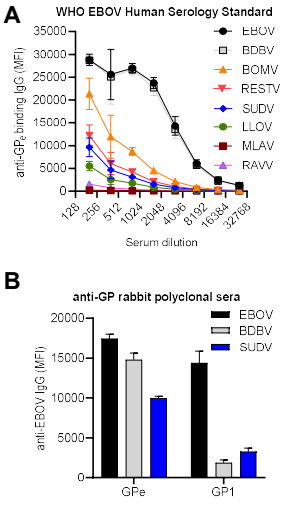
patients EBOV IgG negative, suggest possible exposure from an animal (e.g., bat) reservoir. We also anticipate seeing reactivity ~~to~~ to Bombali virus (BOMV), given the presence of the reservoir species in Liberia (*Mops condylurus*) and reactivity to the African henipaviruses (GhV and AngV), although this may represent reactivity to an antigenically related virus. Serological results from this and the bat study will inform our strategy for prioritizing which bat samples we test by PCR.

C.2.5. Potential problems and solutions. While our preliminary data suggest there will be detection of immune responses to filoviruses including EBOV-Zaire and, significantly, MARV, it is possible that evidence of exposure to other pathogens, including henipaviruses will not be detected. This would be an important finding in this large cohort, especially if these viruses are detected in local bat species, that would warrant further investigation. It is also possible that there will be few or no seroconversion events among the cohort during the 24-month period of follow-up. This too would be reassuring but would suggest a need for longer follow-up of this cohort. To address the possibility that reactivity to EBOV-Zaire virus may be explained by infection during the 2013 Ebola epidemic, we will create a polyclonal sera reference standard utilizing convalescent sera collected from human survivors of EVD. This internal reference standard will be calibrated to the WHO EBOV human serology standard (**Fig 6A**) permitting direct interpolation of anti-EBOV IgG as an MFI to a standard antibody concentration (binding antibody units/mL). The internal reference standard will then be included in our human serology testing strategy allowing semi- or quantitative measurement of anti-EBOV IgG. These EBOV human serology standards will permit us to establish positive and negative predictive performance values for the multiplex assay, furthermore, we will be able to investigate immunoreactivity of EVD survivors against heterotypic ebolaviruses. Our preliminary data indicates that EBOV+ sera IgG is highly cross-reactive with soluble native-like trimeric envelope glycoprotein (GP) antigens from both EBOV and BDBV (Figure 6A). EBOV GP, matrix protein (VP40), and nucleocapsid protein (NP) antigens have all been used in a variety of serological tests, however, the frequency of seroconversion against each antigen has not been fully described. In a longitudinal study of EVD survivors, high seroconversion against GP and VP40 was observed, with affinity maturation occurring overtime against GP[45]. Antigens based on the GP are likely to provide an optimal intrinsic balance of sensitivity and specificity. To limit the degree of heterotypic ebolavirus cross reactions we will test any anti-ebolavirus GP trimer antigen-seropositive samples with a pan-ebolavirus GP1 antigen-based panel. In preliminary studies, GP1 antigens improve specificity and differentiation of antibodies reactive with homotypic ebolaviruses in polyclonal antisera from rabbits immunized with GP trimer immunogens (**Figure 6B**).



Figure~~IGURE 6A & B~~. A) The WHO EBOV human serology standard was testing for immunoreactivity in a filovirid GP-trimer based multiplex assay. Filovirids are indicated along with sera dilution factors; data is the mean of three independent experiments. **B)** Antisera from EBOV GP-trimer immunized rabbits were tested in multiplex panels comprised of GP-trimer and GP1 subunit proteins. Anti-EBOV polyclonal IgG was less reactive with GP1 from BDBV and SUDV.

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C.3. Aim 2. To identify reservoirs and characterize the infection dynamics of filoviruses and henipaviruses in key bat species in Liberia.

C.3.1 *Rationale and preliminary data.* Bats have been established as important hosts for filoviruses and henipaviruses in Africa and Asia, with combinations of viral isolation, molecular detection or serology providing substantial evidence [30,46-49]. MARV was isolated from Egyptian rousette bats in Uganda, and RNA has since been detected in these bats in Sierra Leone, which borders Liberia [50]. To date, EBOV has never been isolated from bats, although molecular and serological evidence suggest that multiple frugivorous bats may carry EBOV in Central Africa[50,51]. Prior to the 2014 epidemic, Zaire ebolavirus had not been reported in West Africa, and the animal reservoir remains unknown[32]. From 2016-2019, our group (PI Epstein & KP Desmond) implemented a large-scale project to identify reservoirs for EBOV in West Africa, under the USAID PREDICT Project[52]. This effort led to the discovery of a new species of Ebola (Bombali virus)[53], the detection of MARV in ERBs in Sierra Leone[33], and **in 2018, we detected, for the first time in West Africa, EBOV RNA and anti-EBOV IgG antibodies in bats (Figure 7A-D)**.

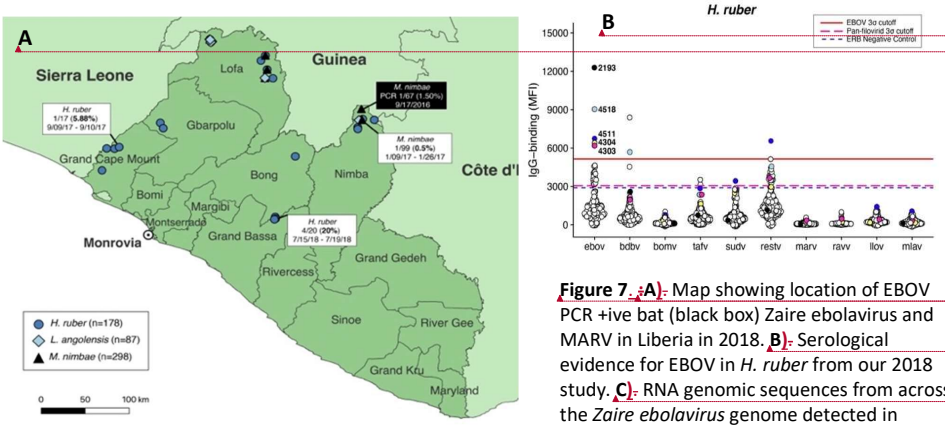
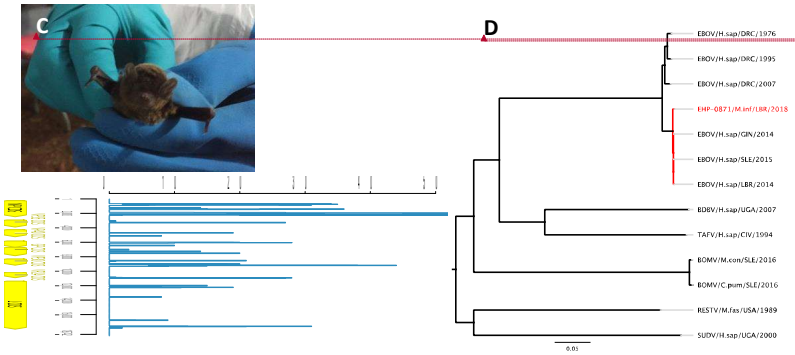


Figure 7. A) Map showing location of EBOV PCR +ive bat (black box) Zaire ebolavirus and MARV in Liberia in 2018. **B)** Serological evidence for EBOV in *H. ruber* from our 2018 study. **C)** RNA genomic sequences from across the *Zaire ebolavirus* genome detected in *Miniopterus nimbae*, Liberia in 2018. **D)** Phylogenetic analysis supports relatedness to 2013 EBOV epidemic virus.



While this finding generated the first evidence of which bats

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may be reservoirs for Zaire ebolavirus, longitudinal studies are required to more completely understand the role these species play in EBOV circulation – either as reservoirs that maintain the virus within a local population, or as incidental hosts, infected by an unknown bat species. One-off, cross-sectional studies have a low probability of detecting any specific filovirus or henipavirus given their low prevalence and short viremic periods [34,54], and thus provide a poor characterization of viral diversity. The lack of understanding of **filovirus and henipavirus diversity and their natural reservoirs in West Africa significantly impedes risk-based public health interventions to prevent human outbreaks.** Longitudinal sampling is also needed to separate reservoir hosts from spillover hosts. For example, MARV has been isolated repeatedly from *R. aegyptiacus* in Uganda, which, when paired with experimental infection studies showing infection and shedding of MARV by *R. aegyptiacus,* strongly suggests that *R. aegyptiacus* is indeed a Marburg virus reservoir[35,55,56]. Similarly, our longitudinal studies of Nipah virus in *Pteropus medius* bats in Bangladesh, which included the same multiplexed serological assay proposed here, confirmed them as a reservoir for NIV and identified important mechanisms driving viral circulation[29]. **Understanding which species persistently carry filoviruses and henipaviruses will provide important insights into how they are maintained in nature and what species should be targeted for follow-up studies on bat habitat use and human-bat contact patterns.**

C.3.2. *Sampling design*. We will repeatedly sample three bat species associated with filoviruses or henipaviruses, based on our preliminary data: *M. nimbae* (EBOV), *H. ruber* (EBOV), and *R. aegyptiacus* (MARV & Henipaviruses). We will sample bats at roost sites in Bong county, as close as possible to the human study sites in Aim 1. In Y2-Y4, we will sample 40 individuals of each species every two months for 36 months and collect excreta using plastic tarps placed underneath bat roosts (details below and in C3.3).

Bat capture and sampling. Briefly, we will use either harp traps or mist nets to capture bats, depending on the capture site. Personnel will wear appropriate personal protective equipment (e.g., Tyvek suits or dedicated long clothing; double nitrile gloves; an N95 respirator and safety glasses or a PAPR) [57]. Traps and nets will be attended continuously during capture periods. Bats will be removed from mist nets immediately upon entanglement to avoid injury. Bats will be removed from harp traps after approximately 1 hour and ~~bats will be~~ placed in individual cloth bags until processed. Body measurements, blood, oropharyngeal swabs, and rectal swabs (or fecal pellets) will be collected from each bat. Swabs will be collected in duplicate; one swab will be placed in a cryovial containing Trizol lysis buffer and the other in viral transport medium (VTM). For bats <50g, blood will be collected in a microhematocrit tube and spun down using a portable centrifuge. A bulb will be used to expel serum from the tube into a cryovial. For larger bats >50g, a tuberculin syringe or 1ml syringe and 27g needle will be used to draw blood from the radial artery or vein[57]. Blood will be placed into a conical vial and centrifuged. Serum will be aliquoted into a cryovial using a pipette. During site visits we will also lay out plastic tarps to collect pooled environmental excreta in Trizol and VTM to improve opportunity to detect viral RNA. All samples will be placed in a vapor phase liquid nitrogen dry shipper or portable battery-powered ultracold freezer, immediately upon collection and then transferred to a -80C freezer at the Phebe lab until testing.

C.3.3. *Testing plan, Data analysis, sample size, and power analysis*.

Serology: At the end of Y3, we will screen all serum samples collected to date using the MAGPIX multiplex assay for IgG against filoviruses and henipaviruses. We will analyze temporal dynamics in seroprevalence using general additive models (GAMs), which are analyses that we have used successfully in many previous studies of similar design[29]. These methods are well adapted for capturing long-term temporal fluctuations in prevalence and moderately sensitive at capturing short-term changes in seroprevalence which can be used to identify population-level disease outbreaks. For example, we estimated that with 40 bats sampled every two months, we would be able to statistically detect an increase in seroprevalence (at a 95% CI limit) of greater than 25% between consecutive sampling events > 80% of the time, assuming a bat population seroprevalence of at least 10% in advance of a bat colony infection (estimated by, in brief, by simulating a fluctuating true seroprevalence value, simulating samples given this underlying seroprevalence, fitting a GAM to these samples, and then calculating if CI on true prevalence between the two sampling occasions over which the change in seroprevalence occurred were non-overlapping). While this is a large change, it is within a range to

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be expected if infection were to sweep through a bat community[29]. MAGPIX-positive bat sera will be sent to RML for serum neutralization assays using viral isolates or VSV pseudoviruses under BSL4 conditions.

VSV-pseudotype neutralization assays: Serological results will be confirmed using VSV-pseudotype neutralization assays for the following filoviruses EBOV, SUDV, BDBV, RETV, BOMV, MARV and RAVN. If novel henipavirus sequences are detected, additional assays will be developed to test positive sera. Serum samples will be inactivated using γ-irradiation (4 MRad). Neutralization of irradiated and heat-inactivated serum samples will be assessed in Vero 352 E6 cells. Briefly, cells were seeded in 96-well round-bottom plates for 24 hours, serial dilutions of heat-inactivated serum samples will be performed in DMEM supplemented with 2% FBS, penicillin/streptomycin, and L-glutamine. Each plate will contain negative serum control, cell-only control, and virus-only control. VSV EBOV-GFP will be added to each well of the serum dilution plate and the serum-virus mix 357 was incubated at 37˚C for 1-hour. The mix was added to the cells and incubated at 37˚C for 24 hours. The cells will then fixed with 4% paraformaldehyde at room temperature 359 for 15-minutes and centrifuged at 600 x g for 5-minutes at room temperature. The supernatant will be discarded and FACS+EDTA buffer was added. Samples will be run on the FACSymphony A5 Cell Analyzer (BD Biosciences, Mississauga, ON, Canada) and FITC MFI 362 was measured. Data will be analyzed using FlowJo.

Molecular testing: We will use specific filovirus taqman real-time PCR assays to screen oropharyngeal, fecal and environmental samples from bats for EBOV and MARV RNA at the UNC-Phebe lab[55,58]. Testing bat samples by PCR may allow us to detect viral RNA during active infection, providing important information about the prevalence and timing of infection within target species. **Because we expect viral prevalence to be significantly lower than seroprevalence, estimated at <5%[25,27,49],** we developed a sampling scheme focused on maximizing our probability of detecting viral RNA with qPCR. To compare power under alternative sampling designs, we built simulation-based power analyses that estimated power to detect: 1) viral EBOV or MARV RNA in at least one bat; and 2) the expected number of positive cases, in each bat species. In brief, we estimated power for total sample sizes between 1500-3000 (between 500 and 1000 bats sampled per species) spread across a variable number of visits per year (2-6), while allowing for uncertainty in true infection dynamics in the bat populations, specifically: 1) prevalence during one three-week period (with unknown timing) per year of high prevalence ranging from 1-5%); and 2) an otherwise low background prevalence (0-1%). Given these estimates of yearly infection dynamics, these analyses showed that with 672 samples per species (minimum 32 individual bats per sampling event), spread across 6 sampling occasions per year, we would obtain a per-species probability of acquiring 0 positive samples of <4% (<1% at 40 bats per event – our target number) and an expectation of ~3-6 RNA positive results (~5-10 positives at 40 bats per event). Because henipaviruses have not been previously described in Liberia, we will focus our resources on filovirus testing by PCR. However, if henipavirus serology suggests active circulation, we may send a set of samples to RML for pathogen discovery (see RML letter of support).

Viral isolation: PCR-positive cDNA samples and their aliquot in VTM will be sent to RML for further sequencing and attempts at viral isolation under BSL4 conditions. If attempts to culture do not work, we will use next generation sequencing techniques to get whole genome sequence and further characterize the virus at RML. Novel viruses will be characterized and rescued (reverse engineered using a minigenome system to synthetically create infectious viral particles [59]) at RML under secure, biosafety level 4 conditions, then used for serum neutralization assays in bat and human sera to determine more precise exposure rates. Sequences from the glycoproteins of any novel viruses discovered will be used by USU (Co-I Laing) to generate purified proteins and added to the Luminex assay, which will improve its specificity.

VirCapSeq-VERT and myBaits probe-based enrichment for recovery of full-length genome sequences of filoviruses: Although shotgun sequencing provides opportunities to analyze all host and virus genetic material, the larger average genome size of bacteria and hosts compared to viral genomes complicates a detailed analysis of the virome, particularly vertebrate viruses. We have established a positive selection probe capture-

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based system to enrich for viral sequences. Here, we will take advantage of these probe capture-based methods (VirCapSeq-VERT and myBaits probe library) to enrich for vertebrate viral sequences of interest. Sample libraries will be normalized and combined in 4- to 12-plex reactions for solution capture hybridization using either the HyperExplore custom bait library version of VirCapSeq-VERT probe set or our custom myBaits probe library. For HyperExplore custom bait library probe set, libraires will be enriched for virus following the SeqCap EZ HyperCap Workflow User's Guide, version 2.3, while for custom myBaits probe library, the myBaits Hybridization Capture for Targeted NGS protocol, Version 4.01 will be used. Sequencing libraries will be normalized and sequenced as 2 X 150 bp fragments on Illumina's MiSeq or NextSeq sequencing platforms, following Illumina's standard procedure (Illumina, San Diego, CA). NGS data will be analyzed using metavirs (https://openomics.github.io/metavirs/), a comprehensive viral metagenomics pipeline to assemble, annotate, and classify viruses. It relies on technologies like Singularity1 to maintain the highest-level of reproducibility. The pipeline consists of a series of data processing and quality-control steps orchestrated by Snakemake2, a flexible and scalable workflow management system, to submit jobs to a cluster. The pipeline is compatible with data generated from Illumina short-read sequencing technologies. As input, it accepts a set of FastQ files and will be run on-premises using the NIAID RML Big Sky supercomputing cluster.

C.3.5. *Expected outcomes.* -Based on our prior work, our multifaceted analysis approach, and calculated power given our proposed temporal sampling strategy, we expect to be able to use temporal serological data to describe filovirus and henipavirus viral dynamics over time. Our molecular screening will primarily focus on filoviruses because we have known targets, and we expect to detect EBOV or MARV RNA, despite low prevalence and short-lived infections. Sequence data from positive samples will 1) provide substantial evidence for the identification of EBOV reservoirs and help explain which virus is responsible for the observed IgG response in bats (and possibly people); and 2) provide valuable information about viral diversity, about which little is known. Henipavirus serology, in the absence of sequence data, will allow us to identify potential bat reservoirs, analyze temporal trends, and generate hypotheses for future targeted studies.

C.3.6. *Potential problems and solutions.*
Serological tests are greatly hindered by the inherent antibody cross-reactivity between antigens from known ebolaviruses, challenging data interpretations that antibodies are EBOV-specific or EBOV, BDBV, and SUDV cross-reactive [40]. This is also true for the henipaviruses in the panel. The advantage of the multiplexed platform is that we can simultaneously see relative MFI values for all the filoviruses and henipaviruses. Glycoproteins from the homologous virus should cause the most intense reaction, particularly at higher dilutions. Co-Is Laing and Munster have demonstrated that homotypic and heterotypic ebolavirus-antibody reactions can be simultaneously measured and accounted for in ERBs challenged with replication-competent VSV-filoGP (**Figure 8**). Still, there is some cross reactivity, and we will use additional tools such as viral neutralization assays as a secondary test, and our statistical approach can correct for a portion of cross reactivity. Past studies have relied on individual ELISAs which had varying degrees of specificity depending on which test was used, but often couldn't rule out cross-reactivity. The Luminex filovirus multiplex assay has predictable patterns of cross-reactivity, but we will still be able to discern to which virus the serum is most reactive.

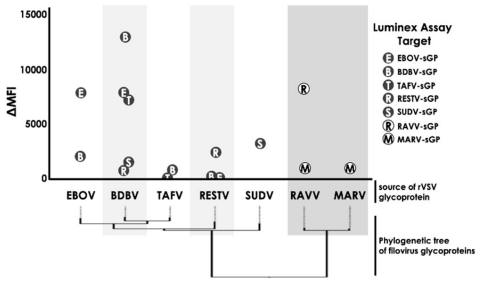

Figure 8. Captive-bred ERBs were experimentally challenged with VSV-filoGPs, the GP source is indicated on the x-axis. In a GP-trimer multiplex test the homotypic ebolavirus bat antisera were most reactive with the homotypic ebolavirus GP-trimer antigen.

C4. Aim 3: To characterize behaviors associated with exposure to filoviruses and henipaviruses.

C.4.1 Background and rationale. As described above, there is substantial interaction between wildlife, domestic animals, and people living in rural Liberia. ~~-~~Our group collected questionnaire data from 585 people across Liberia as part of the USAID-funded Ebola Host Project between 2016 and 2019. ~~-~~When asked about animal exposures during the 2013-2016 Ebola outbreak, participants frequently reported contact with both domestic and wild animals. Common types of animal contact included animals coming inside the dwelling (58%), cooking or handling animal meat (44%), and eating raw or undercooked meat (31%). Additionally, 26% reported slaughtering and 12% reported hunting or trapping an animal. In the ENABLE Study of 5,005 residents of Bong County, survey questions mainly focused on rodent exposures relevant to Lassa fever, but 12% of participants reported hunting or preparing wild animal meat. Little is known about the prevalence and type of other potential bat exposures in this region of Liberia. Elsewhere in West Africa, EcoHealth Alliance participated in the response to Ghana's 2022 cluster of MARV cases, working with local partners to administer a behavioral risk questionnaire characterizing bat exposures in three affected communities[16]. Many participants lived in close proximity to bats, with one fifth reported exposure to bats inside a dwelling or other building in the previous four months. When asked about exposures more relevant to Egyptian Rousette bats, a known MARV reservoir and a target species in this proposed study, participants commonly reported bats feeding on fruit trees on their home compound, eating fruit bearing bite marks, and exposure to bats inside a cave or mine.

C.4.2. *Research design*. As described in Aim 1, serum collected at baseline (in 2021) and at month 24 (in 2023) during the ENABLE Study will be screened for filoviruses and henipaviruses, enabling us to calculate seroprevalence at baseline and seroconversion over a two-year period. In addition to providing blood specimens, the 1,024 participants who initially enrolled responded to baseline questionnaires on demographics, healthcare-seeking behaviors, health status, and animal exposures. At month 24, the 921 participants still actively enrolled are administered repeat questionnaires. This final survey includes additional items based on the Ghanaian questionnaire and asks specifically about bats including the respondents' activities in their habitats (entering forests, caves, and mines), direct contact (hunting, eating handling), indirect contact (presence in home or other used structure), and observed characteristics of bats encountered (fruit tree dwelling). By pairing this existing survey data with the new serology results generated from archived samples under Aim 1, as well as new data on nearby bat infection from Aim 2, we will be able to develop comprehensive risk profiles for exposure to filoviruses and henipaviruses in this community.

C.4.3. *Data analysis.* The primary outcome for this analysis will be testing positive for IgG antibodies against any known filoviruses or henipaviruses. For both families of viruses, and for each species of virus within those families, we will use the serology results generated under Aim 1 to examine risk factors for seropositivity at baseline and seroconversion between baseline and month 24. We plan to conduct a multivariate logistic regression, with appropriate weights applied to account for the single-stage cluster sampling design of the ENABLE Study, in which members of randomly selected households were surveyed and tested. We will use data from both the baseline and month 24 questionnaires to examine the effects of sampling site, participant demographics (e.g., age, gender, occupation), environmental exposures (e.g., animals present in and around the dwelling), and behavioral risks (e.g., direct contact with bats through hunting, preparing, or eating them, entering caves or mines where bats live).

Power analysis: To assess our power to identify risk metrics for seroconversion (or overall seropositivity), we built simulation-based power analyses that consisted of, in brief: 1) simulating survey responses for N (400-1000) individuals, while allowing for moderate unevenness in representation across five binary categories (as largegreat as 80%-20,% for eachexample); 2) examining a range of effect sizes (odds ratios ranging from 1-2.5) for these five binary categorical predictors (e.g., sex, encountered a bat, handled a bat, etc.). Summarizing across 1,200 simulations of surveys of 400-1000 individuals given joint uncertainty in the representation within these surveys and effect sizes, we estimated that we would have at least 80% power

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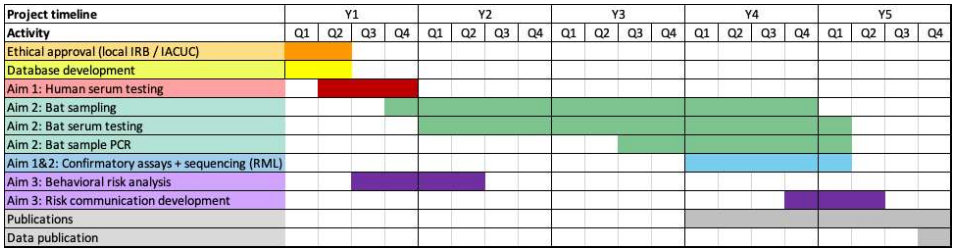
to detect significant effects for each predictor given an odds ratio of 1.65 or greater with 1,000 survey responses or ~1.80 (for example, with a rate just under 5% for Group1 = 0, just over 8% for Group1 = 1) with 750 responses.

C.4.4. *Expected outcomes.* By combining serology results with detailed questionnaire data on participant demographics and animal exposures, we will be able to use odds ratios to identify behaviors significantly associated with seropositivity (e.g., exposure to filoviruses or henipaviruses). We expect to be able to also identify age and gender-based risk factors given our sample size. Based on previous experience conducting behavioral risk surveys, including a recent study in Ghana following a Marburg outbreak, we expect to be able to develop risk mitigation messaging that uses evidence from Aims 1 and 2 as well as results from this aim.

C.4.5. *Potential problems and solutions*. We anticipate that most IgG positive individuals for either set of viruses will be adults, with the likelihood of exposure and having antibodies increasing with age. Because we don't know how long IgG antibodies persist at detectable levels, it will not be possible to accurately determine when exposure occurred, with the exception being EBOV antibodies that most likely occurred as a result of contact with another infected person during the 2014-2016 outbreak. We will address this in two ways: by looking at respondents 1-6 yrs old, we will know that any filovirus antibodies they have are most likely not due to the epidemic, and we will be able to determine whether there are any significant associations with particular behavior that promotes direct animal contact, consumption of contaminated fruit, or exposure to bat habitats (e.g. caves or mines). For adult respondents, we will focus on exposures (based on relative MFI values) specific to non-EBOV filoviruses and henipaviruses and see whether there are associations with specific behaviors.

C.5. Project timeline



D. Summary and Conclusions.

West Africa is a hotspot for emerging zoonotic viruses such as Ebola, Marburg and potentially Nipah-related henipaviruses – groups that have been identified by WHO as among the most significant pathogens that threaten global health. Liberia is also a biodiversity hotspot, with some of the largest tracts of primary forest remaining in the region, and its population relies on wildlife as a primary source of protein, creating continued risk of spillover of high consequence zoonotic pathogens. This project has the potential to determine the origins of Zaire ebolavirus in West Africa, characterize the circulation of Ebola and other filoviruses and henipaviruses in common bat species, identify background rates of spillover in rural human populations, and identify high-risk behaviors associated with exposure to these bat-borne zoonoses. Our group comprises an unparalleled multidisciplinary group of leading experts in epidemiology, clinical medicine, virology, serology, and disease ecology all with deep experience working in Liberia and implementing zoonotic virus research, giving this project a high chance of success in generating impactful results that will help prevent future epidemics in West Africa.

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From: Morgan Kain on behalf of Morgan Kain <kain@ecohealthalliance.org>
To: Wohl, David A
Cc: Jon Epstein; Fischer, William A. II; Eric Laing; Munster, Vincent (NIH/NIAID) [E]; Shannon Ball; Madeline Salino
Subject: Re: Complete draft 7 for rapid review
Date: Thursday, February 2, 2023 4:23:11 PM
Attachments: Liberia R01 Technical proposal d7_clean MPK.docx

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Jon,

Minor edits and tidying throughout + a few comments. Happy to go through v8 tomorrow morning (if you would like me to) to conduct any needed cleanup that slips through (you will see the sort of thing I am talking about when you open the document).

--- Morgan

On Thu, Feb 2, 2023 at 3:02 PM Wohl, David A <david_wohl@med.unc.edu> wrote:

> On it. Will review and send back shortly. Thanks
>
> D
>
> ---
>
> **From:** Jon Epstein <epstein@ecohealthalliance.org>
> **Date:** Thursday, February 2, 2023 at 2:31 PM
> **To:** Fischer, William A. II <william_fischer@med.unc.edu>, Wohl, David A <david_wohl@med.unc.edu>, Eric Laing <eric.laing@usuhs.edu>, Munster, Vincent (NIH/NIAID) [E] <vincent.munster@nih.gov>, Shannon Ball <ball@ecohealthalliance.org>, Morgan Kain <kain@ecohealthalliance.org>
> **Cc:** Madeline Salino <salino@ecohealthalliance.org>
> **Subject:** Complete draft 7 for rapid review
>
> Hi all,
>
> I've compiled a near final draft, incorporating the text you each provided and then cutting it down to fit within the 13 page (1 page specific aims and 12 page research strategy) limit. Thank you all for excellent contributions to this. I'm sure it's not perfect, but I think it's solid.
>
> If you're able to go through it today and check it for overall readability and accuracy where you have preliminary data, that would be fantastic. Please do use track changes and change the file name to add your initials when you edit, and please do not add too much text, as there's not much space at the end.
>
> I do think Aim 3 might be a little thin, so if Shannon, Dave and Billy could look through

that and see if you think it's missing anything, there's space for a few extra lines there.

Please return your edits to me by 7pm, if possible, so I can finalize the draft. Our plan is to submit at noon tomorrow and deal with any errors that may pop up.

While you're reviewing this, I'll work on the summary and narrative, and other peripheral pieces.

Thanks again. We're in the home stretch!

Cheers,
Jon

--

Jonathan H. Epstein DVM, MPH, PhD

Vice President for Science and Outreach

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

--

Morgan Kain, PhD

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

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: Assistant Professor

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. (hons)	05/2008	Biology
Uniformed Services University, Bethesda, MD	Ph.D.	10/2016	Emerging Infectious Diseases
Uniformed Services University, Bethesda, MD	Postdoc	04/2019	Virology

A. Personal Statement

The spillover of zoonotic viruses into human populations remains an ever-prevalent threat to public and global health. Continual outbreaks of known and unknown zoonotic viruses highlights the paucity of our understanding of the viral diversity, geographic distribution, wildlife hosts, and human populations at-risk for spillover. I am a recently appointed assistant professor dedicated to biosurveillance and virological research of emergent zoonotic viruses. I have completed virological training and spent my time as a post-doctoral fellow applying *in vitro* techniques to characterize the replication and mammalian ephrin receptors that mediate cellular entry of emergent henipaviruses. Since the start of the COVID-19 pandemic, I pivoted the focus of my research team to serological analysis of SARS-CoV-2 infection, and then to COVID-19 vaccine-induced antibody durability. In my ongoing research, I develop serological-based approaches to identify the infectome of wildlife and human communities for the assessment of virus spillovers. Since my position as a postdoctoral fellow I have worked collaboratively with national and international researchers including institutional partners at Duke-NUS, Chulalongkorn University, University of Pretoria, and Rocky Mountain Labs. Across all of these international collaborations, we have aimed to identify known and unknown viruses, the wildlife reservoirs of priority pathogens, the high-risk interfaces for spillover and the biotic/abiotic drivers of virus emergence. Throughout these research projects, we have detected unexpected serological profiles in communities of bats and humans that have not fit our prior understanding of the viral diversity established or detected by genetic techniques. This proposal aims to better understand these serological profiles of wildlife and humans with a targeted focus on henipaviruses and filoviruses. Ongoing projects that I would like to highlight include:

- HDTRA12110037. DTRA BRTP, E. Laing (Co-PI). 08/2021-07/2026,"Informing biosurveillance, contribution of pteropodid fruit bats to virus spillover in the Philippines."
- U01AI151797. NIH, Centers for Research in Emerging Infectious Diseases, E. Laing (Co-I). 02/2020 – 03/2025, "EID-Southeast Asia Research Collaborative Hub."
- HDTRA12010025. DTRA BTRP, E. Laing (Co-I). 7/2020 – 6/2025, "Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa"
- HU00012020067, HU00011920111. Defense Health Program, NIAID, E. Laing (Associate Investigator). 03/2020 – 09/2023, "Epidemiology, Immunology, and Clinical Characteristics of Emerging Infectious Diseases with Pandemic Potential (EpICC-EID)."

B. Positions, Scientific Appointments and Honors

Positions

2021-pres. Assistant Professor, Department of Microbiology and Immunology, School of Medicine, Uniformed Services University, Bethesda, MD.

2021-pres. Joint Appointment, Emerging Infectious Diseases Graduate Program, School of Medicine, Uniformed Services University, Bethesda, MD.

2019-21	Research Assistant Professor, Department of Microbiology and Immunology, Uniformed Services University, Bethesda, MD.
2016-19	Postdoctoral fellow, Henry M. Jackson Foundation, Department of Microbiology, Uniformed Services University, Bethesda, MD.
2010-16	Graduate research student, Department of Microbiology, Uniformed Services University, Bethesda, MD.

Other Experiences and Professional Memberships

2022-pres.	Executive committee member, Emerging Infectious Diseases Graduate Program, USUHS
2021-pres.	Research center representative, Laboratory Assays Oversight and Quality Working Group, Emerging Infectious Diseases: Southeast Asia Research Collaboration Hub (EID-SEARCH), Centers for Research in Emerging Infectious Diseases (CREID), NIAID, DMID, NIH
2021-pres.	Review editor, Frontiers in Virology - Emerging and Reemerging Viruses
2021-pres.	Ad hoc reviewer, Frontiers in Immunology, Journal of Clinical Immunology
2021-pres.	Steering committee member, JPI/Military Infectious Diseases Research Program, Emerging Infectious Diseases
2019	Ad hoc reviewer, EcoHealthNet 2.0 Program, EcoHealth Alliance
2018	Ad hoc reviewer, Pakistan One-Health Fellowship Program, National Academy of Sciences & Pakistan Academy of Sciences
2014-2019	Member, American Society of Tropical Medicine and Hygiene
2014-2019	Member, American Society of Microbiology
2014-2019	Volunteer, AAAS STEM K-12 Volunteer Program

Mentoring

Postdoctoral fellows	Si'Ana A. Coggins, PhD, 2020 - 2022
Graduate students	Marana S. Tso, BS, 2021 -
	McKenna Roe, BS, 2022 -
Committee member	Celeste Huaman, BS, 2021 -
	2LT Connor Perry, BS, 2021 -

Honors

2021-2022	Impact Award, USUHS School of Medicine
2021	Outstanding Research Accomplishment/Team/SARS-CoV-2, The EPICC COVID-19 Cohort Team, Military Health System Research Symposium
2020-2021	Impact Award, USUHS School of Medicine
2015-2016	Val G. Hemming Fellowship, Henry M. Jackson Foundation
2015	East Asia and Pacific Summer Institutes Fellowship, National Science Foundation

C. Contributions to Science

1. Lyssaviruses and the prototype, rabies virus, remain a public health concern. Beginning with my PhD thesis work, I've researched the virus host-interactions between a rabies-related lyssavirus, Australian bat lyssavirus (ABLV), and its bat host (*Pteropus alecto*). Research has focused on ABLV cellular entry mechanisms, the development of an animal model and ABLV reporters and exploration of novel monoclonal antibodies that neutralize ABLV and other phylogroup I lyssaviruses. Furthermore, comparative bat immunology research was conducted using black flying fox cell lines and ABLV as a model virus/host interaction. Physiological adaptations that accompanied the evolution of flight in bats have been proposed to contribute to the frequent role of bats as asymptomatic hosts of highly pathogenic zoonotic viruses. Comparatively studying the autophagy pathway in bat cell lines revealed that bat cells had elevated levels of basal autophagy and experienced significantly less cell death when challenged with high virus doses.

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. Virol J. 2013. 11:40. doi: 10.1186/1743-422X-11-40. PMID: 24576301, PMCID: PMC3946599

b. **Laing E.D.**, Sterling S.L., Weir D.L., Beauregard C.R., Smith I.L., Larsen S.E., Wang L-F., Snow A.L., Schaefer B.C., and Broder C.C. Enhanced autophagy contributes to reduced viral infection in black flying fox cells. Viruses. 2019. Mar 14;11(3). pii: E260. doi: 10.3390/v11030260. PMID: 30875748, PMCID: PMC6466025

c. Mastraccio K.E., Huaman C., Warrilow D., Smith G.A., Craig S.B., Weir D.L., **Laing E.D.**, Smith I., Broder C.C. and B.C. Schaefer. Establishment of a longitudinal pre-clinical model of lyssavirus infection. J Virol Methods. 2020 Jul; 281:113882. doi: 10.1016/j.jviromet.2020.113882. Epub 2020 May 12. PMID: 32407866

d. Weir D.L., Coggins S.A., Vu B.K., Coertse J., Yan L., Smith I.L., **Laing E.D.**, Markotter W., Broder C.C., and Schaefer B.C. Isolation and characterization of cross-reactive human monoclonal antibodies that potently neutralize Australian bat lyssavirus variants and other phylogroup 1 lyssaviruses. Viruses. 2021 Mar 1;13(3):391. doi: 10.3390/v13030391. PMID: 33804519; PMCID: PMC8001737.

2. My research experience as a postdoctoral fellow furthered my training in molecular virology techniques. I constructed a recombinant Cedar virus cDNA plasmid and optimized a reverse genetics approach to rescue a recombinant Cedar virus reporter virus, a non-pathogenic *Henipavirus* species. A molecular biology methods chapter detailing recombinant Cedar virus reverse genetics has been submitted and in press. Using this recombinant Cedar virus, we determined that Cedar virus can utilize several non-canonical henipavirus ephrin receptors for cellular entry and explored the structure of the receptor-binding pocket to understand the receptor promiscuity. The non-pathogenic phenotype of CedV creates a potential for CedV to act as a model henipavirus to explore host-pathogen interactions, cellular tropism and factors that determine henipaviral disease pathogenesis. Additionally, I have collaborated on projects detailing henipavirus infection and replication in bat hosts with colleagues at the Rocky Mountain Labs, studying whether specific species of bats are more competent hosts and whether virus-host restriction exists.

a. Amaya M, Broder CC, **Laing ED**. Recombinant Cedar virus: a henipavirus reverse genetics platform. In: Freiberg A.N. and B. Rockx, Nipah Virus: Methods and Protocols, Methods Mol. Biol. (in press)

b. Seifert SN, Letko MC, Bushmaker T, **Laing ED**, Saturday G, Meade-White K, van Doremalen N, Broder CC, Munster VJ. Rousettus aegyptiacus Bats Do Not Support Productive Nipah Virus Replication. J Infect Dis. 2020 May 11;221(Suppl 4):S407-S413. doi: 10.1093/infdis/jiz429. PMID: 31682727; PMCID: PMC7199784.

c. **Laing ED,** Navaratnarajah CK, Cheliout Da Silva S, Petzing SR, Xu Y, Sterling SL, Marsh GA, Wang LF, Amaya M, Nikolov DB, Cattaneo R, Broder CC, Xu K. Structural and functional analyses reveal promiscuous and species specific use of ephrin receptors by Cedar virus. Proc Natl Acad Sci U S A. 2019 Oct 8;116(41):20707-20715. doi: 10.1073/pnas.1911773116. Epub 2019 Sep 23. PMID: 31548390; PMCID: PMC6789926.

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. PMID: 29587789; PMCID: PMC5869790.

3. Bats are increasingly identified as animal reservoirs of emerging zoonotic viruses (e.g. Nipah virus, Ebola virus and SARS-coronavirus). I lead collaborative biosurveillance and research preparedness training including data analysis and interpretations at international partner institutes with lab technicians, field and lab scientists, and masters, doctoral and postdoctoral trainees. Collaborative biosurveillance is presently underway in Thailand (Chulalongkorn University, Bangkok) Malaysia (National Wildlife and Forensic Lab, Universti Purtra Malaysi, National Public Health Lab) via NIH Centers for Research in Emerging Infectious Diseases, EID-Southeast Asia Research Collaborative Hub. As a collaborator within the DARPA PREEMPT network I supported surveillance for coronaviruses and other priority emerging zoonotic viruses, henipaviruses and filoviruses, in Ghana (Zoological Society of London), Australia (Black Mountain Labs) and Bangladesh (icddr,b). We aim to characterize the geographic distribution of zoonotic filoviruses/henipaviruses/coronaviruses, transmission dynamics in wildlife hosts and generate risk-models for Ebola virus, Nipah virus and SARS-related CoV outbreaks. Results discovered so far suggest a wider

geographical footprint of Asiatic filoviruses and have identified several fruit bat species that act as natural reservoirs for these viruses.

a. Paskey AC, Ng JHJ, Rice GK, Chia WN, Philipson CW, Foo RJH, Cer RZ, Long KA, Lueder MR, Lim XF, Frey KG, Hamilton T, Anderson DE, **Laing ED**, Mendenhall IH, Smith GJ, Wang LF, Bishop-Lilly KA. Detection of Recombinant Rousettus Bat Coronavirus GCCDC1 in Lesser Dawn Bats (Eonycteris spelaea) in Singapore. Viruses. 2020 May 14;12(5):539. doi: 10.3390/v12050539. PMID: 32422932; PMCID: PMC7291116.

b. Schulz JE, Seifert SN, Thompson JT, Avanzato V, Sterling SL, Yan L, Letko MC, Matson MJ, Fischer RJ, Tremeau-Bravard A, Seetahal JFR, Ramkissoon V, Foster J, Goldstein T, Anthony SJ, Epstein JH, **Laing ED**, Broder CC, Carrington CVF, Schountz T, Munster VJ. Serological Evidence for Henipa-like and Filo-like Viruses in Trinidad Bats. J Infect Dis. 2020 May 11;221(Suppl 4):S375-S382. doi: 10.1093/infdis/jiz648. PMID: 32034942; PMCID: PMC7213578.

c. Dovih P, **Laing ED**, Chen Y, Low DHW, Ansil BR, Yang X, Shi Z, Broder CC, Smith GJD, Linster M, Ramakrishnan U, Mendenhall IH. Filovirus-reactive antibodies in humans and bats in Northeast India imply zoonotic spillover. PLoS Negl Trop Dis. 2019 Oct 31;13(10):e0007733. doi: 10.1371/journal.pntd.0007733. Erratum in: PLoS Negl Trop Dis. 2021 Nov 16;15(11):e0009836. PMID: 31671094; PMCID: PMC6822707.

d. **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. PMID: 29260678; PMCID: PMC5749470.

4. Responding to the emergence of SARS-CoV-2, our lab developed multiplex serology strategies to identify SARS-CoV-2 antibodies and address research questions related to whether pre-existing antibody memory induced by prior infection with seasonal human coronaviruses effects COVID-19 severity. Our lab's serology efforts supported NIH and DHA collaboratively funded protocols including prospective, longitudinal serological analysis of hospital and community subjects, and health-care workers; and cross-sectional analyses of SARS-CoV-2 infection among deployed military health-care workers.

a. Goguet E, Powers JH 3rd, Olsen CH, Tribble DR, Davies J, Illinik L, Jackson-Thompson BM, Hollis-Perry M, Maiolatesi SE, Pollett S, Duplessis CA, Wang G, Ramsey KF, Reyes AE, Alcorta Y, Wong MA, Ortega O, Parmelee E, Lindrose AR, Moser M, Samuels EC, Coggins SA, Graydon E, Robinson S, Campbell W, Malloy AMW, Voegtly LJ, Arnold CE, Cer RZ, Malagon F, Bishop-Lilly KA, Burgess TH, Broder CC, **Laing ED**, Mitre E. Prospective Assessment of Symptoms to Evaluate Asymptomatic SARS-CoV-2 Infections in a Cohort of Health Care Workers. Open Forum Infect Dis. 2022 Feb 14;9(3):ofac030. doi: 10.1093/ofid/ofac030. PMID: 35198647; PMCID: PMC8860153.

b. Ramos I, Goforth C, Soares-Schanoski A, Weir DL, Samuels EC, Phogat S, Meyer M, Huang K, Pietzsch CA, Ge Y, Pike BL, Regeimbal J, Simons MP, Termini MS, Vangeti S, Marjanovic N, Lizewski S, Lizewski R, George MC, Nair VD, Smith GR, Mao W, Chikina M, Broder CC, **Laing ED**, Bukreyev A, Sealfon SC, Letizia AG. Antibody Responses to SARS-CoV-2 Following an Outbreak Among Marine Recruits With Asymptomatic or Mild Infection. Front Immunol. 2021 Jun 9;12:681586. doi: 10.3389/fimmu.2021.681586. PMID: 34177926; PMCID: PMC8220197.

c. Clifton GT, Pati R, Krammer F, **Laing ED**, Broder CC, Mendu DR, Simons MP, Chen HW, Sugiharto VA, Kang AD, Stadlbauer D, Pratt KP, Bandera BC, Fritz DK, Millar EV, Burgess TH, Chung KK. SARS-CoV-2 Infection Risk Among Active Duty Military Members Deployed to a Field Hospital - New York City, April 2020. MMWR Morb Mortal Wkly Rep. 2021 Mar 5;70(9):308-311. doi: 10.15585/mmwr.mm7009a3. PMID: 33661864; PMCID: PMC7948931.

d. Lalani T, Lee TK, **Laing ED**, Ritter A, Cooper E, Lee M, Baker M, Baldino T, Mcadoo T, Phogat S, Samuels E, Nguyen H, Broder CC, Epsi N, Richard SA, Warkentien TE, Millar EV, Burgess T, Kronmann KC. SARS-CoV-2 Infections and Serologic Responses Among Military Personnel Deployed on the USNS COMFORT to New York City During the COVID-19 Pandemic. Open Forum Infect Dis. 2021 Jan 23;8(2):ofaa654. doi: 10.1093/ofid/ofaa654. PMID: 33553482; PMCID: PMC7856331.

5. In addition to providing serologic assessment of SARS-CoV-2 infection, my research team is actively engaged in examining the durability of COVID-19 vaccine induced humoral immunity. Antibody responses, particularly neutralizing antibodies, are frequently cited as a predictive correlate of protection. With the emergence of variants of concern and waning circulating antibodies, the timing of booster shots remains an important measure for controlling the pandemic. In my lab we evaluate the duration of neutralizing antibodies, durability and breadth of antibody responses against emerging variants of concern, hybrid immune responses, and post-vaccination infections.

a. **Laing ED**, Weiss CD, Samuels EC, Coggins SA, Wang W, Wang R, Vassell R, Sterling SL, Tso MS, Conner T, Goguet E, Moser M, Jackson-Thompson BM, Illinik L, Davies J, Ortega O, Parmelee E, Hollis-Perry M, Maiolatesi SE, Wang G, Ramsey KF, Reyes AE, Alcorta Y, Wong MA, Lindrose AR, Duplessis CA, Tribble DR, Malloy AMW, Burgess TH, Pollett SD, Olsen CH, Broder CC, Mitre E. Durability of Antibody Response and Frequency of SARS-CoV-2 Infection 6 Months after COVID-19 Vaccination in Healthcare Workers. Emerg Infect Dis. 2022 Feb 24;28(4). doi: 10.3201/eid2804.212037. Epub ahead of print. PMID: 35203111.

b. Lusvarghi S, Pollett SD, Neerukonda SN, Wang W, Wang R, Vassell R, Epsi NJ, Fries AC, Agan BK, Lindholm DA, Colombo CJ, Mody R, Ewers EC, Lalani T, Ganesan A, Goguet E, Hollis-Perry M, Coggins SAA, Simons MP, Katzelnick LC, Wang G, Tribble DR, Bentley L, Eakin AE, Broder CC, Erlandson KJ, **Laing ED**, Burgess TH, Mitre E, Weiss CD. SARS-CoV-2 Omicron neutralization by therapeutic antibodies, convalescent sera, and post-mRNA vaccine booster. bioRxiv [Preprint]. 2021 Dec 28:2021.12.22.473880. doi: 10.1101/2021.12.22.473880. PMID: 34981057; PMCID: PMC8722594.

c. Coggins SA, **Laing ED**, Olsen CH, Goguet E, Moser M, Jackson-Thompson BM, Samuels EC, Pollett SD, Tribble DR, Davies J, Illinik L, Hollis-Perry M, Maiolatesi SE, Duplessis CA, Ramsey KF, Reyes AE, Alcorta Y, Wong MA, Wang G, Ortega O, Parmelee E, Lindrose AR, Snow AL, Malloy AMW, Letizia AG, Ewing D, Powers JH, Schully KL, Burgess TH, Broder CC, Mitre E. Adverse Effects and Antibody Titers in Response to the BNT162b2 mRNA COVID-19 Vaccine in a Prospective Study of Healthcare Workers. Open Forum Infect Dis. 2021 Nov 20;9(1):ofab575. doi: 10.1093/ofid/ofab575. PMID: 35047649; PMCID: PMC8759445.

d. Pollett SD, Richard SA, Fries AC, Simons MP, Mende K, Lalani T, Lee T, Chi S, Mody R, Madar C, Ganesan A, Larson DT, Colombo CJ, Colombo R, Samuels EC, Broder CC, **Laing ED**, Smith DR, Tribble D, Agan BK, Burgess TH. The SARS-CoV-2 mRNA vaccine breakthrough infection phenotype includes significant symptoms, live virus shedding, and viral genetic diversity. Clin Infect Dis. 2021 Jun 12:ciab543. doi: 10.1093/cid/ciab543. Epub ahead of print. PMID: 34117878.

BUDGET JUSTIFICATION

The Henry M. Jackson Foundation for the Advancement of Military Medicine Inc. (HJF) in partnership with the Uniformed Services University of the Health Sciences (USUHS) will manage this proposal, if awarded.

Personnel

Key Personnel:

Eric Laing, Ph.D., Multi-Principal Investigator (.96 calendar month; 8% effort). Dr. Laing will be responsible for the overall coordination of this project. Dr. Laing will oversee the lab activities pertaining to the production of recombinant virus protein antigens and the establishment of serological profile standards. Dr. Laing will provide mentorship to graduate students and assist with data analysis. He will provide the necessary concepts and practice of viral glycoprotein antigen preparation for serological application to co-investigators and collaborators. Dr. Laing will also provide the virological and immunological expertise necessary to understand the serological profiles of control animal and wildlife antisera. Dr. Laing will be a government employee and no salary support is requested.

Other Personnel:

Dr. Si'Ana Coggins, PhD, Scientist (6 calendar months; 50% effort, YR1-5). Dr. Coggins will assist with the supervision of all lab activities at USU. As trained biochemist Dr. Coggins will oversee all protein expression and maintain the quality and rigor of the research. Dr. Coggins will work closely with the research assistant, Mr. Lucas, and graduate student, Ms. Roe, to execute the lab experiments described in the grant application. Dr. Coggins is an employee of the Henry M. Jackson Foundation and salary support is requested for year 1-5.

Ms. Marana Tso, BS, Doctoral Candidate (1.8 calendar months; 15% effort, YR1-2). Ms. Tso will assist statistical serological analysis and the development of multidimensional spatial models. Ms. Tso will be an employee of the Henry M. Jackson Foundation and salary support is requested for year 1-2.

Ms. McKenna Roe, BS, Doctoral Student (3 calendar months; 25% effort, YR2-3). Ms. Roe will conduct the *in vitro* experiments outlined in aim 1 of the grant application. Ms. Roe participate in serology statistical analysis and contribute to the development of the *in silico* tools for analysis in aims 2 and 3. Ms. Roe will be an employee of the Henry M. Jackson Foundation and salary support is requested for year 2-3.

Mr. Brendan Lucas, BS, Res Asst I (6 calendar months; 50% effort, YR1-2; 12 calendar months; 100% effort YR3-4). Mr. Lucas will assist Dr. Coggins in the production of soluble glycoproteins and be responsible for microsphere coupling in YR1-5. Mr. Lucas will participate in serological testing rabbit immunization antisera, and assist Ms. McKenna Roe in antigenic cartography and testing of hammer-headed fruit bat and Madagascan rousette sera in YR1-3. He will also prepare shipments of materials to the regional partner lab, at Chulalongkorn University and University of Pretoria in YR4-5. Mr. Lucas is an employee of the Henry M. Jackson Foundation and salary support is requested for year 1-4.

Mr. Spencer Sterling, MpH, Res Assoc II (3 calendar month; 25% effort, YR3-4; 6 calendar months; 50% effort, YR5) Mr. Sterling will assist with the project in YR3-4 when testing and analysis begin at Chulalongkorn University. Mr. Sterling will participate in serological data analysis in YR5. Mr. Sterling is an employee of the Henry M. Jackson Foundation and salary support is requested for year 3-5.

Domestic Travel: $16,900 YR1-5

Based on FY23 government per diem rates and historical travel to New York City (NYC), we estimate $3,400 should cover the travel costs of Dr. Laing, Dr. Coggins, Ms. Tso, and Mr. Lucas to travel to NYC in YR1 to meet with the other PI and initiate the *in silico* analysis. In YR2-3 we anticipate traveling twice to NYC to continue statical analysis with our partner and have budget $5,500 and $5,000, respectively, in travel for each year. In YR4-5, travel will be reduced to Dr. Laing and Dr. Coggins and projection that one trip per year ($1,500) for coordination and analysis will be required.

Equipment:- $32,000 YR1

Year 1 - $32,000

To perform the multiplex serologies outline in the research application we will require a Luminex MAGPIX multiplexing system. These systems remain the only field deployable multiplexing system, are cost-effective,

and remain widely used by several of our research collaborators in resource-limited settings.

Supplies: $371,325 YR1-5
Based on current prices, the following equipment and supplies will be needed to support this project: MAGPIX multiplexing system, recombinant production of 38 protein antigens, rabbit immunizations, magnetic beads, plastic consumables, cell culture materials, affinity matrices and control antibody materials.

Year 1 - $114,475
MAGPIX multiplexing system
Recombinant protein production
Multiplex serologies and consumables
Rabbit immunizations

Year 2 - $81,424
Recombinant protein production
Multiplex serologies and consumables
Rabbit immunizations

Year 3-5 - $58,475
Recombinant protein production (*de novo*)
Multiplex serologies and consumables

**Note* Dr. Laing is seeking a commercial vendor for assistance with custom protein production. If this materializes, supply budget reserved for recombinant protein production will be reallocated to pay for the invoices.*

HJF 2023 INDIRECT COSTS AND FRINGE BENEFIT RATES:

FY23 Henry M. Jackson Foundation (HJF) Fringe Benefits and Indirect Costs:

Henry M. Jackson Foundation (HJF) Fringe Benefits and Indirect Costs: The Henry M. Jackson 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 36.74% (USU On-site Overhead) for all allowable direct costs, less subawards. Additionally, a 16.7% (Companywide G&A) rate is applied on the total direct costs less subawards plus the USU On-site Overhead rate. For proposals including subawards, 1.93% is applied to the total subaward cost. The fringe rate is 29.42% for Tier 1 HJF employees, and 7.29% for Tier 2 HJF employees. The provisional rate agreement for FY 2023 covering the fringe benefits and indirect cost rates was approved by the U.S. Army Medical Research Acquisition Activity on September 1, 2022.

EQUIPMENT (LAING - USU/HJF)

Laing Laboratory

BioRad BioPlex 200 HTF multiplexing system (3)
centrifuges: Eppendorf 5425 microfuges (2)
inverted microscopes for tissue culture (1)
gel electrophoresis equipment for protein (2) and DNA (2) gels
semi-dry protein transfer apparatus (2)
refrigerator
freezers (-20 C, -80 C)
platform orbital shakers (5)
sonicator with cup horn and microtip
heating blocks (2)
waterbaths (1)
biosafety cabinets (2)
CO_2-tissue culture incubators (2)
GE-ATKA low pressure chromatography systems, including UV detectors and fraction collectors (2)

Departmental

Fuji LAS-4010 chemiluminescence/fluorescence CCD camera imaging system
Epifluorescence microscopy system (Fully-automated Zeiss AxioObserver epifluorescence microscope)
IVIS in vivo imaging system (equipped to detect luminescence and fluorescence)
2D gel electrophoresis system (protein)
Alpha-Innotech gel documentation system (EtBr-stained gels)
MilliQ ultra-pure water system
Nanodrop spectrophotometer
Luminometer
Fluorescence plate readers (2)
bacterial shakers (6)
Perkin-Elmer and Eppendorf thermal cyclers (5)
ELISA plate readers (2)
balances (6)
pH meter (2)
Lonza Nucleofector 4D Shuttle – core unit and X-unit
Coulter Z1-Dual cell counter
Beckman Allegra 25R; Thermo-Fisher Jouan GR4i; various rotors
BioRad iQ5 real-time PCR system
Luminex MagPIX multiplexing system

Core facilities (USU BIC – fee for service or free)

Translational Imaging Facility

Siemens Inveon SPECT/PET/CT Scanner
Bruker In-Vivo Xtreme II: supports bioluminescence, X-ray, and direct radioisotopic imaging
Bruker Biospec 70/20 USR MRI
Image Guided Therapy 7T MR guided Focused Ultrasound System for neurological applications

Flow Cytometry

Becton-Dickinson (10- and 13-parameter) LSRII FACS analyzers (2)
15-parameter FACSAria II FACS sorter, including a 541nm laser upgrade for sorting of RFP-expressing cells
Amnis Image Stream X Mark II imaging flow cytometer with IDEAS statistical image analysis software
Bio-Rad Bio-Plex 200 multi-analyte bioassay detection system
Offline data analysis stations (3) with software packages including: FlowJo, WinList, and ModFit

Microscopy

Zeiss 710NLO confocal system with 405/458/488/514/561/633 conventional lasers and a Coherent Ultra2 Ti-Sapphire laser for multiphoton excitation, continuously tunable over the range of 690 to 1080 nm
Zeiss 700 confocal system with 405/458/488/514/561/633 laser lines

Zeiss Elyra PS.1 Superresolution microscope for superresolution structured illumination microscopy (SR-SIM), photoactivated localization microscopy (PALM), and direct stochastic optical reconstruction microscopy (dSTORM). System is fully equipped, including the 3D-PALM/dSTORM option.
Becker-Hickl 2-detector FLIM system (for FRET – attached to Zeiss 710NLO)
Zeiss AxioExaminer.Z1 upright confocal system with Coherent Chameleon multiphoton laser for in vivo imaging and electrophysiology
Zeiss AxioImager.M2 upright microscope and MicroBrightField Stereo Investigator software (stereology analysis).
Leica AF6000 system, including an inverted microscope equipped with a fully motorized 3-axis stage plus atmosphere and temperature control
Wide-field fluorescence microscopes (3)
Transmission electron microscope (Philips CM100 transmission EM)
Ultramicrotome (Leica EM UC6 with EM FC6 cryo attachment)
Offline data analysis stations with software packages including: Zeiss Zen software and full Physiology package; Media Cybernetics' 3D Constructor, Image Pro Analyzer, Autodeblur, and Autovisualize; Metamorph.

Genomics
Fuji FLA-5000/LAS-3000 chemiluminescence/fluorescence CCD/laser scanning imaging systems
ABI 9800 Fast Thermal Cycler
ABI 7500 Real Time PCR System
ABI 3500xl Genetic Analyzer
ABI 394 DNA/RNA Synthesizer (2)
ABI 3900 DNA Synthesizer
Eppendorf epMotion 5075 VAC Liquid handling robot
Thermo Scientific Evolution 300 UV/Vis Spectrophotometer
Systec MediaPrep & Plate Pourer

Proteomics
Agilent 1100 HPLC (2)
AB SCIEX Voyager DSTR MALDI-TOF mass spectrometer
AB SCIEX Q-TOF tandem mass spectrometer
Genetix QArray2 Micro array printer

Histopathology
Fee-for-service histology and immunohistochemistry
Nanozoomer digital slide scanner

Structural Biology
X-ray crystallography facility with a Rigaku HighFlux HomeLab X-ray diffraction system, including:
MicroMax-007 HF, a microfocus rotating anode generator
R-AXIS Imaging Plate detector
X-stream 2000 cryogenic system
AB Sciex 4000 QTRAP LC/MS/MS System

Facilities and Other Resources

Uniformed Services University of the Health Sciences
Dr. Eric Laing (Principal-Investigator)

Overall Scientific Environment: Uniformed Services University (USU) is the medical school at which approximately half of the physicians in the Armed Services receive their graduate training. Research at USU is supported primarily by extramural grants, as in other medical schools. Dr. Laing is an Assistant Professor in the Department of Microbiology and Immunology, which includes 12 full-time Faculty members. The overall focus of the Department is 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. Dr. Laing is currently involved in active collaborations within the University, in areas of viral immunology and vaccine and antiviral therapeutics and animal model development. USU is also physically located directly across from the main NIH campus in Bethesda. The overall broad scientific environment at both USU and the NIH is highly conducive to productive collaborations. The PI often uses these resources to his advantage, both for his research objectives and interests and in assisting in adjunct faculty appointments within the Emerging Infectious Diseases graduate program for both on and off-campus scientists interested in participating in graduate education and graduate student training. Overall, the available technical resources (and University support for continually improving technical resources) is exceptional. In addition, multi-lab Immunology journal club, Immunology data club, Virology journal club, and Virology data club exist. Approximately 10 groups regularly participate in the immunology journal/data club and 5 groups regularly participate in the virology journal/data club.

Dr. Laing has been well-supported by the Department of Microbiology and Immunology and the USU Office of Research. Although USU is a modest-sized medical school, the technical resources are outstanding, rivaling many very large institutions (see below). The modest size of the school is an advantage, as key pieces of equipment (e.g., Bruker In Vivo Xtreme II; Siemens Inveon SPECT/PET/CT Scanner) are not overbooked and therefore readily available to the laboratory personnel. In addition to University funds, we have successfully used the NIH S10 mechanism to purchase advanced and very expensive equipment, including the Zeiss 710NLO system and the FACSAria 15-parameter cell sorter. Overall, the available technical resources (and University support for continually improving technical resources) is exceptional, as is illustrated by the most recent USU purchase of cutting-edge equipment: the Zeiss Elyra PS.1 super-resolution microscope.

Laboratory: Dr. Laing's laboratory is 453 sq. ft. and is currently supported by two graduate students, two research assistants, two research associates, and one scientist. Dr. Laing also has access to 3 rooms within the department totaling 1,440 sq. ft. where BSL-2 virus rescues are performed and blood specimen samples are handled. Dr. Laing has access to or possesses routine equipment for microbiology, molecular biology, and biochemistry including incubators, centrifuges, inverted microscopes, gel electrophoresis equipment for protein and DNA gels, thermal cyclers, balances, pH meters, refrigerators, freezers, platform orbital shakers, sonicator, UV transilluminator, shakers, heating blocks, waterbaths, free-standing biosafety cabinets, and CO_2-tissue culture incubators. Additionally, Dr. Laing has access to three BioPlex 200 HTF multiplexing systems, one MAGPIX multiplexing system, an epifluorescence microscopy system (a Zeiss AxioObserver inverted fluorescence microscope, upgraded in 2015), and two complete GE-ATKA low pressure chromatography systems, with integrated UV detectors, fraction collectors, and pump systems, and gradient fractionator apparatus.

Animal: Animals are maintained in University facilities under the supervision of a full-time veterinarian. The USUHS maintains a modern AAALC Accredited, Central Animal Facility of about 50,000 square feet. It, and the University's animal care and use program, is managed by the Center for Laboratory Animal Medicine, which is directed by a veterinarian who is an ACLAM Diplomate and staffed with three other veterinarians, a graduate animal husbandryman, and about 30 technicians. The University is able to provide appropriate care for a wide variety of laboratory animal species, from invertebrates to lower vertebrates, to higher vertebrates including non-human primates and domestic livestock, as well as the more commonly used species such as rodents and rabbits. The facility also includes a number of properly equipped ABSL-2 rooms.

Computer: Windows-based computers (Intel i5/i7 or similar processors and ≥2GB of RAM) are available for routine use by postdoctoral fellows, graduate students, and technicians located in the laboratories of both investigators. The University maintains site licenses for other image and data analysis software, including Zeiss Axiovision, VisioPharm, and OriginPro.

Office: Dr. Laing has an office separate from but proximal to the laboratories. Trainees and technicians have desks in the laboratory or in a separate office area proximal to the laboratory. The department employees one senior program manager, administrative officer, administrative specialist and a microbiologist.

Clinical: N/A

Other: The Biostatistics Consulting Center (BCC), a service of the Department of Preventive Medicine and Biometrics, provides statistical consulting to USUHS scientific investigators. We routinely consult with Cara Olsen, Research Assistant Professor (the full-time Biostatistics Consultant of the BCC), regarding proper design of experiments for statistical testing and for statistical analysis of the resulting data.
The USU Translational Imaging Facility (TIF) houses state-of-the art equipment for live animal imaging, including a Siemens Inveon SPECT/PET/CT Scanner, a Bruker Biospec 70/20 USR Magnetic Resonance Imaging system, and a Bruker In-Vivo Xtreme II bioluminescence and X-ray imaging system.

The USU Biomedical Instrumentation Center (BIC) houses core equipment for use by investigators throughout the University. Instrumentation is available either free or on a fee-for-service basis, depending on which instruments have annual service contracts (which are paid largely through per-hour use fees). The BIC Flow Cytometry Core includes two Becton-Dickinson (10- and 13-parameter) LSRII FACS analyzers, one 15-parameter FACSAria FACS sorter, and one Amnis Image Stream X Mark II imaging flow cytometer, as well as off-line analysis workstations.

The BIC Imaging Core houses three confocal microscopes, including a Zeiss 700 inverted system with 405/458/488/514/561/633 laser excitation; a Zess 710NLO inverted system with 405/458/488/514/561/633 conventional lasers and a Coherent Ultra2 Ti-Sapphire laser for multiphoton excitation, continuously tunable over the range of 690 to 1080 nm; and a Zeiss AxioExaminer-Z1 upright microscope equipped with a direct-coupled Coherent Chameleon tunable infrared laser for ex vivo and in vivo mutliphoton imaging projects. A Becker-Hickl two-detector FLIM system (for FRET analyses) is connected to the inverted Zeiss 710NLO system. Recently, the BIC has also acquired a Zeiss Elyra PS.1 super-resolution microscope, which is capable of 4-parameter SR-SIM (super-resolution structured illumination) imaging, 3-parameter PALM (Photoactivation localization microscopy) and dSTORM (direct stochastic optical reconstruction microscopy), as well as 3D-PALM/dSTORM. The BIC also houses a Leica AF6000 system, consisting of an inverted microscope equipped with a fully motorized 3-axis stage plus atmosphere and temperature control, allowing extended term (days) live cell analyses. Additionally, there is a stereology system consisting of a Zeiss AxioImager.M2 upright microscope connected to MicroBrightField's Stereo Investigator software package. The facility also includes several additional wide-field fluorescence microscopes, and three offline data analysis stations with software packages including: Zeiss Zen software and full Physiology package; Media Cybernetics' 3D Constructor, Image Pro Analyzer, Autodeblur, and Autovisualize; Metamorph Basic. The Imaging Core also includes a transmission electron microscope (Philips CM100 transmission EM) and an ultramicrotome (Leica EM UC6 with EM FC6 cryo attachment).

The BIC Genomics core includes an ABI 3900 DNA synthesizer, an ABI3500xl Genetic Analyzer (for sequencing), a RochLightCycler 480 for real-time PCR, and Systec Mediaprep and Plate Pourer instrument. There is also an integrated Fuji FLA-5000/LAS-3000 imaging system for many applications that involve fluorescence and chemiluminescence imaging of gels and blots. The BIC Proteomics Core includes two Agilent 1100 HPLCs, an AB SCIEX Voyager DSTR MALDI-TOF mass spectrometer, and an AB SCIEX Q-TOF tandem mass spectrometer.

The BIC Structural Biology Core includes a Rigaku HighFlux HomeLab X-ray diffraction system, with a MicroMax-007 HF microfocus rotating anode generator, an R-AXIS Imaging Plate detector, and an X-stream 2000 cryogenic system. Other available BIC instruments and services include histopathology and PET/CT instrumentation for small animal research.

The University also has an equipment repair service, central duplicating service, audiovisual service, and microcomputer support service. The University Learning Resource Center is a high quality medical and scientific library with additional microcomputers and support. A wide variety of scientific journals are available in print and via remote computer access. A central autoclave/glassware washroom serves the Department of Microbiology and Immunology and is maintained through extramural grant support.

Authentication of Key Resources and Scientific Rigor

Laboratory (USUHS)

Our project aims to optimize multiplex serology protein antigen panels and serological profile standards for henipavirus (e.g. Nipah virus) and ebolaviruses (e.g. Ebola virus). To achieve the aims outlined in the technical proposal, we propose to utilize established stable cell lines to express native-like soluble ectodomain tetramers of all presently described henipaviruses and filoviruses, and purify native-like proteins via size-exclusion and affinity chromatography. Purified envelope glycoproteins will be used to immunize rabbits as animal models to investigate the how antibody response to these protein antigens is shaped after three sequential immunizations. Protein antigens will be coupled to microspheres for multiplex serology applications utilizing Luminex xMAP-based technologies, and testing will be conducted to look at the magnitude and breadth of antigen-specific and cross-reactive antibody responses. These data will be utilized to develop a comprehensive antigen cartographic map, defining the serogroups of henipaviruses and filoviruses based on two envelope protein antigen per virus. Our custom multiplex serology assay will be optimized, qualified, and verified for non-clinical research application in our USUHS lab. Then materials and knowledge will be transferred to the regional partners at Chulalongkorn University and the University of Pretoria where testing of field collected samples will occur. Our goals are to develop *in vitro* and *in silico* models to identify and characterize known and unknown henipaviruses and filoviruses leveraging sera banks collected by collaborators, and co-investigators participating in active field surveillance.

USU will actively engage with EcoHealth Alliance (PI: Ross, N) to ensure that the highest quality science, public accountability, and social responsibility in the conduct of science are maintained throughout**.** The overall goal is to ensure that the underlying scientific foundation of the project from conception to completion is scientifically sound.

The Laing laboratory at USUHS employs quality systems to insure rigor, reproducibility, and robust and unbiased results in all experiments.

The reagents that are used in our lab are routinely authenticated as follows:

Expression constructs: All new expression constructs are verified by (1) restriction digest and electrophoretic separation of fragments to ensure fragments of the predicted size are generated and (2) sequencing to ensure the correct sequence is present with no spurious mutations.

Cell lines: Cell lines will be routinely validated using either flow cytometry and/or functional tests. SOPs, as detailed documents, for work with cell lines been established in the Laing laboratory and all laboratory staff are required to be familiar with this documentation.

Commercial chemical reagents: Such reagents are validated by one or both of the following: (1) performance comparison to previous lots of the same reagent; (2) use in an established assay that incorporates one or more validated performance controls.

Rigor and Reproducibility (all investigators)

The premise of our investigation is based on both basic science discoveries and non-clinical data. Our in vitro mechanistic studies are invariably correlated with in vivo cell-based assays so as to establish biological relevance. All findings in our work have been and will be validated by multiple independent lines of evidence to ensure scientific rigor. All experiments are carried out with several internal controls with multiple replications to guarantee reproducibility.

USU Investigator: Eric D. Laing, Ph.D. (PI), Assistant Professor, Department of Microbiology and Immunology
Prime Investigator: Noam Ross, Ph.D. (PI), Principal Scientist for Computational Research, EcoHealth Alliance
Grant Type: NIH R01; Multi-PI, Early Stage Investigators
Title: Characterizing unknown emerging viruses through targeted serological profiling

Statement of Work (USU-Laing) - **Period of Performance 01/01/2024 - 12/31/2028**

Major Milestones, Tasks and Timeline.

The spillover of zoonotic viruses into human populations remains an ever-prevalent threat to public and global health. Among the WHO list of priority pathogens that are threats to global health and security, Nipah virus (NiV), Ebola virus (EBOV), Marburg virus (MARV) are in the top ten. NiV, EBOV, and MARV, are prototypical viruses of their respective genera, *Henipavirus*, *Ebolavirus*, *Marburgvirus*, and the *Henipavirus* and *Ebolavirus* genera include other species of viruses and isolates that range in their confirmed, e.g. NiV, Langya virus, EBOV, MARV; or inconclusive pathogenic potential, e.g. Bombali virus, Ghana virus. In recent years, we have witnessed spillover of henipaviruses, ebolaviruses, and marburgviruses in regions not previously associated with outbreaks. For example, the 2014 outbreak of Ebola virus disease in western Africa was the largest recorded and highlighted the need for a global health security agenda. In 2022, MARV caused fatal disease in Ghana, a region with no historical outbreaks; and SUDV re-emerged in Uganda. In 2021, a never before described henipavirus, Langya virus, was isolated from patients with acute febrile illness in southern China and then detected in shrews; and since 2019 annual spillovers of NiV in Kerala, India have occurred, well outside the recognized hot-spots in Bangladesh. These outbreaks of known and unknown zoonotic viruses demonstrates our paucity of understanding the geographic distribution, wildlife hosts, at-risk human populations, and viral diversity in these three genera.

We have laid-out a series of aims and hypotheses that will contribute significant insight into identifying and characterizing known and importantly unknown viruses through targeted serological profiling and multidimensional spatial statistical modeling. Serological profiling of wildlife hosts and at-risk human communities can provide a wealth of data about the virus infectome, however, the interpretation of this data requires stringent *in vitro* and *in silico* tools. In this grant application we aim to develop and apply lab and computational tools necessary to identify and characterize known and unknown henipaviruses and filovirids that have been detected in bat and humans hosts. 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 (Format: Year #. Task #. Sub-task#)

Milestone 1: *Develop ebolavirus, marburgvirus, and henipavirus envelope glycoprotein serologic standards*. YR1-2. We will create comprehensive serological profiles for filoviruses and henipaviruses to characterize the spatial antigenic relationships between specific virus protein antigens and across related viruses. In doing so we will establish critical priors that will be used to test and develop statistical models focused on exploring virus antigenic and phylogenetic relationship. The individual subtasks linked to this specific *Milestone 1* are:

1.1.1 Purification of filovirid and henipavirus envelope glycoprotein antigens as native-like ectodomain
1.1.2 Purified native-like filovirid GP protein antigen immunizations of rabbits
1.1.3 Establish antigen cartographic relationships among filovirids based of the envelope glycoprotein antigens, GP (trimer) and GP1 subunit protein
2.1.1 Purified native-like henipavirus G+F protein antigen immunizations of rabbits

USU Investigator: Eric D. Laing, Ph.D. (PI), Assistant Professor, Department of Microbiology and Immunology
Prime Investigator: Noam Ross, Ph.D. (PI), Principal Scientist for Computational Research, EcoHealth Alliance
Grant Type: NIH R01; Multi-PI, Early Stage Investigators
Title: Characterizing unknown emerging viruses through targeted serological profiling

2.1.2 Establish antigen cartographic relationships among henipaviruses based of the envelope glycoprotein antigens, G/RBP (tetramer) and F (trimer)
2.1.3 Leave-one-out, filovirid/henipavirus sequential immunizations

Milestone 2. Perform multiplex serologic testing of fruit bat sera collected in the Republic of Congo and Madagascar. YR2-3. A wildlife host for Bundibugyo virus (BDBV) has not yet been identified, yet we have detected BDBV reactive sera in populations of hammerhead fruit bats collected in the Republic of Congo and Madagascan rousettes collected in Madagascar with collaborators, Drs. Vincent Munster (RML) and Cara Book (U. Chicago), respectively. We aim to refine the serological profiles of these bat populations, which will permit us to characterize the antigenic and phylogenetic relatedness of these BDBV-like viruses. The individual subtasks linked to this specific *Milestone 2* are:

2.2.1 Identify sera samples of hammerheaded fruit bats and Madagascan rousettes that were immunoreactive with Bundibugyo virus glycoprotein antigens
2.2.2 Test individual bat serum samples with an optimized panel of virus envelope glycoprotein antigens
3.2.3 Create multidimensional spatial models of filovirus serological profiles in hammerheaded fruit bat and Madagascan rousettes

Milestone 3. Examine the targeted serological profiles of guano farmers and Egyptian fruit bats. YR4-5. Through present work within the Emerging Infectious Diseases-Southeast Asia Research Collaborative Hub (EID-SEARCH) and South African Bat Research Network (SABRENET) we have detected unique serological profiles to henipaviruses in humans and filovirids in Egyptian fruit bats. In this proposal we aim to develop new *in vitro* and *in silico* serological profiling techniques to improve our understanding of these henipavirus and filovirus viral shadows. The individual subtasks linked to this specific *Milestone 3* are:

4.3.1 Transfer of optimized antigen panel to co-investigator, Dr. Wanda Markotter, lab at the University of Pretoria (SABRENET)
4.3.2 Transfer of optimized antigen panel to co-investigator, Dr. Supaporn Wacharapluesadee, at the Chulalongkorn University (EID-SEARCH)
4.3.3-5.3.3 Testing and analysis of EID-SEARCH collected human guano farmer sera to improve our understanding of the Mojiang virus-like serological profile
4.3.3-5.3.4 Testing and analysis of SABRENET collected Egyptian fruit bat sera with Ebola virus and Bombali virus serologic profiles to discover the intermediate ebolavirus this bat community hosts.

Milestone 4. Disseminate reports to relevant stakeholders. YR1-5. The individual subtasks linked to this specific *Milestone 4* are:

1.4.1-5.4.1 Submit progress reports to NIH
1.4.2-5.4.2 Conduct presentations/meetings at times and places specified in the grant schedule
2.4.3-5.4.3 Upload immunological and serological data and multiplex standard-operating-procedures to ImmPort
2.4.4-5.4.4 Prepare publications

RESEARCH & RELATED BUDGET - Budget Period 1

OMB Number: 4040-0001
Expiration Date: 12/31/2022

UEI: UYLKBRENAPG5 **Enter name of Organization:** The Henry M. Jackson Foundation

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 1** **Start Date:** 01/01/2024 **End Date:** 12/31/2024

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Eric		Laing	PhD		0.96			0.00	0.00	0.00

Project Role: Site PD/PI

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						
1	Graduate Students	1.80			6,077.00	1,788.00	7,865.00
	Undergraduate Students						
	Secretarial/Clerical						
1	Scientist	6.00			42,000.00	12,356.00	54,356.00
1	Research Assistant	6.00			20,000.00	5,884.00	25,884.00

3 **Total Number Other Personnel**

Total Other Personnel 88,105.00

Total Salary, Wages and Fringe Benefits (A+B) 88,105.00

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment item	Funds Requested ($)
Luminex MAGPIX	32,000.00

Additional Equipment: Add Attachment | Delete Attachment | View Attachment

Total funds requested for all equipment listed in the attached file	
Total Equipment	32,000.00

D. Travel

	Funds Requested ($)
1. Domestic Travel Costs (Incl. Canada, Mexico and U.S. Possessions)	3,400.00
2. Foreign Travel Costs	
Total Travel Cost	3,400.00

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	114,475.00
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	
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17.		
	Total Other Direct Costs	114,475.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	237,980.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
FY23 USU Onsite Indirect	36.74	237,980.00	87,434.00
FY23 HJF Companywide G&A	16.70	325,414.00	54,344.00

Total Indirect Costs 141,778.00

Cognizant Federal Agency
(Agency Name, POC Name, and POC Phone Number)
USAMRAA, Jennifer C. Jackson, 301-619-2054

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	379,758.00

J. Fee

	Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	379,758.00

L. Budget Justification

(Only attach one file.) Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Budget Period 2

OMB Number: 4040-0001
Expiration Date: 12/31/2022

UEI: UYLKBRENAPG5 **Enter name of Organization:** The Henry M. Jackson Foundation

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 2** **Start Date:** 01/01/2025 **End Date:** 12/31/2025

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Eric		Laing	PhD		0.96			0.00	0.00	0.00

Project Role: PD/PI

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						
1	Graduate Students	1.80			6,314.00	1,858.00	8,172.00
	Undergraduate Students						
	Secretarial/Clerical						
1	Scientist	6.00			43,638.00	12,838.00	56,476.00
1	Research Assistant	6.00			20,780.00	6,113.00	26,893.00
1	Graduate Student	3.00			10,524.00	3,096.00	13,620.00

4 **Total Number Other Personnel**

Total Other Personnel: 105,161.00

Total Salary, Wages and Fringe Benefits (A+B): 105,161.00

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)	5,500.00
2. Foreign Travel Costs	
Total Travel Cost	5,500.00

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	81,425.00
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	
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17.		
	Total Other Direct Costs	81,425.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	192,086.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
FY23 USU Onsite Indirect	36.74	192,086.00	70,572.00
FY23 HJF Companywide G&A	16.70	262,658.00	43,864.00

Total Indirect Costs 114,436.00

Cognizant Federal Agency
(Agency Name, POC Name, and POC Phone Number)

USAMRAA, Jennifer C. Jackson, 301-619-2054

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	306,522.00

J. Fee

	Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	306,522.00

L. Budget Justification

(Only attach one file.)

Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Budget Period 3

OMB Number: 4040-0001
Expiration Date: 12/31/2022

UEI: UYLKBRENAPG5 **Enter name of Organization:** The Henry M. Jackson Foundation

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 3** **Start Date:** 01/01/2026 **End Date:** 12/31/2026

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Eric		Laing	PhD		0.96			0.00	0.00	0.00

Project Role: PD/PI

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						
1	Graduate Students	3.00			10,934.00	3,217.00	14,151.00
	Undergraduate Students						
	Secretarial/Clerical						
1	Scientist	6.00			45,340.00	13,339.00	58,679.00
1	Research Assistant	12.00			43,181.00	12,704.00	55,885.00
1	Research Associate	3.00			21,591.00	6,352.00	27,943.00

4 **Total Number Other Personnel**

Total Other Personnel 156,658.00

Total Salary, Wages and Fringe Benefits (A+B) 156,658.00

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)	5,000.00
2. Foreign Travel Costs	
Total Travel Cost	5,000.00

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	58,475.00
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	
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	Total Other Direct Costs	58,475.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	220,133.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
FY23 USU Onsite Indirect	36.74	220,133.00	80,877.00
FY23 HJF Companywide G&A	16.70	301,010.00	50,269.00

Total Indirect Costs 131,146.00

Cognizant Federal Agency (Agency Name, POC Name, and POC Phone Number): USAMRAA, Jennifer C. Jackson, 301-619-2054

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	351,279.00

J. Fee

	Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	351,279.00

L. Budget Justification

(Only attach one file.) Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Budget Period 4

OMB Number: 4040-0001
Expiration Date: 12/31/2022

UEI: UYLKBRENAPG5 **Enter name of Organization:** The Henry M. Jackson Foundation

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 4** **Start Date:** 01/01/2027 **End Date:** 12/31/2027

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Eric		Laing	PhD		0.96			0.00	0.00	0.00

Project Role: PD/PI

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	Scientist	6.00			47,109.00	13,859.00	60,968.00
1	Research Assistant	12.00			44,865.00	13,199.00	58,064.00
1	Research Associate	3.00			22,433.00	6,600.00	29,033.00

3 **Total Number Other Personnel**

Total Other Personnel 148,065.00

Total Salary, Wages and Fringe Benefits (A+B) 148,065.00

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)	1,500.00
2. Foreign Travel Costs	
Total Travel Cost	1,500.00

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	58,475.00
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	
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	Total Other Direct Costs	58,475.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	208,040.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
FY23 USU Onsite Indirect	36.74	208,040.00	76,434.00
FY23 HJF Companywide G&A	16.70	284,474.00	47,507.00

Total Indirect Costs 123,941.00

Cognizant Federal Agency
(Agency Name, POC Name, and POC Phone Number)

USAMRAA, Jennifer C. Jackson, 301-619-2054

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	331,981.00

J. Fee

Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	331,981.00

L. Budget Justification

(Only attach one file.)

Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Budget Period 5

OMB Number: 4040-0001
Expiration Date: 12/31/2022

UEI: UYLKBRENAPG5 **Enter name of Organization:** The Henry M. Jackson Foundation

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 5** **Start Date:** 01/01/2028 **End Date:** 12/31/2028

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Eric		Laing	PhD		0.96			0.00	0.00	0.00

Project Role: PD/PI

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	Scientist	6.00			48,946.00	14,400.00	63,346.00
1	Research Associate	6.00			46,615.00	13,714.00	60,329.00

2 **Total Number Other Personnel**

Total Other Personnel 123,675.00

Total Salary, Wages and Fringe Benefits (A+B) 123,675.00

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)	1,500.00
2. Foreign Travel Costs	
Total Travel Cost	1,500.00

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	58,475.00
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	
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17.		
	Total Other Direct Costs	58,475.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	183,650.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
FY23 USU Onsite Indirect	36.74	183,650.00	67,473.00
FY23 HJF Companywide G&A	16.70	251,123.00	41,938.00

Total Indirect Costs 109,411.00

Cognizant Federal Agency
(Agency Name, POC Name, and POC Phone Number)

USAMRAA, Jennifer C. Jackson, 301-619-2054

I. Total Direct and Indirect Costs

	Funds Requested ($)
Total Direct and Indirect Institutional Costs (G + H)	293,061.00

J. Fee

	Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	293,061.00

L. Budget Justification

(Only attach one file.)

Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Cumulative Budget

		Totals ($)
Section A, Senior/Key Person		0.00
Section B, Other Personnel		621,664.00
Total Number Other Personnel	16	
Total Salary, Wages and Fringe Benefits (A+B)		621,664.00
Section C, Equipment		32,000.00
Section D, Travel		16,900.00
1. Domestic	16,900.00	
2. Foreign		
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		371,325.00
1. Materials and Supplies	371,325.00	
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		
9. Other 2		
10. Other 3		
11. Other 4		
12. Other 5		
13. Other 6		
14. Other 7		
15. Other 8		
16. Other 9		
17. Other 10		

Section G, Direct Costs (A thru F)	1,041,889.00
Section H, Indirect Costs	620,712.00
Section I, Total Direct and Indirect Costs (G + H)	1,662,601.00
Section J, Fee	
Section K, Total Costs and Fee (I + J)	1,662,601.00

Investigator Initiatives-Business Development
Department Line: (240) 694-4016
Email: proposals@hjf.org

LETTER OF INTENT TO COLLABORATE

February 1, 2023

Noam Ross, PhD
Principal Scientist, Computational Research
EcoHealth Alliance
520 Eighth Avenue, Suite 1200
New York, NY 10018

Dear Dr. Ross:

This letter confirms The Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc.'s (HJF) intent to collaborate with EcoHealth Alliance in response to the NIH PA-20-185, and is offered on the condition EcoHealth Alliance agrees that if it receives an award for the subject project, it will issue a subaward to HJF for the work hereby proposed.

Confidentiality Notice: *This letter of Intent, including all proposal documents attached hereto, contains HJF confidential and proprietary information and is provided for use solely in connection with HJF's and the (Prime Institution) joint pursuit of the project listed below. Any unauthorized use, disclosure or distribution is prohibited.*

Prime Institution:	EcoHealth Alliance
Prime Principal Investigator:	Noam Ross, PhD
Project Title:	Characterizing unknown emerging viruses through targeted serological profiling
Proposed Principal Investigator:	Eric Laing, PhD; USU-MIC
Proposed Project Period:	01/01/2024 – 12/31/2028
Proposed Budget Amount:	$1,041,889 Direct Costs $ 620,712 Indirect Costs $1,662,601 Total Cost

HJF confirms that it has in place a written and enforced financial conflict of interest policy that applies to all HJF investigators and any other HJF employees responsible for the design, conduct or reporting of the budgeted activities. U.S. Government Principal Investigator(s) (if any) are subject to federal regulations governing conflicts of interest.

Your submission of a prime proposal that includes the subaward activities hereby proposed by HJF will constitute your acknowledgment of, and agreement to, the terms set forth in this letter.

Sincerely,

(b) (6)

Lisa Straker
Deputy Director
Investigator Initiatives-Business Development

cc: Eric Laing, PhD

From:	AIMS-DoNotReply@hjf.org
To:	Noam Ross
Cc:	ERIC LAING; Judy Richardson; Lisa M Straker
Subject:	Message sent on behalf of "Jacquelyne M Ford" <jford@hjf.org>: Eric Laing subaward to EcoHealth Alliance
Date:	Wednesday, February 1, 2023 4:16:30 PM
Attachments:	Biosketch_Laing.pdf Budget Justification.pdf Equipment.pdf Facilities.pdf Key Biologicals.pdf SOW.pdf RR_Budget_3_0-V3.0 UPDATE.pdf HJF LETTER OF INTENT - E.LAING (PRO4479)-NIH Subaward-2-1-23-LMS signed.pdf

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Hello Dr. Ross,

On behalf of the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc., attached are the documents for Dr. Eric Laing, Uniformed Services University of the Health Sciences to collaborate with EcoHealth Alliance in response to PA-20-185 Funding Opportunity. Please let me know if you need additional information and/or you have any questions. Also, please reply to this email to confirm receipt of this proposal submission.

Yours in good health,

Jacquelyne M. Ford

Sr. Proposal Specialist, Domestic Portfolio

Strategic Initiatives Department

Investigator Initiatives – Business Development

Phone: 240-694-2271

Email: jford@hjf.org

Henry M. Jackson Foundation for the

Advancement of Military Medicine

6720A Rockledge Drive, Suite 100

Bethesda, MD 20817

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: Vincent J. Munster

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6)

POSITION TITLE: Chief, Virus Ecology Unit

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
Utrecht University	MSc	1999 - 2001	Molecular Microbiology
Erasmus University	PhD	2002 - 2006	Molecular Virology
Erasmus Medical Center	Postdoctoral	2006 - 2009	Influenza transmission
Rocky Mountain Laboratories, NIH/NIAID	Postdoctoral	2009 - 2012	Virus Ecology

A. Personal statement

Past outbreaks of bat-borne zoonotic viruses such as coronaviruses, henipaviruses and filoviruses, have had an enormous impact on human and wildlife health. The unpredictability of the zoonotic introductions of these bat-borne limits the potential for effective intervention strategies. One of the main reasons for the absence of successful pre-emptive strategies is our lack of understanding of the ecology, evolution and interaction with the immune system of these pathogens in their respective natural reservoirs. I have gained extensive laboratory and field experience. I have expanded my expertise over the last year towards the field of bat-borne zoonotic diseases (Ebola, Marburg, Nipah and MERS-CoV), conducting field studies in the Republic of Congo and Mali and experimental studies at the Rocky Mountain Laboratories high and maximum containment facility (BSL3 and BSL4). Within the NIAID Virus Ecology unit (http://www.niaid.nih.gov/labsandresources/labs/aboutlabs/lv/virusecology/Pages/default.aspx#niaid_inlineNav_Anchor) my work focuses on natural reservoirs of emerging viruses and elucidation of the underlying biotic and abiotic drivers of zoonotic and cross-species transmission events. Over two decades, I have a demonstrated record of accomplished and productive research resulting in over 190 peer-reviewed publications.

B. Positions and Honors

Positions

2013 – Present Chief, Virus Ecology Unit, Laboratory of Virology, Rocky Mountain Laboratories, NIAID/NIH
2009 – 2012 Post-doctoral fellow, Disease Modelling and Transmission Section, NIAID/NIH.
2006 – 2009 Postdoctoral research fellow at the Department of Virology, Erasmus Medical Center.

Other Experiences and Professional Memberships

2021 Member WHO virus evolution working group
2020 Member WHO animal working group
2020 Member Operation Warp Speed / CAG
2020 NIAID coronavirus response
2018 DRC Ebola Outbreak: NIAID Internal Coordination Working Group
2018 NIAID Viral Pathogen Preparedness Working Group
2018 Scientific Advisory Board, DTRA Western Asia bat research project
2017 FAO-OIE-WHO global technical meeting on MERS-CoV, Geneva
2017 WHO Global Outbreak and Alert Network workshop meeting, Hong Kong
2017 WHO Environmental Contamination of MERS-CoV meeting, Hong Kong
2016 Graduate faculty appointment, Marshall University, Joan C. Edwards School of Medicine

2016	PhD thesis examiner, University of Melbourne, Australia
2015-2016	Member the Scientific Advisory Group of the NIAID workshop on MERS animal models
2015	Member the Scientific Advisory Group organizing the NIAID workshop on MERS animal models.
2015	Member of the ASPR SPIRiT Ebola environmental working group.
2015	Member of the ASPR Science Disaster Preparedness working group.
2014-2015	Team lead of the combined WHO - CDC/NIH diagnostic laboratory during the Ebola virus outbreak, Monrovia, Liberia.
2014	American Society for Virology, Program Planning Committee.
2014	Moderator, IOM/NRC Workshop on Research Priorities to Inform Public Health and Medical Practice for Domestic Ebola Virus Disease, Institute of Medicine of the National academy of Sciences.
2014	Organizer of the fifth ESWI Influenza Conference in Riga, Latvia.
2014	Scientific organizing committee of the Endemic and Emerging Viral Diseases of Priority in the Middle East and North Africa.
2013	WHO-ISARIC joint MERS-CoV Outbreak Readiness Workshop.
2013	Member Coronavirus Therapeutics Interagency Working Group (NIH, CDC, BARDA and DoD).
2013	Moderator, NIAID MERS-CoV Research: Current Status and Future Priorities Meeting.
2013	Editor for PLoS One, One Health and Frontiers in Cellular and Infection Microbiology
2011-2014	Board member of the European Scientific Working group on Influenza.
2008	Member of the OIE ad hoc Group on Wildlife Disease Surveillance.
2005-present	Reviewer for journals including: Lancet Infectious Diseases, Nature, Nature Medicine, PNAS and Science.

Grant application reviewer

2019	AAAS for the Saudi Arabia's Ministry of Education Research
2018	BBSRC, UK
2018	Italian Ministry of Health
2017	BBSRC (Biotechnology and Biological Sciences Research Council), UK
2016	USAID Combating Zika and Future Threats Grand Challenge
2016	FINOVI Foundation, France
2015	Italian Ministry of Health
2015	Referee Panel for Health and Medical research Fund, HKSAR
2015	Human Frontier Science Program
2012	Referee Panel for Health and Medical research Fund, HKSAR
2008-2009	National Medical Research Council, Singapore
2008	Minnesota Center of Excellence for Influenza Research and Surveillance, USA
2007	Department for Environment, Food and Rural Affairs, UK

Miscellaneous

2014-2015	Team lead of the combined CDC/NIH diagnostic laboratory under GOARN/WHO during the Ebola virus outbreak, Monrovia, Liberia, providing diagnostic services to several Ebola treatment units in the Monrovia area
2015-2018	Thompson Reuters / Clarivate highly cited researcher 2015, 2016, 2017 and 2018

Honors

2020	AAAS Golden Goose award, COVID-19 response
2016	European Society for Virology young investigator award
2015	NIH Director's award, in recognition of the establishment and running of diagnostic field laboratory during the Ebola virus outbreak in West Africa.
2014	NIAID merit award for the development of a nonhuman primate disease model and a treatment strategy for MERS-CoV
2014	ASM IAAC young investigator award
2014	NIH Director's award, in recognition of the exceptional and rapid response to the emergence of MERS-coronavirus.
2011	European Scientific Working group on Influenza, Best Body of Work award for Young Scientists.

C. Contributions to Science

1 During my PhD working on avian influenza at Erasmus Medical Center, it became clear to me that there was a lack of integration between bench virology and fieldwork. Being trained as a "classical" molecular virologist I could very well answer questions on what mutations would make a virus more pathogenic, but the translation of this knowledge to real world situations remained elusive. Integrating various disciplines helped me to understand the ecology and drivers of avian influenza and pandemic influenza outbreaks.

- **Munster VJ**, Wallensten A, Baas C, Rimmelzwaan GF, Schutten M, Olsen B, Osterhaus AD, Fouchier RA. Mallards and highly pathogenic avian influenza ancestral viruses, northern Europe. Emerg Infect Dis. 2005;11(10):1545-51
- Olsen B, **Munster VJ**, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA. Global patterns of influenza a virus in wild birds. Science. 2006;312(5772):384-8
- **Munster VJ**, Baas C, Lexmond P, Waldenstrom J, Wallensten A, Fransson T, Rimmelzwaan GF, Beyer WE, Schutten M, Olsen B, Osterhaus AD, Fouchier RA. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog. 2007;3(5):e61
- **Munster VJ**, de Wit E, van den Brand JM, Herfst S, Schrauwen EJ, Bestebroer TM, van de Vijver D, Boucher CA, Koopmans M, Rimmelzwaan GF, Kuiken T, Osterhaus AD, Fouchier RA. Pathogenesis and transmission of swine-origin 2009 A(H1N1) influenza virus in ferrets. Science. 2009;325(5939):481-3
- Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, de Wit E, **Munster VJ**, Sorrell EM, Bestebroer TM, Burke DF, Smith DJ, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. Airborne transmission of influenza A/H5N1 virus between ferrets. Science. 2012;336(6088):1534-41.

2 My research group at the Rocky Mountain Laboratories of the NAID/NIH is built around the concept of complete integration of fieldwork, experimental work and computational modeling to allow study of emerging viruses in their natural, intermediate and human hosts. It is clear that past outbreaks of zoonotic viruses have had an enormous impact on human and wildlife health and that the unpredictability of cross-species transmission events limits the potential for effective intervention strategies. My lab aims to identify the underlying changes in virus-host ecology that allow these viral pathogens to cross the species barrier. Recognizing the strengths and weaknesses of a unilateral focus on field research on one hand and experimental research on the other, we have combined both approaches in one research program. This integrated approach has contributed greatly to the rapid advancements in our knowledge on the emergence of MERS-CoV. Using a combination of fundamental concepts of host species restriction of MERS-CoV and protein-protein binding interaction modeling we predicted potential MERS-CoV host species, which were tested in experimental infections. Our field studies in the Middle East confirmed camels as the primary reservoir for MERS-CoV.

- **Munster VJ**, de Wit E, Feldmann H. Pneumonia from human coronavirus in a macaque model. N Engl J Med. 2013;368(16):1560-2.
- de Wit E, Rasmussen AL, Falzarano D, Bushmaker T, Feldmann F, Brining DL, Fischer ER, Martellaro C, Okumura A, Chang J, Scott D, Benecke AG, Katze MG, Feldmann H, **Munster VJ**. Middle East respiratory syndrome coronavirus (MERS-CoV) causes transient lower respiratory tract infection in rhesus macaques. Proc Natl Acad Sci U S A. 2013;110(41):16598-603
- Alagaili AN, Briese T, Mishra N, Kapoor V, Sameroff SC, Burbelo PD, de Wit E, **Munster VJ**, Hensley LE, Zalmout IS, Kapoor A, Epstein JH, Karesh WB, Daszak P, Mohammed OB, Lipkin WI. Middle East respiratory syndrome coronavirus infection in dromedary camels in Saudi Arabia. MBio. 2014;5(2):e00884-14
- van Doremalen N, Miazgowicz KL, Milne-Price S, Bushmaker T, Robertson S, Scott D, Kinne J, McLellan JS, Zhu J, **Munster VJ**. Host species restriction of Middle East respiratory syndrome coronavirus through its receptor, dipeptidyl peptidase 4. J Virol. 2014;88(16):9220-32.
- Adney DR, van Doremalen N, Brown VR, Bushmaker T, Scott D, de Wit E, Bowen RA, **Munster VJ**. Replication and shedding of MERS-CoV in upper respiratory tract of inoculated dromedary camels. Emerg Infect Dis. 2014;20(12):1999-2005.
- **Munster VJ**, Adney DR, van Doremalen N, Brown VR, Miazgowicz KL, Milne-Price S, Bushmaker T, Rosenke R, Scott D, Hawkinson A, de Wit E, Schountz T, Bowen RA. Replication and shedding of MERS-CoV in Jamaican fruit bats (*Artibeus jamaicensis*). Sci Rep. 2016;6:21878.
- de Wit E, van Doremalen N, Falzarano D, **Munster VJ**. SARS and MERS: recent insights into emerging coronaviruses. Nat Rev Microbiol. 2016. doi: 10.1038/nrmicro.2016.81.
- van Doremalen N, Falzarano D, Ying T, de Wit E, Bushmaker T, Feldmann F, Okumura A, Wang Y, Scott DP, Hanley PW, Feldmann H, Dimitrov DS, **Munster VJ**. Efficacy of antibody-based therapies against Middle East respiratory syndrome coronavirus (MERS-CoV) in common marmosets. Antiviral Res. 2017. doi: 10.1016/j.antiviral.2017.03.025.
- van Doremalen N, Hijazeen ZS, Holloway P, Omari BA, McDowell C, Adney D, Talafha HA, Guitian J, Steel J, Amarin N, Tibbo M, Abu-Basha E, Al-Majali AM, **Munster VJ**, Richt JA. High Prevalence of Middle East Respiratory Coronavirus in Young Dromedary Camels in Jordan. Vector Borne Zoonotic Dis. 2016. doi: 10.1089/vbz.2016.2062.
- Schountz T, Baker ML, Butler J, **Munster VJ**. Immunological Control of Viral Infections in Bats and the Emergence of Viruses Highly Pathogenic to Humans. Front Immunol. 2017;8:1098. doi: 10.3389/fimmu.2017.01098.

3 My research group was directly involved in the response to several outbreaks including the Ebola virus outbreak in West Africa by providing diagnostic support at the request of the WHO for several Ebola treatment units in Monrovia, Liberia and SARS-CoV-2. The emergence of Ebola virus in West Africa highlighted significant gaps in our knowledge, including fundamental ecological questions surrounding zoonotic and human-to-human transmission. In order to understand the drivers of transmission, we examined the stability of the virus within tissues and on body surfaces and determined the potential for transmission. The results from this study directly aided the interpretation of epidemiologic data collected from human corpses and are also applicable to interpreting samples collected from remains of wildlife infected with Ebola virus, especially nonhuman primates, and helped to assess the risk of zoonotic transmission. With the current COVID19 pandemic we are actively involved in the development of medical countermeasures and providing critical experimental data supporting direct public health decisions and interventions.

- Mate SE, Kugelman JR, Nyenswah TG, Ladner JT, Wiley MR, Cordier-Lassalle T, Christie A, Schroth GP, Gross SM, Davies-Wayne GJ, Shinde SA, Murugan R, Sieh SB, Badio M, Fakoli L, Taweh F, de Wit E, van Doremalen N, **Munster VJ**, Pettitt J, Prieto K, Humrighouse BW, Stroher U, DiClaro JW, Hensley LE, Schoepp RJ, Safronetz D, Fair J, Kuhn JH, Blackley DJ, Laney AS, Williams DE, Lo T, Gasasira A, Nichol ST, Formenty P, Kateh FN, De Cock KM, Bolay F, Sanchez-Lockhart M, Palacios G. Molecular Evidence of Sexual Transmission of Ebola Virus. N Engl J Med. 2015;373(25):2448-54
- de Wit E, Falzarano D, Onyango C, Rosenke K, Marzi A, Ochieng M, Juma B, Fischer RJ, Prescott JB, Safronetz D, Omballa V, Owuor C, Hoenen T, Groseth A, van Doremalen N, Zemtsova G, Self J, Bushmaker T, McNally K, Rowe T, Emery SL, Feldmann F, Williamson B, Nyenswah TG, Grolla A, Strong JE, Kobinger G, Stroeher U, Rayfield M, Bolay FK, Zoon KC, Stassijns J, Tampellini L, de Smet M, Nichol ST, Fields B, Sprecher A, Feldmann H, Massaquoi M, **Munster VJ**. The Merits of Malaria Diagnostics during an Ebola Virus Disease Outbreak. Emerg Infect Dis. 2016;22(2)
- Miller MR, McMinn RJ, Misra V, Schountz T, Muller MA, Kurth A, **Munster VJ**. Broad and Temperature Independent Replication Potential of Filoviruses on Cells Derived From Old and New World Bat Species. J Infect Dis. 2016
- **Munster V,** Munoz-Fontela C, Olson SH, Seifert SN, Sprecher A, Ntoumi F, Massaquoi M, Mombouli JV. Outbreaks in a Rapidly Changing Central Africa - Lessons from Ebola. N Engl J Med. 2018.
- van Doremalen N, Bushmaker T, Morris DH, Holbrook MG, Gamble A, Williamson BN, Tamin A, Harcourt JL, Thornburg NJ, Gerber SI, Lloyd-Smith JO, de Wit E, **Munster VJ**. Aerosol and Surface Stability of SARS-CoV-2 as Compared with SARS-CoV-1. N Engl J Med. 2020.
- Letko M, Marzi A, **Munster V**. Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. Nat Microbiol. 2020.
- **Munster VJ**, Koopmans M, van Doremalen N, van Riel D, de Wit E. A Novel Coronavirus Emerging in China - Key Questions for Impact Assessment. N Engl J Med. 2020;382(8):692-4.
- **Munster VJ**, Feldmann F, Williamson BN, van Doremalen N, Perez-Perez L, Schulz J, Meade-White K, Okumura A, Callison J, Brumbaugh B, Avanzato VA, Rosenke R, Hanley PW, Saturday G, Scott D, Fischer ER, de Wit E. Respiratory disease in rhesus macaques inoculated with SARS-CoV-2. Nature. 2020
- van Doremalen N, Lambe T, Spencer A, Belij-Rammerstorfer S, Purushotham JN, Port JR, Avanzato VA, Bushmaker T, Flaxman A, Ulaszewska M, Feldmann F, Allen ER, Sharpe H, Schulz J, Holbrook M, Okumura A, Meade-White K, Perez-Perez L, Edwards NJ, Wright D, Bissett C, Gilbride C, Williamson BN, Rosenke R, Long D, Ishwarbhai A, Kailath R, Rose L, Morris S, Powers C, Lovaglio J, Hanley PW, Scott D, Saturday G, de Wit E, Gilbert SC, **Munster VJ**. ChAdOx1 nCoV-19 vaccine prevents SARS-CoV-2 pneumonia in rhesus macaques. Nature. 2020
- Victoria A. Avanzato, M. Jeremiah Matson, Stephanie N. Seifert, Rhys Pryce, Brandi N. Williamson, Sarah L. Anzick, Kent Barbian, Seth D. Judson, Elizabeth R. Fischer, Craig Martens, Thomas A. Bowden, Emmie de Wit, Francis X. Riedo, **Vincent J. Munster**. Prolonged infectious SARS-CoV-2 shedding from an immunocompromised patient with chronic lymphocytic leukemia and acquired hypogammaglobulinemia. Cell 2021.

Publications in peer-reviewed journals: 190
Citations: >28000
H-factor: 61

ORCID: 0000-0002-2288-3196
ResearcherID: I-7607-2018

Research Support

2019 CEPI ChAd-Ox vaccine development for MERS-CoV, Nipah and Lassa virus, $1,281,810
2018 CEPI Lassa mRNA vaccine, CureVac, $998,000

2018	EDCTP (European & Developing Countries Clinical Trial Partnership). EDCTP2 Call for Proposals - Mobilization of research funds in case of Public Health Emergencies - RIA2018 Emergency Funding Mechanism Proposal: RIA2018EF-2082 — EPIRISK-EBOV. €500,000.
2018	Department of Defense, DARPA grant 'PREEMPT', $1,358,133
2018	CEPI MERS-DNA vaccine "Translational portfolio program encompassing cGMP manufacturing and clinical development of DNA vaccine candidates against both Lassa virus and MERS coronavirus." $143,980.
2016	Department of Defense, DARPA grant 'Thunder', $414,000
2015-2016	ARCUS foundation, Improving Laboratory Capacity in the Republic of Congo as a Foundation for Understanding and Mitigating the Threat of Ebola to Great Apes and People, Co-PI.
2013-2020	NIH, NIAID Division of Intramural Research support for the Virus Ecology Unit, PI.
2013-2020	International Centers of Excellence in Research Center, Brazzaville, Republic of Congo, PI.
2014-2015	Biomedical Advanced Research and Development Authority, MERS-CoV countermeasures, PI.
2013-2014	US Fish and Wildlife, wildlife without borders –Africa program. "Satellite telemetry and the landscape ecology of Hammer-headed fruit bats (*Hypsignathus monstrosus*) in Odzala National Park, Republic of Congo", PI.

From: Laing, Eric on behalf of Laing, Eric <eric.laing@usuhs.edu>
To: Noam Ross
Subject: Re: [EXTERNAL] R01_Applied serology methods and statistics
Date: Tuesday, January 31, 2023 9:06:19 PM
Attachments: biosketchMunsternew.docx
image001.png

Biosketch.

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, Jan 31, 2023 at 12:48 PM Noam Ross <ross@ecohealthalliance.org> wrote:

> Thanks, Vincent!
> --
>
> Dr. Noam Ross
> *Principal Scientist, Computational Research*
>
> EcoHealth Alliance
> 520 Eighth Avenue, Suite 1200
> New York, NY 10018
> www.ecohealthalliance.org
>
> +1.212.380.4471 (direct)
> +1.212.380.4465 (fax)
> @noamross@ecoevo.social (fediverse)
> noamross.net
>
> *EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.*

On Tue, Jan 31, 2023 at 12:40 PM Munster, Vincent (NIH/NIAID) [E] <vincent.munster@nih.gov> wrote:

> Here it is guys,
>
> Cheers,
>
> Vincent Munster, PhD
>
> Chief Virus Ecology Section
>
> Rocky Mountain Laboratories
>
> NIAID/NIH
>
> ---
>
> **From:** Laing, Eric <eric.laing@usuhs.edu>
> **Sent:** Tuesday, January 31, 2023 7:40 AM
> **To:** Munster, Vincent (NIH/NIAID) [E] <vincent.munster@nih.gov>
> **Cc:** Noam Ross <ross@ecohealthalliance.org>; Van Tol, Sarah (NIH/NIAID) [F] <sarah.vantol@nih.gov>
> **Subject:** Re: [EXTERNAL] R01_Applied serology methods and statistics
>
> Hi Vincent,
>
> Sorry for the late reply to this email and follow-up for R01 Letter of Support. Noam and I are hoping to submit by this Friday. I've drafted a LOS (attached), can you make any edits, add your letterhead, sign and send back to us? You sent Jon a biosketch for the Liberia-focused R01, I can grab that from the email thread if that works for you.
>
> Also, there's a good chance my research group will be participating in surveillance in Jordan, Oman, and Republic of Georgia so I'll probably need to increase the breadth of MERS-like CoVs in the multiplex. We're still finalizing the IAA with NCI FNL but I'll prob loop back to you for aliquots of bat sera from Jordan to cross-verify antigen performance.
>
> - Eric
>
> Eric D. Laing, Ph.D.
>
> Assistant Professor
>
> Department of Microbiology and Immunology
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> Uniformed Services University
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> 4301 Jones Bridge Road
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> Bethesda, MD 20814
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> cell: (301) 980-8192
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> office: (301) 295-3419
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> lab: (301) 295-9618

eric.laing@usuhs.edu

On Tue, Jan 10, 2023 at 4:42 PM Laing, Eric <eric.laing@usuhs.edu> wrote:

> Hi Vincent,
>
> Really appreciate your support.
>
> One of my ideas was to write that we could leverage the R24 Bat ID model to verify/validate rabbit-immunizations. So it's awesome to hear that you are already on the same page. Wanda Markotter even brought this up during a meeting this morning and I think it's an obvious limitation that a reviewer might pick at, if we only propose antigen immunization in rabbits. Wanda has also offered to do some bat-infection experiments so I'll write that in and make sure there is money in a budget for her lab to do that as well.
>
> RE: CoVs
>
> Chris/my lab pulled back from expressing CoV antigens in-house and have been working with Dominic Esposito's Protein Expression lab at NCI FNLCR. We're in the process of re-establishing an IAA with his group and he's interested in de novo expression of bat CoV antigens. Do you have particular RBD, S1, or spike (S-2P) antigens that you would want for a custom panel? We're going to build out something related to Wanda's work, and work with Supaporn and Gavin Smith in southeast Asia.
>
> - Eric

Eric D. Laing, Ph.D.

Assistant Professor

Department of Microbiology and Immunology

Uniformed Services University

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Bethesda, MD 20814

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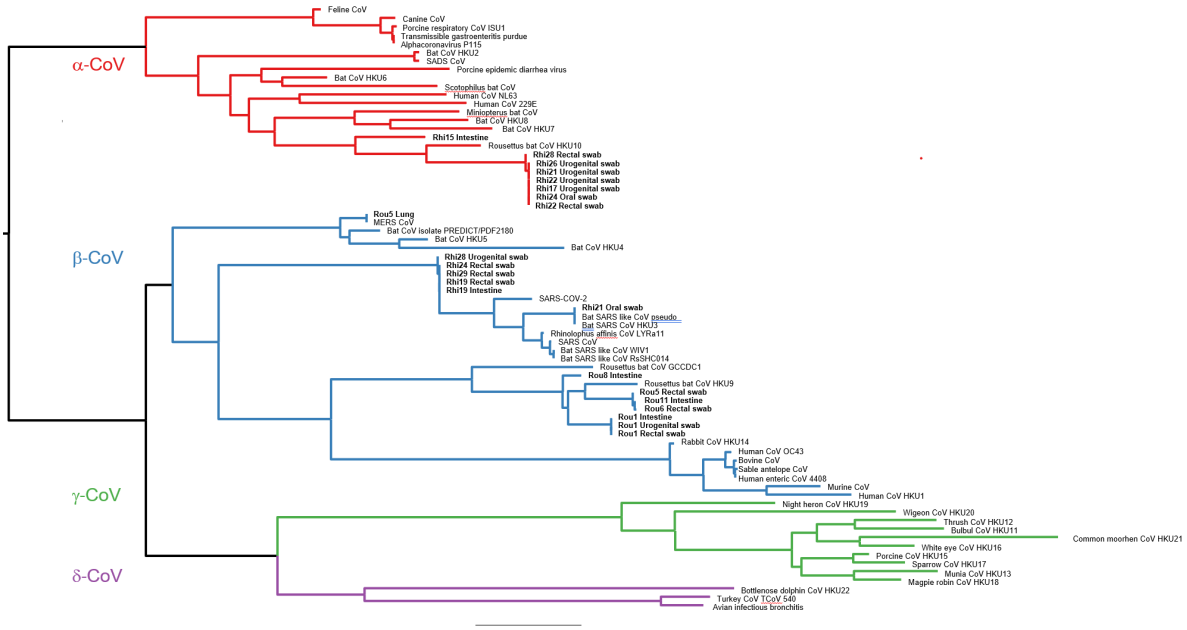
lab: (301) 295-9618

eric.laing@usuhs.edu

On Tue, Jan 10, 2023 at 2:44 PM Munster, Vincent (NIH/NIAID) [E] <vincent.munster@nih.gov> wrote:

> Sounds great,
>
> Looping in my postdoc Dr. Sarah van Tol as well, as she will be doing some bat infection studies soon here at RML and we could share some sera (Artibeus) from these experiments as well.
>
> We could also in the future (my colony is still at CSU, but will be moving to RML) do some bat protein immunizations and use those as controls to validate your rabbit antisera.
>
> Speaking of which, I have a nice serum set of bats from Jordan (Rhinolophus and Rousettus) for which we detected a variety of corona's in (the darker ones), would like to run these through the corona luminex and see what their serum cross reactivity looks like. I could do it here on the magpix, but could share an aliquot of the sere with you as well.



Vincent Munster, PhD

Chief Virus Ecology Section

Rocky Mountain Laboratories

NIAID/NIH

From: Laing, Eric <eric.laing@usuhs.edu>
Sent: Tuesday, January 10, 2023 10:10 AM
To: Munster, Vincent (NIH/NIAID) [E] <vincent.munster@nih.gov>; Noam Ross <ross@ecohealthalliance.org>
Subject: [EXTERNAL] R01_Applied serology methods and statistics

Hi Vincent,

Noam and I are putting together a "methods" focused R01 that leverages ongoing work via a NIH center and DTRA BTRP project with Wanda Markotter. My lab is going to further optimize a 28plex panel of antigens (henipa/filo-focused) that balances two goals: a) discovery of new viruses through serology and b) specific detection of homotypic viruses. We are going to include a ton of protein-immunizations of rabbits to establish cross-reaction matrices for each virus and standard antigenic cartography. Noam is working on novel multidimensional spatial analyzes for Aim 2. In Aim 3 we are proposing to re-test/re-analyze data with the in vitro and in silico tools developed through Aims 1 and 2.

I was wondering whether you would be willing to share with my lab some of the sera collected from bats in the Republic of Congo that has interesting sero-positives for the research being outlined in Aim 1 and 3, and be listed as a collaborator on the project? If so, I would include the restesting of those sera with the optimized panel and downstream analysis in the proposal, and I could provide a draft LOS. The Specific Aims are below:

Specific Aims

1. Create multiplex serological panels optimized for viral discovery

2. Develop novel statistical approaches for identifying and characterize novel virus signals from multiplex data

3. Determine epidemiological patterns of novel virus exposure in human and bat populations.

Kind regards,

Eric

Eric D. Laing, Ph.D.

Assistant Professor

Department of Microbiology and Immunology

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eric.laing@usuhs.edu

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OMB No. 0925-0001 and 0925-0002 (Rev. 03/2020 Approved Through 02/28/2023)

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: Jonathan H. Epstein

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6)

POSITION TITLE: Vice President for Science and Outreach

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
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, Mailman SPH, NY	Fellowship	2006-2009	Molecular Virology
Kingston University, UK	PhD	2017	Disease Ecology

A. Personal Statement.

The goal of this research is to create and validate novel laboratory and statistical methods for detecting, identifying, and characterizing zoonotic viruses via serological techniques, and apply these methods to better understand reservoirs and spillover processes of viruses at the human-wildlife interface. The work will include re-analysis of longitudinal data of Nipah Virus circulation in Bangladesh. I am well positioned to contribute and provide expertise to this work, as this data emerges from research I led over nearly 20 years involving epidemiological studies of bat-origin viruses in Bangladesh, including as current PI of an NIH NIAID-funded U01 studying Nipah virus spillover. This is part of over 20 years of experience conducting epidemiological and immunological studies of bat-origin viruses and the last 15 years managing large-scale multidisciplinary surveillance in wildlife and humans in Liberia, South Africa, Malaysia, India, as well, under various US federal agency-funded projects. I have trained multidisciplinary teams to conduct fieldwork in Malaysia, India, Bangladesh, Saudi Arabia, Cambodia, The Philippines, and Liberia and conducted ecological and epidemiological studies of zoonotic viruses in bats, including henipaviruses, filoviruses, and coronaviruses. 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, using molecular and serological techniques, bioinformatics, and phylogenetic analysis, all of which has given me a strong understanding of the links between immunology and epidemiology in wildlife and humans and the challenges and constraints of serological diagnostics and quantitative analysis in these systems. I have extensively collaborated with PIs

Ross and Laing as well as Co-Investigators Markotter and Wacharapluesadee over the last 10 years and have active projects with each. Collectively, we bring unprecedented data, resources and comprehensive expertise to this project to transform how serology is used in zoonotic disease surveillance.

B. Positions and Honors

Positions and Employment

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,
2008- Adjunct Assoc. Prof. Mt Sinai School of Medicine, Infectious Dis. Dept. 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
2020 - Adjunct Professor, University of Pretoria School of Medicine, Dept. Medical Virology, RSA

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 2022);
Board of Advisors, Tufts University Cummings School of Veterinary Medicine (2017-)
2018 - WHO Blueprint R&D Nipah virus SME; Scientific review committee for IMED 2018
2019 - Global Health SME for Aspen Institute Congressional Delegation: Rwanda;
Technical lead, WHO SEARO Nipah virus prevention strategy development for South Asia (2019-2023)
2020- Expert witness, US Senate Committee on Environment and Public Works (wildlife trade & epidemics)
2020-21 Founder, Jackson Heights Science Communication Initiative (@jhscicomms) – community outreach group communicating COVID-19 science to local public in NYC
2022-23 Guest Editor – *Viruses* journal special issue

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

2021 Pensinger Lecture in Comparative Medicine at the University of Illinois College of Veterinary Medicine; Griffin Circle Award; Garden School, NYC; for technical guidance and assistance developing COVID-19 safety protocols and policies

C. Contributions to Science

1. Research on the epidemiology of emerging zoonotic viruses, including SARS-CoV, Nipah virus and Reston ebolavirus. 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, Reston ebolavirus, SARS-CoV, and MERS-CoV. I have developed and led field teams in Asia and Africa that 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 and South Africa, I helped characterize henipavirus 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 managed field and laboratory teams in Liberia**, Malaysia, India, and Bangladesh that conducted human and wildlife surveillance for novel viruses as well as important viral zonooses such as Ebola, Nipah virus, MERS-CoV, 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.***, S. J. Anthony, A. Islam, A. M. Kilpatrick, S. A. Khan, M. D. Balkey, N. Ross, I. Smith, C. Zambrana-Torrelio and Y. Tao (2020). *"Nipah virus dynamics in bats and implications for spillover to humans."* ***PNAS*** *117(46): 29190-29201.* *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. Mortlock, Marinda, Marike Geldenhuys, Muriel Dietrich, **Jonathan H. Epstein**, Jacqueline Weyer, Janusz T. Pawęska, and Wanda Markotter. "Seasonal shedding patterns of diverse henipavirus-related paramyxoviruses in Egyptian rousette bats." ***Scientific Reports*** 11, no. 1 (2021): 24262.

e. Jayme, S., Field, H.E., de Jong, C., Olival, K.J., Marsh, G., Tagtag, A., Hughes, T., Bucad, A., Barr, J., Azul, R., Retes,L., Foord, A., Yu, M., Cruz, M., Santos, I., Catbagan, D., Lim, M., Benigno, C., Epstein, J.H., Wang, LF., Daszak, P., Newman, S. Molecular evidence of Ebola Reston virus infection in Philippine bats. ***Virology Journal*** (2015) 12:107

2. Experimental studies of bat ecology and immunology to understand emerging zoonoses. In an effort to approach epidemiological studies more comprehensively, we have integrated field and laboratory experimental studies, including experimental infections, into larger field 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 viral maintenance at low incidence and observed patterns of viral genetic diversity. I have used experimental NiV infections (BSL 4) and immunological experiments in bats to generate actual data about viral shedding, pathogenesis, and antibody persistence in

bats, which has informed empirical field data and mechanistic models of viral dynamics. This work has enhanced our ability to understand how Nipah and other viruses circulate in bat populations and how specific age groups 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. **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
c. 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
d. Kim Halpin, Alex D. Hyatt, Rhys Fogarty, Deborah Middleton, John Bingham, Jonathan H. Epstein, Sohayati Abdul Rahman, Tom Hughes, Craig Smith, Hume E. Field, Peter Daszak and the Henipavirus Ecology Research Group. Pteropid Bats are Confirmed as the Reservoir Hosts of Henipaviruses: A Comprehensive Experimental Study of Virus Transmission. ***Am J Trop Med Hyg*** 2011 85:946-951; doi:10.4269/ajtmh.2011.10-0567

3. 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*, as well as a novel morbillivirus in Brazilian bats. 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 led work in Liberia, Bangladesh, India, and Malaysia. As a collaborativegroup, we discovered more than 1200 novel viruses in 25 countries. I served as a technical lead throughout the 10-year project, designing SOPs for wildlife surveillance, and training dozens of scientists and government officials from wildlife and agricultural ministries in more than 25 countries. In South Africa, we are working to understand the diversity of henipaviruses, filoviruses and coronaviruses in Egyptian rousette bats and use serology to determine rates of spillover of these groups of viruses into livestock and human populations.

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. Mortlock, Marinda, Marike Geldenhuys, Muriel Dietrich, **Jonathan H. Epstein**, Jacqueline Weyer, Janusz T. Pawęska, and Wanda Markotter. "Seasonal shedding patterns of diverse henipavirus-related paramyxoviruses in Egyptian rousette bats." ***Scientific Reports*** 11, no. 1 (2021): 24262.
e. Satoshi Ikegame, Jillian Carmichael, Heather Wells, Robert L. Furler, Joshua Acklin, Hsin-Ping Chiu,

Kasopefoluwa Y. Oguntuyo, Robert M. Cox, Aum Patel, Shreyas Kowdle, Christian Stevens, Jean Lim, Takao Hashiguchi, Edison Durigon, Tony Shountz, **Jonathan H. Epstein**, Richard K. Plemper, Peter Daszak, Simon J. Anthony, Benhur Lee. Zoonotic potential of a novel bat morbillivirus. 2023. ***Nature microbiology*** *(in press).*

D. Additional Information: Research Support and/or Scholastic Performance

Ongoing research support related to this proposal

NIH NIAID 1U01AI153420 Epstein (PI) 9/15/2020 – 6/30/2025
The Study of Nipah virus dynamics and genetics in its bat reservoir and of human exposure to NiV across Bangladesh to understand patterns of human outbreaks.

DTRA BTRP HDTRA1-20-1-0026 Epstein (PI)
Biosurveillance for henipaviruses and filoviruses in rural communities in India. 10/1/2020- 09/30/2023
Serological survey of bats, domestic animals and people at high risk interfaces to assess whether spillover of filoviruses or henipaviruses has occurred.

Role: Project director

DTRA BTRP HDTRA1-20-1-0025 P00001 Markotter (PI) 10/1/2020 – 9/30/2023
Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa.
Role: Co-PI, project co-director

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: Ross, Noam

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6)

POSITION TITLE: Principal 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
Brown University	BS	2006	Environmental Science
University of California, Davis	PhD	2015	Ecology

A. Personal Statement

The goal of this research is to create and validate novel laboratory and statistical methods for detecting, identifying, and characterizing zoonotic viruses via serological techniques, and apply these methods to better understand reservoirs and spillover processes of viruses at the human-wildlife interface. I am uniquely suited to lead this work as a mathematical and statistical disease ecologist with extensive experience collaborating with field epidemiologists and virologists in zoonotic disease surveillance. As a Principal Scientist for Computational Research at EcoHealth Alliance, I lead work in ecological and epidemiological modeling, statistical design and interpretation, and data management for our work across multiple USAID, NIH, NSF, and DTRA-supported disease surveillance projects in South and Southeast Asia, Sub-Saharan and Western Africa. This includes disease systems such as Nipah virus, Rift Valley Fever, and emerging filoviruses and sarbecoviruses. Central to this work is modeling and collaborative interpretation of serological and molecular data to infer underlying infections and disease dynamics and evolution. This experience has given me a strong understanding of the ecological, biological, and laboratory processes that serological data. My expertise in dynamic epidemiological processes and linking mechanistic and statistical models allows me represent the underlying biology in tractable, interpretable ways. Through my work with rOpenSci, I lead peer-review of statistical software tools, overseeing the publication of over 300 software packages and the leading standards for the correctness, accessibility, and documentation of research software, which will enable me to ensure that output of this project will be useful and accessible tools for research and surveillance. I am also PI for multiple projects that focuses on machine learning to forecast the emergence and movement veterinary disease outbreaks. I have collaborated with PI Laing and Co-Investigators Epstein, Markotter, Wacharapluesadee extensively and we bring complementary and collectively unique expertise to allow us to execute this project.

Current supported projects that highlight my work and collaborations includes:
As Principal Investigator:

- **Predicting biothreat impacts from early-stage data via transfer learning** (DTRA, 08/2021 – 07/2023)
- **An open source framework for Rift Valley Fever forecasting** (Wellcome Trust, 08/2021 – 07/2023)
- **Expanding statistical software peer review** (Sloan Foundation, 08/2019-06/2024)

As Collaborator:

- **Study of Nipah virus dynamics and genetics in its bat reservoir and of human exposure to NiV across Bangladesh to understand patterns of human outbreaks** (NIH, 09/2020 – 06/2025)
    - Role: Co-I, with PI Jonathan Epstein and Senior Personnel Eric Laing

- **Biosurveillance for viral zoonoses around bat-livestock-human interfaces in South Africa** (DTRA, 07/2020 – 07/2025)
    - Role: Co-I, with PI Wanda Morkotter, Co-I Jonathan Epstein, and Co-I Laing
- **Biosurveillance for spillover of henipaviruses and filoviruses in rural communities in India**
    - Role: Project Scientist, with PI Jonathan Epstein

B. Positions, Scientific Appointments, and Honors

2020 - Present	Principal Scientist, Computational Research, EcoHealth Alliance
2016 – 2019	Senior Research Scientist Ecologist, EcoHealth Alliance
2015 – 2016	Disease Ecologist, EcoHealth Alliance
2010 – 2015	Graduate Researcher, University of California-Davis
2007 – 2009	Senior Analyst, Environmental Strategy and Markets, GreenOrder
2006 – 2007	Analyst, Environmental Strategy and Markets, GreenOrder
2006	Contract Researcher: Energy Efficient Products Initiative, Wal-Mart

C. Contributions to Science

1. **Detecting emerging diseases and the drivers of their dynamics:** A heavy component of my research is collaborating with field surveillance teams to identify spillovers of novel emerging diseases by devising sampling designs, interpreting molecular and serological data, and using mechanistic and nonparametric models to best detect disease emergence events and elucidate predictive factors. Importantly this has included developing statistical approaches to deal with rare events and sparse predictors. This work has helped identify novel spillover events and actionable behavioral risk factors.

   Epstein JE, Anthony SJ, … **Ross N** *et. al.* (2020) Nipah virus dynamics in bats and implications for spillover to humans. PNAS 117(46):29190-29201

   Rostal MK, Cleaveland S,…, **Ross N** *et. al.* (2020) Farm-Level Risk Factors of Increased Abortion and Mortality in Domestic Ruminants during the 2010 Rift Valley Fever Outbreak in Central South Africa. Pathogens 9(11):914.

   Li, H, Mendelsohn, E, Zong, C, Zhang, W, Hagan, E, Wang, N., … **Ross N.**, … Daszak P (2019). Human-animal interactions and bat coronavirus spillover potential among rural residents in Southern China. Biosafety and Health, 1(2), 84–90.

   Salerno J, **Ross N**, Ghai R, Mahero M, Travis DA, Gillespie TR, Hartter J. (2017) Self-reported fever associated with human-wildlife interactions across park landscapes in western Uganda. EcoHealth 14(4):675-690.

2. **Modeling dynamics of Heterogeneity:** I have worked on both theoretical and applied approaches of dealing with individual-and system-level heterogeneity ecological-epidemiological dynamics. This work includes complex size structure of populations modeled with integral projection models, and heterogenous disease states in multi-infection fungal systems, and is directly applicable to this proposal's aims of capturing the complexity of multidimensional immune states to identify viruses. My work showed how and where these models diverged from other, traditional models in their properties, and identified statistical patterns that could be used to identify processes of disease amplification in hosts. I developed numerical tools for modeling and simulation of controls and intervention, making it tractable to use such statistical and simulation models in ecology, and this work has been used in applied disease management.

   Pedersen, EJ, Miller, DL, Simpson, G. L., & **Ross, N**. (2019). Hierarchical generalized additive models in ecology. PeerJ, (7) e6876.

   Cobb R, Hartsough P, **Ross N**, Klein J, LaFever DH, Frankel SJ, Rizzo DM (2017) Resiliency or restoration: management of sudden oak death before and after outbreak. Forest Phytophthoras (7):1

Schreiber S, **Ross N**. (2016) Individual-based integral projection models: The role of size-structure on extinction risk and establishment success. Methods in Ecology and Evolution 7(7):867-74.

Ross N (2015). Disease with Multiple Infections: Population Structure, Dynamics, and Control. University of California, Davis. Dissertation.

3. **The macroecology of disease emergence:** I have worked in understanding global patterns and drivers of disease distributions, host-sharing and spillover. This work involved integrating models of macroecological processes, host and disease phylogenies, climate as well and human travel and behavior. This also includes using modern data science tools to extract information about disease events from unstructured and textual sources to assemble sufficient data to produce such analyses.

Carlson CJ, Albery GF, Merow C, Trisos CH, Zipfel CM, Eskew EA, **Ross N**, et al. (2022) Climate change will drive novel cross-species viral transmission. Nature 607 (7919).

Grange ZL, Goldstein T, Johnson CK, Anthony S…**Ross N,** *S Wacharapluesadee* et al. (2021). Ranking the risk of animal-to-human spillover for newly discovered viruses. PNAS (118), dx.doi.org/10.1073/pnas.2002324118

Albery, G.F., Eskew, E.A., **Ross, N**. *et al.* Predicting the global mammalian viral sharing network using phylogeography. Nature Communications 11, 2260 (2020).

Olival KJ, Hosseini P, Zambrana-Torrelio C, **Ross N**, Bogich T, Daszak P. (2017) Host and viral traits predict zoonotic spillover from mammals. Nature 546:646–650.

4. **Statistical software and reproducibility:** As co-lead of the rOpenSci project for software peer-review, and a member of the Software Carpentry foundation, I develop, evaluate, and set standards and for the correctness and quality of open-source statistical software, overseeing the publication of over 300 scientific software packages in the past five years. I am also partner of the Rocker Project developing reproducible containerized computer environments for scientific research, and co-lead of a mentorship program to increase the participation of historically underrepresented groups in scientific computing.

Nüst D, Eddelbuettel…**Ross N et al.** (2020). The Rockerverse: Packages and Applications for Containerisation with R. The R Journal 12:1, p437-461.

Ram K, Boettiger C,…, **Ross N** et al. (2019) A Community of Practice Around Peer Review for Long-Term Research Software Sustainability. Computing Science & Engineering, 21(2):59-65

Ross, N., Eskew, E., & Ray, N. (2019). citesdb: An R package to support analysis of CITES Trade Database shipment-level data. Journal of Open Source Software, 4(37), 1483.

Ross N (2016) fasterize: high performance raster conversion for modern spatial data. https://github.com/ecohealthalliance/fasterize

5. **Modeling decision-making in complex systems:** A long-standing theme of my work has been linking ecological dynamics to social systems and decision-making under uncertainty. This has included determining whether statistical signals of ecological changes are sufficient to justify management changes in fisheries, and has extended to optimizing investment in disease surveillance and intervention.

Machalaba C, Smith KM…**Ross N…** et al. (2017) *One Health Economics to confront disease threats*. **Transactions of the Royal Society of Tropical Medicine and Hygiene**

Boettiger C, **Ross N,** Hastings A. (2013) Early warning signals: The charted and uncharted territories. Theoretical Ecology 6(3):255-64.

Kate Fuller K, Kling D, Kroetz K, **Ross N**, and Sanchirico JN (2013) Economics and Ecology of Open-Access Fisheries. In: Shogren, J.F., (ed.) ***Encyclopedia of Energy, Natural Resource, and Environmental Economics, Vol. 2*** Encyclopedia of Energy, Natural Resource, and Environmental Economics p.39-49. Amsterdam: Elsevier. http://dx.doi.org/10.1016/B978-0-12-375067-9.00114-5

From: Noam Ross on behalf of Noam Ross <ross@ecohealthalliance.org>
To: Wanda Markotter
Cc: Laing, Eric
Subject: Re: NIH submission proposed budget
Date: Tuesday, January 31, 2023 1:58:31 PM
Attachments: nih-serostats_BioSketch_Epstein_TOSUMBIT.docx
nih-serostats_BioSketch_Ross_TOREVIEW.docx

Many thanks, Wanda!

The NIH biosketch section (C) is a little more expansive and narrative than just the list of four papers, unfortunately. They ask for descriptions of major contributions, each with up to four references as examples. I'm attaching Jon's and mine for reference. (Sorry! I am always embarrassed for how much of a to-do NIH and NSF make these.)

Would you be able to expand on this? If you just take a few minutes to put in some bullets or pasted text and references to additional papers/topics, we're happy to with stitching it together and formatting!

Noam

--

Dr. Noam Ross
Principal Scientist, Computational Research

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noamross.net

On Tue, Jan 31, 2023 at 7:52 AM Wanda Markotter <wanda.markotter@up.ac.za> wrote:

> Afternoon Noam
>
> See the attached documents requested. Let me know if you need anything else.
>
> Wanda
>
> On Sat, 28 Jan 2023 at 00:28, Noam Ross <ross@ecohealthalliance.org> wrote:
>
> > Dear Wanda,
> >
> > Find attached two documents for your review: A letter of support and a budget justification.

- The LoS requires only your review or edits, signature and to be on UP letterhead.
- The budget justification just requires your review. I do note that it describes a 35% fringe benefits rate, as the budget did, but this should be based on the institutional rate. If this should be different, described differently, and/or we should modify the budget to incorporate this into base salary or time, please let me know. Thank you!

Best,
Noam
--

Dr. Noam Ross
Principal Scientist, Computational Research

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

On Thu, Jan 26, 2023 at 1:50 PM Noam Ross <ross@ecohealthalliance.org> wrote:

> Great, Wanda. I've moved your salary to two lines for the postdoc and lab technician. We will still list you as committing 2 weeks/yr as the institutional lead at no salary.
>
> If we could have the BioSketch and Facilities description by the end of your Tuesday, that would be great.
>
> I'm doing a last pass over all the paperwork requirements to make sure there isn't other info I'll need from any partners. There will be a Letter of Support and Budget Justification, both of which I'll draft before next week for you to review.
>
> Noam
> --
>
> Dr. Noam Ross
> *Principal Scientist, Computational Research*
>
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EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.

On Thu, Jan 26, 2023 at 9:47 AM Wanda Markotter <wanda.markotter@up.ac.za> wrote:

> Afternoon Noam
>
> Thanks for this. Overall it looks fine. I usually do not claim direct salary, but it can be directed to a postdoc salary, so you can keep it as is or split a technician and a postdoc and have both. I think both will be needed.
>
> The sample costs are fine.
>
> I still owe you a bio sketch and equipment description. When do you need that at the latest?
>
> W
>
> On Thu, 26 Jan 2023 at 02:10, Noam Ross <ross@ecohealthalliance.org> wrote:
>
> > Dear Wanda,
> >
> > Find attached a rough proposed budget for your participation in our serology methods proposal, based on the per-plate costs you provided. My apologies for getting this to you late. We've estimated that we want to re-test up to 6,000 samples from SABRENET and related projects. This is based on current positive hit rates, and including matched negative samples. We propose to do this across years 3 and 4 of the project, after the bulk of our panel modifications and statistical testing take place in years 1 and 2. We include budget for personnel time extending into year 5 of the grant for you and a postdoc/tech to continue to participate in analysis and interpretation.
> >
> > I have made some very rough guesses in the attached to understand the order of magnitude. For instance, I'm unsure if you need to take direct salary and don't know at rate that would be (other PI partners doing testing/re-analysis are taking 2 weeks/yr). Please let us know how this looks and adjust as appropriate.
> >
> > Best,
> > Noam
> > --
> >
> > Dr. Noam Ross
> > *Principal Scientist, Computational Research*
> >
> > EcoHealth Alliance
> > 520 Eighth Avenue, Suite 1200
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On Sun, Jan 15, 2023 at 6:17 AM Wanda Markotter <wanda.markotter@up.ac.za> wrote:

> Afternoon
>
> Thank you for the discussion. It will be an interesting project
>
> Attached are some costs for consideration
>
> To run one plate (about 80 sample, considering controls also) costs us about 265 USD labour included. So the total costs will depend on how many samples you want to include and repeats. These costs also assume we are getting coupled beads with antigen from USU, so no costs on that was considered.
>
> I also mention the costs for collecting the samples and costs of a research scientists for consideration.
>
> Let me know if you have questions. Marinda will send you the breakdown of the samples.
>
> Will send the bio and equipment list by the end of this week.
>
> Wanda

On Wed, 11 Jan 2023 at 01:19, Noam Ross <ross@ecohealthalliance.org> wrote:

> Dear Wanda, Marinda, and Marike,
>
> Good to speak with you today. I'm writing with the materials for the biosketch and facilities document for the application. Find attached a template and example NIH format biosketch that we'll need for Wanda. (Further instructions here if needed).
>
> We'll also need a Facilities & Equipment document that summarizes resources available at your institution and lab, with a focus on what is needed to run Luminex and associated we will need (this is actually Supaporn's for a different NIH project), (Instructions)
>
> It would be great if we could get these by **Wednesday, Jan 25th.**
>
> If we get some preliminary figures on samples by species and costs, we'll get budget info to you ASAP next.

Best,
Noam
--

Dr. Noam Ross
Principal Scientist, Computational Research

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Facilities and Other Resources

Uniformed Services University of the Health Sciences
Dr. Eric Laing (Co-Investigator)

Overall Scientific Environment: Uniformed Services University (USU) is the medical school at which approximately half of the physicians in the Armed Services receive their graduate training. Research at USU is supported primarily by extramural grants, as in other medical schools. Dr. Laing is an Assistant Professor in the Department of Microbiology and Immunology, which includes 12 full-time Faculty members. The overall focus of the Department is 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. Dr. Laing is currently involved in active collaborations within the University, in areas of viral immunology and vaccine and antiviral therapeutics and animal model development. USU is also physically located directly across from the main NIH campus in Bethesda. The overall broad scientific environment at both USU and the NIH is highly conducive to productive collaborations. The PI often uses these resources to his advantage, both for his research objectives and interests and in assisting in adjunct faculty appointments within the Emerging Infectious Diseases graduate program for both on and off-campus scientists interested in participating in graduate education and graduate student training. Overall, the available technical resources (and University support for continually improving technical resources) is exceptional. In addition, multi-lab Immunology journal club, Immunology data club, Virology journal club, and Virology data club exist. Approximately 10 groups regularly participate in the immunology journal/data club and 5 groups regularly participate in the virology journal/data club.

Dr. Laing has been well-supported by the Department of Microbiology and Immunology and the USU Office of Research. Although USU is a modest-sized medical school, the technical resources are outstanding, rivaling many very large institutions (see below). The modest size of the school is an advantage, as key pieces of equipment (e.g., Bruker In Vivo Xtreme II; Siemens Inveon SPECT/PET/CT Scanner) are not overbooked and therefore readily available to the laboratory personnel. In addition to University funds, we have successfully used the NIH S10 mechanism to purchase advanced and very expensive equipment, including the Zeiss 710NLO system and the FACSAria 15-parameter cell sorter. Overall, the available technical resources (and University support for continually improving technical resources) is exceptional, as is illustrated by the most recent USU purchase of cutting-edge equipment: the Zeiss Elyra PS.1 super-resolution microscope.

Laboratory: Dr. Laing's laboratory is 453 sq. ft. and is currently supported by two graduate students, two research assistants, two research associates, and one scientist. Dr. Laing also has access to 3 rooms within the department totaling 1,440 sq. ft. where BSL-2 virus rescues are performed and blood specimen samples are handled. Dr. Laing has access to or possesses routine equipment for microbiology, molecular biology, and biochemistry including incubators, centrifuges, inverted microscopes, gel electrophoresis equipment for protein and DNA gels, thermal cyclers, balances, pH meters, refrigerators, freezers, platform orbital shakers, sonicator, UV transilluminator, shakers, heating blocks, waterbaths, free-standing biosafety cabinets, and CO_2-tissue culture incubators. Additionally, Dr. Laing has access to three BioPlex 200 HTF multiplexing systems, one MAGPIX multiplexing system, an epifluorescence microscopy system (a Zeiss AxioObserver inverted fluorescence microscope, upgraded in 2015), and two complete GE-ATKA low pressure chromatography systems, with integrated UV detectors, fraction collectors, and pump systems, and gradient fractionator apparatus.

Animal: Animals are maintained in University facilities under the supervision of a full-time veterinarian. The USUHS maintains a modern AAALC Accredited, Central Animal Facility of about 50,000 square feet. It, and the University's animal care and use program, is managed by the Center for Laboratory Animal Medicine, which is directed by a veterinarian who is an ACLAM Diplomate and staffed with three other veterinarians, a graduate animal husbandryman, and about 30 technicians. The University is able to provide appropriate care for a wide variety of laboratory animal species, from invertebrates to lower vertebrates, to higher vertebrates including non-human primates and domestic livestock, as well as the more commonly used species such as rodents and rabbits. The facility also includes a number of properly equipped ABSL-2 rooms.

Computer: Windows-based computers (Intel i5/i7 or similar processors and ≥2GB of RAM) are available for routine use by postdoctoral fellows, graduate students, and technicians located in the laboratories of both investigators. The University maintains site licenses for other image and data analysis software, including Zeiss Axiovision, VisioPharm, and OriginPro.

Office: Dr. Laing has an office separate from but proximal to the laboratories. Trainees and technicians have desks in the laboratory or in a separate office area proximal to the laboratory. The department employees one senior program manager, administrative officer, administrative specialist and a microbiologist.

Clinical: N/A

Other: The Biostatistics Consulting Center (BCC), a service of the Department of Preventive Medicine and Biometrics, provides statistical consulting to USUHS scientific investigators. We routinely consult with Cara Olsen, Research Assistant Professor (the full-time Biostatistics Consultant of the BCC), regarding proper design of experiments for statistical testing and for statistical analysis of the resulting data.
The USU Translational Imaging Facility (TIF) houses state-of-the art equipment for live animal imaging, including a Siemens Inveon SPECT/PET/CT Scanner, a Bruker Biospec 70/20 USR Magnetic Resonance Imaging system, and a Bruker In-Vivo Xtreme II bioluminescence and X-ray imaging system.

The USU Biomedical Instrumentation Center (BIC) houses core equipment for use by investigators throughout the University. Instrumentation is available either free or on a fee-for-service basis, depending on which instruments have annual service contracts (which are paid largely through per-hour use fees). The BIC Flow Cytometry Core includes two Becton-Dickinson (10- and 13-parameter) LSRII FACS analyzers, one 15-parameter FACSAria FACS sorter, and one Amnis Image Stream X Mark II imaging flow cytometer, as well as off-line analysis workstations.

The BIC Imaging Core houses three confocal microscopes, including a Zeiss 700 inverted system with 405/458/488/514/561/633 laser excitation; a Zess 710NLO inverted system with 405/458/488/514/561/633 conventional lasers and a Coherent Ultra2 Ti-Sapphire laser for multiphoton excitation, continuously tunable over the range of 690 to 1080 nm; and a Zeiss AxioExaminer-Z1 upright microscope equipped with a direct-coupled Coherent Chameleon tunable infrared laser for ex vivo and in vivo mutliphoton imaging projects. A Becker-Hickl two-detector FLIM system (for FRET analyses) is connected to the inverted Zeiss 710NLO system. Recently, the BIC has also acquired a Zeiss Elyra PS.1 super-resolution microscope, which is capable of 4-parameter SR-SIM (super-resolution structured illumination) imaging, 3-parameter PALM (Photoactivation localization microscopy) and dSTORM (direct stochastic optical reconstruction microscopy), as well as 3D-PALM/dSTORM. The BIC also houses a Leica AF6000 system, consisting of an inverted microscope equipped with a fully motorized 3-axis stage plus atmosphere and temperature control, allowing extended term (days) live cell analyses. Additionally, there is a stereology system consisting of a Zeiss AxioImager.M2 upright microscope connected to MicroBrightField's Stereo Investigator software package. The facility also includes several additional wide-field fluorescence microscopes, and three offline data analysis stations with software packages including: Zeiss Zen software and full Physiology package; Media Cybernetics' 3D Constructor, Image Pro Analyzer, Autodeblur, and Autovisualize; Metamorph Basic. The Imaging Core also includes a transmission electron microscope (Philips CM100 transmission EM) and an ultramicrotome (Leica EM UC6 with EM FC6 cryo attachment).

The BIC Genomics core includes an ABI 3900 DNA synthesizer, an ABI3500xl Genetic Analyzer (for sequencing), a RochLightCycler 480 for real-time PCR, and Systec Mediaprep and Plate Pourer instrument. There is also an integrated Fuji FLA-5000/LAS-3000 imaging system for many applications that involve fluorescence and chemiluminescence imaging of gels and blots. The BIC Proteomics Core includes two Agilent 1100 HPLCs, an AB SCIEX Voyager DSTR MALDI-TOF mass spectrometer, and an AB SCIEX Q-TOF tandem mass spectrometer.

The BIC Structural Biology Core includes a Rigaku HighFlux HomeLab X-ray diffraction system, with a MicroMax-007 HF microfocus rotating anode generator, an R-AXIS Imaging Plate detector, and an X-stream 2000 cryogenic system. Other available BIC instruments and services include histopathology and PET/CT instrumentation for small animal research.

The University also has an equipment repair service, central duplicating service, audiovisual service, and microcomputer support service. The University Learning Resource Center is a high quality medical and scientific library with additional microcomputers and support. A wide variety of scientific journals are available in print and via remote computer access. A central autoclave/glassware washroom serves the Department of Microbiology and Immunology and is maintained through extramural grant support.

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: Assistant Professor

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. (hons)	05/2008	Biology
Uniformed Services University, Bethesda, MD	Ph.D.	10/2016	Emerging Infectious Diseases
Uniformed Services University, Bethesda, MD	Postdoc	04/2019	Virology

A. Personal Statement

The spillover of zoonotic viruses into human populations remains an ever-prevalent threat to public and global health. Continual outbreaks of known and unknown zoonotic viruses highlights the paucity of our understanding of the viral diversity, geographic distribution, wildlife hosts, and human populations at-risk for spillover. I am a recently appointed assistant professor dedicated to biosurveillance and virological research of emergent zoonotic viruses. I have completed virological training and spent my time as a post-doctoral fellow applying *in vitro* techniques to characterize the replication and mammalian ephrin receptors that mediate cellular entry of emergent henipaviruses. Since the start of the COVID-19 pandemic, I pivoted the focus of my research team to serological analysis of SARS-CoV-2 infection, and then to COVID-19 vaccine-induced antibody durability. In my ongoing research, I develop serological-based approaches to identify the infectome of wildlife and human communities for the assessment of virus spillovers. Since my position as a postdoctoral fellow I have worked collaboratively with national and international researchers including institutional partners at Duke-NUS, Chulalongkorn University, University of Pretoria, and Rocky Mountain Labs. Across all of these international collaborations, we have aimed to identify known and unknown viruses, the wildlife reservoirs of priority pathogens, the high-risk interfaces for spillover and the biotic/abiotic drivers of virus emergence. Throughout these research projects, we have detected unexpected serological profiles in communities of bats and humans that have not fit our prior understanding of the viral diversity established or detected by genetic techniques. This proposal aims to better understand these serological profiles of wildlife and humans with a targeted focus on henipaviruses and filoviruses. Ongoing projects that I would like to highlight include:

- HDTRA12110037. DTRA BRTP, E. Laing (Co-PI). 08/2021-07/2026,"Informing biosurveillance, contribution of pteropodid fruit bats to virus spillover in the Philippines."
- U01AI151797. NIH, Centers for Research in Emerging Infectious Diseases, E. Laing (Co-I). 02/2020 – 03/2025, "EID-Southeast Asia Research Collaborative Hub."
- HDTRA12010025. DTRA BTRP, E. Laing (Co-I). 7/2020 – 6/2025, "Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa"
- HU00012020067, HU00011920111. Defense Health Program, NIAID, E. Laing (Associate Investigator). 03/2020 – 09/2023, "Epidemiology, Immunology, and Clinical Characteristics of Emerging Infectious Diseases with Pandemic Potential (EpICC-EID)."

B. Positions, Scientific Appointments and Honors

Positions

2021-pres. Assistant Professor, Department of Microbiology and Immunology, School of Medicine, Uniformed Services University, Bethesda, MD.

2021-pres. Joint Appointment, Emerging Infectious Diseases Graduate Program, School of Medicine, Uniformed Services University, Bethesda, MD.

2019-21 Research Assistant Professor, Department of Microbiology and Immunology, Uniformed Services University, Bethesda, MD.

2016-19 Postdoctoral fellow, Henry M. Jackson Foundation, Department of Microbiology, Uniformed Services University, Bethesda, MD.

2010-16 Graduate research student, Department of Microbiology, Uniformed Services University, Bethesda, MD.

Other Experiences and Professional Memberships

2022-pres. Executive committee member, Emerging Infectious Diseases Graduate Program, USUHS

2021-pres. Research center representative, Laboratory Assays Oversight and Quality Working Group, Emerging Infectious Diseases: Southeast Asia Research Collaboration Hub (EID-SEARCH), Centers for Research in Emerging Infectious Diseases (CREID), NIAID, DMID, NIH

2021-pres. Review editor, Frontiers in Virology - Emerging and Reemerging Viruses

2021-pres. Ad hoc reviewer, Frontiers in Immunology, Journal of Clinical Immunology

2021-pres. Steering committee member, JPI/Military Infectious Diseases Research Program, Emerging Infectious Diseases

2019 Ad hoc reviewer, EcoHealthNet 2.0 Program, EcoHealth Alliance

2018 Ad hoc reviewer, Pakistan One-Health Fellowship Program, National Academy of Sciences & Pakistan Academy of Sciences

2014-2019 Member, American Society of Tropical Medicine and Hygiene

2014-2019 Member, American Society of Microbiology

2014-2019 Volunteer, AAAS STEM K-12 Volunteer Program

Mentoring

Postdoctoral fellows Si'Ana A. Coggins, PhD, 2020 - 2022

Graduate students Marana S. Tso, BS, 2021 -
McKenna Roe, BS, 2022 -

Committee member Celeste Huaman, BS, 2021 -
2LT Connor Perry, BS, 2021 -

Honors

2021-2022 Impact Award, USUHS School of Medicine

2021 Outstanding Research Accomplishment/Team/SARS-CoV-2, The EPICC COVID-19 Cohort Team, Military Health System Research Symposium

2020-2021 Impact Award, USUHS School of Medicine

2015-2016 Val G. Hemming Fellowship, Henry M. Jackson Foundation

2015 East Asia and Pacific Summer Institutes Fellowship, National Science Foundation

C. Contributions to Science

1. Lyssaviruses and the prototype, rabies virus, remain a public health concern. Beginning with my PhD thesis work, I've researched the virus host-interactions between a rabies-related lyssavirus, Australian bat lyssavirus (ABLV), and its bat host (*Pteropus alecto*). Research has focused on ABLV cellular entry mechanisms, the development of an animal model and ABLV reporters and exploration of novel monoclonal antibodies that neutralize ABLV and other phylogroup I lyssaviruses. Furthermore, comparative bat immunology research was conducted using black flying fox cell lines and ABLV as a model virus/host interaction. Physiological adaptations that accompanied the evolution of flight in bats have been proposed to contribute to the frequent role of bats as asymptomatic hosts of highly pathogenic zoonotic viruses. Comparatively studying the autophagy pathway in bat cell lines revealed that bat cells had elevated levels of basal autophagy and experienced significantly less cell death when challenged with high virus doses.

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. Virol J. 2013. 11:40. doi: 10.1186/1743-422X-11-40. PMID: 24576301, PMCID: PMC3946599

b. **Laing E.D.**, Sterling S.L., Weir D.L., Beauregard C.R., Smith I.L., Larsen S.E., Wang L-F., Snow A.L., Schaefer B.C., and Broder C.C. Enhanced autophagy contributes to reduced viral infection in black flying fox cells. Viruses. 2019. Mar 14;11(3). pii: E260. doi: 10.3390/v11030260. PMID: 30875748, PMCID: PMC6466025

c. Mastraccio K.E., Huaman C., Warrilow D., Smith G.A., Craig S.B., Weir D.L., **Laing E.D.**, Smith I., Broder C.C. and B.C. Schaefer. Establishment of a longitudinal pre-clinical model of lyssavirus infection. J Virol Methods. 2020 Jul; 281:113882. doi: 10.1016/j.jviromet.2020.113882. Epub 2020 May 12. PMID: 32407866

d. Weir D.L., Coggins S.A., Vu B.K., Coertse J., Yan L., Smith I.L., **Laing E.D.**, Markotter W., Broder C.C., and Schaefer B.C. Isolation and characterization of cross-reactive human monoclonal antibodies that potently neutralize Australian bat lyssavirus variants and other phylogroup 1 lyssaviruses. Viruses. 2021 Mar 1;13(3):391. doi: 10.3390/v13030391. PMID: 33804519; PMCID: PMC8001737.

2. My research experience as a postdoctoral fellow furthered my training in molecular virology techniques. I constructed a recombinant Cedar virus cDNA plasmid and optimized a reverse genetics approach to rescue a recombinant Cedar virus reporter virus, a non-pathogenic *Henipavirus* species. A molecular biology methods chapter detailing recombinant Cedar virus reverse genetics has been submitted and in press. Using this recombinant Cedar virus, we determined that Cedar virus can utilize several non-canonical henipavirus ephrin receptors for cellular entry and explored the structure of the receptor-binding pocket to understand the receptor promiscuity. The non-pathogenic phenotype of CedV creates a potential for CedV to act as a model henipavirus to explore host-pathogen interactions, cellular tropism and factors that determine henipaviral disease pathogenesis. Additionally, I have collaborated on projects detailing henipavirus infection and replication in bat hosts with colleagues at the Rocky Mountain Labs, studying whether specific species of bats are more competent hosts and whether virus-host restriction exists.

a. Amaya M, Broder CC, **Laing ED**. Recombinant Cedar virus: a henipavirus reverse genetics platform. In: Freiberg A.N. and B. Rockx, Nipah Virus: Methods and Protocols, Methods Mol. Biol. (in press)

b. Seifert SN, Letko MC, Bushmaker T, **Laing ED**, Saturday G, Meade-White K, van Doremalen N, Broder CC, Munster VJ. Rousettus aegyptiacus Bats Do Not Support Productive Nipah Virus Replication. J Infect Dis. 2020 May 11;221(Suppl 4):S407-S413. doi: 10.1093/infdis/jiz429. PMID: 31682727; PMCID: PMC7199784.

c. **Laing ED,** Navaratnarajah CK, Cheliout Da Silva S, Petzing SR, Xu Y, Sterling SL, Marsh GA, Wang LF, Amaya M, Nikolov DB, Cattaneo R, Broder CC, Xu K. Structural and functional analyses reveal promiscuous and species specific use of ephrin receptors by Cedar virus. Proc Natl Acad Sci U S A. 2019 Oct 8;116(41):20707-20715. doi: 10.1073/pnas.1911773116. Epub 2019 Sep 23. PMID: 31548390; PMCID: PMC6789926.

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. PMID: 29587789; PMCID: PMC5869790.

3. Bats are increasingly identified as animal reservoirs of emerging zoonotic viruses (e.g. Nipah virus, Ebola virus and SARS-coronavirus). I lead collaborative biosurveillance and research preparedness training including data analysis and interpretations at international partner institutes with lab technicians, field and lab scientists, and masters, doctoral and postdoctoral trainees. Collaborative biosurveillance is presently underway in Thailand (Chulalongkorn University, Bangkok) Malaysia (National Wildlife and Forensic Lab, Universti Purtra Malaysi, National Public Health Lab) via NIH Centers for Research in Emerging Infectious Diseases, EID-Southeast Asia Research Collaborative Hub. As a collaborator within the DARPA PREEMPT network I supported surveillance for coronaviruses and other priority emerging zoonotic viruses, henipaviruses and filoviruses, in Ghana (Zoological Society of London), Australia (Black Mountain Labs) and Bangladesh (icddr,b). We aim to characterize the geographic distribution of zoonotic filoviruses/henipaviruses/coronaviruses, transmission dynamics in wildlife hosts and generate risk-models for Ebola virus, Nipah virus and SARS-related CoV outbreaks. Results discovered so far suggest a wider

geographical footprint of Asiatic filoviruses and have identified several fruit bat species that act as natural reservoirs for these viruses.

a. Paskey AC, Ng JHJ, Rice GK, Chia WN, Philipson CW, Foo RJH, Cer RZ, Long KA, Lueder MR, Lim XF, Frey KG, Hamilton T, Anderson DE, **Laing ED**, Mendenhall IH, Smith GJ, Wang LF, Bishop-Lilly KA. Detection of Recombinant Rousettus Bat Coronavirus GCCDC1 in Lesser Dawn Bats (Eonycteris spelaea) in Singapore. Viruses. 2020 May 14;12(5):539. doi: 10.3390/v12050539. PMID: 32422932; PMCID: PMC7291116.

b. Schulz JE, Seifert SN, Thompson JT, Avanzato V, Sterling SL, Yan L, Letko MC, Matson MJ, Fischer RJ, Tremeau-Bravard A, Seetahal JFR, Ramkissoon V, Foster J, Goldstein T, Anthony SJ, Epstein JH, **Laing ED**, Broder CC, Carrington CVF, Schountz T, Munster VJ. Serological Evidence for Henipa-like and Filo-like Viruses in Trinidad Bats. J Infect Dis. 2020 May 11;221(Suppl 4):S375-S382. doi: 10.1093/infdis/jiz648. PMID: 32034942; PMCID: PMC7213578.

c. Dovih P, **Laing ED**, Chen Y, Low DHW, Ansil BR, Yang X, Shi Z, Broder CC, Smith GJD, Linster M, Ramakrishnan U, Mendenhall IH. Filovirus-reactive antibodies in humans and bats in Northeast India imply zoonotic spillover. PLoS Negl Trop Dis. 2019 Oct 31;13(10):e0007733. doi: 10.1371/journal.pntd.0007733. Erratum in: PLoS Negl Trop Dis. 2021 Nov 16;15(11):e0009836. PMID: 31671094; PMCID: PMC6822707.

d. **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. PMID: 29260678; PMCID: PMC5749470.

4. Responding to the emergence of SARS-CoV-2, our lab developed multiplex serology strategies to identify SARS-CoV-2 antibodies and address research questions related to whether pre-existing antibody memory induced by prior infection with seasonal human coronaviruses effects COVID-19 severity. Our lab's serology efforts supported NIH and DHA collaboratively funded protocols including prospective, longitudinal serological analysis of hospital and community subjects, and health-care workers; and cross-sectional analyses of SARS-CoV-2 infection among deployed military health-care workers.

a. Goguet E, Powers JH 3rd, Olsen CH, Tribble DR, Davies J, Illinik L, Jackson-Thompson BM, Hollis-Perry M, Maiolatesi SE, Pollett S, Duplessis CA, Wang G, Ramsey KF, Reyes AE, Alcorta Y, Wong MA, Ortega O, Parmelee E, Lindrose AR, Moser M, Samuels EC, Coggins SA, Graydon E, Robinson S, Campbell W, Malloy AMW, Voegtly LJ, Arnold CE, Cer RZ, Malagon F, Bishop-Lilly KA, Burgess TH, Broder CC, **Laing ED**, Mitre E. Prospective Assessment of Symptoms to Evaluate Asymptomatic SARS-CoV-2 Infections in a Cohort of Health Care Workers. Open Forum Infect Dis. 2022 Feb 14;9(3):ofac030. doi: 10.1093/ofid/ofac030. PMID: 35198647; PMCID: PMC8860153.

b. Ramos I, Goforth C, Soares-Schanoski A, Weir DL, Samuels EC, Phogat S, Meyer M, Huang K, Pietzsch CA, Ge Y, Pike BL, Regeimbal J, Simons MP, Termini MS, Vangeti S, Marjanovic N, Lizewski S, Lizewski R, George MC, Nair VD, Smith GR, Mao W, Chikina M, Broder CC, **Laing ED**, Bukreyev A, Sealfon SC, Letizia AG. Antibody Responses to SARS-CoV-2 Following an Outbreak Among Marine Recruits With Asymptomatic or Mild Infection. Front Immunol. 2021 Jun 9;12:681586. doi: 10.3389/fimmu.2021.681586. PMID: 34177926; PMCID: PMC8220197.

c. Clifton GT, Pati R, Krammer F, **Laing ED**, Broder CC, Mendu DR, Simons MP, Chen HW, Sugiharto VA, Kang AD, Stadlbauer D, Pratt KP, Bandera BC, Fritz DK, Millar EV, Burgess TH, Chung KK. SARS-CoV-2 Infection Risk Among Active Duty Military Members Deployed to a Field Hospital - New York City, April 2020. MMWR Morb Mortal Wkly Rep. 2021 Mar 5;70(9):308-311. doi: 10.15585/mmwr.mm7009a3. PMID: 33661864; PMCID: PMC7948931.

d. Lalani T, Lee TK, **Laing ED**, Ritter A, Cooper E, Lee M, Baker M, Baldino T, Mcadoo T, Phogat S, Samuels E, Nguyen H, Broder CC, Epsi N, Richard SA, Warkentien TE, Millar EV, Burgess T, Kronmann KC. SARS-CoV-2 Infections and Serologic Responses Among Military Personnel Deployed on the USNS COMFORT to New York City During the COVID-19 Pandemic. Open

Forum Infect Dis. 2021 Jan 23;8(2):ofaa654. doi: 10.1093/ofid/ofaa654. PMID: 33553482; PMCID: PMC7856331.

5. In addition to providing serologic assessment of SARS-CoV-2 infection, my research team is actively engaged in examining the durability of COVID-19 vaccine induced humoral immunity. Antibody responses, particularly neutralizing antibodies, are frequently cited as a predictive correlate of protection. With the emergence of variants of concern and waning circulating antibodies, the timing of booster shots remains an important measure for controlling the pandemic. In my lab we evaluate the duration of neutralizing antibodies, durability and breadth of antibody responses against emerging variants of concern, hybrid immune responses, and post-vaccination infections.

a. **Laing ED**, Weiss CD, Samuels EC, Coggins SA, Wang W, Wang R, Vassell R, Sterling SL, Tso MS, Conner T, Goguet E, Moser M, Jackson-Thompson BM, Illinik L, Davies J, Ortega O, Parmelee E, Hollis-Perry M, Maiolatesi SE, Wang G, Ramsey KF, Reyes AE, Alcorta Y, Wong MA, Lindrose AR, Duplessis CA, Tribble DR, Malloy AMW, Burgess TH, Pollett SD, Olsen CH, Broder CC, Mitre E. Durability of Antibody Response and Frequency of SARS-CoV-2 Infection 6 Months after COVID-19 Vaccination in Healthcare Workers. Emerg Infect Dis. 2022 Feb 24;28(4). doi: 10.3201/eid2804.212037. Epub ahead of print. PMID: 35203111.

b. Lusvarghi S, Pollett SD, Neerukonda SN, Wang W, Wang R, Vassell R, Epsi NJ, Fries AC, Agan BK, Lindholm DA, Colombo CJ, Mody R, Ewers EC, Lalani T, Ganesan A, Goguet E, Hollis-Perry M, Coggins SAA, Simons MP, Katzelnick LC, Wang G, Tribble DR, Bentley L, Eakin AE, Broder CC, Erlandson KJ, **Laing ED**, Burgess TH, Mitre E, Weiss CD. SARS-CoV-2 Omicron neutralization by therapeutic antibodies, convalescent sera, and post-mRNA vaccine booster. bioRxiv [Preprint]. 2021 Dec 28:2021.12.22.473880. doi: 10.1101/2021.12.22.473880. PMID: 34981057; PMCID: PMC8722594.

c. Coggins SA, **Laing ED**, Olsen CH, Goguet E, Moser M, Jackson-Thompson BM, Samuels EC, Pollett SD, Tribble DR, Davies J, Illinik L, Hollis-Perry M, Maiolatesi SE, Duplessis CA, Ramsey KF, Reyes AE, Alcorta Y, Wong MA, Wang G, Ortega O, Parmelee E, Lindrose AR, Snow AL, Malloy AMW, Letizia AG, Ewing D, Powers JH, Schully KL, Burgess TH, Broder CC, Mitre E. Adverse Effects and Antibody Titers in Response to the BNT162b2 mRNA COVID-19 Vaccine in a Prospective Study of Healthcare Workers. Open Forum Infect Dis. 2021 Nov 20;9(1):ofab575. doi: 10.1093/ofid/ofab575. PMID: 35047649; PMCID: PMC8759445.

d. Pollett SD, Richard SA, Fries AC, Simons MP, Mende K, Lalani T, Lee T, Chi S, Mody R, Madar C, Ganesan A, Larson DT, Colombo CJ, Colombo R, Samuels EC, Broder CC, **Laing ED**, Smith DR, Tribble D, Agan BK, Burgess TH. The SARS-CoV-2 mRNA vaccine breakthrough infection phenotype includes significant symptoms, live virus shedding, and viral genetic diversity. Clin Infect Dis. 2021 Jun 12:ciab543. doi: 10.1093/cid/ciab543. Epub ahead of print. PMID: 34117878.

RESEARCH & RELATED BUDGET - Budget Period 1

OMB Number: 4040-0001
Expiration Date: 12/31/2022

UEI: UYLKBRENAPG5 **Enter name of Organization:** HENRY M. JACKSON FDN FOR THE ADV MIL/MED

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 1** **Start Date:** 09/01/2023 **End Date:** 08/30/2024

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Eric		Laing	Ph.D.		0.80			0.00	0.00	0.00
Project Role:	PD/PI										
	Si'Ana		Coggins	Ph.D.		6.00			37,000.00	10,885.00	47,885.00
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: 47,885.00

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	Research Assistant	6.00			20,600.00	6,061.00	26,661.00

1 **Total Number Other Personnel**

Total Other Personnel: 26,661.00

Total Salary, Wages and Fringe Benefits (A+B): 74,546.00

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	3,640.00
Total Travel Cost	3,640.00

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	66,954.00
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	
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17.		
	Total Other Direct Costs	66,954.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	145,140.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
On Site USU FY23	36.74	145,140.00	53,324.00
Companywide G&A FY23	16.70	198,464.00	33,143.00

Total Indirect Costs	86,467.00

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)	231,607.00

J. Fee

Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	231,607.00

L. Budget Justification

(Only attach one file.) R01 LiberiaEBOV Laing Budget Justifica | Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Budget Period 2

OMB Number: 4040-0001
Expiration Date: 12/31/2022

UEI: UYLKBRENAPG5 **Enter name of Organization:** HENRY M. JACKSON FDN FOR THE ADV MIL/MED

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 2** **Start Date:** 09/01/2024 **End Date:** 08/30/2025

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Eric		Laing	Ph.D.		0.80			0.00	0.00	0.00
Project Role:	PD/PI										
	Si'Ana		Coggins	Ph.D.		6.00			38,110.00	11,212.00	49,322.00
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 49,322.00

B. Other Personnel

Number of Personnel	Project Role	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
1	Post Doctoral Associates	12.00			55,000.00	16,181.00	71,181.00
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						

1 **Total Number Other Personnel**

Total Other Personnel 71,181.00

Total Salary, Wages and Fringe Benefits (A+B) 120,503.00

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	3,640.00
Total Travel Cost	3,640.00

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	20,682.00
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	
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14.		
15.		
16.		
17.		
	Total Other Direct Costs	20,682.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	144,825.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
On Site USU FY23	36.74	144,825.00	53,209.00
Companywide G&A FY23	16.70	198,034.00	33,072.00

Total Indirect Costs	86,281.00

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)	231,106.00

J. Fee

	Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	231,106.00

L. Budget Justification

(Only attach one file.) R01 LiberiaEBOV Laing Budget Justifica | Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Budget Period 3

OMB Number: 4040-0001
Expiration Date: 12/31/2022

UEI: UYLKBRENAPG5 **Enter name of Organization:** HENRY M. JACKSON FDN FOR THE ADV MIL/MED

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 3** **Start Date:** 09/01/2025 **End Date:** 08/30/2026

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Eric		Laing	Ph.D.		0.80			0.00	0.00	0.00
Project Role:	PD/PI										
	Si'Ana		Coggins	Ph.D.		6.00			39,253.00	11,548.00	50,801.00
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 50,801.00

B. Other Personnel

Number of Personnel	Project Role	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
1	Post Doctoral Associates	12.00			56,650.00	16,666.00	73,316.00
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						

1 **Total Number Other Personnel**

Total Other Personnel 73,316.00

Total Salary, Wages and Fringe Benefits (A+B) 124,117.00

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	3,640.00
Total Travel Cost	3,640.00

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	20,682.00
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	
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12.		
13.		
14.		
15.		
16.		
17.		
	Total Other Direct Costs	20,682.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	148,439.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
On Site USU FY23	36.74	148,439.00	54,537.00
Companywide G&A FY23	16.70	202,975.00	33,897.00

Total Indirect Costs 88,434.00

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)	236,873.00

J. Fee

	Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	236,873.00

L. Budget Justification

(Only attach one file.) R01 LiberiaEBOV Laing Budget Justifica | Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Budget Period 4

OMB Number: 4040-0001
Expiration Date: 12/31/2022

UEI: UYLKBRENAPG5 **Enter name of Organization:** HENRY M. JACKSON FDN FOR THE ADV MIL/MED

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 4** **Start Date:** 09/01/2026 **End Date:** 08/30/2027

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Eric		Laing	Ph.D.		0.80			0.00	0.00	0.00
Project Role:	PD/PI										
	Si'Ana		Coggins	Ph.D.		3.00			20,215.00	5,947.00	26,162.00
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: 26,162.00

B. Other Personnel

Number of Personnel	Project Role	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
1	Post Doctoral Associates	12.00			58,350.00	17,167.00	75,517.00
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						

1 **Total Number Other Personnel**

Total Other Personnel: 75,517.00

Total Salary, Wages and Fringe Benefits (A+B): 101,679.00

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)	1,500.00
2. Foreign Travel Costs	3,640.00
Total Travel Cost	5,140.00

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	11,673.00
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	
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17.		
	Total Other Direct Costs	11,673.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	118,492.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
On Site USU FY23	36.74	118,492.00	43,534.00
Companywide G&A FY23	16.70	162,026.00	27,058.00

Total Indirect Costs 70,592.00

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)	189,084.00

J. Fee

	Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	189,084.00

L. Budget Justification

(Only attach one file.) R01 LiberiaEBOV Laing Budget Justifica | Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Budget Period 5

OMB Number: 4040-0001
Expiration Date: 12/31/2022

UEI: UYLKBRENAPG5 **Enter name of Organization:** HENRY M. JACKSON FDN FOR THE ADV MIL/MED

Budget Type: ☐ Project ☒ Subaward/Consortium **Budget Period: 5** **Start Date:** 09/01/2027 **End Date:** 08/30/2028

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
	Eric		Laing	Ph.D.		0.80			0.00	0.00	0.00
Project Role:	PD/PI										
	Si'Ana		Coggins	Ph.D.		3.00			20,215.00	5,947.00	26,162.00
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: 26,162.00

B. Other Personnel

Number of Personnel	Project Role	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
1	Post Doctoral Associates	12.00			60,101.00	17,682.00	77,783.00
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						

1 **Total Number Other Personnel**

Total Other Personnel: 77,783.00

Total Salary, Wages and Fringe Benefits (A+B): 103,945.00

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)	1,500.00
2. Foreign Travel Costs	3,640.00
Total Travel Cost	5,140.00

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	11,673.00
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	
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17.		
	Total Other Direct Costs	11,673.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	120,758.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
On Site USU FY23	36.74	120,758.00	44,366.00
Companywide G&A FY23	16.70	165,124.00	27,576.00

Total Indirect Costs	71,942.00

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)	192,700.00

J. Fee

	Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	192,700.00

L. Budget Justification

(Only attach one file.) R01 LiberiaEBOV Laing Budget Justifica | Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Cumulative Budget

		Totals ($)
Section A, Senior/Key Person		200,332.00
Section B, Other Personnel		324,458.00
Total Number Other Personnel	5	
Total Salary, Wages and Fringe Benefits (A+B)		524,790.00
Section C, Equipment		
Section D, Travel		21,200.00
1. Domestic	3,000.00	
2. Foreign	18,200.00	
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		131,664.00
1. Materials and Supplies	131,664.00	
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		
9. Other 2		
10. Other 3		
11. Other 4		
12. Other 5		
13. Other 6		
14. Other 7		
15. Other 8		
16. Other 9		
17. Other 10		

Section G, Direct Costs (A thru F)	677,654.00
Section H, Indirect Costs	403,716.00
Section I, Total Direct and Indirect Costs (G + H)	1,081,370.00
Section J, Fee	
Section K, Total Costs and Fee (I + J)	1,081,370.00

BUDGET JUSTIFICATION

The Henry M. Jackson Foundation for the Advancement of Military Medicine Inc. (HJF) in partnership with the Uniformed Services University of the Health Sciences (USUHS) will manage this proposal, if awarded.

Personnel

Key Personnel:

Eric Laing, Ph.D., Co-Investigator (0.8 calendar month). Dr. Laing will be responsible for the coordination of the project activities with the principal investigator and other co-investigators. Dr. Laing will oversee the lab activities pertaining to the production of recombinant virus protein antigens and the establishment of the multiplex serology test. Dr. Laing will provide mentorship to the postdoctoral student and assist with data analysis. Dr. Laing will oversee serological training activities at the partner lab in Bong, Liberia and assist with in-country testing and data interpretation. He will provide the necessary concepts and practice of viral glycoprotein antigen preparation for serological application to co-investigators and collaborators. Dr. Laing will also provide the virological and immunological expertise necessary to understand the serological profiles of humans and wildlife antisera. Dr. Laing will be a government employee and no salary support is requested.

Other Personnel:

Dr. Si'Ana Coggins, PhD, Associate Scientist (6 calendar months, YR1-3; 3 calendar months, YR4-5). Dr. Coggins will assist with the supervision of all lab activities at USU and interface with the project management teams. As a trained biochemist Dr. Coggins will oversee all protein expression and maintain the quality and rigor of the research. Dr. Coggins will work closely with the research assistant, Mr. Lucas in year 1 to prepare protein antigens, verification of material quality, coordinate the transfer for materials to the partner institute in Bong, Liberia, and assist in the project activities. Dr. Coggins is an employee of the Henry M. Jackson Foundation and salary support is requested for years 1-5.

Mr. Brendan Lucas, BS, Res Asst I (6 calendar months, YR1). Mr. Lucas will assist Dr. Coggins in the production of soluble glycoproteins and be responsible for microsphere coupling in YR1. Mr. Lucas will participate in serological testing and quality verification of the material. He will also prepare shipments of materials to the regional partner lab. Mr. Lucas is an employee of the Henry M. Jackson Foundation and salary support is requested for year 1.

TBD, Postdoctoral Fellow (12 calendar month, YR2-5). A postdoctoral fellow will be hired to participate in the scientific activities described in this grant application. The postdoctoral fellow will assist with the preparation of materials for testing of human and wildlife samples, and be the statistical analysis of the serological data. The postdoctoral fellow will develop statistical models to analyze serologic, host, and ecological data. The postdoctoral fellow will work closely with epidemiologists at the prime institute to generate data for yearly reports. The postdoctoral fellow will be an employee of the Henry M. Jackson Foundation and salary support is requested for year 2-5.

International Travel:

Based on FY23 Department of State per diem rates and historical travel to Liberia (outside of Monrovia), we estimate $3,640 should cover the travel costs of two lab members to travel to initiate and maintain project activities in the partner lab over one week in all years.

Domestic Travel:

Based on FY23 GSA per diem rates, we request $1500 for multiple trips to the Principal Investigator home office in New York City for collaboration and meetings for years 4-5.

Supplies:

Based on current prices, the following equipment and supplies will be needed to support this project: Recombinant production of 34 protein antigens, magnetic beads, plastic consumables, cell culture materials, affinity matrices and control antibody materials.

Year 1 - $66,957
Recombinant protein production
Multiplex serologies and consumables

Year 2-3 - $20,683
Recombinant protein production (reduced)
Multiplex serologies and consumables

Year 4-5 - $11,673
Multiplex serologies and consumables

Indirect Costs:

HJF indirect cost (IDC) is calculated based on the value-added cost base overhead rates. The fringe rate used is 29.42 % for Tier 1 employees and 7.29% for Tier 2 employees (all employees in this proposal are Tier 1).

The HJF indirect cost is calculated based on the value-added cost base overhead rates. The IDC rate is 36.74% USU Onsite Overhead. Additionally, 16.70% Companywide G&A and applied on the total direct cost less subaward plus the USU Onsite Overhead. For proposals including subawards, an additional 1.93% is applied on total external subaward costs.

The above fringe benefits and indirect cost rates for FY2023 were approved by the U.S. Army Medical Research Acquisition Activity on September 1, 2022.

From: Boxley, Kimberly on behalf of Boxley, Kimberly <kimberly.boxley.ctr@usuhs.edu>
To: Madeline Salino
Cc: Laing, Eric; Jon Epstein
Subject: Re: R01 itemized budgets & justifications!
Date: Monday, January 30, 2023 7:07:01 PM
Attachments: R01_LiberiaEBOV_Laing_Facilities_20JAN2023.docx.pdf
R01_LiberiaEBOV_Laing_Biosketch_22JAN2023.docx.pdf
RR_Budget_LiberiaR01_Laing.pdf
R01_LiberiaEBOV_Laing_Budget Justification_25JAN2023.docx.pdf

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Attached!

The only things missing per your instructions are the authentication of key resources and letter of support.

On Mon, Jan 30, 2023 at 4:48 PM Madeline Salino <salino@ecohealthalliance.org> wrote:

> Hi, all,
>
> Please send me your itemized budgets & justifications ASAP. I am looking for updated budgets to meet the new (reduced) yearly totals for USU, UNC, and NPHIL.
>
> I need to have enough time to get them approved by EHA and input into NIH ASSIST, so please have them to me by Wednesday morning at the latest.
>
> Please let me know if I can answer questions about yearly budget targets or anything else.
>
> Best,
> Madeline
>
> --
> **Madeline Salino**
> *Science and Outreach Administrative Assistant*
> EcoHealth Alliance
> 520 Eighth Avenue, Ste. 1200
> New York, NY 10018
> Office: (212) 380-4460 x4513
>
> *EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.*

--
Kim N. Boxley l Program Manager 3 l Henry M Jackson Foundation
Uniformed Services University of the Health Sciences l Microbiology and Immunology
4301 Jones Bridge Road, Room B4122 l Bethesda, Maryland 20814
Phone: **301-295-1941** l Fax: **301-295-1545** l Cell: **301-793-1883**
Email: **KIMBERLY.BOXLEY.CTR@USUHS.EDU**

From:	Krongkan Srimuang on behalf of Krongkan Srimuang <krongkan.sr@gmail.com>
To:	info@creid-network.org
Cc:	Peter Daszak; Eric Laing; spwa; Spencer Sterling; Hongying Li
Subject:	Applying for Pilot Research Program 2023 - EID-SEARCH (Thailand)
Date:	Monday, January 30, 2023 11:01:48 AM
Attachments:	CREID Pilot Research Program 2023 - Full application_Thailand.pdf RR_Budget_Form_Thailand.pdf

---

To whom it may concern,

We are part of EID-SEARCH research group and we would like to submit for the CREID Pilot Research Program 2023.
Please find attached the two documents including;
1. Full application
2. Budget

If you require any further information, please do not hesitate to contact us.

Best regards,
Krongkan Srimuang
Thai Red Cross Emerging Infectious Diseases Clinical Center,
King Chulalongkorn Memorial Hospital,
Rama4 road, Patumwan, Bangkok, Thailand 10330

Proposal Cover Sheet

Project Title	Immune memory bait & capture to identify emerging henipavirus origins
Principal Investigator Name	Krongkan Srimuang, Ph.D.
Position/Title	Research Scientist
Department	Thai Red Cross Emerging Infectious Diseases Clinical Center
Institution Name	King Chulalongkorn Memorial Hospital
Street	Rama 4 Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	Krongkan.sr@gmail.com
Phone	+66 (0) 92-453-0808
Country(ies) where work will be conducted	Thailand
Pathogen(s) focus	Mojiang Virus, Henipavirus, Paramyxovirus
Co-Principal Investigator Name (*If applicable*)	Spencer Lee Sterling, MPH
Position/Title	Scientific Project Coordinator
Department	Microbiology and Immunology
Institution Name	Uniformed Services University of the Health Sciences
Street	Jones Bridge Road
City, State, Zip Code	Bethesda, MD 20814
Country	USA
Email	spencer.sterling.ctr@usuhs.edu
Phone	+66 (0) 83-494-5980

Contact information for institution(s) that would financially manage the award *(please note: changing the managing institution upon award may result in substantial funding delays)*	
Contractual Contact, Title	Chula Unisearch
Institution Name	Chulalongkorn University
Street	254 Phyathai Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	unisearch@chula.ac.th
Phone	0 2218 2880
For Co-Principal Investigator (If applicable):	
Contractual Contact, Title	
Institution Name	
Street	
City, State, Zip Code	
Country	
Email	
Phone	

Name of Mentor	Eric Laing, Ph.D.
Mentor Institution	Uniformed Services University of the Health Sciences
Institution Address	Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814
Permanent location of Mentor	4301 Jones Bridge Road, Bethesda, MD 20814
Mentor email	eric.laing@usuhs.edu
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Total Budget	***Repeat this table if there are two Co-PIs***
Direct Costs	$138,831
Indirect Costs	$11,106
Proposed Start Date	1 May 2023
Proposed End Date	30 April 2024

Project Abstract (250 words)

Prototypical henipaviruses (Hendra and Nipah virus) are bat-borne zoonotic viruses and cause high mortality. The recent detection of closely related Mojiang and Langya viruses in rats and shrews, and isolation of Langya virus from individuals with acute febrile illness, challenges the dogma that fruit bats are the sole henipavirus reservoirs. The recently expanded *Henipavirus* genus includes two other shrew-borne viruses (Gamak and Daeryong viruses), and the Madagascan fruit bat-borne Angovkeely virus. As part of EID-SEARCH activities, we found serological evidence of infection by a Mojiang-related virus in a community of Thai bat guano collectors who have occupational exposure to microbats and rodents. Identifying the wildlife reservoir of this likely novel virus will assist intervention strategies to reduce spillover risk. However, PCR detection or virus isolation in wildlife is challenging, and new serological approaches may be more successful in identifying reservoir hosts. This project aims to identify novel viruses and zoonotic reservoirs through serologic and cellular immune discovery. We will conduct follow-up human and wildlife shrews and rodents) surveillance in the province where the seropositive humans were identified and apply an expanded serological test and genomic sequencing to detect this novel MojV-like henipavirus. Further, we

plan to isolate anti-MojV monoclonal antibodies (mAb) from peripheral blood mononuclear cells collected from human study participants we have identified as immunoreactive with MojV antigens, and characterize the cross reactive and cross neutralizing potential of these mAbs to LayV.

Project Title **Immune memory bait & capture to identify emerging henipavirus origins**

Table of Contents

Study Personnel

Krongkan Srimuang, Ph.D., is a researcher at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital and supports the Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia (EID-SEARCH) research. Dr. Srimuang is also a medical technologist working to identify, detect and characterize the spill-over risk of high zoonotic potential viruses from wildlife and humans using serology techniques, Next-generation sequencing, and PCR assays to identify novel viruses. Dr. Srimuang also performs the project Smart Sentinel Surveillance System: Emerging Viral Pneumonia and Respiratory tract infections (RTIs) to identify, detect and characterize the novel/known Respiratory Viruses in our country, Thailand by using multiplex real-time PCR, family PCR and Next-generation sequencing of novel viral pathogens. Dr. Srimuang graduated with Ph.D. in Tropical Medicine from the department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University.

Spencer Sterling, MPH, is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region. Mr. Sterling will planning, sample processing, data analysis, and report preparation for this project.

Dr. Eric Laing will act as mentor for Dr. Srimuang and Mr. Sterling under the Centers for Research in Emerging Infectious Diseases (CREID) Pilot Research Program. Dr. Laing is an assistant professor dedicated to biosurveillance and virological research of emergent zoonotic viruses at Uniformed Services University, Bethesda, MD. Part of Dr. Laing's research focus in on the development of serological-based approaches to identify the infectome of wildlife and human communities for the assessment of virus spillovers. Dr. Laing has worked collaboratively with national and international researchers including institutional partners at Duke-NUS, Chulalongkorn University, University of Pretoria, and Rocky Mountain Labs towards these goals. Through these collaborations, Dr. Laing's research group aims to identify known and unknown viruses, the wildlife reservoirs of priority pathogens, the high-risk interfaces for spillover and the biotic/abiotic drivers of virus emergence.

Dr. Supaporn Wacharapluesadee, Ph.D. is the head laboratory at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, a WHO Collaborating Centre for Research and Training on Viral Zoonoses. Dr. Wacharapluesadee research interests include emerging infectious diseases in bats (including Nipah, Rabies, Coronavirus, and novel pathogens) as well as molecular diagnoses and sequencing of viruses. During the past 10 years, Dr. Wacharapluesadee got funding from USAID PREDICT to conduct the surveillance for novel virus in wildlife and human, and from Biological Threat Reduction Program US DTRA to conduct the EID surveillance in bat using the molecular technology and multiplex serology panel (Luminex technology). TRC-EIDCC is responsible for molecular diagnoses services to the hospital, and is the reference laboratory for rabies virus, MERS-CoV, Ebola, Zika, and other infectious diseases diagnoses for Ministry of Public Health, Thailand and laboratory training center on national and regional level. Dr. Wacharapluesadee has served and consulted on several WHO and Thai government committees.

Specific Aims

The recent discovery of novel shrew and rodent-borne henipaviruses in East and Southeast Asia has highlighted gaps in our understanding of henipavirus transmission and underscores the importance of surveillance activities in the region. In particular the isolation of Langya virus from acutely ill humans and shrews demonstrated the need to further surveillance and therapeutic development against emergent henipaviruses. Therefore, **this project aims to expand the study of known reservoirs of zoonotic pathogen origin by using genetic and serologic techniques, and to use cellular immune discovery in seropositive humans as a tool to determine henipaviral antigenic relatedness to assist with the functional assessment of current therapeutics as and their effect on these novel viruses.**

In our study, we detected that a high seroprevalence against MojV in human participants near the Khao Chong Phran Non-hunting Area in Ratchaburi province who we identified as representing a high risk population for zoonotic spillover. Yet, no virus sequences were detected. We hypothesize that (i) there is a novel henipavirus present in the local wildlife population that has spilled over into the human population at least once, and (ii) the antibodies from these seropositive people will react with the novel shrew-borne henipaviruses in a way that can inform us on therapeutic approaches for future outbreaks. We will follow-up with persons who had high anti-MojV IgG levels, sample new participants within this community who interact with the local wildlife, and sample shrews and rodents to identify the wildlife source of the MojV-like henipavirus. Additionally, in collaboration with NIH VRC PREMISE, MojV receptor binding protein (RBP) and fusion protein-reactive B cell will be isolated from previously-identified seropositive human participants. MojV-like virus-specific B-cell receptors will be sequences and monoclonal antibodies specific to the infecting MojV-like virus RBP and F protein will be generated and tested for cross reactivity against presently described rodent associated henipavirus envelope glycoproteins, and cross neutralization against Langya virus.

The proposed project has two Aims:

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population.

Aim 2. Characterization of monoclonal antibodies from humans with serological profiles consistent with MojV infection.

The proposed project has two Objectives:

Objective 1. To use metagenomic and targeted sequencing approaches in human and wildlife populations to detect the Thai MojV-like virus.

Objective 2. To isolate and sequence memory B-cells from humans positive for Mojiang virus in Ratchaburi province, Thailand for antibody discovery.

Study Rationale

Nipah virus (NiV) and henipaviral diseases are among the most virulent of zoonotic diseases. The genus *Henipavirus* is currently composed of nine viruses including the prototypes, NiV and Hendra virus (HeV), and Cedar virus (CedV), Ghana virus (GhV), Mojiang virus (MojV) (McLinton et al., 2017), Langya virus (LayV) (Zhang X et al., 2022), Gamak virus (GAKV), Daeryong virus (DARV) (Lee et al., 2021), and Angavokely virus (AngV) (Madera et al., 2022) (Table 1). HeV was first identified in Australia in 1994 as a cause of respiratory illness and fatal encephalitis in humans and horses. Closely following the discovery of HeV, NiV was identified in 1999 in Malaysia after outbreaks of fatal encephalitis in humans, with swine determined to be the intermediate host. Annual outbreaks of NiV have occurred in Bangladesh and are associated with the consumption of raw date palm sap, and since 19 May 2018 annual outbreaks of NiV associated encephalitis of been recorded in Kerala, India (Uwishema et al., 2022). Both HeV and NiV have a broad host range capable of infecting several mammalian orders, and can enter and infect endothelial cells, vasculature, and the central nervous system leading to severe multisystem organ diseases and high mortality. The broad host and tissue tropism of these viruses has been attributed to the use of the ephrinB2 and ephrinB3 ligands, highly conserved proteins with critical functions in evolutionary development, as virus receptors (Bossart et al., 2008). There are currently no vaccines or therapeutics and NiV and henipaviral diseases remain WHO Blueprint list priority pathogens (Middleton and Weingartl, 2012).

A close relative of HeV, CedV was isolated from Australian bats in 2012 and found to be non-pathogenic, failing to cause any clinical symptoms in ferrets and guinea pig animal models (Marsh GA et al., 2012). The natural reservoirs of HeV, NiV, and CedV are flying foxes (*Pteropus sp.*) (Middleton and Weingartl, 2012). GhV was detected in Ghana in straw-colored fruit bats (*Eidolon helvum*) during routine surveillance in 2015 and was the first henipavirus found outside of the Indo-Pacific region. GhV showed a close phylogenetic relationship with HeV and NiV, and like HeV, NiV, and CedV the GhV RBP can mediate binding to ephrinB2 ligand and mediate cellular fusion (Drexler, J. F., 2009). Recent investigations into Madagascan fruit bats (*Eidolon duprenum*) detected a second African henipavirus, AngV. As neither GhV nor AngV have been isolated or rescued, the pathogenic and zoonotic potential of these African fruit bat associated henipaviruses remains unknown.

Multiple genera of small mammals were tested for viral RNA, and MojV was detected in yellow-breasted rats (*R. flavipectus*) (Wu Z et al., 2014). The discovery of the MojV genome occurred during follow-up investigations into lethal pneumonia from three miners in 2012 in China, however the pathogenic nature of this virus has only been suggested and no isolates have been discovered or tested in an animal model. Additionally, the novel shrew-borne henipaviruses, GAKV and DARV were found in the Republic of Korea in 2017-2018. (Lee et al., 2021). Most recently, a close relative of MojV, Langya henipavirus (LayV) was recently isolated from febrile patients in eastern China. Subsequently, LayV was identified in shrews (Zhang et al., 2022), increasing the number of Asiatic rodent-associated henipaviruses to four. These recent discoveries are challenging the paradigms surrounding henipaviruses and their associations with bats and may suggest a novel rodent sub-genus of henipaviruses.

The henipaviral genome consists of six structural proteins including nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), receptor-binding protein (RBP), and large (L) protein (approximately length of 18,000 nucleotides) (Luby, S. P., & Gurley, E. S., 2012). In HeV and NiV, slippage sites within the P protein lead to the expression of the V, W, and C proteins. These proteins are virulence factors and are absent in CedV.

Table 1 Summary viruses in Henipavirus

Henipavirus	Origin (Year)	Reservoir	Genome (nucleotides)	Symptom for human	Reference
Hendra virus (HeV)	Australia (1994)	Fruit bats	18,234	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Nipah virus (NiV)	Malaysia (1999)	Fruit bat	18,246	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Ghana virus (GhV)	Africa (2009)	Fruit bat (*Eidolon helvum)*	18,530	No data	Drexler, J. F., 2009
Cedar virus (CedV)	Australia (2012)	Fruit bat	18,162	Non-pathogenic	Marsh, G. A et al., 2012
Mojiang virus (MojV)	China (2012)	Rodent	18,404	Febrile	Wu Z et al., 2014
Gamak virus (GAKV)	Korea (2017-2018)	Shrew	18,460	No data	Lee et al., 2021
Daeryong virus (DARV)	Korea (2017-2018)	Shrew	19,471	No data	Lee et al., 2021
Langya virus (LayV)	China (2019)	Shrew	18,402	Acute febrile	Zhang et al., 2022
Angavokely virus	Madagascar (2019)	Fruit bat	16,740	No data	Madera et al., 2022

Viruses in the genus *Henipavirus* have a broad reservoir and may cause high case fatality rates following human infection. The discovery and characterization of the novel Henipavirus is a high public health priority.

Khao Chong Phran, a cave in Ratchaburi Province, has possibly the largest colony of bats in Thailand. There are estimated to be around 2 million bats in the cave and 95% are insect-eating bats (Department of National Parks, Wildlife and Plant Conservation, Thailand). Moreover, Khao Chong Phran has many community sites, such as cave entrances near communities, guano collection, and tourism sites, which risk zoonotic spillover. Bats are widely considered the animal hosts of zoonotic pathogens such as rhabdoviruses, coronaviruses, paramyxoviruses, and filoviruses, and many of the host species can be found in Thailand.

MojV was associated with a severe pneumonia-like illness killing three of six miners in 2012 in a mineshaft Yunnan, China. Interestingly, this mineshaft was also site where RaTG13, a beta-coronavirus most closely related with SARS-CoV-2, was detected in *Rhinolophid* bats (Rahalkar and Bahulikar, 2020). Identifying the wildlife reservoir of this novel virus will assist intervention strategies to reduce spillover risk.

Research Gaps

As a part of Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH), serological investigations using a multiplex microsphere-based immunoassay (MMIA) have detected 19% (54/284) seroprevalence for MojV in a high-risk cohort, including one person with a high magnitude and durability of anti-MojV IgG across longitudinal samples. Despite this, genomic surveillance has not detected MojV or any novel MojV-like viruses. For this result, we would

like to identify and characterize the origin of the Mojiang virus-reactive serum and to explore the novel MojV-like virus in Thailand.

Serological and cellular immunity is an important resource for virus discovery detection by using the specificities and characteristics of memory B cells from peripheral blood mononuclear cells (PBMC). The immune response will recognize and induce antibodies specific to the infecting pathogen. Characterizing the memory B cell response to an unknown pathogen in humans could in the detection of the exposure pathogen in humans. Molecular techniques such as PCR can detect only the genomic material in the body, but memory B cells are long-lived and thus have a much larger window of detection.

Memory B-cell differentiation is characterized by somatic hypermutation in antibody variable region genes (V) and selection of B cells expressing high-affinity variants of this antigen receptor and high sequence similarity in individuals exposed to the same antigen reflect antigen-driven clonal selection and form shared immunological memory between individuals (Yang et al., 2021). Memory B cells are highly abundant in the human spleen, and can proliferate 45% of the total B cell population in this organ (Hauser et al., 2015). Memory B cells can be detected in the Ig variable (IgV) genes in a fraction of IgM+ B cells. In humans, B cells expressing the memory marker CD27+ can be found in the marginal zone of spleen and circulate in the blood. After re-exposure to antigen, memory B cells can quickly proliferate and differentiate into plasma cells. Many studies the antibody diversity and specificity of the naturally paired immunoglobulin heavy and light chains for affinity maturation of B cell receptor (BCR) after virus infection such as SARS-CoV-2 infection (Yang et al., 2021), influenza (Dougan, S et al., 2013), HIV (Moir, S., & Fauci, A. S.,2017), etc. The characterization of memory B cells can be used to develop monoclonal antibodies and produce an effective therapeutic.

Significance and Approach

East and Southeast Asia is one of the world's highest-risk emerging infectious diseases hotspots. From this region emerged both SARS-CoV-1 and SARS-CoV-2, recurrent outbreaks of novel influenza strains, and the spillover of dangerous viral pathogens such as Nipah virus. The diversity of coronaviruses, henipaviruses, and filoviruses are found in wildlife reservoirs in Southeast Asia and likely due to the high diversity of wildlife, and the demography of the human population brings humans and wildlife into close contact. The spillover of viruses from animals to humans is often misdiagnosed and the clinical symptoms from patients is underestimated during the pandemic. This study provides a surveillance strategy in human and wildlife populations to better understand immunity to novel pathogens and identify the origin of these pathogens to serve as an early warning system to prevent outbreaks of emerging viruses.

This study uses immunology (serologic and cellular) to identify a novel virus that can aide in effective vaccine discovery and development, monoclonal antibody production for prevention and therapy, and generate data resources for early detection and diagnostic assays. Whereas PCR detection or virus isolation in wildlife is challenging, this new serological approach may be more successful in identifying reservoir hosts.

The Indo-Pacific region has a high risk of novel viral emergence. This study provides an opportunity for in-country research capacity building by linking local infectious disease scientists with an international collaborative network that has proven capacity to conduct this work and produce significant findings. Moreover, a global sero-surveillance network for surveillance technology extends virologic and immunologic screening to product discovery. This study will benefit and expand the CREID, EID-SEARCH networks, and the global sero-surveillance network to improve our understanding of immunology to identify novel pathogens and their origin to prevent future pandemics.

Research Methods

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population

Serological profiling provides an indirect measurement of the infecting virus. Despite 19% (54/284) seroprevalence in humans, we have not detected nucleic acid sequences of MojV or MojV-like henipaviruses in this human cohort. Additionally, we intend on expanding our understanding of henipavirus prevalence in this population with longitudinal sampling of the wildlife and human populations in this region.

Animal samples collection

200 shrews or rodents in and around the Khao Chong Phran cave in Ratchaburi Province (Figure 1) will be collected for surveillance targeting the Mojiang-like virus. We plan two sampling trips to collect target shrews and rodents. Due to limited knowledge of the local shrew population, we are unsure as to whether we can collect up to 200 samples and will supplement the collection with rodents, as needed. Samples will be collected based on the following enrollment criteria: random age, random weight, random sex. Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and stored. Oral swab and rectal swab will also collect in 200 shrews or rodents. Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and stored.

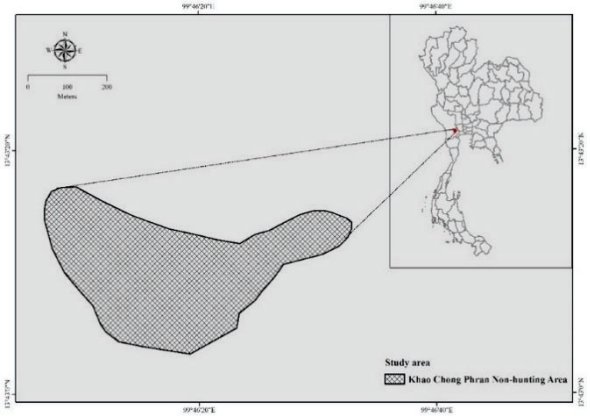

Figure 1: Map of Khao Chong Phran Non-hunting Area, Ratchaburi Province, Thailand (Boonpha N et al., 2019)

Human surveillance

Four of the samples from prior investigations have shown high MojV RBP-reactive antibody titers and will be selected for follow-up sampling. Additionally, 100 human serum samples, nasopharyngeal swabs, throat swabs and questionnaires will be collected from persons deemed to be in close contact with wildlife, and people who present with or have recent history of undiagnosed acute febrile illness using the following enrollment criteria;

- Adults (≥ 18 years of age) who sign in the informed consent
- Who present with or have recent history of illness without diagnosis, such as severe/acute respiratory illness (SARI/ARI), Influenza-like illness (ILI), fever of unknown origin (FUO), or respiratory tract infection
- Who is the bat guano collector in the cave
- Who lives or works around wildlife habitats
- Who is involved with livestock or wildlife farming
- Who hunts or works with wildlife
- Who has not been previously sampled as part of the EID-SEARCH and PREICT projects

Sample size justification

Human population

$$n = \frac{N}{1 + Ne^2}$$

n = sample size of the study
N = population size for finite populations: 8,150 (data from 2018)
e = error: 10%

$$n = \frac{8{,}150}{1 + (8{,}510 \text{ x } 0.1^2)}$$

n = 98.79 (round up to 100 samples)

Wildlife population
We will target a total of 200 individuals of shrews and/or rodents across two trips for this project. This sample size is estimated based on a realistic detection rate and required sampling efforts from previous wildlife surveillance work, given the rodent/shrew population at the study site. Previous publications regarding Henipavirus discovery in small mammals had an average PCR detection prevalence between 20 and 33% (Wu et al., 2014, Lee et al., 2021, and Zhang et al., 2022). We acknowledge the challenge of identifying positive results, and very limited serology research has been done to provide guidance. However, we will make the best use of previous data and experience to conduct sampling at the selected site where human positives have been. We are confident that this targeted sampling and testing strategy offers the best chance to identify positive results and potentially novel henipaviruses.

Targeted viral sequencing and serologic testing
Targeted sequencing
For samples from shrews, rodents, and acute individuals, we will use targeted sequencing and metagenomic approaches in an attempt to detect the Thai MojV-like virus. The nucleic acid from oral swab and rectal swab will do target sequencing in henipavirus Family PCR and Sanger sequencing.

Serological testing
Serum samples will be tested for RBP and envelope fusion protein (F) binding to the known henipavirus species, including the Nipah, Hendra, Cedar, Mojiang, Ghana, Langya, Gamak, and Daerong viruses to further investigate the seroprevalence in this region. Additionally, prior positive samples will be re-tested with the expanded panel to enhance our understanding of the antigenic relationships between the novel shrew-borne henipavirus and the Thai virus.

Data analysis
Sequencing will be analyzed assembling reads in MEGA11. The consensus sequences will compare to the references strains available in the GenBank data-base using the Basic Local Alignment Software Tool (BLAST) (National Center for Bio-technology Information). Serological data will be analyzed using cluster analysis in RStudio.

Aim 2. Characterization of monoclonal antibodies from humans with serological profiles consistent with MojV infection.

In collaboration with NIH VRC PREMISE, MojV RBP and F-reactive B cells will be captured, and the B cell receptor will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific mAbs will be generated from the corresponding sequences and will be assessed for binding potential to LayV RBP and F, as well as their neutralization potential.

Importance: The isolation of these mAbs represents a potential therapeutic for MojV, MojV-like henipas, and LayV, depending on how well these cross react.

Target human population

Human positive serum for MojV was found close to Khao Chong Phran Non-hunting Area in Ratchaburi Province (Figure 2).

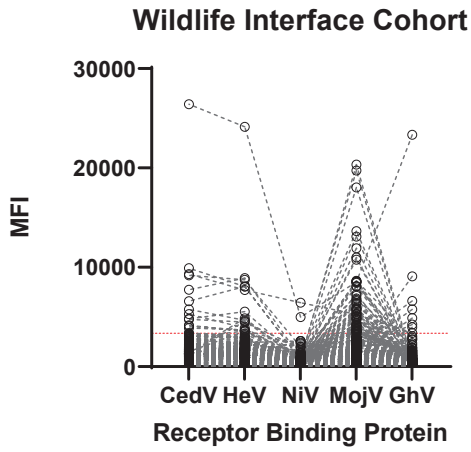

Figure 2: Seroprevalence of henipavirus proteins from a community with a large wildlife interface during 2017-2018. MFI is the median fluorescence intensity for IgG reactivity to each protein. Red-dashed line is at 3,357 MFI, a threshold for seropositivity.

PBMC sorting and B cell isolation

Whole blood from MojV-positive people will be collected in individual heparin tubes and processed for PBMC isolation using Density Gradient Centrifugation (SepMate™ PBMC Isolation Tubes). Human PBMCs will be stained with antibodies for positive markers of IgG memory B cells, and antibodies to negatively select and avoid contamination with dead cells, T cells, NK cells or monocytes (live, CD3 ,CD14 ,CD56 , IgM , IgA , CD19+,CD20+,CD27+). Avi-tag for biotinylation and 6xHIS tag for Nickel purification using the HisPur™ Ni-NTA Spin Purification Kit (Thermo Fisher Scientific, Waltham, MA). Single Memory B Cells will be sorted, cells will proliferate, cDNA will synthesized, then Heavy and light chain variable domains will be amplified (Waltari E et al., 2019).

B cell receptor amplicon preparation and recombinant antibody sorting

Total RNA yields from the PBMC will be determined by absorbance at 260 nm on the NanoDrop™ One (Thermo Fisher Scientific). Total RNA will be used for first strand cDNA synthesis with gene-specific reverse transcription (RT) primers directed to the constant regions. The RT primers for IgG, IgM, IgA, lambda, and kappa will be included. Light chain and heavy chains will be amplified into amplicon. Double-stranded cDNA will be sequenced. After completion of MiSeq sequencing, antibody repertoires will analyze using methods based on the pipeline. Heavy and light-chain sequences will be assembled into an expression vector and transfected into cells for monoclonal antibody expression. Recombinant antibody will be isolated and purified for downstream applications.

Functional assessment

We will first determine the extent to which isolated mAbs can bind binding to native-like MojV RBP and F proteins. We will also characterize the cross-reaction potential of these mAbs will be assessed against GamV, DarV, and LayV RBP and F. Neutralization of viruses remains a critical correlate of protection for most vaccine strategies. However, MojV has not been isolated or rescued, and the virus receptor for MojV has not yet been discovered (Rissanen et al., 2017; Cheliout et al., 2021). Thus, a functional assessment of MojV neutralizing potential is not possible. Though, we will test the cross-neutralization potential of these anti-MojV RBP and F mAbs to neutralize LayV utilizing a plaque reduction neutralization test with EID-SEARCH co-investigators at Duke-NUS. As LayV causes acute illness in human, cross neutralization functionality of these mAbs has therapeutic potential.

Project Timeline

Activity	05/23	06/23	07/23	08/23	09/23	10/23	11/23	12/24	01/24	02/24	03/24	04/24
Mentoring and training activities												
1.Training, literature review												
2.Monthly mentoring meeting												
3.Manuscript writing												
4.EID-SEARCH meeting												
Research activities												
1.IRB amendment												
Aim 1												
2. Human and Wildlife samples collection												
3. Identify **Targeted sequencing**												
4. Serological testing												
Aim 2												
5. Collect PBMC human sample and isolation memory B-cell for Mojiang virus												
6. PBMC Sorting and B Cell isolation												
7. Determination of envelope glycoprotein epitope binding												

Research Performance Sites

This proposed study will be implemented at the King Chulalongkorn Memorial Hospital (KCMH) collaborating with the Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC). KCMH is a 1,000-bed, university hospital in close coordination with the Faculty of Medicine, Chulalongkorn University. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. It's one of the center hospitals for Emerging Infectious Diseases in Thailand, especially for domestic and international patients.

TRC-EIDCC was established by the Committee of the Emerging Infectious Disease Center, which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. Our clinical center provides standard medical care to patients with emerging and recurrent diseases and disease transmission. Moreover, the center plans preparedness activities, trains medical and government staff on interventions, hosts conferences for medical and public health professionals, creates networks of medical care for patients with emerging and recurrent diseases, and leads research and development projects for medical care and disease control. Apart from medical innovation in emerging and recurrent diseases, the clinical center strives to be the model of infectious diseases clinical services and pandemic control through research activities surrounding infectious diseases and effective disease control measures.

We also collaborate with the Department of National Parks, Wildlife and Plant Conservation for detecting novel pathogens from animals such as bats, rodents, and shrews in our country. We also collaborate with the Department of Disease Control of Thailand (Thai-DDC) to support the Thai government and national policy makers through molecular diagnosis, including the identification of the first case of SARS-CoV-2 infection in Thailand. TRC-EIDCC prides itself on its high performance in research laboratory services and is a part of the national reference laboratory for COVID-19 and other emerging infectious diseases confirmation in Thailand.

CREID Research Center Collaboration

The goals and objectives of the proposed research project, "Immune memory bait & capture to identify emerging henipavirus origins", are fully consistent with the specific aims of EID-SEARCH. EID-SEARCH takes a One Health approach to understanding zoonotic disease emergence, with a focus on viral diversity in wildlife at high-risk human animal interfaces. EID-SEARCH aims to understand the frequency and causes of virus spillover in high-risk communities across Southeast Asia and identify strategies and tools to reduce this risk. As the proposed research focuses on the links between farming practices, wildlife diversity, virus diversity, and cross-species transmission in Thailand, we expect the results of the project to make a significant contribution towards the overall goals of EID-SEARCH.

The proposed project is well-designed, leverages cutting-edge technology, and includes a robust ecological component that corresponds to the overall research strategy of EID-SEARCH. The EID-SEARCH team is highly motivated to support the application of metagenomics to ecological questions and assist in the analysis of the unique dataset that will be generated from this research. With the heightened focus on identifying and limiting risks associated with the complex interactions between humans, wildlife, and domestic mammals that has resulted from the emergence of SARS-CoV-2 from a wet market in China, the proposed research is both timely and likely to yield highly-cited, impactful publications for EID-SEARCH and Thailand partners.

The EID-SEARCH team includes a number of experts in study design, laboratory-based diagnostics, genetic and genomic analyses, and ecological modelling who will contribute to project implementation, data analysis, and manuscript preparation as required. Communication and coordination mechanisms will be set up between the Thailand research team and scientists at the EID-SEARCH research sites for resource sharing, training, knowledge exchange, and mentorship. In particular, EID-SEARCH will facilitate and encourage relationships between our partners in Southeast Asia (based in Malaysia, Singapore, and Thailand) that will facilitate Thailand team professional development and strengthen research capacity in the region. Dr. Eric Laing, the project Mentor, has an established relationship with both Dr. Krongkan and Spencer Sterling, and will act as the liaison between EID-SEARCH and the TRC-EIDCC research team. Further, Dr. Doeuk who is the director of the VRC PREMISE program is enthusiastic about the opportunity to collaborate and apply their rapid assembly, transfection and production of immunoglobulin (RATP-Ig) workflow as a serologic/immunologic based approached for therapeutic and virus discovery (see Letter of Support). An expected outcome of this research project will be pilot data to support a joint funding application in the future.

Mentoring Plan

Goals for Pilot study and Career

Krongan Srimuang: My career goal is to be an expert scientist in either an academic research laboratory or a lecturer in medical science. My long-term research interests include the understanding of priority emerging infectious diseases, genetics, and epigenetics using molecular and serologic techniques. Detection of novel pathogens in their wildlife vectors can help characterize the pathogenicity, virulence, and evolution of these and other closely related pathogens. I believe that this pilot study can provide training in new techniques for the detection, identification, and characterization of novel pathogens that are currently unavailable in Thailand. My goal for the Pilot study is to gain more knowledge and skills for a lifelong career in public health-related research. I wish to expand my skills in novel virus detection using next-generation serologic techniques such as PBMC studies and memory B-cell isolation and sequencing that can contribute to improved surveillance and research capacity at human-animal interfaces. My mentors Dr. Laing and Dr. Wacharapluesadee can provide me with opportunities to develop and apply project skills and improve my scientific writing.

My education has provided me with a strong foundation to expand for this study. I received my undergraduate degree in Medical Technology from Chulalongkorn University which included training in molecular biology, serology, immunology, chemistry, virology, bacteriology, mycology, parasitology, hematology, clinical microscopy, transfusion medicine, and forensic medicine. My interest in molecular technology led me to pursue a Ph.D. at the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, which provided me with training in several molecular techniques including conventional PCR, real-time PCR, Next Generation Sequencing (NGS), etc. The emergence of COVID-19 motivated me to focus on emerging infectious diseases. I am currently completing my post-doctoral fellowship at the Emerging Infectious Diseases Clinical Center (EIDCC), King Chulalongkorn Memorial Hospital with Dr. Wacharpluesadee. Throughout this fellowship, I have continued learning advanced and innovative techniques to detect, identify, and characterize the novel pathogens. Through this pilot project, I will learn about the Peripheral Blood Mononuclear Cells (PBMC) which are a critical part of many subdivisions of medical research including Infectious diseases, Vaccine development, and Immunology. This project aims to study zoonotic reservoirs for by using serologic and cellular immune discovery in humans, and applying this immune knowledge in wildlife for early detection and identification of novel pathogens. This proposed study will expose me to various techniques and immunology topics that I useful in designing and testing new pipelines as technological advances continue to develop. This study will also allow me to develop, manage, and communicate my research.

Spencer Sterling: My overall career goal is to understand the factors that lead to viral zoonosis and to build epidemiological capacity in regions with the highest risk for zoonotic spillover. Through my training and educational experiences, I have learned laboratory and epidemiological techniques that can assist in the understanding of zoonosis. I wish to continue my education by expanding my repertoire of molecular techniques to include studies of the immune system's response to zoonotic disease in humans and animals, as well as to learn next-generation sequencing techniques. Additionally, I would like to use my experience in epidemiological techniques to train scientists in the Southeast Asian region in order to build local capacity in infectious disease research. Dr. Laing has been a mentor for me throughout my career, and this project would allow for this relationship to expand into new molecular areas, as well as grant writing and project management skills. My professional relationship Dr. Wacharapluesadee with began in 2018 when I participated in a DTRA BTRP funding project that focused on transferring antigen-based multiplex serological technology to her lab group. Currently, I am a Visiting Scientist at TRCEIDCC and I work closely with Dr. Srimuang, and other members of Dr. Wacharapluesadee's research group on EID-SEARCH activities. Additionally,

Dr. Wacharapluesadee has decades of experience in infectious disease research in the region and has mentored scientists that have gone on to careers at the Thai Ministry of Public Health, Department of Disease Control, Department of Parks and Wildlife, academia, and the private sector. Both mentors have encouraged Dr. Srimuang and I to apply for this pilot study as well as pursue other funding mechanisms for our research interests. Both mentors will provide for me the ability to independently pursue my research interests in the zoonotic epidemiology and train me on the best practices of scientific communication.

Vertebrate Animals Section Requirements

Animal samples will be collected in Ratchaburi Province for this project under Aim 1 to understand the antigenic relationships among known and unknown henipaviruses. Protocol to perform animal sample collection has been reviewed and approved by the Institutional Animal Care and Use Committee at Tuft University under the EID-SEARCH project (No. G2020-42) and the Institutional Animal Care and Use Committee at Chulalongkorn University (No. 019/2563)

1. Description of Procedures

Rodents (order: *Rodentia*) and Shrew (order: *Scandentia* and *Eulipotyphla*)

- Species: Free-ranging rodents (family *Hystricidae, Muridae, Sciuridae, Spalacidae*) and shrew (tree shrews, shrews, moles) that present at the sites.
- Age & sex: Adults and juveniles, males and females. Neonates will not be sampled.
- Target Number: 200 individuals
- Capture and Restraint: Free-ranging wild mammal species will be captured with metal box traps (Sherman/Tomahawk traps). Traps will be prepared with food, water, padding and shelter, and be checked at least every 12 hours, in the morning and in the afternoon. If adverse weather (extreme heat, rain) is expected or researchers are working in areas where predation is common, traps will be checked more frequently and closed during adverse weather. Veterinary staff will be prepared to deal with a variety of trap injuries, including lacerations, broken teeth, and crush injuries. Depending on the species, individual size, captive or free-ranging status of the individual, manual or chemical restraint and anesthesia (gas or injectable anesthesia) will be applied. Animals will be manually restrained during sampling utilizing insulated bite gloves to prevent injury to the handler and animal. Based upon past field experience (>20 years of animal capture and release in Southeast Asia), we do not expect to trap sick, debilitated, or young animals that would be too young to cope with capture. Sick and debilitated animals tend not to roam widely, reducing their opportunity to enter traps. Additionally, should sick, debilitated, or young animals enter traps, we anticipate that they will not suffer adverse conditions because the traps contain food (the bait) and liquid (water or fruit as a source of water). These individuals would be examined by a veterinarian, rehydrated and if presenting clinical signs treated by a veterinarian, prior to release or sampling then release.
- Sample Collection: Once anesthetized or safely restrained, whole blood will be collected through the appropriate venipuncture site, no more than 1 ml of blood per 100 g (= 10 ml/kg or 1%) of body weight will be collected at any one time. Anesthetized animals will be monitored regularly during recovery until they can no longer be safely handled, at which point they will be confined in a trap or cage. Swabs will be taken from the oropharynx, urogenital tract, and rectum. No destructive sampling is planned, but any individuals that die during capture or sampling will have a necropsy performed with organ samples collected.
- Release: Wild mammals are held for a maximum of thirteen hours depending on trap timing, but typically less than three hours. Captive animals are held for a maximum of two hours but typically less than one hour. At the completion of sampling, animals may be provided with rehydrating fluids (either subcutaneously if anesthetized or orally if manually handled and accepted). Wild mammals will be released at the site of capture in a location that allows for further recovery or safe escape (e.g., bodies of water, known predatory areas, and human settlements will be avoided). Captive mammals will be released back into their captive setting as is appropriate for the species, either isolated or in social group if it is deemed safe from aggression from enclosure mates while the post-recovery period continues.

2. Justifications

The purpose of this study is to identify zoonotic pathogens through serologic and cellular immune discovery, including developing methods and standards for direct antibody-mediated virus capture in people and wildlife, focusing on rodent/shrew-borne Henipaviruses (HNVs). This will require hands-on fieldwork to collect whole blood and serum samples from rodents and shrews for lab analysis. One technique that avoids the direct capture and sampling of animals is to collect fresh feces or urine, but this will not be able to provide the appropriate samples for serologic analysis, and the research objectives cannot be achieved using an alternative methods (e.g., computational, human, invertebrate, in vitro). Therefore, we believe there are no viable alternatives than the use of live animals.

3. Minimization of Pain and Distress

In every situation, sampling of wildlife will be conducted in the most humane manner while minimizing the impacts on individual animals and their wild populations. In all instances, the fewest number of animals will be sampled that will provide valid information and statistical inference for the pathogen and disease of interest and every effort will be made to minimize stress and discomfort for the animal.

Small mammals may be held for up to 13 hours depending on trap capture timing. Field animal sampling team with zoologists and veterinarians have been well trained and have extensive experience in capture, anesthesia, and sampling of wildlife. In our team's experience, the target species tolerate the described procedure well. Mist nets will be attended continuously during capture periods. This will minimize stress and injury from entanglement.

For rodents and shrews, food, water, padding and shelter will be provided at traps, which will be checked at least every 12 hours. Traps will be checked more frequently or closed if adverse weather (extreme heat, rain) is expected, or researchers are working in areas where predation is common. Veterinary staff will be prepared to deal with a variety of trap injuries, including lacerations, broken teeth, and crush injuries. All animal individuals will be monitored by a veterinarian or experienced field team member during all stages of capture, processing, anesthesia and release. Animals will be kept in a cool place, free from adverse weather conditions and access by non-field team members or other animals while in the pillowcases, trap, or cage for recovery. All recovering individuals will be observed until they are able to effectively ambulate and orient to their surroundings. Animals that are injured during the capture or sampling process will be assessed by an experienced team leader or attending veterinarian, and if the animal is determined to be unlikely to survive if released, it shall be euthanized humanely (see euthanasia section).

4. Method of Euthanasia

In the event that an animal has been injured or is moribund, a determination will be made as to whether it may be treated and released on site by veterinarians or transferred to facility that may treat and rehabilitate the animal, or if, as a last resort, the animal will require euthanasia. Euthanasia methods will vary depending on species; however methods to be used will not deviate from the AVMA “Guidelines for the Euthanasia of Animals” (2020 edition). Any animal that is euthanized using a chemical agent will be disposed of such that it will not be permitted to enter the food supply either through markets or hunting.

Human Subjects Research

1. Risks to the subjects

In this project, we will target populations in one community site who with frequent exposure to bats and other wildlife based on our previous behavioral survey. Subjects will be enrolled voluntarily, and informed consent will be obtained from all participants. Enrolled participants will provide biological samples. Survey data and biological samples will be collected from enrolled participants, and follow-up data collection will be performed among participants whose samples were tested positive.
Biological samples and questionnaire data will be collected from individuals who meet recruitment and inclusion criteria and complete the informed consent process. During data collection, a standardized questionnaire will be administered to all participants. This questionnaire will collect information of demographic background, wildlife contract, travel and daily movement, and unusual SARI/ILI symptoms, and biological specimens will be collected from participants. Both questionnaire and biological data will be analyzed to assess the exposure to coronaviruses and the spread risk among humans. From all participants, a one-time whole blood sample (Max. 15 mL) will be collected during the study period. This sample will allow us to test for historical exposure to bat or rodent-born henipaviruses and collect Peripheral Blood Mononuclear Cells (PBMCs) for those study participants whose serum tests are positive.

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 who are 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, the administration will be conducted privately and confidentially to protect individuals' 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 these risks

2.1 Informed consent and assent
Only consented participants will be enrolled in the study. No research procedures will be undertaken before the participant fully understands the research, agrees to the study procedures, and completes the consent process will be enrolled in the study. Informed consent statements and forms, and project protocols will be translated into the local language of each study site. Research team members involved in this consent process will be required to be fluent in the local language to ensure that the subjects understand the study and all involved procedures.

If participants meet the criteria for enrollment, they will be invited to discuss the details of the study with the research staff. Study staff will review an information sheet and informed consent form with the participant when applicable. Each individual will be provided with a copy of the informed consent form that has been translated into the local language and written with a Flesch–Kincaid readability score equivalent to a 7th-grade reading level or below, to assure that potential participants understand the information being shared. The informed consent form will explain the details of the study, including how and why the individual was selected, the study process and procedures, risks and benefits, financial considerations and the gift of appreciation, confidentiality of data shared, alternatives to participating, and how to obtain more information now or at a later date. The informed consent form will be read in the local language of the site at a location ensuring participant privacy. After which individuals will have as much time as they would like to ask questions and discuss the

study with study staff. The study staff will endeavor to ensure that the participant understands the information provided. The study staff will then ask the participant to consider study participation. Participants will have as much time as required to consider the participation.

Those participants who consent to the study will sign and date two copies of the consent form. These form copies will be countersigned and data by the study staff. A copy of the signed consent form must be provided to the subject and the other copy will be kept by study staff. Informed consent paperwork will be kept until the end of the project in a locked box at the local country project office.

2.2 Protection against risks

The potential risks to study participants as a result of study participation are minimal. Collection of venous blood samples and nasal or oropharyngeal swab samples pose minimal risk to subjects. Potential complications associated with venipuncture include pain and/or hematoma at the site of collection. Trained medical professionals and/or clinic staff will monitor the blood collection site and treat any complications according to existing health facility protocols. A potential complication of nasal/ oropharyngeal sampling is minor irritation at the time of collection. Employing trained medical and/or clinic staff to collect blood and swab samples will minimize the potential for complications.

Another risk that this study may pose concerns the information to be gained on henipaviruses newly recognized in the community. We will provide participating communities, hospitals, and clinicians with information and background data on target zoonotic viruses to ensure up-to-date communication of risk. Because of the timeline for diagnostic testing and results interpretation, we are not likely to provide results to participating clinics within a time frame that would be clinically relevant to outbreaks of undiagnosed diseases. Therefore, the information provided by this project will not impact patient management or outlook.

If an individual decides to participate in this research, his/her participation and all information provided by the participant will be strictly confidential, and personal identifying information will not be shared with anyone outside of the study staff. Participants will not be identified or named in any reports or publications. Questionnaire information and all biological samples will be identified by an alphanumeric code, not by the participant's name. 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 study site in locked files cabinets or password-protected devices in areas with access limited to the research staff of this project. Research databases will be secured with password-protected access systems and controlled distribution web-based certificates and will not contain any identifying characteristics about study participants (e.g., name, address, or telephone number). Access to all data will be limited to the staff involved in this study. The health information disclosed by an individual will not be used by or disclosed (released) to another institution. Any surveillance report that is published or shared with partners will not contain any personally identifying information for individual participants.

3. Potential benefits of the research to the subjects and others

There are no measurable benefits to the individual study participants enrolled in this study. There are benefits to the community and regional healthcare providers to help them understand the risk of zoonotic infections among high-risk populations in the regions they work. At the conclusion of the study, we will deliver an educational workshop reporting aggregate study findings that will be open to both study and non-study participants, describing the health benefits of using personal protection equipment (PPE) and handwashing during animal handling activities throughout the day, as well as to share other prevention/interventions that emerge from the research data.

4. Importance of the knowledge to be gained

There are valuable potential benefits to the public from the knowledge to be gained from this study. One key benefit to the community is sharing information and knowledge to better understand the risk of zoonotic spillover events and related health risks, as well as information sharing with communities on practices that could reduce risks, such as the avoidance of particular animal contacts or the need for PPE and extra care when handling wildlife, that may substantially reduce the risk zoonotic pathogen transmission in the community. 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 create or implement public health interventions to disrupt disease emergence and/or spread in an area that is beneficial to all. Additionally, there are valuable benefits to the general public from the knowledge to be gained from this study. Knowledge gained will 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.

5. Country- / institution-specific ethics / IRB regulations addressed

Main research protocol and materials to conduct human subject research in this project have been reviewed and approved by the Health Medical Lab Institutional Review Board, US (No. 894ECOH21b) and the Institute Review Board of the Faculty of Medicine, Chulalongkorn University, Thailand (No. 211/64). Amendment for PBMCs collection will be made and approved before the start of this project.

Research and Related Other Project Information

1. Are Human Subjects Involved?	Yes	X	No												
1.a. If YES to Human Subjects															
Is the Project Exempt from Federal regulations?	Yes		No	X											
If yes, check appropriate exemption number	1		2		3		4		5		6		7		8	
If no, is the IRB review Pending?	Yes	X	No												
IRB Approval Date:															
Human Subject Assurance Number	FWA00000943														

2. Are Vertebrate Animals Used?	Yes	X	No	
2.a. If YES to Vertebrate Animals				
Is the IACUC review Pending?	Yes		No	X
IACUC Approval Date	3 December 2020			
Animal Welfare Assurance Number				

3. Is proprietary/privileged information included in the application?	Yes		No	X

4.a. Does this project have an Actual or Potential impact -- positive or negative -- on the environment?	Yes		No	X
4.b. If yes, please explain				
4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed?	Yes		No	
4.d. If yes, please explain				

5. If the research performance site designated, or eligible to	Yes		No	X

be designated, as a historic place?				
5.a. If yes, please explain				

6. Does this project involve activities outside of the United States or partnership with international collaborators?	Yes	X	No	
6.a. If yes, identify countries	Thailand			
6.b. Optional explanation				

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: Srimuang, Krongkan

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

POSITION TITLE: Research 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
Chulalongkorn University, Bangkok, Thailand	B.Sc.	03/2012	Medical Technology
Mahidol University, Bangkok, Thailand	Ph.D.	04/2017	Molecular Tropical Medicine and Genetics

A. Personal Statement

I am a medical technologist. My undergraduate training has provided me with a background in multiple biological disciplines including molecular biology, microbiology, and genetics. During my Ph.D., I conducted research with Dr. Mallika Imwong focused on Malaria. My thesis was entitled “Exploring the molecular mechanism of mefloquine resistance in *Plasmodium falciparum*”. I conducted research on drug resistance to malaria in Southeast Asia. Specifically, my focus was on molecular techniques in identifying the genetic mutations involved with drug resistance. Moreover, I got experience in *Plasmodium falciparum* malaria culture. After my Ph.D., I worked at the genetics laboratory at Jetanin Institute of Assisted Conception. My responsibilities included Preimplantation Genetic Testing (PGT) is screen embryos for genetic abnormalities such as chromosome copy number gains or losses (PGT-A, aneuploidy screening), single gene disorders (PGT-M, monogenic or single-gene disease), or structural rearrangement (PGT-SR, structural rearrangement to identify embryos with chromosome imbalanced) by using Next Generation Sequencing. Additionally, I conducted genetic screening in couples for detecting carrier diseases such as glucose-6-phosphate dehydrogenase (G6PD), Alpha thalassemia, Beta thalassemia, and Spinal muscular atrophy (SMA) by using molecular genetics techniques. Currently, I am a Research Scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC EIDCC), King Chulalongkorn Memorial Hospital where I investigate emerging infectious diseases under the guidance of Dr. Supaporn Wacharapluesadee. The research focuses on detecting, identifying, and characterizing pathogens and human genome segments using molecular and serology techniques, including Next Generation Sequencing and Multiplex serology assays as a part of EID-SEARCH project. Additionally, I am the laboratory sequencing lead on the Surveillance System: Emerging Viral Pneumonia and Respiratory tract infections (RTIs) project from patients in Thailand as part of a collaboration with the Department of Disease Control.

1. **Srimuang K**, Miotto O, Lim P, Fairhurst RM, Kwiatkowski DP, Woodrow CJ, Imwong M; Tracking Resistance to Artemisinin Collaboration. “Analysis of anti-malarial resistance markers in *pfmdr1* and *pfcrt* across Southeast Asia in the Tracking Resistance to Artemisinin Collaboration” Malar J. 2016 Nov 8;15(1):541.
2. Reamtong O, **Srimuang K**, Saralamba N, Sangvanich P, Day NP, White NJ, Imwong M. “Protein profiling of mefloquine resistant *Plasmodium falciparum* using mass spectrometry- based proteomics” Int J Mass Spectrom. 2015 Nov 30;391:82-92

3. **Srimuang K**, Udompat P, Thippamom N, Petchrat S, Doung-Ngern P, Kripattanapong S, Supataragul S, Sterling SL, Wacharapluesadee S, Putcharoen O. “Genetic characterization of Norovirus strains from an unusual diarrheal outbreak in the community in Eastern Thailand” (Submitted, January 2023)

B. Positions, Scientific Appointments, and Honors

Positions and Scientific Appointments:

02/2022 – Present	Medical research scientist, Emerging Infectious Diseases Clinical Center (EIDCC), King Chulalongkorn Memorial Hospital
05/2017 - 01/2022	Scientist, Genetics Laboratory, Jetanin Institute for Assisted Reproduction
10/2015 - 03/2016	Fellowship at Laboratory of Molecular and Cellular Parasitology, Department of Microbiology and Immunology, National University of Singapore, Singapore.
11/2011 – 02/2012	Training Medical Technology Programs at Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

Honors:

2012 -2017	Scholarship for Ph.D. from the Royal Golden Jubilee, Ph.D. Grant (14th Batch).

C. Contributions to Science

Undergraduate Research: I participated in research activities under Dr. Attakorn Palasuwan to study in human genetic variants in proteins, such as G6PD, as they relate to the effect of exercise on the reduction of free radicals in the body. We conducted the multiplex PCR to identify the G6PD variants and found 8 variants including Vanua Lava, Mahidol, Mediterranean, Coimbra, Viangchan, Union, Canton, and Kaiping.

Graduate Research: I continued to studies under Dr. Mallika Imwong in the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University. My thesis, “Exploring the molecular mechanism of mefloquine resistance in *Plasmodium falciparum*”, focused on the evolution of resistance to mefloquine, which is commonly used in Southeast Asia in Malaria treatment. The mechanism of mefloquine resistance remains unknown, but I focused on the *Pfmdr1* gene which is involved in the drug resistance mechanism in *Plasmodium falciparum.* I found mutations and polymorphisms in *Pfmdr1* gene.

1. **Srimuang K**, Miotto O, Lim P, Fairhurst RM, Kwiatkowski DP, Woodrow CJ, Imwong M; Tracking Resistance to Artemisinin Collaboration. “Analysis of anti-malarial resistance markers in *pfmdr1* and *pfcrt* across Southeast Asia in the Tracking Resistance to Artemisinin Collaboration” Malar J. 2016 Nov 8;15(1):541.
2. Reamtong O, **Srimuang K**, Saralamba N, Sangvanich P, Day NP, White NJ, Imwong M. “Protein profiling of mefloquine resistant *Plasmodium falciparum* using mass spectrometry- based proteomics” Int J Mass Spectrom. 2015 Nov 30;391:82-92

Post-graduate Research: I was a scientist in genetics lab at Jetanin, a holistic center for infertility, offering counseling and treatment with Assisted Reproductive Technologies such as IVF, ICSI, and Preimplantation Genetic Testing (PGT). My work included the development of standard operating procedures for the lab, including Next Generation Sequencing, for detecting and identifying chromosomes in blastocyst growth to an embryo. Additionally, we assisted couples who had a family history of genetic diseases for screening before pregnancy. The carrier screening focused on highly prevalent diseases in Thailand such as alpha thalassemia, beta thalassemia, G6PD, and SMA. Currently, I am a Research Scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital. I am currently tasked with using molecular and serological techniques in the surveillance of novel, exotic, and known pathogens in wildlife and humans.

1. **Srimuang K**, Udompat P, Thippamom N, Petchrat S, Doung-Ngern P, Kripattanapong S, Supataragul S, Sterling SL, Wacharapluesadee S, Putcharoen O. “Genetic characterization of Norovirus strains from an unusual diarrheal outbreak in the community in Eastern Thailand” (Submitted, January 2023)

PHS OTHER SUPPORT
For All Application Types – DO NOT SUBMIT UNLESS REQUESTED

There is no "form page" for reporting Other Support. Information on Other Support should be provided in the format shown below.

*Name of Individual: Krongkan Srimuang
Commons ID:

Other Support – Project/Proposal

*Title: Immune memory bait & amp; capture to identify emerging henipavirus origins

*Major Goals: This project aims to identify novel viruses and zoonotic reservoirs through serologic and cellular immune discovery.

*Status of Support: Pending

Project Number:

Name of PD/PI: Krongkan Srimuang

*Source of Support: NIH/NIAID/CREID

*Primary Place of Performance: Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital, Thailand

Project/Proposal Start and End Date: (MM/YYYY) (if available): 05/2023 – 04/2024

* Total Award Amount (including Indirect Costs): $149,934

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2023	10.00
2. [enter year 2]	
3. [enter year 3]	
4. [enter year 4]	
5. [enter year 5]	

Name of Individual: Krongkan Srimuang
Commons ID:

Other Support – Project/Proposal

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

*Major Goals: The goals of this project are to identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife; identify evidence of novel virus spillover and key risk pathways for transmission; and identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts.

*Status of Support: Active

Project Number: U01 AI151797

Name of PD/PI: Peter Daszak

*Source of Support: NIH/NIAID

*Primary Place of Performance: EcoHealth Alliance and International Field and Lab Locations

Project/Proposal Start and End Date: (MM/YYYY) (if available): 06/2020 – 05/2025

* Total Award Amount (including Indirect Costs): 7,573,721.35

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2020	02.00
2. 2021	02.00
3. 2022	02.00
4. 2023	02.00
5. 2024	02.00

IN-KIND

*Summary of In-Kind Contribution:

*Status of Support:

*Primary Place of Performance:

Project/Proposal Start and End Date (MM/YYYY) (if available):

*Person Months (Calendar/Academic/Summer) per budget period

Year (YYYY)	Person Months (##.##)
1. [enter year 1]	
2. [enter year 2]	
3. [enter year 3]	
4. [enter year 4]	
5. [enter year 5]	

*Estimated Dollar Value of In-Kind Information:

Name of Individual: Krongkan Srimuang
Commons ID:

***Overlap** (summarized for each individual):

No overlap

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

*Signature: (b) (6)

Date: Jan 30, 2023

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contr butors.
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

PHS OTHER SUPPORT
For All Application Types – DO NOT SUBMIT UNLESS REQUESTED

There is no "form page" for reporting Other Support. Information on Other Support should be provided in the format shown below.

*Name of Individual: Spencer Lee Sterling
Commons ID:

Other Support – Project/Proposal

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

*Major Goals: The goals of this project are to identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife; identify evidence of novel virus spillover and key risk pathways for transmission; and identify and characterize viral etiology of ‘cryptic’ outbreaks in clinical cohorts.

*Status of Support: Active

Project Number: U01 AI151797

Name of PD/PI: Peter Daszak

*Source of Support: NIH/NIAID

*Primary Place of Performance: EcoHealth Alliance

Project/Proposal Start and End Date: (MM/YYYY) (if available): 06/2020-05/2025

* Total Award Amount (including Indirect Costs): 7,573,721.35

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2020	12.00
2. 2021	12.00
3. 2022	12.00
4. 2023	12.00
5. 2024	12.00

Name of Individual:
Commons ID:

***Overlap** (summarized for each individual):

No overlap

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

*Signature:

Date: January 30, 2023

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: Assistant Professor

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. (hons)	05/2008	Biology
Uniformed Services University, Bethesda, MD	Ph.D.	10/2016	Emerging Infectious Diseases
Uniformed Services University, Bethesda, MD	Postdoc	04/2019	Virology

A. Personal Statement

The spillover of zoonotic viruses into human populations remains an ever-prevalent threat to public and global health. Continual outbreaks of known and unknown zoonotic viruses highlights the paucity of our understanding of the viral diversity, geographic distribution, wildlife hosts, and human populations at-risk for spillover. I am a recently appointed assistant professor dedicated to biosurveillance and virological research of emergent zoonotic viruses. I have completed virological training and spent my time as a post-doctoral fellow applying *in vitro* techniques to characterize the replication of, and mammalian ephrin receptors that mediate cellular entry of emergent henipaviruses. Since the start of the COVID-19 pandemic, I pivoted the focus of my research team to serological analysis of SARS-CoV-2 infection, and then to COVID-19 vaccine-induced antibody durability. In my ongoing research, I develop serological-based approaches to identify the infectome of wildlife and human communities for the assessment of virus spillovers. Since my position as a postdoctoral fellow I have worked collaboratively with national and international researchers including institutional partners at Duke-NUS, Chulalongkorn University, University of Pretoria, and Rocky Mountain Labs towards these goals. Across all of these collaborations, we have aimed to identify known and unknown viruses, the wildlife reservoirs of priority pathogens, the high-risk interfaces for spillover and the biotic/abiotic drivers of virus emergence. Throughout these research projects, we have detected unexpected serological profiles in communities of bats and humans that have not fit our prior understanding of the viral diversity established or detected by genetic techniques. This proposal aims to better understand these serological profiles of wildlife and humans with a targeted focus on henipaviruses and filoviruses. Ongoing projects that I would like to highlight include:

- HDTRA12110037. DTRA BRTP, E. Laing (Co-PI). 08/2021-07/2026, “Informing biosurveillance, contribution of pteropodid fruit bats to virus spillover in the Philippines.”
- HU00012020067, HU00012120104, HU00012120094.Defense Health Program/CARES Act, NIAID, E. Laing (Associate Investigator). 9/2020-9/2025, “Prospective Assessment of SARS-CoV-2 Seroconversion.”
- HDTRA12010025. DTRA BTRP, E. Laing (Co-I). 7/2020 – 6/2025, “Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa.”
- U01AI151797. NIH, Centers for Research in Emerging Infectious Diseases, E. Laing (Co-I). 02/2020 – 03/2025, “EID-Southeast Asia Research Collaborative Hub.”
- HU00012020067, HU00011920111. Defense Health Program, NIAID, E. Laing (Associate Investigator). 03/2020 – 09/2023, “Epidemiology, Immunology, and Clinical Characteristics of Emerging Infectious Diseases with Pandemic Potential (EpICC-EID).”

B. Positions, Scientific Appointments and Honors

Positions

2021-pres. Assistant Professor, Department of Microbiology and Immunology, School of Medicine, Uniformed Services University, Bethesda, MD.

2021-pres. Joint Appointment, Emerging Infectious Diseases Graduate Program, School of Medicine, Uniformed Services University, Bethesda, MD.

2019-21 Research Assistant Professor, Department of Microbiology and Immunology, Uniformed Services University, Bethesda, MD.

2016-19 Postdoctoral fellow, Henry M. Jackson Foundation, Department of Microbiology, Uniformed Services University, Bethesda, MD.

2010-16 Graduate research student, Department of Microbiology, Uniformed Services University, Bethesda, MD.

Other Experiences and Professional Memberships

2022-pres. Executive committee member, Emerging Infectious Diseases Graduate Program, USUHS

2021-pres. Research center representative, Laboratory Assays Oversight and Quality Working Group, Emerging Infectious Diseases: Southeast Asia Research Collaboration Hub (EID-SEARCH), Centers for Research in Emerging Infectious Diseases (CREID), NIAID, DMID, NIH

2021-pres. Review editor, Frontiers in Virology - Emerging and Reemerging Viruses

2021-pres. Ad hoc reviewer, Frontiers in Immunology, Journal of Clinical Immunology

2021-pres. Steering committee member, JPI/Military Infectious Diseases Research Program, Emerging Infectious Diseases

2019 Ad hoc reviewer, EcoHealthNet 2.0 Program, EcoHealth Alliance

2018 Ad hoc reviewer, Pakistan One-Health Fellowship Program, National Academy of Sciences & Pakistan Academy of Sciences

2014-2019 Member, American Society of Tropical Medicine and Hygiene

2014-2019 Member, American Society of Microbiology

2014-2019 Volunteer, AAAS STEM K-12 Volunteer Program

Mentoring

Postdoctoral fellows Si'Ana A. Coggins, PhD, 2020 - 2022

Graduate students Marana S. Tso, BS, 2021 -
McKenna Roe, BS, 2022 -

Committee member Celeste Huaman, BS, 2021 -
2LT Connor Perry, BS, 2021 -

Honors

2021-2022 Impact Award, USUHS School of Medicine

2021 Outstanding Research Accomplishment/Team/SARS-CoV-2, The EPICC COVID-19 Cohort Team, Military Health System Research Symposium

2020-2021 Impact Award, USUHS School of Medicine

2015-2016 Val G. Hemming Fellowship, Henry M. Jackson Foundation

2015 East Asia and Pacific Summer Institutes Fellowship, National Science Foundation

C. Contributions to Science (†*mentee*, *corresponding)

1. Lyssaviruses and the prototype, rabies virus, remain a public health concern. Beginning with my PhD thesis work, I've researched the virus host-interactions between a rabies-related lyssavirus, Australian bat lyssavirus (ABLV), and its bat host (*Pteropus alecto*). Research has focused on ABLV cellular entry mechanisms, the development of an animal model and ABLV reporters and exploration of novel monoclonal antibodies that neutralize ABLV and other phylogroup I lyssaviruses. Furthermore, comparative bat immunology research was conducted using black flying fox cell lines and ABLV as a model virus/host interaction. Physiological adaptations that accompanied the evolution of flight in bats have been proposed to contribute to the frequent role of bats as asymptomatic hosts of highly pathogenic zoonotic viruses.

Comparatively studying the autophagy pathway in bat cell lines revealed that bat cells had elevated levels of basal autophagy and experienced significantly less cell death when challenged with high virus doses.

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. Virol J. 2013. 11:40. doi: 10.1186/1743-422X-11-40. PMID: 24576301, PMCID: PMC3946599

b. **Laing E.D.***, Sterling S.L., Weir D.L., Beauregard C.R., Smith I.L., Larsen S.E., Wang L-F., Snow A.L., Schaefer B.C., and Broder C.C. Enhanced autophagy contributes to reduced viral infection in black flying fox cells. Viruses. 2019. Mar 14;11(3). pii: E260. doi: 10.3390/v11030260. PMID: 30875748, PMCID: PMC6466025

c. Mastraccio K.E., Huaman C., Warrilow D., Smith G.A., Craig S.B., Weir D.L., **Laing E.D.**, Smith I., Broder C.C. and B.C. Schaefer. Establishment of a longitudinal pre-clinical model of lyssavirus infection. J Virol Methods. 2020 Jul; 281:113882. doi: 10.1016/j.jviromet.2020.113882. Epub 2020 May 12. PMID: 32407866

d. Weir D.L., Coggins S.A., Vu B.K., Coertse J., Yan L., Smith I.L., **Laing E.D.**, Markotter W., Broder C.C., and Schaefer B.C. Isolation and characterization of cross-reactive human monoclonal antibodies that potently neutralize Australian bat lyssavirus variants and other phylogroup 1 lyssaviruses. Viruses. 2021 Mar 1;13(3):391. doi: 10.3390/v13030391. PMID: 33804519; PMCID: PMC8001737.

2. My research experience as a postdoctoral fellow furthered my training in molecular virology techniques. I constructed a recombinant Cedar virus cDNA plasmid and optimized a reverse genetics approach to rescue a recombinant Cedar virus reporter virus, a non-pathogenic *Henipavirus* species. A molecular biology methods chapter detailing recombinant Cedar virus reverse genetics has been submitted and in press. Using this recombinant Cedar virus, we determined that Cedar virus can utilize several non-canonical henipavirus ephrin receptors for cellular entry and explored the structure of the receptor-binding pocket to understand the receptor promiscuity. The non-pathogenic phenotype of CedV creates a potential for CedV to act as a model henipavirus to explore host-pathogen interactions, cellular tropism and factors that determine henipaviral disease pathogenesis. Additionally, I have collaborated on projects detailing henipavirus infection and replication in bat hosts with colleagues at the Rocky Mountain Labs, studying whether specific species of bats are more competent hosts and whether virus-host restriction exists.

a. Amaya M, Broder CC, **Laing ED***. Recombinant Cedar virus: a henipavirus reverse genetics platform. In: Freiberg A.N. and B. Rockx, Nipah Virus: Methods and Protocols, Methods Mol. Biol. (in press)

b. Seifert SN, Letko MC, Bushmaker T, **Laing ED**, Saturday G, Meade-White K, van Doremalen N, Broder CC, Munster VJ. Rousettus aegyptiacus Bats Do Not Support Productive Nipah Virus Replication. J Infect Dis. 2020 May 11;221(Suppl 4):S407-S413. doi: 10.1093/infdis/jiz429. PMID: 31682727; PMCID: PMC7199784.

c. **Laing ED,** Navaratnarajah CK, Cheliout Da Silva S, Petzing SR, Xu Y, Sterling SL, Marsh GA, Wang LF, Amaya M, Nikolov DB, Cattaneo R, Broder CC, Xu K. Structural and functional analyses reveal promiscuous and species specific use of ephrin receptors by Cedar virus. Proc Natl Acad Sci U S A. 2019 Oct 8;116(41):20707-20715. doi: 10.1073/pnas.1911773116. Epub 2019 Sep 23. PMID: 31548390; PMCID: PMC6789926.

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. PMID: 29587789; PMCID: PMC5869790.

3. Bats are increasingly identified as animal reservoirs of emerging zoonotic viruses (e.g. Nipah virus, Ebola virus and SARS-coronavirus). I lead collaborative biosurveillance and research preparedness training including data analysis and interpretations at international partner institutes with lab technicians, field and lab scientists, and masters, doctoral and postdoctoral trainees. Collaborative biosurveillance is presently underway in Thailand (Chulalongkorn University, Bangkok) Malaysia (National Wildlife and Forensic Lab, Universti Purtra Malaysi, National Public Health Lab) via NIH Centers for Research in

Emerging Infectious Diseases, EID-Southeast Asia Research Collaborative Hub. As a collaborator within the DARPA PREEMPT network I supported surveillance for coronaviruses and other priority emerging zoonotic viruses, henipaviruses and filoviruses, in Ghana (Zoological Society of London), Australia (Black Mountain Labs) and Bangladesh (icddr,b). We aim to characterize the geographic distribution of zoonotic filoviruses/henipaviruses/coronaviruses, transmission dynamics in wildlife hosts and generate risk-models for Ebola virus, Nipah virus and SARS-related CoV outbreaks. Results discovered so far suggest a wider geographical footprint of Asiatic filoviruses and have identified several fruit bat species that act as natural reservoirs for these viruses.

a. Paskey AC, Ng JHJ, Rice GK, Chia WN, Philipson CW, Foo RJH, Cer RZ, Long KA, Lueder MR, Lim XF, Frey KG, Hamilton T, Anderson DE, **Laing ED**, Mendenhall IH, Smith GJ, Wang LF, Bishop-Lilly KA. Detection of Recombinant Rousettus Bat Coronavirus GCCDC1 in Lesser Dawn Bats (Eonycteris spelaea) in Singapore. Viruses. 2020 May 14;12(5):539. doi: 10.3390/v12050539. PMID: 32422932; PMCID: PMC7291116.

b. Schulz JE, Seifert SN, Thompson JT, Avanzato V, Sterling SL, Yan L, Letko MC, Matson MJ, Fischer RJ, Tremeau-Bravard A, Seetahal JFR, Ramkissoon V, Foster J, Goldstein T, Anthony SJ, Epstein JH, **Laing ED**, Broder CC, Carrington CVF, Schountz T, Munster VJ. Serological Evidence for Henipa-like and Filo-like Viruses in Trinidad Bats. J Infect Dis. 2020 May 11;221(Suppl 4):S375-S382. doi: 10.1093/infdis/jiz648. PMID: 32034942; PMCID: PMC7213578.

c. Dovih P, **Laing ED**, Chen Y, Low DHW, Ansil BR, Yang X, Shi Z, Broder CC, Smith GJD, Linster M, Ramakrishnan U, Mendenhall IH. Filovirus-reactive antibodies in humans and bats in Northeast India imply zoonotic spillover. PLoS Negl Trop Dis. 2019 Oct 31;13(10):e0007733. doi: 10.1371/journal.pntd.0007733. Erratum in: PLoS Negl Trop Dis. 2021 Nov 16;15(11):e0009836. PMID: 31671094; PMCID: PMC6822707.

d. **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. PMID: 29260678; PMCID: PMC5749470.

4. Responding to the emergence of SARS-CoV-2, our lab developed multiplex serology strategies to identify SARS-CoV-2 antibodies and address research questions related to whether pre-existing antibody memory induced by prior infection with seasonal human coronaviruses effects COVID-19 severity. Our lab's serology efforts supported NIH and DHA collaboratively funded protocols including prospective, longitudinal serological analysis of hospital and community subjects, and health-care workers; and cross-sectional analyses of SARS-CoV-2 infection among deployed military health-care workers.

a. Epsi NJ, Richard SA, Lindholm DA, Mende K, Ganesan A, Huprikar N, Lalani T, Fries AC, Maves RC, Colombo RE, Larson DT, Smith A, Chi SW, Maldonado CJ, Ewers EC, Jones MU, Berjohn CM, Libraty DH, Edwards MS, English C, Rozman JS, Mody RM, Colombo CJ, Samuels EC, Nwachukwu P, Tso MS, Scher AI, Byrne C, Rusiecki J, Simons MP, Tribble D, Broder CC, Agan BK, Burgess TH, **Laing ED**, Pollett SD; EPICC COVID-19 Cohort Study Group. Understanding 'hybrid immunity': comparison and predictors of humoral immune responses to SARS-CoV-2 infection and COVID-19 vaccines. Clin Infect Dis. 2022 May 24:ciac392. doi: 10.1093/cid/ciac392. Epub ahead of print. PMID: 35608504; PMCID: PMC9213853.

b. Lu Z, **Laing ED**, Pena DaMata J, Pohida K, Tso MS, Samuels EC, Epsi NJ, Dorjbal B, Lake C, Richard SA, Maves RC, Lindholm DA, Rozman JS, English C, Huprikar N, Mende K, Colombo RE, Colombo CJ, Broder CC, Ganesan A, Lanteri CA, Agan BK, Tribble D, Simons MP, Dalgard CL, Blair PW, Chenoweth J, Pollett SD, Snow AL, Burgess TH, Malloy AMW; EPICC COVID-19 Cohort Study Group. Durability of SARS-CoV-2-Specific T-Cell Responses at 12 Months Postinfection. J Infect Dis. 2021 Dec 15;224(12):2010-2019. doi: 10.1093/infdis/jiab543. PMID: 34673956; PMCID: PMC8672777.

c. Pollett SD, Richard SA, Fries AC, Simons MP, Mende K, Lalani T, Lee T, Chi S, Mody R, Madar C, Ganesan A, Larson DT, Colombo CJ, Colombo R, Samuels EC, Broder CC, **Laing ED**, Smith DR, Tribble D, Agan BK, Burgess TH. The SARS-CoV-2 mRNA vaccine breakthrough infection phenotype includes significant symptoms, live virus shedding, and viral genetic diversity. Clin Infect Dis. 2021 Jun 12:ciab543. doi: 10.1093/cid/ciab543. Epub ahead of print. PMID: 34117878.

d. Clifton GT, Pati R, Krammer F, **Laing ED**, Broder CC, Mendu DR, Simons MP, Chen HW, Sugiharto VA, Kang AD, Stadlbauer D, Pratt KP, Bandera BC, Fritz DK, Millar EV, Burgess TH, Chung KK. SARS-CoV-2 Infection Risk Among Active Duty Military Members Deployed to a Field Hospital - New York City, April 2020. MMWR Morb Mortal Wkly Rep. 2021 Mar 5;70(9):308-311. doi: 10.15585/mmwr.mm7009a3. PMID: 33661864; PMCID: PMC7948931.

5. In addition to providing serologic assessment of SARS-CoV-2 infection, my research team is actively engaged in examining the durability of COVID-19 vaccine induced humoral immunity. Antibody responses, particularly neutralizing antibodies, are frequently cited as a predictive correlate of protection. With the emergence of variants of concern and waning circulating antibodies, the timing of booster shots remains an important measure for controlling the pandemic. In my lab we evaluate the duration of neutralizing antibodies, durability and breadth of antibody responses against emerging variants of concern, hybrid immune responses, and post-vaccination infections.

a. Wang W, Lusvarghi S, Subramanian R, Epsi NJ, Wang R, Goguet E, Fries AC, Echegaray F, Vassell R, Coggins SA, Richard SA, Lindholm DA, Mende K, Ewers EC, Larson DT, Colombo RE, Colombo CJ, Joseph JO, Rozman JS, Smith A, Lalani T, Berjohn CM, Maves RC, Jones MU, Mody R, Huprikar N, Livezey J, Saunders D, Hollis-Perry M, Wang G, Ganesan A, Simons MP, Broder CC, Tribble DR, **Laing ED**, Agan BK, Burgess TH, Mitre E, Pollett SD, Katzelnick LC, Weiss CD. Antigenic cartography of well-characterized human sera shows SARS-CoV-2 neutralization differences based on infection and vaccination history. Cell Host Microbe. 2022 Dec 14;30(12):1745-1758.e7. doi: 10.1016/j.chom.2022.10.012. Epub 2022 Oct 21. PMID: 36356586; PMCID: PMC9584854.

b. **Laing ED,** Weiss CD, Samuels EC, Coggins SA, Wang W, Wang R, Vassell R, Sterling SL, Tso MS, Conner T, Goguet E, Moser M, Jackson-Thompson BM, Illinik L, Davies J, Ortega O, Parmelee E, Hollis-Perry M, Maiolatesi SE, Wang G, Ramsey KF, Reyes AE, Alcorta Y, Wong MA, Lindrose AR, Duplessis CA, Tribble DR, Malloy AMW, Burgess TH, Pollett SD, Olsen CH, Broder CC, Mitre E. Durability of Antibody Response and Frequency of SARS-CoV-2 Infection 6 Months after COVID-19 Vaccination in Healthcare Workers. Emerg Infect Dis. 2022 Apr;28(4):828-832. doi: 10.3201/eid2804.212037. Epub 2022 Feb 24. PMID: 35203111; PMCID: PMC8962883.

c. Lusvarghi S, Pollett SD, Neerukonda SN, Wang W, Wang R, Vassell R, Epsi NJ, Fries AC, Agan BK, Lindholm DA, Colombo CJ, Mody R, Ewers EC, Lalani T, Ganesan A, Goguet E, Hollis-Perry M, Coggins SA, Simons MP, Katzelnick LC, Wang G, Tribble DR, Bentley L, Eakin AE, Broder CC, Erlandson KJ, **Laing ED**, Burgess TH, Mitre E, Weiss CD. SARS-CoV-2 BA.1 variant is neutralized by vaccine booster-elicited serum but evades most convalescent serum and therapeutic antibodies. Sci Transl Med. 2022 May 18;14(645):eabn8543. doi: 10.1126/scitranslmed.abn8543. Epub 2022 May 18. PMID: 35380448; PMCID: PMC8995032.

d. [†]*Coggins SA*, **Laing ED**, Olsen CH, Goguet E, Moser M, Jackson-Thompson BM, Samuels EC, Pollett SD, Tribble DR, Davies J, Illinik L, Hollis-Perry M, Maiolatesi SE, Duplessis CA, Ramsey KF, Reyes AE, Alcorta Y, Wong MA, Wang G, Ortega O, Parmelee E, Lindrose AR, Snow AL, Malloy AMW, Letizia AG, Ewing D, Powers JH, Schully KL, Burgess TH, Broder CC, Mitre E. Adverse Effects and Antibody Titers in Response to the BNT162b2 mRNA COVID-19 Vaccine in a Prospective Study of Healthcare Workers. Open Forum Infect Dis. 2021 Nov 20;9(1):ofab575. doi: 10.1093/ofid/ofab575. PMID: 35047649; PMCID: PMC8759445.

Name of Individual: Laing, Eric
Commons ID: (b) (6)

Other Support – Project/Proposal

Title: Efficacy testing of a novel human monoclonal antibody therapy for late-stage rabies/lyssavirus infection

Major Goals: The major goals of this project is to test the efficacy of mAb therapy at times beyond day seven post-infection, to more completely define the degree to which mAb therapy can confer protection from death post-onset of clinically evident severe neurological disease.

Status of Support: Active

Project Number: HU00011920118

Name of PD/PI: Schaefer, B.

Source of Support: USUHS/CGHE

Primary Place of Performance: USUHS, Bethesda, MD

Project/Proposal Start and End Date: (MM/YYYY): 09/30/2019 – 09/29/2023

Total Award Amount (including Indirect Costs): $518,000

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months(##.##)
3. 2022	0.6 calendar
4. 2023	0.6 calendar

Title: Epidemiology, Immunology, and Clinical Characteristics of Emerging Infectious Diseases with Pandemic Potential (EpICC-EID)

Major Goals: The major goals of this project are to utilize SARS-CoV-2 serology to explore hospital and community infection, correlates of COVID-19, and vaccinology.

Status of Support: Active

Project Number: HU00012020067, HU00011920111

Name of PD/PI: Burgess, T.

Source of Support: Defense Health Program

Primary Place of Performance: USUHS, Bethesda, MD

Project/Proposal Start and End Date: (MM/YYYY): 04/01/2020 – 09/30/2023

Total Award Amount (including Indirect Costs): $1,178,876

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months(##.##)
2,3. 2022	1.0 calendar
3,4. 2023	1.0 calendar

Title: Prospective Assessment of SARS-CoV-Seroconversion

Major Goals: The major goals of this project are to monitor SARS-CoV-2 infection, the roleof human coronavirus antibodies in COVID-19 outcomes, and COVID-19 vaccinology

Status of Support: Active

Project Number: HU00012020067, HU00012120104, HU00012120094, HU00011920111

Name of PD/PI: Mitre, E

Source of Support: Defense Health Program/CARES Act, NIAID

Primary Place of Performance: USUHS

Project/Proposal Start and End Date: (MM/YYYY): 04/01/2020-09/30/2024

Total Award Amount (including Indirect Costs): $235,388

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months(##.##)
2, 3. 2022	1.0 calendar
3, 4. 2023	1.0 calendar
4. 2024	1.0 calendar

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

Major Goals: The major goals of this Centers for Research in Emerging Infectious Diseases(CREID) project are to develop multidisciplinary teams of investigators in the program will conduct pathogen/host surveillance, study pathogen transmission, pathogenesis and immunologic responses in the host, and will develop reagents and diagnostic assays for improved detection for important emerging pathogens and their vectors.

Status of Support: Active Project Number: U01AI151797

Name of PD/PI: Daszak, P.

Source of Support: EcoHealth Alliance (NIH/NIAID/CREID 07-049-7012-52338 flow through)

Primary Place of Performance: USUHS, Bethesda, MD

Project/Proposal Start and End Date: (MM/YYYY): 06/17/2020-05/31/2025

Total Award Amount (including Indirect Costs): $539,119

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months(##.##)
2, 3. 2022	1.0 calendar
3, 4. 2023	1.0 calendar
4, 5. 2024	1.0 calendar
5. 2025	1.0 calendar

Title: Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa

Major Goals: This project will focus on building capacity and enhancing surveillance, including early detection programs for henipa-, filo- and zoonotic coronaviruses in Southern Africa.

Status of Support: Active

Project Number: HDTRA12010025

Name of PD/PI: Markotter, W.

Source of Support: University of Pretoria (flow-through DTRA BTRP)

Primary Place of Performance: USUHS, Bethesda, MD

Project/Proposal Start and End Date: (MM/YYYY): 07/31/2020-07/30/2025

Total Award Amount (including Indirect Costs): $1,120,965

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months(##.##)
2, 3. 2022	1.0 calendar
3,4. 2023	1.0 calendar
4, 5. 2024	1.0 calendar
5. 2025	1.0 calendar

Title: Biosurveillance for Spillover of *Henipaviruses* and *Filoviruses* in Rural Communities in India

Major Goals: The major goals of this project are to conduct biosurveillance for evidence of henipavirus and filovirus infection in bats and cryptic infections in at-risk human communities

Status of Support: Active

Project Number: HDTRA12010026

Name of PD/PI: Epstein, J.

Source of Support: EcoHealth Alliance (DTRA – BTRP flow through)

Primary Place of Performance: USUHS, Bethesda, MD

Project/Proposal Start and End Date: (MM/YYYY) (if available): 10/01/2020 – 09/30/2023

Total Award Amount (including Indirect Costs): $1,195,275

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months(##.##)
2. 2022	0.5 calendar
2. 2023	0.5 calendar

Title: Informing Biosurveillance: Contribution of pteropodid fruit bats to virus spillover in the Philippines.

Major Goals: The major goals of this project are to investigate Nipah virus and Reston virus circulation in bat populations in the Philippines, build research capacity for serological biosurveillance, and identify at-risk interfaces of virus zoonosis.

Status of Support: Funded

Project Number: HDTRA12110037

Name of PD/PI: Smith, G.

Source of Support: DTRA

Primary Place of Performance: USUHS, Bethesda, MD

Project/Proposal Start and End Date: (MM/YYYY): 07/2022 – 06/2025

Total Award Amount (including Indirect Costs): $1,213,746

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months(##.##)
1. 2022	1.0 calendar
2. 2023	2.0 calendar
3. 2024	2.0 calendar
4. 2025	2.0 calendar
5. 2026	1.0 calendar

Title: Solving Opportunities for Spillover (SOS): Frequency and Mechanisms of Cross-species Transmission of Henipaviruses in Bangladesh

Major Goals: We aim to better understand and prevent spillovers of bat-borne viruses into intermediate hosts and humans. The knowledge gained from this study will be immediately applicable to human and animal health programs because if we know which henipaviruses infect humans and domesticated animals, and how they are infected, we can advise public health surveillance programs on how to optimize detection and prevention of infections.

Status of Support: Pending

Project Number: 1 R01 AI168287-01A1

Name of PD/PI: Gurley, E.

Source of Support: NIH/NIAID

Primary Place of Performance: Johns Hopkins University

Project/Proposal Start and End Date: 09/01/2022- 08/31/2027

Total Award Amount (including Indirect Costs): $3,356,172

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2023	1.0 calendar
2. 2024	1.0 calendar
3. 2025	1.0 calendar
4. 2026	1.0 calendar
5. 2027	1.0 calendar

Title: Establishment of a Bat Resource for Infectious Disease Research

Major Goals: The major goals of this proposal is to contribute significant insight into the viral determinants that confer henipaviral pathogenesis. The construction and generation of recombinant henipavirus chimeric viruses will be the primary means for determining how virus receptor usage and the expression of virulence factors act individually or in conjunction to cause limited or severe disease. The recombinant constructs generated from the proposed studies will also be used to determine host determinants of immunity in Jamaican fruit bats and animal models.

Status of Support: Pending

Project Number: R24AI165424

Name of PD/PI: Schountz, T.

Source of Support: Colorado State University (NIH / NIAID flow through)

Primary Place of Performance: USUHS, Bethesda, MD

Project/Proposal Start and End Date: (MM/YYYY): 01/01/2023-12/30/2027

Total Award Amount (including Indirect Costs): $695,937

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2023	1.0 calendar
2. 2024	1.0 calendar
3. 2025	1.0 calendar
4. 2026	1.0 calendar
5. 2027	1.0 calendar

IN-KIND

NONE

OVERLAP

In my role on this project, titled: Immune memory bait & capture to identify emerging henipavirus origins, I will be providing mentorship and my group will be providing recombinant antigenic material for multiplex serologies and our expertise in virology and assay development and interpretation to the PI and Co-PI. These research activities and mentorship overlap with my present role as a co-investigator within the EID-SEARCH center.

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

*Signature:

Date: 01/30/2023

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: Wacharapluesadee, Supaporn

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

POSITION TITLE: Senior Researcher

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
Chiang Mai University, Thailand	B.S.	02/1991	Medical Technology
Mahidol University, Thailand	M.S.	01/1994	Biochemistry
Chulalongkorn University, Thailand	Ph.D.	03/2006	Biomedical Sciences

A. Personal Statement

I have 29 years in public health research and 20+ years of experience in emerging viral zoonoses. I have managed many internationally funded research projects that involve working with and managing international and local interdisciplinary teams. Most of my research projects are diagnostics development, pathogen discovery, public health surveillance, field surveillance in wild mammals, human behavioral risk surveys, and clinical research. I conduct workshops on the development of novel diagnostic approaches, appropriate sample collection and handling for different pathogens, and viral characterization *in vitro* and *in vivo*. I am a senior researcher at the Thai Red Cross Emerging Infectious Diseases Clinical Center, which conducts research on emerging zoonoses, clinical research, and public health surveillance research. My research background mainly focuses on understanding the process of zoonotic disease emergence, particularly viral zoonoses. This includes identifying the bat origin of Nipah virus, MERS-CoV, and SARS-CoV-2 and pathogenesis and diagnoses of Rabies. My studies on the emergence of novel betacoronaviruses found in Thai bats and Nipah virus have been published. My laboratory has supported the Department of Disease Control (DDC) in diagnosing emerging infectious diseases, communicable diseases, and wastewater surveillance. I am the DDC instructor on the clinical sampling method and consultant for Ebola, MERS, and COVID-19 diagnosis. I am a committee member of the Global Laboratory Leadership Program (GLLP) Thailand, organized by the Department of Medical Sciences. I led the team to diagnose the first human MERS case in 2015 and the first human COVID-19 case outside China in January 2020. I have been the PI on six multidisciplinary research projects that use epidemiology, laboratory, field science, and bioinformatics to diagnose and monitor the emergence of wildlife-origin viral zoonoses, including SARS-CoV, Nipah, and Hendra viruses, Avian influenza, and novel viruses from bats. I am also the Thailand country coordinator for PREDICT project, large contracts from USAID during 2010-2019 involving the successful management of teams of virologists, field biologists, veterinarians, epidemiologists, hospitals, and laboratorians.

1. **Wacharapluesadee S**, Buathong R, Iamsirithawon S, Chaifoo W, Ponpinit T, Ruchisrisarod C, Sonpee C, Katasrila P, Yomrat S, Ghai S, Sirivichayakul S, Okada P, Mekha N, Karnkawinpong O, Uttayamakul S, Vachiraphan A, Plipat T, Hemachudha T. Identification of a Novel Pathogen Using Family-Wide PCR: Initial Confirmation of COVID-19 in Thailand. Front Public Health. 2020 Oct 7;8:555013. doi: 10.3389/fpubh.2020.555013. PMID: 33134237; PMCID: PMC7579402.

2. Buathong R, Chaifoo W, Iamsirithaworn S, **Wacharapluesadee S**, Joyjinda Y, Rodpan A, Ampoot W, Putcharoen O, Paitoonpong L, Suwanpimolkul G, Jantarabenjakul W, Petcharat S, Bunprakob S, Ghai S, Prasithsirikul W, Mungaomklang A, Plipat T, Hemachudha T. Multiple clades of SARS-CoV-2 were introduced to Thailand during the first quarter of 2020. Microbiol Immunol. 2021 Oct;65(10):405-409. doi: 10.1111/1348-0421.12883. Epub 2021 Sep 1. PMID: 33835528; PMCID: PMC8251142.
3. **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. Nat Commun. 2021 Feb 9;12(1):972. doi: 10.1038/s41467-021-21240-1. Erratum in: **Nat Commun**. 2021 Feb 25;12(1):1430.
4. **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 Jul 5;3(1):12. doi: 10.1186/s42522-021-00044-9. PMID: 34218820; PMCID: PMC8255096.

B. Positions, Scientific Appointments, and Honors

Positions and Employment

1994 -97 Biochemical Technician, Department of Entomology, AFRIMS, Thailand
1997 Researcher, Department of Immunology, Chulabhorn Research Institute, Thailand
1997 -00 Medical Technologist, The HIV/AIDS Collaboration Thai-US, Thailand
2000 -16 Laboratory Chief, Neuroscience Centre for Research and Development & WHO Collaborating Centre for Research and Training on Viral Zoonoses, Faculty of Medicine, Chulalongkorn University Hospital, Thai Red Cross Society, Thailand
2016 -21 Deputy Chief of Thai Red Cross Emerging Infectious Diseases Health Science Centre, Faculty of Medicine, Chulalongkorn University Hospital
2021 - Senior Researcher, Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital
Committee member, School of Global Health, Faculty of Medicine, Chulalongkorn University

Other Experience and Professional Membership

2010 -14 PREDICT Thailand Country Coordinator
2014 - Thai Ministry of Public Health (MOPH) Ebola Diagnostic Committee
2015 -19 PREDICT 2 Thailand Country Coordinator
2016 - Steering committee, Bat One Health Research Network, BTRP DTRA
2021 - WHO Scientific Advisory Group for the Origins on Novel Pathogens (SAGO)

C. Contributions to Science

1. **Research on One Health.** One Health is an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals, and ecosystems, as the definition by WHO. Our projects are multidisciplinary studies on human surveillance, wildlife surveillance, wastewater surveillance, and human behavior.
   a. Tangwangvivat R, **Wacharapluesadee S**, Pinyopornpanish P, Petcharat S, Muangnoicharoen H. S, Thippamom N, Phiancharoen C, Hirunpatrawong P, Duangkaewkart P, Chaiden C, Wechsirisan W, Wandee N, Srimuang K, Paitoonpong L, Buathong R, Pawun V, Hinjoy S, Putcharoen O, Iamsirithaworn S. Assessment of SARS-CoV-2 variant wastewater detection strategies in the Bangkok Metropolitan region. **Preprint Research** 2023

b. Keusch GT, Amuasi JH, Anderson DE, Daszak P, Eckerle I, Field H, Koopmans M, Lam SK, Das Neves CG, Peiris M, Perlman S, **Wacharapluesadee S**, Yadana S, Saif L. Pandemic origins and a One Health approach to preparedness and prevention: Solutions based on SARS-CoV-2 and other RNA viruses. **Proc Natl Acad Sci USA**. 2022 Oct 18;119(42):e2202871119. doi: 10.1073/pnas.2202871119. Epub 2022 Oct 10. PMID: 36215506; PMCID: PMC9586299.
c. 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 Infect Dis**. 2022 May 16;22(1):472. doi: 10.1186/s12879-022-07439-7. PMID: 35578171; PMCID: PMC9109443.

2. **Research on Public Health Surveillance in Thailand.** Effective disease control programs rely on effective surveillance and response systems. Our laboratory collaborates with the Department of Diseases Control for active surveillance on syndromic surveillance and outbreak investigation. Both molecular and serology surveillance systems were conducted to identify the cause of the disease.
   a. Plipat T, Buathong R, **Wacharapluesadee S**, Siriarayapon P, Pittayawonganon C, Sangsajja C, Kaewpom T, Petcharat S, Ponpinit T, Jumpasri J, Joyjinda Y, Rodpan A, Ghai S, Jittmittraphap A, Khongwichit S, Smith DR, Corman VM, Drosten C, Hemachudha T (2017). Imported case of Middle East respiratory syndrome coronavirus (MERS-CoV) infection from Oman to Thailand, June 2015. **Euro Surveill** 22(33):pii: 30598.
   b. Okada P, Buathong R, Phuygun S, Thanadachakul T, Parnmen S, Wongboot W, Waicharoen S, **Wacharapluesadee S**, Uttayamakul S, Vachiraphan A, Chittaganpitch M, Mekha N, Janejai N, Iamsirithaworn S, Lee RT, Maurer-Stroh S. Early transmission patterns of coronavirus disease 2019 (COVID-19) in travellers from Wuhan to Thailand, January 2020. **Euro Surveill**. 2020 Feb;25(8):2000097. doi: 10.2807/1560-7917.ES.2020.25.8.2000097. PMID: 32127124; PMCID: PMC7055038.
   c. 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 Feb 12;16(2):e0246864. doi: 10.1371/journal.pone.0246864. PMID: 33577615; PMCID: PMC7880427.
   d. Sangkakam A, Hemachudha P, Saraya AW, Thaweethee-Sukjai B, Cheun-Arom T, Latinne A, Olival KJ, **Wacharapluesadee S**. Detection of influenza virus in rectal swabs of patients admitted in hospital for febrile illnesses in Thailand. **SAGE Open Med**. 2021 Jan 22;9:2050312121989631. doi: 10.1177/2050312121989631. PMID: 33552519; PMCID: PMC7841862.

3. **Research on diagnostic development.** The molecular technique is a gold standard method for pathogen diagnosis and confirmation. The first COVID-19 case outside China in Thailand was primarily detected by family PCR and further confirmed by next-generation sequencing (NGS). The SARS-CoV-2 variants can be identified by the NGS technique or by identifying the mutation markers by MassARRAY technology.
   a. **Wacharapluesadee S**, Kaewpom T, Ampoot W, Ghai S, Khamhang W, Worachotsueptrakun K, Wanthong P, Nopvichai C, Supharatpariyakorn T, Putcharoen O, Paitoonpong L, Suwanpimolkul G, Jantarabenjakul W, Hemachudha P, Krichphiphat A, Buathong R, Plipat T, Hemachudha T. Evaluating the efficiency of specimen pooling for PCR-based detection of COVID-19. **J Med Virol**. 2020 Oct;92(10):2193-2199. doi: 10.1002/jmv.26005. Epub 2020 Jul 21. PMID: 32401343; PMCID: PMC7272832.
   b. **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 R. A, Fernandez S, Putcharoen O. Simultaneous Detection of Omicron and Other SARS-CoV-2 Variants by Multiplex PCR MassARRAY Technology. **Preprint Research** 2023. DOI: https://doi.org/10.21203/rs.3.rs-2482226/v1

c. Joyjinda Y, Rodpan A, Chartpituck P, Suthum K, Yaemsakul S, Cheun-Arom T, Bunprakob S, Olival KJ, Stokes MM, Hemachudha T, **Wacharapluesadee S**. First Complete Genome Sequence of Human Coronavirus HKU1 from a Nonill Bat Guano Miner in Thailand. **Microbiol Resour Announc**. 2019 Feb 7;8(6):e01457-18. doi: 10.1128/MRA.01457-18. PMID: 30746519; PMCID: PMC6368654.

d. Siriyasatien P, **Wacharapluesadee S**, Kraivichian K, Suwanbamrung C, Sutthanont N, Cantos-Barreda A, Phumee A. Development and evaluation of a visible reverse transcription-loop-mediated isothermal amplification (RT-LAMP) for the detection of Asian lineage ZIKV in field-caught mosquitoes. **Acta Trop**. 2022 Dec;236:106691. doi: 10.1016/j.actatropica.2022.106691. Epub 2022 Sep 11. PMID: 36103950.

4. **Research on coronavirus prevalence in Thailand.** Numerous high-impact emerging viruses appear to have bat reservoirs. Our surveillance projects study the diversity of coronavirus (CoV) in bats in Thailand. We have isolated and characterized CoVs from many bat species and detected and sequenced CoV in bat guano collectors. Our surveillance studies continue to analyze the drivers of their emergence and risk factors for spillover.
   a. **Wacharapluesadee S**, Duengkae P, Chaiyes A, Kaewpom T, Rodpan A, Yingsakmongkon S, Petcharat S, Phengsakul P, Maneeorn P, Hemachudha T (2019). Longitudinal study of age-specific pattern of coronavirus infection in Lyle's flying fox (*Pteropus lylei*) in Thailand. **Virol J** 20;15(1):38.
   b. **Wacharapluesadee S**, Duengkae P, Rodparn A, Kaewpom T, Maneeorn P, Kanchanasaka B, Yingsakmongkon S, Sittidetboripat N, Chareesaen C, Khlangsap N, Pidthong A, Leadprathom K, Ghai S, Epstein JH, Daszak P, Olival KJ, Blair PJ, Callahan MV and Hemachudha T (2015). Diversity of Coronavirus in Bats from Eastern Thailand. **Virol J** 12(1):57.
   c. **Wacharapluesadee S**, Sintunawa C, Kaewpom T, Khongnomnan K, Olival KJ, Epstein JH, Rodpan A, Sangsri P, Intarut N, Chindamporn A, Suksawa K, Hemachudha T (2013). Group C betacoronavirus in bat guano fertilizer, Thailand. **Emerg Infect Dis** 19(8).

5. **Research on Nipah virus prevalence in Thai bats.** Nipah virus outbreaks, previously in Thailand's neighboring country, Malaysia, and ongoing in Bangladesh, have high mortality rates. Our surveillance projects study the characterization of Nipah Virus (NiV) in bats in Thailand. In addition, our surveillance studies continue to analyze the drivers of their emergence, understanding their seasonal preferences and risk factors for spillover.
   a. **Wacharapluesadee S**, Samseeneam P, Phermpool M, Kaewpom T, Rodpan A, Maneeorn P, Srongmongkol P, Kanchanasaka B, Hemachudha T (2016). Molecular characterization of Nipah virus from Pteropus hypomelanus in Southern Thailand. **Virol J** 13(1):53
   b. **Wacharapluesadee S**, Jittmittraphap A, Yingsakmongkon S, and Hemachudha T (2016). Molecular Detection of Animal Viral Pathogens. Nipah Virus. **CRC Press**.
   c. **Wacharapluesadee S**, Ngamprasertwong T, Kaewpom T, Kattong P, Rodpana A, Wanghongsa S, Hemachudha T (2013). Genetic characterization of Nipah virus from Thai fruit bats (*Pteropus lylei*). **Asian Biomedicine** 7(6);813-819.
   d. 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 Jun;19(2):175-189. doi: 10.1007/s10393-022-01588-6. Epub 2022 Jun 3. PMID: 35657574.
   e. Breed AC, Meers J, Sendow I, Bossart KN, Barr JA, Smith I, **Wacharapluesadee S**, Wang L, Field HE (2013). The Distribution of Henipaviruses in Southeast Asia and Australasia: Is Wallace's Line a Barrier to Nipah Virus? **PLoS One** 8(4):e61316.

6. **Rabies Neuropathogenesis, diagnosis and management.** The center worked for many years on molecular analyses of rabies, including mutational effects and designing primers to detect the Thai street rabies virus. I regularly organize workshops to teach laboratories in the region how to correctly collect specimens and test for rabies.
   a. Hemachudha T, Ugolini G, Sungkarat W, Laothamatas J, Shuangshoti S, **Wacharapluesadee S** (2013). Human Rabies: neuropathogenesis, diagnosis and management. **Lancet Neurology** 498-513.
   b. Shuangshoti S, Thepa N, Phukpattaranont P, Jittmittraphap A, Intarut N, Tepsumethanon V, **Wacharapluesadee S**, Thorner PS, Hemachudha T (2013). Reduced viral burden in paralytic compared to furious canine rabies is associated with prominent inflammation at the brainstem level. **BMC Vet Res** 14;9(1):31.
   c. Virojanapirom P, Khawplod P, Sawangvaree A, **Wacharapluesadee S**, Hemachudha T, Yamada K, Morimoto K, Nishizono A (2012). Molecular analysis of the mutational effects of Thai street rabies virus with increased virulence in mice after passages in the BHK cell line. **Arch Virol** 157(11):2201-5.
   d. Wilde H, Hemachudha T, **Wacharapluesadee S**, Lumlertdacha B, Tepsumethanon V (2013). Rabies in Asia: The Classical Zoonosis. **Curr Top Microbiol Immunol** 365:185-203.

PHS OTHER SUPPORT
For All Application Types – DO NOT SUBMIT UNLESS REQUESTED

There is no "form page" for reporting Other Support. Information on Other Support should be provided in the format shown below.

*Name of Individual: Supaporn Wacharapluesadee
Commons ID:

Other Support – Project/Proposal

*Title: Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of South East Asia

*Major Goals: The goals of this project are to identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife; identify evidence of novel virus spillover and key risk pathways for transmission; and identify and characterize viral etiology of ‘cryptic’ outbreaks in clinical cohorts.

*Status of Support: Active

Project Number: U01 AI151797

Name of PD/PI: Peter Daszak

*Source of Support: NIH/NIAID

*Primary Place of Performance: EcoHealth Alliance and International Field and Lab Locations

Project/Proposal Start and End Date: (MM/YYYY) (if available): 06/2020 – 05/2025

* Total Award Amount (including Indirect Costs): 7,573,721.35

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2020	03.00
2. 2021	03.00
3. 2022	03.00
4. 2023	03.00
5. 2024	03.00

Name of Individual: Supaporn Wacharapluesadee
Commons ID:

*Name of Individual: Supaporn Wacharapluesadee
Commons ID:

Other Support – Project/Proposal

*Title: Evaluation of GeneXpert® Bacterial Vs. Viral in a Clinical Setting Alpha Study

*Major Goals: Demonstrate that the clinical performance of GeneXpert Bacterial vs. Viral is not inferior to that of procalcitonin (PCT) and/or C-reactive protein (CRP) as compared to the infection status determined by 3 different reference methods: an algorithm of clinical standardized testing results, clinical adjudication with forced classification, and clinical adjudication with consensus.

*Status of Support: Active

Project Number:

Name of PD/PI:

*Source of Support: Defense Threat Reduction Agency, USA

*Primary Place of Performance: Chulalongkorn University

Project/Proposal Start and End Date: (MM/YYYY) (if available): 04/2022 – 08/2023

* Total Award Amount (including Indirect Costs): 307,746.39

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2022	01.20
2. 2023	01.20
3. [enter year 3]	
4. [enter year 4]	
5. [enter year 5]	

Name of Individual: Supaporn Wacharapluesadee
Commons ID:

IN-KIND

*Summary of In-Kind Contribution:

*Status of Support:

*Primary Place of Performance:

Project/Proposal Start and End Date (MM/YYYY) (if available):

*Person Months (Calendar/Academic/Summer) per budget period

Year (YYYY)	Person Months (##.##)
1. [enter year 1]	
2. [enter year 2]	
3. [enter year 3]	
4. [enter year 4]	
5. [enter year 5]	

*Estimated Dollar Value of In-Kind Information:

***Overlap** (summarized for each individual):

No overlap

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

*Signature: (b) (6)

Date: Jan 30, 2023

Co-PI Plan (only needed if applying as Co-PIs):

What each Co-PI will contribute to the proposed research study?

As stated in the Mentoring Plan, the co-PIs, Krongkan Srimuang and Spencer Sterling have different educational and training backgrounds. This Co-PI plan is proposed to fill the gaps in knowledge of each scientist and supports a collaborative working relationship for project success. Moreover, this plan was developed to maximize the strengths of each PI. Overall, the project is separated into two objectives, the proposal of this project will be equally managed by Dr. Srimuang and Mr. Sterling. Dr. Srimuang will be responsible for PBMC isolation, sorting, and characterization. Mr. Sterling will be responsible for the technical serological aspects of the project. Both will work closely with the Research Center Mentor, Dr. Eric Laing, to develop this project, protocol development, and network connections. Dr. Supaporn Wacharapluesade, the Co-mentor, will support the wildlife sampling, samples collection, and molecular aspects within Thailand.

How the Co-PIs will jointly work with the affiliated Research Center?

Dr. Srimuang is currently employed at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University and supports the molecular biology investigations in the EID-SEARCH project. Mr. Sterling is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region. Both Co-PIs will work closely with Dr. Laing, Dr. Wacharapluesadee, and the staff at EcoHealth Alliance to coordinate field sampling, research activities, data analysis, and manuscript production.

How the Co-PIs will jointly manage the proposed study?

This is a great opportunity for both PIs to undergo training in advanced techniques and develop skills in next-generation serology and molecular sequencing. Dr. Srimuang will learn and practice serologic techniques and data analysis from Mr. Sterling. Mr. Sterling will develop skills in cell isolation and molecular sequencing techniques from Dr. Srimuang. Moreover, Mr. Sterling will coordinate planning, sample processing, data analysis, and report preparation for this project with Dr. Srimuang.

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List of abbreviations, acronyms, symbols

AngV: Angavokely virus
BCR: B cell receptor
BSc: Bachelor of Science
BSL2: Biological Safety level 2
BSL3: Biological Safety level 3
cDNA: Complementary DNA
CedV: Cedar virus
Co-PI: Co-principal investigator
CREID: Centers for Research in Emerging Infectious Diseases
DARV: Daeryong virus
DNA: Deoxyribonucleic acid
EHA: EcoHealth Alliance
EID: Emerging Infectious Disease
EID-SEARCH: The Emerging Infectious Disease – South East Asia Research Collaboration Hub
ELISA: Enzyme-linked immunosorbent assay
F: Fusion protein
FACS: Fluorescence-activated cell sorting
FUO: Fever of unknown origin
GAKV: Gamak virus
GhV: Ghana virus
HeV: Hendra virus
HNVs: Henipaviruses
ILI: Influenza-like illness
KCMH: King Chulalongkorn Memorial Hospital
L: large protein
LayV: Langya virus
M: Matrix protein
mAb: Monoclonal antibody
MD: Maryland
MMIA: Multiplex microsphere-based immunoassay
MojV: Mojiang virus
MSc: Master of Science
N: Nucleocapsid
NGS: Next-generation sequencing
NIAID: National Institute of Allergy and Infectious Diseases
NIH: National Institutes of Health
NiV: Nipah virus
P: Phosphoprotein
PBMC: Peripheral blood mononuclear cells
PCR: Polymerase Chain Reaction
PhD: Doctor of Philosophy
PI: Principal investigator
RBP: Receptor-binding protein
RNA: Ribonucleic acid
RT-PCR: Real-time PCR
SARI/ARI: Severe/acute respiratory illness
SARS-CoV-1: Severe acute respiratory syndrome coronavirus 1
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2
SEA: Southeast Asia

Thai-DDC: Department of Disease Control of Thailand
TRC-EIDCC: Thai Red Cross. Emerging Infectious Diseases. Clinical Center
USA: United States
WGS: Whole Genome Sequencing

Facilities and Resources

FACILITIES: Specify the facilities to be used for the conduct of the proposed research.

Laboratory:

The Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC), King Chulalongkorn Memorial Hospital (KCMH) was established by the Committee of the Emerging Infectious Disease Center which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. TRC-EIDCC laboratory facilities include 3 biosafety cabinets (BSL 2, BSL 2+, BSL2+), 3 automated extraction machines, 2 thermocyclers (Proflex), 2 real-time PCR machines (CFX, Bio-rad), 3 Freezers (-80 °C), 2 refrigerators; 4 °C, 2 autoclaves. Our EIDCC uses the Pathogen Asset Control System (PACS) system for sample tracking. Moreover, our Faculty of Medicine, Chulalongkorn University has a central laboratory, the Chula Medical Research Center (Chula MRC), which provide many instruments for medical, masters, and graduate students, Post-doctoral fellows, and researchers at Chulalongkorn University and KCMH. The Chula MRC laboratory facilities are divided into molecular diagnostics (PCR Thermal Cycler, QStudio6 Flex Real-Time PCR, Droplet Digital PCR, GeneXpert, Film array, Illumina MiSeq), immunology facilities (ELISA testing equipment (reader & washer), Luminex, Flow Cytometry), cell sorting machine and a BSL3 laboratory. Moreover, the facility provides general equipment, such as autoclaves, balances, CO2 incubators, Dual Fluorescence Cell Counter, 4 °C refrigerators, -20°C, and -80°C freezers.

Clinical:

KCMH is a 1,000-bed university hospital, in close coordination with the Faculty of Medicine, Chulalongkorn University. The Thai Red Cross Society provides medical treatment, rehabilitation, health promotion and disease prevention services to the general public and the vulnerable. It also provides biopharmaceutical and sterile medicines that are sufficient to meet the needs of patients and safe in accordance with international standards. It serves as a center for providing eye and organ donation services to patients nationwide in line with international standards. It has specialized doctors and public health personnel and also produces specialized nurses. The TRC-EIDCC in the KCMH provides standard medical care to patients with emerging and recurrent diseases and disease spreading prevention.

Animal:

TRC-EIDCC also collaborates with the Department of National Parks, Wildlife and Plant Conservation for detecting the emerging novel pathogens from animals such as bats, rodents, and pangolins in our country.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer, Lee

Computer:

TRC-EIDCC used the service from the Office of Information Technology, Chulalongkorn University that provides the application, network, and software such as Office 365, VPN, WIFI, Microsoft Office, Microsoft Windows, SPSS, Adobe, Zoom Meeting, etc. Moreover, our EIDCC use the Pathogen Asset Control System (PACS) for sample tracking.

Office:

The office and laboratory space are located at the EIDCC at 4th Floor, Jongkolnee Watwong Building and 6th Floor, Aor Por Ror Building, respectively. The applicants will have dedicated desks in both buildings.

Other:

PI Name: Srimuang, Krongkan and Sterling, Spencer, Lee **Foreign Site:** Thailand
CREID CC Grant: 1U01AI151378

Foreign Site Information

If the Pilot Award will have multiple foreign sites, complete this form for EACH research site

Principal Investigator Name:	Krongkan Srimuang
Project Title:	Immune memory bait & capture to identify emerging henipavirus origins
Institution:	King Chulalongkorn Memorial Hospital
Foreign Research Site:	Rama 4 Road, Pathumwan, Bangkok 10330 Thailand
Point of Contact for Research Site:	Krongkan Srimuang Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital Rama 4 Road, Pathumwan, Bangkok 10330 Thailand krongkan.sr@gmail.com

Introduction (3-4 sentences):
Provide a simple description of the overall goals of the project including the work that will be done at the foreign site. Indicate if any of the NIAID grant funds will be sent to the foreign site.

The goal of this project is to identify the origin of zoonotic pathogens by applying serologic and cellular immune tools in humans and animals for early detection and identification of novel pathogens to prevent the next pandemic.

To achieve this, the project will conduct serological surveillance among humans and rodents/shrews at this site, and perform lab analysis collaborating with NIH PREMISE for antibody-mediated bait & capture of Thai MojV-like virus.

$149,937 of grant funds will be sent to the site for these research activities.

Foreign component (3-4 sentences):
Describe the specific role of the foreign site (i.e. conduct critical experiments?, conduct data analyses?, provide consultations?, provide samples?, study human and/or animal populations?, conduct observational or interventional clinical trials?, collect samples and/or data to be brought to the US?).

The site will 1) conduct all field work to collect human and animal samples from the study sites; 2) perform laboratory analysis using collected samples; and 3) conduct data analysis as proposed in the project.

Human Subjects (1 word or 1 sentence per bullet):

- **Parent Study IRB approval**
  - **IRB approval number for parent study:** #894ECOH21b (US); #221/64 (Thailand)
  - **IRB approval date:** 12-05-2021 (US); 08-06-2021 (Thailand)
  - **Human Subject Assurance Number: (FWA)#:** #00001102 (external IRB); #00022431 (EcoHealth Alliance); #00000943 (Chulalongkorn University)
- **Does this study require a modification to the IRB approval of a parent study (Yes or No)?**
  - **Yes**
- **Will existing samples from human subjects will be used: (Yes or No)?**
  - **No**
  - **How many subjects provided the existing samples to be used?** N/A
- **Will human subjects be recruited (Yes or No)**
  - **Yes**
  - **Number of human subjects that will be recruited:** 110

- **Population parameters:**
  - **Gender:** 55 males, 55 females
  - **Age Group:** Age >=18 years who provide informed consent.
  -
  - **Race/Ethnicity:** 110 Asian

- **Sample collection will include:**
  - **Blood:** Yes
  - **Urine:** No
  - **Tissues:** No
  - **Other samples (describe):** Nasopharyngeal swab and throat swab

- **Sample collection will be completed in how many visits:** 2 trips

- **Will samples be de-identified (Yes or No)? If No, describe how they will be protected.**
  - Yes

- **Will local IRB approval be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals being sought or state why local IRB approval is not required.**
  - Yes

- **Will informed consent be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals/consents being sought or state why informed consent is not required.**
  - Yes

- **Will samples be brought back to the US (Yes or No)?**
  - No

- **Will data be brought back to the US (Yes or No)?**
  - Yes, some of the data will be shared with EID-SEARCH partners in the US for relevant analysis.

Animal Subjects (1 word or 1 sentence per bullet):

- **Parent study IACUC approval**
  - **IACUC approval number for parent study:** #G2020-42 (US); #019/2563 (Thailand)
  - **IACUC approval date:** 05-05-2020 (US); 03-12-2020 (Thailand)
    Animal Welfare Assurance Number: D16-00572 (A4059-01)
- **Does this study require a modification to the IACUC approval of a parent study (Yes or No)?**
  - **No**

- **Will existing samples from animal subjects will be used: (Yes or No)?**
  - **No**
  - **How many animal subjects provided the existing samples to be used?** N/A
- **Will vertebrate animals be collected (Yes or No)?**
  - **Yes**

- **Species of animals (e.g. rats, mice, rabbits, monkeys):** Rodents, and shrews
- **Animal parameters:**
  - **Total number of animals:** 200 (200 rodents and shrews)
  - **Gender:** 100 males, 100 females
  - **Age range:** 4 - 12 months and elder depending upon species
  - **Lab strain (e.g. Sprague-Dawley rats, Balb/C mice):** None
  - **Wild animals procured in country (e.g. Rhesus monkeys from a reserve):** No

- **What will be done to them or with them and how often?**
  - Free-ranging rodents and shrews will be captured through pit traps and box traps.

- Anesthesia will be conducted for captive rodents and shrews.
- Once anesthetized blood will be collected.
- All actions will only be performed one time for each animal individual.
- All animals will be released after sampling.

- **What are the follow-ups?**
  - No follow-up relevant as per protocol, since all animals will be released after sampling ups

- **What will be their fate at the end of the experiments – will they be euthanized?**
  - All animals will not be held longer than 6 hours (typically less than 3 hours) during the sampling process and released after sampling.

- **Will in-country clearances be obtained prior to engaging in any animal research (Yes or No)? If No, describe alternate approvals or state why in-country clearances are not required.**
  - Yes
- **Will samples be brought back to the US (Yes or No)?**
  - No
- **Will data be brought back to the US (Yes or No)?**
  - Yes, some of the data will be shared with EID-SEARCH partners in the US for relevant analysis.

27th January 2022

Krongkan Srimuang Ph.D.
Research Scientist
Thai Red Cross Emerging Infectious Diseases Clinical Center
King Chulalongkorn Memorial Hospital
Rama 4 Road
Pathumwan, Bangkok 10330
Thailand

Subject: Letter of Support for CREID Network Pilot Research Program

Dear Dr. Srimuang,

I am writing to express my support for your CREID Network Pilot Research Program proposal titled, "Immune memory bait and capture to identify emerging henipavirus origins." Your preliminary data demonstrating immunoreactivity against Mojiang henipavirus in individuals sampled as part of the Emerging Infectious Diseases Southeast Asia Research Collaborative Hub (EID-SEARCH) center, provides compelling serologic evidence that novel henipaviruses spill over into human populations. Point-of-care diagnostics and nucleic acid-amplification tests rely on our present understanding of medically relevant pathogens. Thus, emergence of novel zoonotic viruses may go undetected. Serological profiling provides us an ability to measure the human infectome through broad-scale peptide arrays or targeted antigen-based detection.

The research activities conducted at the Thai Red Cross Emerging Infectious Diseases Clinical Center are complementary to the focus of the Vaccine Research Center's PREMISE (Pandemic REsponse REpository through Microbial and Immunological Surveillance and Epidemiology) program. PREMISE works closely with intramural and extramural NIH programs and in partnership with a global network of investigators and collaborators to achieve its objectives through 3 core activities: (1) genetic analysis of zoonotic reservoirs and symptomatic humans for pathogen discovery; (2) development of high throughput, multiplexed serologic and cellular assays; and (3) global blood sampling to identify antigen-specific and cross-reactive immune responses to known and previously unknown viruses of pandemic potential. Biological samples including serum and PBMC are obtained from broad-based population cohorts as well as targeted populations at risk from emerging infections. At the same time, sequence data from known and unknown (through virus discovery) pathogens are analyzed to design and express candidate antigenic proteins of interest. These proteins are then used in high throughput multiplexed assays to screen sera for antibody reactivity. Serum reactivity to candidate antigens is followed by further interrogation of the adaptive immune response at the cellular level using corresponding PBMC samples. Proteins used in the serological arrays are conjugated to fluorophores and used as probes to sort antigen-specific B cells by flow cytometry. In addition, the sequence of such candidate antigens provides the template for the design of overlapping peptide pools to assess T cell responses. Further analysis allows for the identification of

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neutralizing antibodies specific for the target antigens as well as specific T cell epitopes which may be tested in animal models. Epitope identification informs and guides immunogen design. At the conclusion of this pipeline of assays and analysis, PREMISE delivers the following:

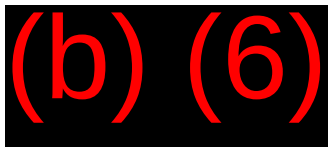
1. reagent and data resources for early detection and diagnosis
2. monoclonal antibodies with therapeutic potential
3. candidate immunogens for further vaccine development

Thus, PREMISE serves as a translational vehicle to integrate serologic and cellular immune discovery, targeting a broad array of pathogens, into product development, and constitutes an anticipatory reagent repository to accelerate the global response to pandemic threats.

As investigators in the Vaccine Research Center at the National Institute of Allergy and Infectious Diseases (NIAID), we are not eligible to be listed as a "co-investigator" on the application but look forward to participating as collaborators. All materials from us will be provided under an approved material transfer or other collaborative agreement. This collaboration is part of our official duties as employees at the NIAID, and no funds from the grant will be used in intramural research, neither will we accept any form of renumeration, whether in the form of salary, honoraria, or travel expenses. We will provide scientific input and mentoring but will not have any duties associated with programmatic stewardship, which will be performed by NIAID extramural program officials. Further, in keeping with the mission of NIAID to promote and facilitate biomedical research and the dissemination of new knowledge, we would supply requested research materials and technical expertise not only to you, but also to other interested and qualified parties for research purposes.

My group has met with Dr. Eric Laing (USU) one of the two mentors for this pilot research program to discuss ways we can collaborate and support your research application. We are enthusiastic about providing the technological and subject-expertise in immunology and translational therapeutic discovery afforded by the PREMISE program that would enhance your knowledge base and broaden your own scientific training.

Sincerely,



Daniel C. Douek

Daniel Douek MD PhD | Chief, Human Immunology Section | Vaccine Research Center
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30 January 2023

Dr. Krongkan Srimuang & Mr. Spencer Sterling

Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC),

King Chulalongkorn Memorial Hospital

Object: Thai Red Cross Emerging Infectious Diseases Clinical Center support for your application to the CREID Pilot Research Program, entitled "Immune memory bait & capture to identify emerging henipavirus origins."

Dear Dr. Srimuang & Mr. Sterling,

Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC) is pleased to support your application to the **CREID Pilot Research Program**, entitled "Immune memory bait & capture to identify emerging henipavirus origins." We are fully committed to executing the statement of work to study zoonotic reservoirs for pathogen origins by using serologic and cellular immune discovery in humans and wildlife and their application as a tool for early detection and identification of novel pathogens in pandemic prevention.

The work proposed in this project is a great opportunity for collaboration with our team, it serves to enhance the use of serology and immunology for the detection of novel virus pathogens in Thailand using new technology and techniques. Moreover, the building of capacity and capability in our laboratory and Faculty will enhance the regional capacity, and the Chulalongkorn network will be used to ensure the success and completion of this project.

This letter expresses my strong interest in you pursuing this application and I fully support the project. I look forward to collaborating with you on this project and I wish you success in the CREID Pilot Research Program application.

Sincerely,

(b) (6)

Assistant Professor Opass Putcharoen, MD, Msc

Head of Thai Red Cross Emerging Infectious Diseases Clinical Center

King Chulalongkorn Memorial Hospital

EID-SEARCH
Emerging Infectious Diseases
South East Asia Research Collaboration Hub

Dr. Krongkan Srimuang, Mr. Spencer Sterling
Thai Red Cross Emerging Infectious Diseases Clinical Center
King Chulalongkorn Memorial Hospital
1873, Rama 4 Road, Pathumwan
Bangkok, Thailand 10330

Dear Krongkan and Spencer,

This letter conveys a strong interest from the Emerging Infectious Disease-Southeast Asia Research Collaboration Hub (EID-SEARCH) at EcoHealth Alliance to collaborate with you at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital on the proposed project titled "*Immune memory bait & capture to identify emerging henipavirus origins"* in Thailand.

The question of the origin of emerging Henipaviruses (HPVs) was identified from the EID-SEARCH human surveillance when a Mojiang-related virus was identified in a local population highly exposed to bats in Thailand. Faced with the challenges of PCR detection and viral isolation, this project will establish and validate a new serological approach to develop MojV-like virus-specific MAbs and define the antigenic spatial relationships between all known rodent- and bat-borne HPVs, revealing the serologic signature of all related undiscovered HPVs. This will significantly contribute to the objectives of EID-SEARCH to conduct targeted and strategic surveillance to identify emerging pathogens with zoonotic potential at the high-risk human-animal interfaces. The research population and site for this project – bat guano collection – represents one of the key scenarios where zoonotic spillover is mostly concerned that requires further in-depth investigation to identify the reservoir hosts. In addition to the gained knowledge, established methods from this project will be shared and used as standards in zoonotic HPVs surveillance and prevention strategies in Thailand and regionally through collaborative and coordinative cooperation among multiple stakeholders.

I am very excited to work with you both on this project, given your critical roles in the previous surveillance work to identify the question and develop the research idea. This project will bring valuable insights to advance disease surveillance and early warning systems for endemic and emerging infectious diseases in Thailand. Members of EID-SEARCH are committed to working closely with you to develop the research project and support the efforts necessary for the success of this project. You will be invited to join all training conducted by EID-SEARCH and EcoHealth Alliance regarding emerging infectious disease surveillance, relevant statistical analysis, and grant writing to expand your skill set. You'll also be supported to present the findings from this research at international conferences, the CREID Network meetings, and external partners and produce high-quality publications from this research.

Developing and implementing this project will advance your skills in project management for future research as an independent researcher. Co-Investigators of EID-SEARCH, Dr. Eric Laing from the Uniformed Services University of the Health Sciences and Dr. Supaporn Wacharapluesadee from the Thai Red Cross Emerging Infectious Diseases Clinical Center, have enthusiastically joined the project as your Mentors. Dr. Laing will advise on the study design and provide training on data analysis, and Dr. Wacharapluesadee from your institute will work closely with you to advise on the day-to-day project implementation. Both Mentors will work with you for manuscript writing and provide pertinent and timely career advice to assist in your professional development. They will bring a well-established

EID-SEARCH
Emerging Infectious Diseases
South East Asia Research Collaboration Hub

network of leaders and experts in emerging infectious disease research in Thailand, Southeast Asia, and North America for your network building.

I am confident that this proposed project will bring genuine opportunities for your professional development. And the combined experience in the field and lab and interdisciplinary expertise in serology and public health from the co-PIs will make this a successful and purposeful project to bring public health impacts in a broad region. I look forward to collaborating with you and your team on all phases of this proposed project. I wish you success in the CREID Pilot Research Program application.

Sincerely,

(b) (6)

Peter [redacted]asza
Principal Investigator, EID-SEARCH
President, EcoHealth Alliance

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799
www.usuhs.edu

January 27, 2023

Krongkan Srimuang, Ph.D.
Research Scientist
Thai Red Cross Emerging Infectious Diseases Clinical Center
King Chulalongkorn Memorial Hospital
Rama 4 Road
Pathumwan, Bangkok 10330

Subject: Letter of Support for CREID Network Pilot Research Program

Dear Dr. Srimaung,

I am writing to express my enthusiastic support for your CREID Network Pilot Research Program proposal titled, “Immune memory bait & capture to identify emerging henipavirus origins.” In 2018, I led a training and multiplex serology technology transfer to your mentor Dr. Wacharapluesadee’s research group via DTRA-funded project Chulalongkorn Luminex Training and Research Preparedness (HDTRA1-17-C-0019), and was excited to leverage those activities into ongoing research within the EID-SEARCH. Since 2015 I have been developing serological-approaches for biosurveillance of zoonotic viruses in wildlife and human populations, and I am excited to serve as a co-mentor for this pilot program application.

I hope to provide you with the research and career developmental support for the activities outlined in the proposal. The serological data on henipavirus exposure in Thai guano farmers provides compelling evidence that Mojiang-like henipaviruses can cross the species barrier and may be widely distributed in Southeast Asia. The research aims detailed in the proposal will facilitate critical follow-up sampling of this human cohort to identify whether acute infection causes clinical disease, estimate the sero-attack rate of this novel henipavirus, and will apply a cutting-edge serologic and immunologic approach for monoclonal antibody development and novel emergent virus immunogenic characterization. If awarded, a collaboration with VRC PREMISE program scientists will facilitate an important opportunity to broaden your knowledge base of cellular immunology and B cell biology. You have an extensive background in next-generation sequencing application, and through this proposal would gain a considerable understanding of how next-generation sequencing is used for BCR-sequencing, IgG characterization and monoclonal antibody discovery.

Your proposal is an excellent extension of the EID-SEARCH activities and significant training opportunity.

Sincerely,

(b) (6)

Eric D. Laing
Assistant Professor
Department of Microbiology and Immunology
Uniformed Services University

30th January, 2022

Dr. Krongkan Srimuang & Mr. Spencer Sterling

Thai Red Cross Emerging Infectious

Diseases Clinical Centre (TRC-EIDCC),

King Chulalongkorn Memorial Hospital

Dear Dr. Srimuang & Mr. Sterling,

I am writing in support as Research Center Mentor for your application to the CREID Pilot Research Program, entitled "Immune memory bait & capture to identify emerging henipavirus origins."

With my 20 years of experience in conducting surveillance for novel viruses in wildlife, humans, and bats using molecular technology, I will support the growth of the scientists in the CREID Pilot Research Program. The research aims have the potential to significantly enhance our approach to pathogen surveillance at high-risk human-animal interfaces. Given the understanding of pathogen origins by using serologic and cellular immune discovery in humans, and applying these techniques as a tool for early detection and identification of novel pathogens.

As the head laboratory cheif at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, I believe that this is a great opportunity for our Thailand team to train in the characterization of memory B cells from peripheral blood mononuclear cells (PBMC) and enhanced serological surveillance in novel virus pathogens. Moreover, our laboratory has supported the Department of Disease Control (DDC) in diagnosing emerging infectious diseases and communicable diseases such as Ebola, MERS, and COVID-19. I am also a committee member of the Global Laboratory Leadership Program (GLLP) Thailand, organized by the Department of Medical Sciences. With my experience in diagnosing the first human MERS case in Thailand in 2015 and the first human COVID-19 case outside China in January 2020, I will support multidisciplinary projects involving epidemiology, laboratory, field science, and bioinformatics to diagnose and monitor the emergence of wildlife-origin viral zoonoses, including SARS-CoV, Nipah and Hendra viruses, Avian influenza, and novel viruses from bats. I was also the Thailand country coordinator for the PREDICT project, large contracts from USAID during 2010-2019 involving the successful management of teams of virologists, field biologists, veterinarians, epidemiologists, hospitals, and laboratory staff. I will enthusiastically support the development of a strong knowledge base to build capacity and capability in this project and our team in Thailand.

The CREID Network and the Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH) will support scientists in regions around the world where emerging and reemerging infectious disease outbreaks. Many Southeast Asian partnerships in EID-SEARCH have strong potential to enhance innovations in this area.

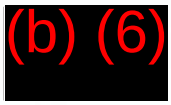
I believe that Dr. Laing, and I will provide scientific skills, expertise, and networks for this project.

Towards that aim, I am committed to supporting your professional development in the following ways:

1. I will maintain regular bi-weekly meetings for the project progresses.
2. I will support the resources in our country and research team in this project.
3. I will create opportunities for scientists network to ensure the success of research project.
4. I will provide and share advice and technical expertise in specific knowledge for this project.

I'm very much looking forward to working closely with you on this 1-year research project and beyond as you continue to develop your surveillance zoonotic disease research program.

Best regards,



Supaporn Wacharapluesadee, Ph.D.

Head Laboratory,

Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC),

King Chulalongkorn Memorial Hospital,

Faculty of Medicine,

Chulalongkorn University.

Pilot Program Application Submission Checklist – Final Application Submission
Title of Application: Immune memory bait & capture to identify emerging henipavirus origins
Submitted by: Krongkan Srimuang and Spencer Sterling
Submission Date: 1/30/2023

Full Application Submission Requirements

- ☒ Proposal Cover Sheet
- ☒ Title and Table of Contents
- ☒ Study Personnel *(1-page limit)*
- Main Application Body Section Requirements *(7-page limit):*
  - ☒ Research Aims & Objectives
  - ☒ Study Rationale/Research Gap/Impact
  - ☒ Significance and Approach
  - ☒ Research Methods
- ☒ Project Timeline (*1-page limit*)
- ☒ Research Performance Sites *(1-page limit)*
- ☒ CREID Research Center Collaboration *(1-page limit)*
- ☒ Mentoring Plan *(2-page limit)*
- Vertebrate Animals Section Requirements *(3-page limit):*
  - ☒ Description of Procedures
  - ☒ Justifications
  - ☒ Minimization of Pain and Distress
  - ☒ Method of Euthanasia (Cover Page Supplement / PHS Fellowship Supplemental Form)
- Human Subjects Research *(3-page limit):*
  - ☒ Summary of the parent study and IRB approval information for the study
  - ☒ Risks to the subjects
  - ☒ Adequacy of protection against these risks
  - ☒ Potential benefits of the research to the subjects and others
  - ☒ Importance of the knowledge gained or to be gained
  - ☒ Country / institution-specific ethics / IRB regulations addressed
- Research, Related Project Information, and Budget/Budget Justification
  - ☒ R&R Other Project Information Form
  - ☒ Full budget, with total costs of no more than $150,000
  - ☒ Budget justification which describes the labor and other direct costs
  - ☐ If your institution does not have adequate funds for a cost-reimbursement award and requires pre-payment of funds during the award year, please note this in your budget justification and outline a payment schedule that will function for your project.

  Supporting Documentation
  - Biographical Sketch and Other Support. All applications must include:
    - ☒ Applicant PI Biographical Sketch (*4-page limit*)
    - ☒ Applicant PI Previous/Current/Pending Support (Include funding amounts, *no page limit*)
    - ☒ Mentor Biographical Sketch (*4-page limit*)
    - ☒ Mentor Current/Pending Support (*no page limit*)
    - ☒ Key Personnel Biographical Sketches (*4-page limit each*)
    - ☒ Key Personnel Current/Pending Support (*no page limit*)
  - Co-PI Plan (only needed if applying as Co-PIs) (*1-page limit*)

Pilot Program Application Submission Checklist – Final Application Submission
Title of Application: Immune memory bait & capture to identify emerging henipavirus origins
Submitted by: Krongkan Srimuang and Spencer Sterling
Submission Date: 1/30/2023

- ☒ What each Co-PI will contribute to the proposed research study
- ☒ How the Co-PIs will jointly work with the affiliated Research Center
- ☒ How the Co-PIs will jointly manage the proposed study

☒ References Cited (*no page limit*)
☒ List of Abbreviations, Acronyms, and Symbols
☒ Facilities, Existing Equipment, and Other Resources (*template provided*)
☒ NIH Foreign Clearance form (*template provided*)
☒ Letters of Organizational Support *(2-page limit per letter)*
☒ Letter of Collaboration from CREID Research Center PI *(2-page limit per letter)*
☒ Letter from Research Center Mentor (*2-page limit)*
☒ Letter from Primary Scientific Mentor (if different than Research Center mentor) (*2-page limit)*

RESEARCH & RELATED BUDGET - Budget Period 1

OMB Number: 4040-0001
Expiration Date: 12/31/2022

ORGANIZATIONAL DUNS: 6598088360000 **Enter name of Organization:** Chula Unisearch, Chulalongkorn University

Budget Type: ☒ Project ☐ Subaward/Consortium **Budget Period: 1** **Start Date:** 05/01/2023 **End Date:** 04/30/2024

A. Senior/Key Person

Prefix	First	Middle	Last	Suffix	Base Salary ($)	Months Cal.	Months Acad.	Months Sum.	Requested Salary ($)	Fringe Benefits ($)	Funds Requested ($)
Dr.	Krongkan		Srimuang			10.00			24,000.00	0.00	24,000.00
Project Role:	PD/PI										
Mr.	Spencer		Sterling			6.00			0.00	0.00	0.00
Project Role:	Co-PI										

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 24,000.00

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	Laboratory Technician	12.00			15,000.00	0.00	15,000.00
1	Field Coordinator	4.00			5,000.00	0.00	5,000.00

2 **Total Number Other Personnel**

Total Other Personnel 20,000.00

Total Salary, Wages and Fringe Benefits (A+B) 44,000.00

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)	11,005.00
2. Foreign Travel Costs	5,023.00
Total Travel Cost	16,028.00

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	72,803.00
2.	Publication Costs	3,000.00
3.	Consultant Services	3,000.00
4.	ADP/Computer Services	
5.	Subawards/Consortium/Contractual Costs	
6.	Equipment or Facility Rental/User Fees	
7.	Alterations and Renovations	
8.		
9.		
10.		
	Total Other Direct Costs	78,803.00

G. Direct Costs

	Funds Requested ($)
Total Direct Costs (A thru F)	138,831.00

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
Indirect cost at 8%	8.00		11,106.00

Total Indirect Costs	11,106.00

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)	149,937.00

J. Fee

Funds Requested ($)

K. Total Costs and Fee

	Funds Requested ($)
Total Costs and Fee (I + J)	149,937.00

L. Budget Justification

(Only attach one file.) Budget Justification Srimuang and Ste1 | Add Attachment | Delete Attachment | View Attachment

RESEARCH & RELATED BUDGET - Cumulative Budget

		Totals ($)
Section A, Senior/Key Person		24,000.00
Section B, Other Personnel		20,000.00
Total Number Other Personnel	2	
Total Salary, Wages and Fringe Benefits (A+B)		44,000.00
Section C, Equipment		
Section D, Travel		16,028.00
1. Domestic	11,005.00	
2. Foreign	5,023.00	
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		78,803.00
1. Materials and Supplies	72,803.00	
2. Publication Costs	3,000.00	
3. Consultant Services	3,000.00	
4. ADP/Computer Services		
5. Subawards/Consortium/Contractual Costs		
6. Equipment or Facility Rental/User Fees		
7. Alterations and Renovations		
8. Other 1		
9. Other 2		
10. Other 3		
Section G, Direct Costs (A thru F)		138,831.00
Section H, Indirect Costs		11,106.00
Section I, Total Direct and Indirect Costs (G + H)		149,937.00
Section J, Fee		
Section K, Total Costs and Fee (I + J)		149,937.00

Name of Individual: Laing, Eric
Commons ID: (b) (6)

Other Support – Project/Proposal

Title: Efficacy testing of a novel human monoclonal antibody therapy for late-stage rabies/lyssavirus infection

Major Goals: The major goals of this project is to test the efficacy of mAb therapy at times beyond day seven post-infection, to more completely define the degree to which mAb therapy can confer protection from death post-onset of clinically evident severe neurological disease.

Status of Support: Active

Project Number: HU00011920118

Name of PD/PI: Schaefer, B.

Source of Support: USUHS/CGHE

Primary Place of Performance: USUHS, Bethesda, MD

Project/Proposal Start and End Date: (MM/YYYY): 09/30/2019 – 09/29/2023

Total Award Amount (including Indirect Costs): $518,000

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months(##.##)
3. 2022	0.6 calendar
4. 2023	0.6 calendar

Title: Epidemiology, Immunology, and Clinical Characteristics of Emerging Infectious Diseases with Pandemic Potential (EpICC-EID)

Major Goals: The major goals of this project are to utilize SARS-CoV-2 serology to explore hospital and community infection, correlates of COVID-19, and vaccinology.

Status of Support: Active

Project Number: HU00012020067, HU00011920111

Name of PD/PI: Burgess, T.

Source of Support: Defense Health Program

Primary Place of Performance: USUHS, Bethesda, MD

Project/Proposal Start and End Date: (MM/YYYY): 04/01/2020 – 09/30/2023

Total Award Amount (including Indirect Costs): $1,178,876

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months(##.##)
2,3. 2022	1.0 calendar
3,4. 2023	1.0 calendar

Title: Prospective Assessment of SARS-CoV-Seroconversion

Major Goals: The major goals of this project are to monitor SARS-CoV-2 infection, the roleof human coronavirus antibodies in COVID-19 outcomes, and COVID-19 vaccinology

Status of Support: Active

Project Number: HU00012020067, HU00012120104, HU00012120094, HU00011920111

Name of PD/PI: Mitre, E

Source of Support: Defense Health Program/CARES Act, NIAID

Primary Place of Performance: USUHS

Project/Proposal Start and End Date: (MM/YYYY): 04/01/2020-09/30/2024

Total Award Amount (including Indirect Costs): $235,388

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months(##.##)
2, 3. 2022	1.0 calendar
3, 4. 2023	1.0 calendar
4. 2024	1.0 calendar

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

Major Goals: The major goals of this Centers for Research in Emerging Infectious Diseases(CREID) project are to develop multidisciplinary teams of investigators in the program will conduct pathogen/host surveillance, study pathogen transmission, pathogenesis and immunologic responses in the host, and will develop reagents and diagnostic assays for improved detection for important emerging pathogens and their vectors.

Status of Support: Active Project Number: U01AI151797

Name of PD/PI: Daszak, P.

Source of Support: EcoHealth Alliance (NIH/NIAID/CREID 07-049-7012-52338 flow through)

Primary Place of Performance: USUHS, Bethesda, MD

Project/Proposal Start and End Date: (MM/YYYY): 06/17/2020-05/31/2025

Total Award Amount (including Indirect Costs): $539,119

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months(##.##)
2, 3. 2022	1.0 calendar
3, 4. 2023	1.0 calendar
4, 5. 2024	1.0 calendar
5. 2025	1.0 calendar

Title: Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa

Major Goals: This project will focus on building capacity and enhancing surveillance, including early detection programs for henipa-, filo- and zoonotic coronaviruses in Southern Africa.

Status of Support: Active

Project Number: HDTRA12010025

Name of PD/PI: Markotter, W.

Source of Support: University of Pretoria (flow-through DTRA BTRP)

Primary Place of Performance: USUHS, Bethesda, MD

Project/Proposal Start and End Date: (MM/YYYY): 07/31/2020-07/30/2025

Total Award Amount (including Indirect Costs): $1,120,965

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months(##.##)
2, 3. 2022	1.0 calendar
3,4. 2023	1.0 calendar
4, 5. 2024	1.0 calendar
5. 2025	1.0 calendar

Title: Biosurveillance for Spillover of *Henipaviruses* and *Filoviruses* in Rural Communities in India

Major Goals: The major goals of this project are to conduct biosurveillance for evidence of henipavirus and filovirus infection in bats and cryptic infections in at-risk human communities

Status of Support: Active

Project Number: HDTRA12010026

Name of PD/PI: Epstein, J.

Source of Support: EcoHealth Alliance (DTRA – BTRP flow through)

Primary Place of Performance: USUHS, Bethesda, MD

Project/Proposal Start and End Date: (MM/YYYY) (if available): 10/01/2020 – 09/30/2023

Total Award Amount (including Indirect Costs): $1,195,275

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months(##.##)
2. 2022	0.5 calendar
2. 2023	0.5 calendar

Title: Informing Biosurveillance: Contribution of pteropodid fruit bats to virus spillover in the Philippines.

Major Goals: The major goals of this project are to investigate Nipah virus and Reston virus circulation in bat populations in the Philippines, build research capacity for serological biosurveillance, and identify at-risk interfaces of virus zoonosis.

Status of Support: Funded

Project Number: HDTRA12110037

Name of PD/PI: Smith, G.

Source of Support: DTRA

Primary Place of Performance: USUHS, Bethesda, MD

Project/Proposal Start and End Date: (MM/YYYY): 07/2022 – 06/2025

Total Award Amount (including Indirect Costs): $1,213,746

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months(##.##)
1. 2022	1.0 calendar
2. 2023	2.0 calendar
3. 2024	2.0 calendar
4. 2025	2.0 calendar
5. 2026	1.0 calendar

Title: Solving Opportunities for Spillover (SOS): Frequency and Mechanisms of Cross-species Transmission of Henipaviruses in Bangladesh

Major Goals: We aim to better understand and prevent spillovers of bat-borne viruses into intermediate hosts and humans. The knowledge gained from this study will be immediately applicable to human and animal health programs because if we know which henipaviruses infect humans and domesticated animals, and how they are infected, we can advise public health surveillance programs on how to optimize detection and prevention of infections.

Status of Support: Pending

Project Number: 1 R01 AI168287-01A1

Name of PD/PI: Gurley, E.

Source of Support: NIH/NIAID

Primary Place of Performance: Johns Hopkins University

Project/Proposal Start and End Date: 09/01/2022- 08/31/2027

Total Award Amount (including Indirect Costs): $3,356,172

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2023	1.0 calendar
2. 2024	1.0 calendar
3. 2025	1.0 calendar
4. 2026	1.0 calendar
5. 2027	1.0 calendar

Title: Establishment of a Bat Resource for Infectious Disease Research

Major Goals: The major goals of this proposal is to contribute significant insight into the viral determinants that confer henipaviral pathogenesis. The construction and generation of recombinant henipavirus chimeric viruses will be the primary means for determining how virus receptor usage and the expression of virulence factors act individually or in conjunction to cause limited or severe disease. The recombinant constructs generated from the proposed studies will also be used to determine host determinants of immunity in Jamaican fruit bats and animal models.

Status of Support: Pending

Project Number: R24AI165424

Name of PD/PI: Schountz, T.

Source of Support: Colorado State University (NIH / NIAID flow through)

Primary Place of Performance: USUHS, Bethesda, MD

Project/Proposal Start and End Date: (MM/YYYY): 01/01/2023-12/30/2027

Total Award Amount (including Indirect Costs): $695,937

Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2023	1.0 calendar
2. 2024	1.0 calendar
3. 2025	1.0 calendar
4. 2026	1.0 calendar
5. 2027	1.0 calendar

IN-KIND

NONE

OVERLAP

In my role on this project, titled: Immune memory bait & capture to identify emerging henipavirus origins, I will be providing mentorship and my group will be providing recombinant antigenic material for multiplex serologies and our expertise in virology and assay development and interpretation to the PI and Co-PI. These research activities and mentorship overlap with my present role as a co-investigator within the EID-SEARCH center.

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

*Signature:

(b) (6)

Date: 01/30/2023

From: Laing, Eric on behalf of Laing, Eric <eric.laing@usuhs.edu>
To: Hongying Li
Cc: Sterling, Spencer; Krongkan Srimuang; Supaporn Wacharapluesadee; Sasiprapa Ninwattana
Subject: Re: Don"t forget to fill and sign your Other Support form
Date: Monday, January 30, 2023 9:45:37 AM
Attachments: Laing_Other support_30JAN2023-signed.docx

Nope, not your misunderstanding. I forgot to update this title for this pilot project.

Attached doc with updated language

- 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 Mon, Jan 30, 2023 at 9:35 AM Hongying Li <li@ecohealthalliance.org> wrote:

> Hi Eric,
>
> In the overlap section *"In my role on this project, titled: Establishment of a Bat Resource for Infectious Disease Research, my group will be providing recombinant antigenic material for multiplex serologies and our expertise in virology and assay development and interpretation..."* is the project title correct, or it's my misunderstanding?
>
> **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
>
> ***EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.***
>
> On Mon, Jan 30, 2023 at 8:56 PM Laing, Eric <eric.laing@usuhs.edu> wrote:
>
> > signature in word doc

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 Mon, Jan 30, 2023 at 7:18 AM Laing, Eric <eric.laing@usuhs.edu> wrote:

Eric D. Laing, Ph.D.
Assistant Professor
Department of Microbiology and Immunology
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On Mon, Jan 30, 2023 at 7:06 AM Sterling, Spencer <spencer.sterling.ctr@usuhs.edu> wrote:

> Please see mine attached.
>
> On Mon, Jan 30, 2023 at 12:53 PM Hongying Li <li@ecohealthalliance.org> wrote:
>
> > Kio - your draft is attached, since you are paid by EID-SEARCH already for 2 months, you can only ask for 10-month salaries on the pilot, we can adjust everything after we get the award. Please double-check and sign it for submission.
> >
> > For All, EID-SEARCH information is in the template for Kio.
> >
> > Dr. Supaporn - I have your Other Support, but it's in the NSF format, maybe someone can convert it into NIH format with the template?
> >
> > **Hongying Li, MPH**
> > *Senior Program Manager & Senior Research Scientist*
> >
> > EcoHealth Alliance
> > 520 Eighth Avenue, Ste. 1200
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> > 1.917.573.2178 (mobile)
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EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.

--
Spencer Sterling, MPH (he/him)
Scientific Project Coordinator
Broder/Laing Lab
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Proposal Cover Sheet

Project Title	Immune memory bait & capture to identify emerging henipavirus origins
Principal Investigator Name	Krongkan Srimuang, Ph.D.
Position/Title	Research Scientist
Department	Thai Red Cross Emerging Infectious Diseases Clinical Center
Institution Name	King Chulalongkorn Memorial Hospital
Street	Rama 4 Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	Krongkan.sr@gmail.com
Phone	+66 (0) 92-453-0808
Country(ies) where work will be conducted	Thailand
Pathogen(s) focus	Mojiang Virus, Henipavirus, Paramyxovirus
Co-Principal Investigator Name (*If applicable*)	Spencer Lee Sterling, MPH
Position/Title	Scientific Project Coordinator
Department	Microbiology and Immunology
Institution Name	Uniformed Services University of the Health Sciences
Street	Jones Bridge Road
City, State, Zip Code	Bethesda, MD 20814
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Email	spencer.sterling.ctr@usuhs.edu
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Contact information for institution(s) that would financially manage the award *(please note: changing the managing institution upon award may result in substantial funding delays)*	
Contractual Contact, Title	Chula Unisearch
Institution Name	Chulalongkorn University
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Institution Name	
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Name of Mentor	Eric Laing, Ph.D.
Mentor Institution	Uniformed Services University of the Health Sciences
Institution Address	Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814
Permanent location of Mentor	4301 Jones Bridge Road, Bethesda, MD 20814
Mentor email	eric.laing@usuhs.edu
Mentor phone	+1 (301) 980-8192
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Name of Mentor	Supaporn Wacharapluesadee, Ph.D.
Mentor Institution	Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital
Institution Address	1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Permanent location of Mentor	4th Floor, Jongkolnee Watwong Building, Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital, 1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Mentor email	spwa@hotmail.com
Mentor phone	+66 (0) 89-920-6292
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Total Budget	***Repeat this table if there are two Co-PIs***
Direct Costs	$138,831
Indirect Costs	$11,106
Proposed Start Date	1 May 2023
Proposed End Date	30 April 2024

Project Abstract (250 words)

Prototypical henipaviruses (Hendra and Nipah virus) are bat-borne zoonotic viruses and cause high mortality. The recent detection of closely related Mojiang and Langya viruses in rats and shrews, and isolation of Langya virus from individuals with acute febrile illness, challenges the dogma that fruit bats are the sole henipavirus reservoirs. The recently expanded *Henipavirus* genus includes two other shrew-borne viruses (Gamak and Daeryong viruses), and the Madagascan fruit bat-borne Angovkeely virus. As part of EID-SEARCH activities, we found serological evidence of infection by a Mojiang-related virus in a community of Thai bat guano collectors who have occupational exposure to microbats and rodents. Identifying the wildlife reservoir of this likely novel virus will assist intervention strategies to reduce spillover risk. However, PCR detection or virus isolation in wildlife is challenging, and new serological approaches may be more successful in identifying reservoir hosts. This project aims to identify novel viruses and zoonotic reservoirs through serologic and cellular immune discovery. We will conduct follow-up human and wildlife shrews and rodents) surveillance in the province where the seropositive humans were identified and apply an expanded serological test and genomic sequencing to detect this novel MojV-like henipavirus. Further, we

plan to isolate anti-MojV monoclonal antibodies (mAb) from peripheral blood mononuclear cells collected from human study participants we have identified as immunoreactive with MojV antigens, and characterize the cross reactive and cross neutralizing potential of these mAbs to LayV.

Study Personnel

Krongkan Srimuang, Ph.D., is a researcher at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital and supports the Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia (EID-SEARCH) research. Dr. Srimuang is also a medical technologist working to identify, detect and characterize the spill-over risk of high zoonotic potential viruses from wildlife and humans using serology techniques, Next-generation sequencing, and PCR assays to identify novel viruses. Dr. Srimuang also performs the project Smart Sentinel Surveillance System: Emerging Viral Pneumonia and Respiratory tract infections (RTIs) to identify, detect and characterize the novel/known Respiratory Viruses in our country, Thailand by using multiplex real-time PCR, family PCR and Next-generation sequencing of novel viral pathogens. Dr. Srimuang graduated with Ph.D. in Tropical Medicine from the department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University.

Spencer Sterling, MPH, is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region. Mr. Sterling will planning, sample processing, data analysis, and report preparation for this project.

Dr. Eric Laing will act as mentor for Dr. Srimuang and Mr. Sterling under the Centers for Research in Emerging Infectious Diseases (CREID) Pilot Research Program. Dr. Laing is an assistant professor dedicated to biosurveillance and virological research of emergent zoonotic viruses at Uniformed Services University, Bethesda, MD. Part of Dr. Laing's research focus in on the development of serological-based approaches to identify the infectome of wildlife and human communities for the assessment of virus spillovers. Dr. Laing has worked collaboratively with national and international researchers including institutional partners at Duke-NUS, Chulalongkorn University, University of Pretoria, and Rocky Mountain Labs towards these goals. Through these collaborations, Dr. Laing's research group aims to identify known and unknown viruses, the wildlife reservoirs of priority pathogens, the high-risk interfaces for spillover and the biotic/abiotic drivers of virus emergence.

Dr. Supaporn Wacharapluesadee, Ph.D. is the head laboratory at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, a WHO Collaborating Centre for Research and Training on Viral Zoonoses. Dr. Wacharapluesadee research interests include emerging infectious diseases in bats (including Nipah, Rabies, Coronavirus, and novel pathogens) as well as molecular diagnoses and sequencing of viruses. During the past 10 years, Dr. Wacharapluesadee got funding from USAID PREDICT to conduct the surveillance for novel virus in wildlife and human, and from Biological Threat Reduction Program US DTRA to conduct the EID surveillance in bat using the molecular technology and multiplex serology panel (Luminex technology). TRC-EIDCC is responsible for molecular diagnoses services to the hospital, and is the reference laboratory for rabies virus, MERS-CoV, Ebola, Zika, and other infectious diseases diagnoses for Ministry of Public Health, Thailand and laboratory training center on national and regional level. Dr. Wacharapluesadee has served and consulted on several WHO and Thai government committees.

Specific Aims

The recent discovery of novel shrew and rodent-borne henipaviruses in East and Southeast Asia has highlighted gaps in our understanding of henipavirus transmission and underscores the importance of surveillance activities in the region. In particular the isolation of Langya virus from acutely ill humans and shrews demonstrated the need to further surveillance and therapeutic development against emergent henipaviruses. Therefore, **this project aims to expand the study of known reservoirs of zoonotic pathogen origin by using genetic and serologic techniques, and to use cellular immune discovery in seropositive humans as a tool to determine henipaviral antigenic relatedness to assist with the functional assessment of current therapeutics as and their effect on these novel viruses.**

In our study, we detected that a high seroprevalence against MojV in human participants near the Khao Chong Phran Non-hunting Area in Ratchaburi province who we identified as representing a high risk population for zoonotic spillover. Yet, no virus sequences were detected. We hypothesize that (i) there is a novel henipavirus present in the local wildlife population that has spilled over into the human population at least once, and (ii) the antibodies from these seropositive people will react with the novel shrew-borne henipaviruses in a way that can inform us on therapeutic approaches for future outbreaks. We will follow-up with persons who had high anti-MojV IgG levels, sample new participants within this community who interact with the local wildlife, and sample shrews and rodents to identify the wildlife source of the MojV-like henipavirus. Additionally, in collaboration with NIH VRC PREMISE, MojV receptor binding protein (RBP) and fusion protein-reactive B cell will be isolated from previously-identified seropositive human participants. MojV-like virus-specific B-cell receptors will be sequences and monoclonal antibodies specific to the infecting MojV-like virus RBP and F protein will be generated and tested for cross reactivity against presently described rodent associated henipavirus envelope glycoproteins, and cross neutralization against Langya virus.

The proposed project has two Aims:

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population.

Aim 2. Characterization of monoclonal antibodies from humans with serological profiles consistent with MojV infection.

The proposed project has two Objectives:

Objective 1. To use metagenomic and targeted sequencing approaches in human and wildlife populations to detect the Thai MojV-like virus.

Objective 2. To isolate and sequence memory B-cells from humans positive for Mojiang virus in Ratchaburi province, Thailand for antibody discovery.

Study Rationale

Nipah virus (NiV) and henipaviral diseases are among the most virulent of zoonotic diseases. The genus *Henipavirus* is currently composed of nine viruses including the prototypes, NiV and Hendra virus (HeV), and Cedar virus (CedV), Ghana virus (GhV), Mojiang virus (MojV) (McLinton et al., 2017), Langya virus (LayV) (Zhang X et al., 2022), Gamak virus (GAKV), Daeryong virus (DARV) (Lee et al., 2021), and Angavokely virus (AngV) (Madera et al., 2022) (Table 1). HeV was first identified in Australia in 1994 as a cause of respiratory illness and fatal encephalitis in humans and horses. Closely following the discovery of HeV, NiV was identified in 1999 in Malaysia after outbreaks of fatal encephalitis in humans, with swine determined to be the intermediate host. Annual outbreaks of NiV have occurred in Bangladesh and are associated with the consumption of raw date palm sap, and since 19 May 2018 annual outbreaks of NiV associated encephalitis of been recorded in Kerala, India (Uwishema et al., 2022). Both HeV and NiV have a broad host range capable of infecting several mammalian orders, and can enter and infect endothelial cells, vasculature, and the central nervous system leading to severe multisystem organ diseases and high mortality. The broad host and tissue tropism of these viruses has been attributed to the use of the ephrinB2 and ephrinB3 ligands, highly conserved proteins with critical functions in evolutionary development, as virus receptors (Bossart et al., 2008). There are currently no vaccines or therapeutics and NiV and henipaviral diseases remain WHO Blueprint list priority pathogens (Middleton and Weingartl, 2012).

A close relative of HeV, CedV was isolated from Australian bats in 2012 and found to be non-pathogenic, failing to cause any clinical symptoms in ferrets and guinea pig animal models (Marsh GA et al., 2012). The natural reservoirs of HeV, NiV, and CedV are flying foxes (*Pteropus sp.*) (Middleton and Weingartl, 2012). GhV was detected in Ghana in straw-colored fruit bats (*Eidolon helvum*) during routine surveillance in 2015 and was the first henipavirus found outside of the Indo-Pacific region. GhV showed a close phylogenetic relationship with HeV and NiV, and like HeV, NiV, and CedV the GhV RBP can mediate binding to ephrinB2 ligand and mediate cellular fusion (Drexler, J. F., 2009). Recent investigations into Madagascan fruit bats (*Eidolon duprenum*) detected a second African henipavirus, AngV. As neither GhV nor AngV have been isolated or rescued, the pathogenic and zoonotic potential of these African fruit bat associated henipaviruses remains unknown.

Multiple genera of small mammals were tested for viral RNA, and MojV was detected in yellow-breasted rats (*R. flavipectus*) (Wu Z et al., 2014). The discovery of the MojV genome occurred during follow-up investigations into lethal pneumonia from three miners in 2012 in China, however the pathogenic nature of this virus has only been suggested and no isolates have been discovered or tested in an animal model. Additionally, the novel shrew-borne henipaviruses, GAKV and DARV were found in the Republic of Korea in 2017-2018. (Lee et al., 2021). Most recently, a close relative of MojV, Langya henipavirus (LayV) was recently isolated from febrile patients in eastern China. Subsequently, LayV was identified in shrews (Zhang et al., 2022), increasing the number of Asiatic rodent-associated henipaviruses to four. These recent discoveries are challenging the paradigms surrounding henipaviruses and their associations with bats and may suggest a novel rodent sub-genus of henipaviruses.

The henipaviral genome consists of six structural proteins including nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), receptor-binding protein (RBP), and large (L) protein (approximately length of 18,000 nucleotides) (Luby, S. P., & Gurley, E. S., 2012). In HeV and NiV, slippage sites within the P protein lead to the expression of the V, W, and C proteins. These proteins are virulence factors and are absent in CedV.

Table 1 Summary viruses in Henipavirus

Henipavirus	**Origin (Year)**	**Reservoir**	**Genome (nucleotides)**	**Symptom for human**	**Reference**
Hendra virus (HeV)	Australia (1994)	Fruit bats	18,234	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Nipah virus (NiV)	Malaysia (1999)	Fruit bat	18,246	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Ghana virus (GhV)	Africa (2009)	Fruit bat (*Eidolon helvum)*	18,530	No data	Drexler, J. F., 2009
Cedar virus (CedV)	Australia (2012)	Fruit bat	18,162	Non-pathogenic	Marsh, G. A et al., 2012
Mojiang virus (MojV)	China (2012)	Rodent	18,404	Febrile	Wu Z et al., 2014
Gamak virus (GAKV)	Korea (2017-2018)	Shrew	18,460	No data	Lee et al., 2021
Daeryong virus (DARV)	Korea (2017-2018)	Shrew	19,471	No data	Lee et al., 2021
Langya virus (LayV)	China (2019)	Shrew	18,402	Acute febrile	Zhang et al., 2022
Angavokely virus	Madagascar (2019)	Fruit bat	16,740	No data	Madera et al., 2022

Viruses in the genus *Henipavirus* have a broad reservoir and may cause high case fatality rates following human infection. The discovery and characterization of the novel Henipavirus is a high public health priority.

Khao Chong Phran, a cave in Ratchaburi Province, has possibly the largest colony of bats in Thailand. There are estimated to be around 2 million bats in the cave and 95% are insect-eating bats (Department of National Parks, Wildlife and Plant Conservation, Thailand). Moreover, Khao Chong Phran has many community sites, such as cave entrances near communities, guano collection, and tourism sites, which risk zoonotic spillover. Bats are widely considered the animal hosts of zoonotic pathogens such as rhabdoviruses, coronaviruses, paramyxoviruses, and filoviruses, and many of the host species can be found in Thailand.

MojV was associated with a severe pneumonia-like illness killing three of six miners in 2012 in a mineshaft Yunnan, China. Interestingly, this mineshaft was also site where RaTG13, a beta-coronavirus most closely related with SARS-CoV-2, was detected in *Rhinolophid* bats (Rahalkar and Bahulikar, 2020). Identifying the wildlife reservoir of this novel virus will assist intervention strategies to reduce spillover risk.

Research Gaps

As a part of Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH), serological investigations using a multiplex microsphere-based immunoassay (MMIA) have detected 19% (54/284) seroprevalence for MojV in a high-risk cohort, including one person with a high magnitude and durability of anti-MojV IgG across longitudinal samples. Despite this, genomic surveillance has not detected MojV or any novel MojV-like viruses. For this result, we would to further

identify and characterize the origin of the Mojiang virus-reactive serum and to explore the novel MojV-like virus in Thailand.

Serological and cellular immunity is an important resource for virus discovery detection by using the specificities and characteristics of memory B cells from peripheral blood mononuclear cells (PBMC). The immune response will recognize and induce antibodies specific to the infecting pathogen. Characterizing the memory B cell response to an unknown pathogen in humans could in the detection of the exposure pathogen in humans. Molecular techniques such as PCR can detect only the genomic material in the body, but memory B cells are long-lived and thus have a much larger window of detection.

Memory B-cell differentiation is characterized by somatic hypermutation in antibody variable region genes (V) and selection of B cells expressing high-affinity variants of this antigen receptor and high sequence similarity in individuals exposed to the same antigen reflect antigen-driven clonal selection and form shared immunological memory between individuals (Yang et al., 2021). Memory B cells are highly abundant in the human spleen, and can proliferate 45% of the total B cell population in this organ (Hauser et al., 2015). Memory B cells can be detected in the Ig variable (IgV) genes in a fraction of IgM+ B cells. In humans, B cells expressing the memory marker CD27+ can be found in the marginal zone of spleen and circulate in the blood. After re-exposure to antigen, memory B cells can quickly proliferate and differentiate into plasma cells. Many studies the antibody diversity and specificity of the naturally paired immunoglobulin heavy and light chains for affinity maturation of B cell receptor (BCR) after virus infection such as SARS-CoV-2 infection (Yang et al., 2021), influenza (Dougan, S et al., 2013), HIV (Moir, S., & Fauci, A. S.,2017), etc. The characterization of memory B cells can be used to develop monoclonal antibodies and produce an effective therapeutic.

Significance and Approach

East and Southeast Asia is one of the world's highest-risk emerging infectious diseases hotspots. From this region emerged both SARS-CoV-1 and SARS-CoV-2, recurrent outbreaks of novel influenza strains, and the spillover of dangerous viral pathogens such as Nipah virus. The diversity of coronaviruses, henipaviruses, and filoviruses are found in wildlife reservoirs in Southeast Asia and likely due to the high diversity of wildlife, and the demography of the human population brings humans and wildlife into close contact. The spillover of viruses from animals to humans is often misdiagnosed and the clinical symptoms from patients is underestimated during the pandemic. This study provides a surveillance strategy in human and wildlife populations to better understand immunity to novel pathogens and identify the origin of these pathogens to serve as an early warning system to prevent outbreaks of emerging viruses.

This study uses immunology (serologic and cellular) to identify a novel virus that can aide in effective vaccine discovery and development, monoclonal antibody production for prevention and therapy, and generate data resources for early detection and diagnostic assays. Whereas PCR detection or virus isolation in wildlife is challenging, this new serological approach may be more successful in identifying reservoir hosts.

The Indo-Pacific region has a high risk of novel viral emergence. This study provides an opportunity for in-country research capacity building by linking local infectious disease scientists with an international collaborative network that has proven capacity to conduct this work and produce significant findings. Moreover, a global sero-surveillance network for surveillance technology extends virologic and immunologic screening to product discovery. This study will benefit and expand the CREID, EID-SEARCH networks, and the global sero-surveillance network to improve our understanding of immunology to identify novel pathogens and their origin to prevent future pandemics.

Research Methods

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population

Serological profiling provides an indirect measurement of the infecting virus. Despite 19% (54/284) seroprevalence in humans, we have not detected nucleic acid sequences of MojV or MojV-like henipaviruses in this human cohort. Additionally, we intend on expanding our understanding of henipavirus prevalence in this population with longitudinal sampling of the wildlife and human populations in this region.

Animal samples collection

200 shrews or rodents in and around the Khao Chong Phran cave in Ratchaburi Province (Figure 1) will be collected for surveillance targeting the Mojiang-like virus. We plan two sampling trips to collect target shrews and rodents. Due to limited knowledge of the local shrew population, we are unsure as to whether we can collect up to 200 samples and will supplement the collection with rodents, as needed. Samples will be collected based on the following enrollment criteria: random age, random weight, random sex. Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and stored. Oral swab and rectal swab will also collect in 200 shrews or rodents. Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and stored.

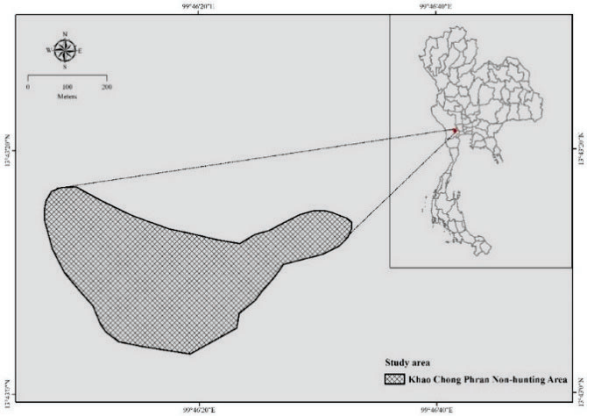

Figure 1: Map of Khao Chong Phran Non-hunting Area, Ratchaburi Province, Thailand (Boonpha N et al., 2019)

Human surveillance

Four of the samples from prior investigations have shown high MojV RBP-reactive antibody titers and will be selected for follow-up sampling. Additionally, 100 human serum samples, nasopharyngeal swab and throat swab will be collected from persons deemed to be in close contact with wildlife, and people who present with or have recent history of undiagnosed acute febrile illness using the following enrollment criteria;

- Adults (≥ 18 years of age) who sign in the informed consent
- Who present with or have recent history of illness without diagnosis, such as severe/acute respiratory illness (SARI/ARI), Influenza-like illness (ILI), fever of unknown origin (FUO), or respiratory tract infection
- Who is the bat guano collector in the cave
- Who lives or works around wildlife habitats
- Who is involved with livestock or wildlife farming
- Who hunts or works with wildlife
- Who has not been previously sampled as part of the EID-SEARCH and PREICT projects

Sample size justification

<u>*Human population*</u>

$$n = \frac{N}{1 + Ne^2}$$

n = sample size of the study
N = population size for finite populations: 8,150 (data from 2018)
e = error: 10%

$$n = \frac{8{,}150}{1 + (8{,}510 \text{ x } 0.1^2)}$$

n = 98.79 (round up to 100 samples)

Wildlife population
We will target a total of 200 individuals of shrews and/or rodents across two trips for this project. This sample size is estimated based on a realistic detection rate and required sampling efforts from previous wildlife surveillance work, given the rodent/shrew population at the study site. Previous publications regarding Henipavirus discovery in small mammals had an average PCR detection prevalence between 20 and 33% (Wu et al., 2014, Lee et al., 2021, and Zhang et al., 2022). We acknowledge the challenge of identifying positive results, and very limited serology research has been done to provide guidance. However, we will make the best use of previous data and experience to conduct sampling at the selected site where human positives have been. We are confident that this targeted sampling and testing strategy offers the best chance to identify positive results and potentially novel henipaviruses.

Targeted viral sequencing and serologic testing
Targeted sequencing
For samples from shrews, rodents, and acute individuals, we will use targeted sequencing and metagenomic approaches in an attempt to detect the Thai MojV-like virus. The nucleic acid from oral swab and rectal swab will do target sequencing in henipavirus Family PCR and Sanger sequencing.

Serological testing
Serum samples will be tested for RBP and envelope fusion protein (F) binding to the known henipavirus species, including the Nipah, Hendra, Cedar, Mojiang, Ghana, Langya, Gamak, and Daerong viruses to further investigate the seroprevalence in this region. Additionally, prior positive samples will be re-tested with the expanded panel to enhance our understanding of the antigenic relationships between the novel shrew-borne henipavirus and the Thai virus.

Data analysis
Sequencing will be analyzed assembling reads in MEGA11. The consensus sequences will compare to the references strains available in the GenBank data-base using the Basic Local Alignment Software Tool (BLAST) (National Center for Bio-technology Information). Serological data will be analyzed using cluster analysis in RStudio.

Aim 2. Characterization of monoclonal antibodies from humans with serological profiles consistent with MojV infection.

In collaboration with NIH VRC PREMISE, MojV RBP and F-reactive B cells will be captured, and the B cell receptor will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific mAbs will be generated from the corresponding sequences and will be assessed for binding potential to LayV RBP and F, as well as their neutralization potential.
Importance: The isolation of these mAbs represents a potential therapeutic for MojV, MojV-like henipas, and LayV, depending on how well these cross react.

Target human population
Human positive serum for MojV was found close to Khao Chong Phran Non-hunting Area in Ratchaburi Province (Figure 2).

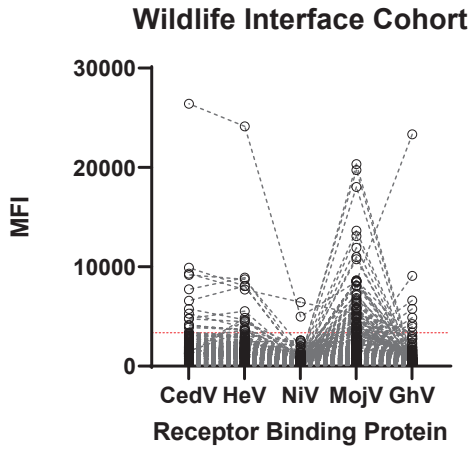

Figure 2: Seroprevalence of henipavirus proteins from a community with a large wildlife interface during 2017-2018. MFI is the median fluorescence intensity for IgG reactivity to each protein. Red-dashed line is at 3,357 MFI, a threshold for seropositivity.

PBMC sorting and B cell isolation

Whole blood from MojV-positive people will be collected in individual heparin tubes and processed for PBMC isolation using Density Gradient Centrifugation (SepMate™ PBMC Isolation Tubes). Human PBMCs will be stained with antibodies for positive markers of IgG memory B cells, and antibodies to negatively select and avoid contamination with dead cells, T cells, NK cells or monocytes (live, CD3 ,CD14 ,CD56 , IgM , IgA , CD19+,CD20+,CD27+). Avi-tag for biotinylation and 6xHIS tag for Nickel purification using the HisPur™ Ni-NTA Spin Purification Kit (Thermo Fisher Scientific, Waltham, MA). Single Memory B Cells will be sorted, cells will proliferate, cDNA will synthesized, then Heavy and light chain variable domains will be amplified (Waltari E et al., 2019).

B cell receptor Amplicon Preparation and Recombinant Antibody sorting

Total RNA yields from the PBMC will be determined by absorbance at 260 nm on the NanoDrop™ One (Thermo Fisher Scientific). Total RNA will be used for first strand cDNA synthesis with gene-specific reverse transcription (RT) primers directed to the constant regions. The RT primers for IgG, IgM, IgA, lambda, and kappa will be included. Light chain and heavy chains will be amplified into amplicon. Double-stranded cDNA will be sequenced. After completion of MiSeq sequencing, antibody repertoires will analyze using methods based on the pipeline. Heavy and light-chain sequences will be assembled into an expression vector and transfected into cells for monoclonal antibody expression. Recombinant antibody will be isolated and purified for downstream applications.

Functional assessment

We will first determine the extent to which isolated mAbs can bind binding to native-like MojV RBP and F proteins. We will also characterize the cross-reaction potential of these mAbs will be assessed against GamV, DarV, and LayV RBP and F. Neutralization of viruses remains a critical correlate of protection for most vaccine strategies. However, MojV has not been isolated or rescued, and the virus receptor for MojV has not yet been discovered (Rissanen et al., 2017; Cheliout et al., 2021). Thus, a functional assessment of MojV neutralizing potential is not possible. Though, we will test the cross-neutralization potential of these anti-MojV RBP and F mAbs to neutralize LayV utilizing a plaque reduction neutralization test with EID-SEARCH co-investigators at Duke-NUS. As LayV causes acute illness in human, cross neutralization functionality of these mAbs has therapeutic potential.

Project Timeline

Activity	05/23	06/23	07/23	08/23	09/23	10/23	11/23	12/24	01/24	02/24	03/24	04/24
Mentoring and training activities												
1.Training, literature review												
2.Monthly mentoring meeting												
3.Manuscript writing												
4.EID-SEARCH meeting												
Research activities												
1.IRB amendment												
Aim 1												
2. Human and Wildlife samples collection												
3. Identify **Targeted sequencing**												
4. Serological testing												
Aim 2												
5. Collect PBMC human sample and isolation memory B-cell for Mojiang virus												
6. PBMC Sorting and B Cell isolation												
7. Determination of envelope glycoprotein epitope binding												

Research Performance Sites

This proposed study will be implemented at the King Chulalongkorn Memorial Hospital (KCMH) collaborating with the Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC). KCMH is a 1,000-bed, university hospital in close coordination with the Faculty of Medicine, Chulalongkorn University. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. It's one of the center hospitals for Emerging Infectious Diseases in Thailand, especially for domestic and international patients.

TRC-EIDCC was established by the Committee of the Emerging Infectious Disease Center, which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. Our clinical center provides standard medical care to patients with emerging and recurrent diseases and disease transmission. Moreover, the center plans preparedness activities, trains medical and government staff on interventions, hosts conferences for medical and public health professionals, creates networks of medical care for patients with emerging and recurrent diseases, and leads research and development projects for medical care and disease control. Apart from medical innovation in emerging and recurrent diseases, the clinical center strives to be the model of infectious diseases clinical services and pandemic control through research activities surrounding infectious diseases and effective disease control measures.

We also collaborate with the Department of National Parks, Wildlife and Plant Conservation for detecting novel pathogens from animals such as bats, rodents, and shrews in our country. We also collaborate with the Department of Disease Control of Thailand (Thai-DDC) to support the Thai government and national policy makers through molecular diagnosis, including the identification of the first case of SARS-CoV-2 infection in Thailand. TRC-EIDCC prides itself on its high performance in research laboratory services and is a part of the national reference laboratory for COVID-19 and other emerging infectious diseases confirmation in Thailand.

CREID Research Center Collaboration

The goals and objectives of the proposed research project, "Immune memory bait & capture to identify emerging henipavirus origins", are fully consistent with the specific aims of EID-SEARCH. EID-SEARCH takes a One Health approach to understanding zoonotic disease emergence, with a focus on viral diversity in wildlife at high-risk human animal interfaces. EID-SEARCH aims to understand the frequency and causes of virus spillover in high-risk communities across Southeast Asia and identify strategies and tools to reduce this risk. As the proposed research focuses on the links between farming practices, wildlife diversity, virus diversity, and cross-species transmission in Thailand, we expect the results of the project to make a significant contribution towards the overall goals of EID-SEARCH.

The proposed project is well-designed, leverages cutting-edge technology, and includes a robust ecological component that corresponds to the overall research strategy of EID-SEARCH. The EID-SEARCH team is highly motivated to support the application of metagenomics to ecological questions and assist in the analysis of the unique dataset that will be generated from this research. With the heightened focus on identifying and limiting risks associated with the complex interactions between humans, wildlife, and domestic mammals that has resulted from the emergence of SARS-CoV-2 from a wet market in China, the proposed research is both timely and likely to yield highly-cited, impactful publications for EID-SEARCH and Thailand partners.

The EID-SEARCH team includes a number of experts in study design, laboratory-based diagnostics, genetic and genomic analyses, and ecological modelling who will contribute to project implementation, data analysis, and manuscript preparation as required. Communication and coordination mechanisms will be set up between the Thailand research team and scientists at the EID-SEARCH research sites for resource sharing, training, knowledge exchange, and mentorship. In particular, EID-SEARCH will facilitate and encourage relationships between our partners in Southeast Asia (based in Malaysia, Singapore, and Thailand) that will facilitate Thailand team professional development and strengthen research capacity in the region. Dr. Eric Laing, the project Mentor, has an established relationship with both Dr. Krongkan and Spencer Sterling, and will act as the liaison between EID-SEARCH and the TRC-EIDCC research team. Further, Dr. Doeuk who is the director of the VRC PREMISE program is enthusiastic about the opportunity to collaborate and apply their rapid assembly, transfection and production of immunoglobulin (RATP-Ig) workflow as a serologic/immunologic based approached for therapeutic and virus discovery (see Letter of Support). An expected outcome of this research project will be pilot data to support a joint funding application in the future.

Mentoring Plan

Goals for Pilot study and Career

Krongan Srimuang: My career goal is to be an expert scientist in either an academic research laboratory or a lecturer in medical science. My long-term research interests include the understanding of priority emerging infectious diseases, genetics, and epigenetics using molecular and serologic techniques. Detection of novel pathogens in their wildlife vectors can help characterize the pathogenicity, virulence, and evolution of these and other closely related pathogens. I believe that this pilot study can provide training in new techniques for the detection, identification, and characterization of novel pathogens that are currently unavailable in Thailand. My goal for the Pilot study is to gain more knowledge and skills for a lifelong career in public health-related research. I wish to expand my skills in novel virus detection using next-generation serologic techniques such as PBMC studies and memory B-cell isolation and sequencing that can contribute to improved surveillance and research capacity at human-animal interfaces. My mentors Dr. Laing and Dr. Wacharapluesadee can provide me with opportunities to develop and apply project skills and improve my scientific writing.

My education has provided me with a strong foundation to expand for this study. I received my undergraduate degree in Medical Technology from Chulalongkorn University which included training in molecular biology, serology, immunology, chemistry, virology, bacteriology, mycology, parasitology, hematology, clinical microscopy, transfusion medicine, and forensic medicine. My interest in molecular technology led me to pursue a Ph.D. at the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, which provided me with training in several molecular techniques including conventional PCR, real-time PCR, Next Generation Sequencing (NGS), etc. The emergence of COVID-19 motivated me to focus on emerging infectious diseases. I am currently completing my post-doctoral fellowship at the Emerging Infectious Diseases Clinical Center (EIDCC), King Chulalongkorn Memorial Hospital with Dr. Wacharpluesadee. Throughout this fellowship, I have continued learning advanced and innovative techniques to detect, identify, and characterize the novel pathogens. Through this pilot project, I will learn about the Peripheral Blood Mononuclear Cells (PBMC) which are a critical part of many subdivisions of medical research including Infectious diseases, Vaccine development, and Immunology. This project aims to study zoonotic reservoirs for by using serologic and cellular immune discovery in humans, and applying this immune knowledge in wildlife for early detection and identification of novel pathogens. This proposed study will expose me to various techniques and immunology topics that I useful in designing and testing new pipelines as technological advances continue to develop. This study will also allow me to develop, manage, and communicate my research.

Spencer Sterling: My overall career goal is to understand the factors that lead to viral zoonosis and to build epidemiological capacity in regions with the highest risk for zoonotic spillover. Through my training and educational experiences, I have learned laboratory and epidemiological techniques that can assist in the understanding of zoonosis. I wish to continue my education by expanding my repertoire of molecular techniques to include studies of the immune system's response to zoonotic disease in humans and animals, as well as to learn next-generation sequencing techniques. Additionally, I would like to use my experience in epidemiological techniques to train scientists in the Southeast Asian region in order to build local capacity in infectious disease research. Dr. Laing has been a mentor for me throughout my career, and this project would allow for this relationship to expand into new molecular areas, as well as grant writing and project management skills. My professional relationship Dr. Wacharapluesadee with began in 2018 when I participated in a DTRA BTRP funding project that focused on transferring antigen-based multiplex serological technology to her lab group. Currently, I am a Visiting Scientist at TRCEIDCC and I work closely with Dr. Srimuang, and other members of Dr. Wacharapluesadee's research group on EID-SEARCH activities. Additionally,

Dr. Wacharapluesadee has decades of experience in infectious disease research in the region and has mentored scientists that have gone on to careers at the Thai Ministry of Public Health, Department of Disease Control, Department of Parks and Wildlife, academia, and the private sector. Both mentors have encouraged Dr. Srimuang and I to apply for this pilot study as well as pursue other funding mechanisms for our research interests. Both mentors will provide for me the ability to independently pursue my research interests in the zoonotic epidemiology and train me on the best practices of scientific communication.

Vertebrate Animals Section Requirements

Animal samples will be collected in Ratchaburi Province for this project under Aim 1 to understand the antigenic relationships among known and unknown henipaviruses. Protocol to perform animal sample collection has been reviewed and approved by the Institutional Animal Care and Use Committee at Tuft University under the EID-SEARCH project (No. G2020-42) and the Institutional Animal Care and Use Committee at Chulalongkorn University (No. 019/2563)

1. Description of Procedures

Rodents (order: *Rodentia*) and Shrew (order: *Scandentia* and *Eulipotyphla*)

- Species: Free-ranging rodents (family *Hystricidae, Muridae, Sciuridae, Spalacidae*) and shrew (tree shrews, shrews, moles) that present at the sites.
- Age & sex: Adults and juveniles, males and females. Neonates will not be sampled.
- Target Number: 200 individuals
- Capture and Restraint: Free-ranging wild mammal species will be captured with metal box traps (Sherman/Tomahawk traps). Traps will be prepared with food, water, padding and shelter, and be checked at least every 12 hours, in the morning and in the afternoon. If adverse weather (extreme heat, rain) is expected or researchers are working in areas where predation is common, traps will be checked more frequently and closed during adverse weather. Veterinary staff will be prepared to deal with a variety of trap injuries, including lacerations, broken teeth, and crush injuries. Depending on the species, individual size, captive or free-ranging status of the individual, manual or chemical restraint and anesthesia (gas or injectable anesthesia) will be applied. Animals will be manually restrained during sampling utilizing insulated bite gloves to prevent injury to the handler and animal. Based upon past field experience (>20 years of animal capture and release in Southeast Asia), we do not expect to trap sick, debilitated, or young animals that would be too young to cope with capture. Sick and debilitated animals tend not to roam widely, reducing their opportunity to enter traps. Additionally, should sick, debilitated, or young animals enter traps, we anticipate that they will not suffer adverse conditions because the traps contain food (the bait) and liquid (water or fruit as a source of water). These individuals would be examined by a veterinarian, rehydrated and if presenting clinical signs treated by a veterinarian, prior to release or sampling then release.
- Sample Collection: Once anesthetized or safely restrained, whole blood will be collected through the appropriate venipuncture site, no more than 1 ml of blood per 100 g (= 10 ml/kg or 1%) of body weight will be collected at any one time. Anesthetized animals will be monitored regularly during recovery until they can no longer be safely handled, at which point they will be confined in a trap or cage. Swabs will be taken from the oropharynx, urogenital tract, and rectum. No destructive sampling is planned, but any individuals that die during capture or sampling will have a necropsy performed with organ samples collected.
- Release: Wild mammals are held for a maximum of thirteen hours depending on trap timing, but typically less than three hours. Captive animals are held for a maximum of two hours but typically less than one hour. At the completion of sampling, animals may be provided with rehydrating fluids (either subcutaneously if anesthetized or orally if manually handled and accepted). Wild mammals will be released at the site of capture in a location that allows for further recovery or safe escape (e.g., bodies of water, known predatory areas, and human settlements will be avoided). Captive mammals will be released back into their captive setting as is appropriate for the species, either isolated or in social group if it is deemed safe from aggression from enclosure mates while the post-recovery period continues.

2. Justifications

The purpose of this study is to identify zoonotic pathogens through serologic and cellular immune discovery, including developing methods and standards for direct antibody-mediated virus capture in people and wildlife, focusing on rodent/shrew-borne Henipaviruses (HNVs). This will require hands-on fieldwork to collect whole blood and serum samples from rodents and shrews for lab analysis. One technique that avoids the direct capture and sampling of animals is to collect fresh feces or urine, but this will not be able to provide the appropriate samples for serologic analysis, and the research objectives cannot be achieved using an alternative methods (e.g., computational, human, invertebrate, in vitro). Therefore, we believe there are no viable alternatives than the use of live animals.

3. Minimization of Pain and Distress

In every situation, sampling of wildlife will be conducted in the most humane manner while minimizing the impacts on individual animals and their wild populations. In all instances, the fewest number of animals will be sampled that will provide valid information and statistical inference for the pathogen and disease of interest and every effort will be made to minimize stress and discomfort for the animal.

Small mammals may be held for up to 13 hours depending on trap capture timing. Field animal sampling team with zoologists and veterinarians have been well trained and have extensive experience in capture, anesthesia, and sampling of wildlife. In our team's experience, the target species tolerate the described procedure well. Mist nets will be attended continuously during capture periods. This will minimize stress and injury from entanglement.

For rodents and shrews, food, water, padding and shelter will be provided at traps, which will be checked at least every 12 hours. Traps will be checked more frequently or closed if adverse weather (extreme heat, rain) is expected, or researchers are working in areas where predation is common. Veterinary staff will be prepared to deal with a variety of trap injuries, including lacerations, broken teeth, and crush injuries. All animal individuals will be monitored by a veterinarian or experienced field team member during all stages of capture, processing, anesthesia and release. Animals will be kept in a cool place, free from adverse weather conditions and access by non-field team members or other animals while in the pillowcases, trap, or cage for recovery. All recovering individuals will be observed until they are able to effectively ambulate and orient to their surroundings. Animals that are injured during the capture or sampling process will be assessed by an experienced team leader or attending veterinarian, and if the animal is determined to be unlikely to survive if released, it shall be euthanized humanely (see euthanasia section).

4. Method of Euthanasia

In the event that an animal has been injured or is moribund, a determination will be made as to whether it may be treated and released on site by veterinarians or transferred to facility that may treat and rehabilitate the animal, or if, as a last resort, the animal will require euthanasia. Euthanasia methods will vary depending on species; however methods to be used will not deviate from the AVMA “Guidelines for the Euthanasia of Animals” (2020 edition). Any animal that is euthanized using a chemical agent will be disposed of such that it will not be permitted to enter the food supply either through markets or hunting.

Human Subjects Research

1. Risks to the subjects

In this project, we will target populations in one community site who with frequent exposure to bats and other wildlife based on our previous behavioral survey. Subjects will be enrolled voluntarily, and informed consent will be obtained from all participants. Enrolled participants will provide biological samples. Survey data and biological samples will be collected from enrolled participants, and follow-up data collection will be performed among participants whose samples were tested positive.
Biological samples and questionnaire data will be collected from individuals who meet recruitment and inclusion criteria and complete the informed consent process. During data collection, a standardized questionnaire will be administered to all participants. This questionnaire will collect information of demographic background, wildlife contract, travel and daily movement, and unusual SARI/ILI symptoms, and biological specimens will be collected from participants. Both questionnaire and biological data will be analyzed to assess the exposure to coronaviruses and the spread risk among humans. From all participants, a one-time whole blood sample (Max. 15 mL) will be collected during the study period. This sample will allow us to test for historical exposure to bat or rodent-born henipaviruses and collect Peripheral Blood Mononuclear Cells (PBMCs) for those study participants whose serum tests are positive.

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 who are 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, the administration will be conducted privately and confidentially to protect individuals' 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 these risks

2.1 Informed consent and assent
Only consented participants will be enrolled in the study. No research procedures will be undertaken before the participant fully understands the research, agrees to the study procedures, and completes the consent process will be enrolled in the study. Informed consent statements and forms, and project protocols will be translated into the local language of each study site. Research team members involved in this consent process will be required to be fluent in the local language to ensure that the subjects understand the study and all involved procedures.

If participants meet the criteria for enrollment, they will be invited to discuss the details of the study with the research staff. Study staff will review an information sheet and informed consent form with the participant when applicable. Each individual will be provided with a copy of the informed consent form that has been translated into the local language and written with a Flesch–Kincaid readability score equivalent to a 7th-grade reading level or below, to assure that potential participants understand the information being shared. The informed consent form will explain the details of the study, including how and why the individual was selected, the study process and procedures, risks and benefits, financial considerations and the gift of appreciation, confidentiality of data shared, alternatives to participating, and how to obtain more information now or at a later date. The informed consent form will be read in the local language of the site at a location ensuring participant privacy. After which individuals will have as much time as they would like to ask questions and discuss the

study with study staff. The study staff will endeavor to ensure that the participant understands the information provided. The study staff will then ask the participant to consider study participation. Participants will have as much time as required to consider the participation.

Those participants who consent to the study will sign and date two copies of the consent form. These form copies will be countersigned and data by the study staff. A copy of the signed consent form must be provided to the subject and the other copy will be kept by study staff. Informed consent paperwork will be kept until the end of the project in a locked box at the local country project office.

2.2 Protection against risks

The potential risks to study participants as a result of study participation are minimal. Collection of venous blood samples and nasal or oropharyngeal swab samples pose minimal risk to subjects. Potential complications associated with venipuncture include pain and/or hematoma at the site of collection. Trained medical professionals and/or clinic staff will monitor the blood collection site and treat any complications according to existing health facility protocols. A potential complication of nasal/ oropharyngeal sampling is minor irritation at the time of collection. Employing trained medical and/or clinic staff to collect blood and swab samples will minimize the potential for complications.

Another risk that this study may pose concerns the information to be gained on henipaviruses newly recognized in the community. We will provide participating communities, hospitals, and clinicians with information and background data on target zoonotic viruses to ensure up-to-date communication of risk. Because of the timeline for diagnostic testing and results interpretation, we are not likely to provide results to participating clinics within a time frame that would be clinically relevant to outbreaks of undiagnosed diseases. Therefore, the information provided by this project will not impact patient management or outlook.

If an individual decides to participate in this research, his/her participation and all information provided by the participant will be strictly confidential, and personal identifying information will not be shared with anyone outside of the study staff. Participants will not be identified or named in any reports or publications. Questionnaire information and all biological samples will be identified by an alphanumeric code, not by the participant's name. 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 study site in locked files cabinets or password-protected devices in areas with access limited to the research staff of this project. Research databases will be secured with password-protected access systems and controlled distribution web-based certificates and will not contain any identifying characteristics about study participants (e.g., name, address, or telephone number). Access to all data will be limited to the staff involved in this study. The health information disclosed by an individual will not be used by or disclosed (released) to another institution. Any surveillance report that is published or shared with partners will not contain any personally identifying information for individual participants.

3. Potential benefits of the research to the subjects and others

There are no measurable benefits to the individual study participants enrolled in this study. There are benefits to the community and regional healthcare providers to help them understand the risk of zoonotic infections among high-risk populations in the regions they work. At the conclusion of the study, we will deliver an educational workshop reporting aggregate study findings that will be open to both study and non-study participants, describing the health benefits of using personal protection equipment (PPE) and handwashing during animal handling activities throughout the day, as well as to share other prevention/interventions that emerge from the research data.

4. Importance of the knowledge to be gained

There are valuable potential benefits to the public from the knowledge to be gained from this study. One key benefit to the community is sharing information and knowledge to better understand the risk of zoonotic spillover events and related health risks, as well as information sharing with communities on practices that could reduce risks, such as the avoidance of particular animal contacts or the need for PPE and extra care when handling wildlife, that may substantially reduce the risk zoonotic pathogen transmission in the community. 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 create or implement public health interventions to disrupt disease emergence and/or spread in an area that is beneficial to all. Additionally, there are valuable benefits to the general public from the knowledge to be gained from this study. Knowledge gained will 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.

5. Country- / institution-specific ethics / IRB regulations addressed

Main research protocol and materials to conduct human subject research in this project have been reviewed and approved by the Health Medical Lab Institutional Review Board, US (No. 894ECOH21b) and the Institute Review Board of the Faculty of Medicine, Chulalongkorn University, Thailand (No. 211/64). Amendment for PBMCs collection will be made and approved before the start of this project.

Research and Related Other Project Information

1. Are Human Subjects Involved?	Yes	X	No												
1.a. If YES to Human Subjects															
Is the Project Exempt from Federal regulations?	Yes		No	X											
If yes, check appropriate exemption number	1		2		3		4		5		6		7		8	
If no, is the IRB review Pending?	Yes	X	No												
IRB Approval Date:															
Human Subject Assurance Number	FWA00000943														

2. Are Vertebrate Animals Used?	Yes	X	No	
2.a. If YES to Vertebrate Animals				
Is the IACUC review Pending?	Yes		No	X
IACUC Approval Date	3 December 2020			
Animal Welfare Assurance Number				

3. Is proprietary/privileged information included in the application?	Yes		No	X

4.a. Does this project have an Actual or Potential impact -- positive or negative -- on the environment?	Yes		No	X
4.b. If yes, please explain				
4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed?	Yes		No	
4.d. If yes, please explain				

5. If the research performance site designated, or eligible to	Yes		No	X

be designated, as a historic place?				
5.a. If yes, please explain				

6. Does this project involve activities outside of the United States or partnership with international collaborators?	Yes	X	No	
6.a. If yes, identify countries	Thailand			
6.b. Optional explanation				

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: Srimuang, Krongkan

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

POSITION TITLE: Research 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
Chulalongkorn University, Bangkok, Thailand	B.Sc.	03/2012	Medical Technology
Mahidol University, Bangkok, Thailand	Ph.D.	04/2017	Molecular Tropical Medicine and Genetics

A. Personal Statement

I am a medical technologist. My undergraduate training has provided me with a background in multiple biological disciplines including molecular biology, microbiology, and genetics. During my Ph.D., I conducted research with Dr. Mallika Imwong focused on Malaria. My thesis was entitled “Exploring the molecular mechanism of mefloquine resistance in *Plasmodium falciparum*”. I conducted research on drug resistance to malaria in Southeast Asia. Specifically, my focus was on molecular techniques in identifying the genetic mutations involved with drug resistance. Moreover, I got experience in *Plasmodium falciparum* malaria culture. After my Ph.D., I worked at the genetics laboratory at Jetanin Institute of Assisted Conception. My responsibilities included Preimplantation Genetic Testing (PGT) is screen embryos for genetic abnormalities such as chromosome copy number gains or losses (PGT-A, aneuploidy screening), single gene disorders (PGT-M, monogenic or single-gene disease), or structural rearrangement (PGT-SR, structural rearrangement to identify embryos with chromosome imbalanced) by using Next Generation Sequencing. Additionally, I conducted genetic screening in couples for detecting carrier diseases such as glucose-6-phosphate dehydrogenase (G6PD), Alpha thalassemia, Beta thalassemia, and Spinal muscular atrophy (SMA) by using molecular genetics techniques. Currently, I am a Research Scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC EIDCC), King Chulalongkorn Memorial Hospital where I investigate emerging infectious diseases under the guidance of Dr. Supaporn Wacharapluesadee. The research focuses on detecting, identifying, and characterizing pathogens and human genome segments using molecular and serology techniques, including Next Generation Sequencing and Multiplex serology assays as a part of EID-SEARCH project. Additionally, I am the laboratory sequencing lead on the Surveillance System: Emerging Viral Pneumonia and Respiratory tract infections (RTIs) project from patients in Thailand as part of a collaboration with the Department of Disease Control.

1. **Srimuang K**, Miotto O, Lim P, Fairhurst RM, Kwiatkowski DP, Woodrow CJ, Imwong M; Tracking Resistance to Artemisinin Collaboration. “Analysis of anti-malarial resistance markers in *pfmdr1* and *pfcrt* across Southeast Asia in the Tracking Resistance to Artemisinin Collaboration” Malar J. 2016 Nov 8;15(1):541.
2. Reamtong O, **Srimuang K**, Saralamba N, Sangvanich P, Day NP, White NJ, Imwong M. “Protein profiling of mefloquine resistant *Plasmodium falciparum* using mass spectrometry- based proteomics” Int J Mass Spectrom. 2015 Nov 30;391:82-92

3. **Srimuang K**, Udompat P, Thippamom N, Petchrat S, Doung-Ngern P, Kripattanapong S, Supataragul S, Sterling SL, Wacharapluesadee S, Putcharoen O. “Genetic characterization of Norovirus strains from an unusual diarrheal outbreak in the community in Eastern Thailand” (Submitted, January 2023)

B. Positions, Scientific Appointments, and Honors

Positions and Scientific Appointments:

02/2022 – Present	Medical research scientist, Emerging Infectious Diseases Clinical Center (EIDCC), King Chulalongkorn Memorial Hospital
05/2017 - 01/2022	Scientist, Genetics Laboratory, Jetanin Institute for Assisted Reproduction
10/2015 - 03/2016	Fellowship at Laboratory of Molecular and Cellular Parasitology, Department of Microbiology and Immunology, National University of Singapore, Singapore.
11/2011 – 02/2012	Training Medical Technology Programs at Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

Honors:

2012 -2017	Scholarship for Ph.D. from the Royal Golden Jubilee, Ph.D. Grant (14th Batch).

C. Contributions to Science

Undergraduate Research: I participated in research activities under Dr. Attakorn Palasuwan to study in human genetic variants in proteins, such as G6PD, as they relate to the effect of exercise on the reduction of free radicals in the body. We conducted the multiplex PCR to identify the G6PD variants and found 8 variants including Vanua Lava, Mahidol, Mediterranean, Coimbra, Viangchan, Union, Canton, and Kaiping.

Graduate Research: I continued to studies under Dr. Mallika Imwong in the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University. My thesis, “Exploring the molecular mechanism of mefloquine resistance in *Plasmodium falciparum*”, focused on the evolution of resistance to mefloquine, which is commonly used in Southeast Asia in Malaria treatment. The mechanism of mefloquine resistance remains unknown, but I focused on the *Pfmdr1* gene which is involved in the drug resistance mechanism in *Plasmodium falciparum.* I found mutations and polymorphisms in *Pfmdr1* gene.

1. **Srimuang K**, Miotto O, Lim P, Fairhurst RM, Kwiatkowski DP, Woodrow CJ, Imwong M; Tracking Resistance to Artemisinin Collaboration. “Analysis of anti-malarial resistance markers in *pfmdr1* and *pfcrt* across Southeast Asia in the Tracking Resistance to Artemisinin Collaboration” Malar J. 2016 Nov 8;15(1):541.
2. Reamtong O, **Srimuang K**, Saralamba N, Sangvanich P, Day NP, White NJ, Imwong M. “Protein profiling of mefloquine resistant *Plasmodium falciparum* using mass spectrometry- based proteomics” Int J Mass Spectrom. 2015 Nov 30;391:82-92

Post-graduate Research: I was a scientist in genetics lab at Jetanin, a holistic center for infertility, offering counseling and treatment with Assisted Reproductive Technologies such as IVF, ICSI, and Preimplantation Genetic Testing (PGT). My work included the development of standard operating procedures for the lab, including Next Generation Sequencing, for detecting and identifying chromosomes in blastocyst growth to an embryo. Additionally, we assisted couples who had a family history of genetic diseases for screening before pregnancy. The carrier screening focused on highly prevalent diseases in Thailand such as alpha thalassemia, beta thalassemia, G6PD, and SMA. Currently, I am a Research Scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital. I am currently tasked with using molecular and serological techniques in the surveillance of novel, exotic, and known pathogens in wildlife and humans.

1. **Srimuang K**, Udompat P, Thippamom N, Petchrat S, Doung-Ngern P, Kripattanapong S, Supataragul S, Sterling SL, Wacharapluesadee S, Putcharoen O. “Genetic characterization of Norovirus strains from an unusual diarrheal outbreak in the community in Eastern Thailand” (Submitted, January 2023)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contr butors.
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

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: Assistant Professor

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. (hons)	05/2008	Biology
Uniformed Services University, Bethesda, MD	Ph.D.	10/2016	Emerging Infectious Diseases
Uniformed Services University, Bethesda, MD	Postdoc	04/2019	Virology

A. Personal Statement

The spillover of zoonotic viruses into human populations remains an ever-prevalent threat to public and global health. Continual outbreaks of known and unknown zoonotic viruses highlights the paucity of our understanding of the viral diversity, geographic distribution, wildlife hosts, and human populations at-risk for spillover. I am a recently appointed assistant professor dedicated to biosurveillance and virological research of emergent zoonotic viruses. I have completed virological training and spent my time as a post-doctoral fellow applying *in vitro* techniques to characterize the replication of, and mammalian ephrin receptors that mediate cellular entry of emergent henipaviruses. Since the start of the COVID-19 pandemic, I pivoted the focus of my research team to serological analysis of SARS-CoV-2 infection, and then to COVID-19 vaccine-induced antibody durability. In my ongoing research, I develop serological-based approaches to identify the infectome of wildlife and human communities for the assessment of virus spillovers. Since my position as a postdoctoral fellow I have worked collaboratively with national and international researchers including institutional partners at Duke-NUS, Chulalongkorn University, University of Pretoria, and Rocky Mountain Labs towards these goals. Across all of these collaborations, we have aimed to identify known and unknown viruses, the wildlife reservoirs of priority pathogens, the high-risk interfaces for spillover and the biotic/abiotic drivers of virus emergence. Throughout these research projects, we have detected unexpected serological profiles in communities of bats and humans that have not fit our prior understanding of the viral diversity established or detected by genetic techniques. This proposal aims to better understand these serological profiles of wildlife and humans with a targeted focus on henipaviruses and filoviruses. Ongoing projects that I would like to highlight include:

- HDTRA12110037. DTRA BRTP, E. Laing (Co-PI). 08/2021-07/2026, “Informing biosurveillance, contribution of pteropodid fruit bats to virus spillover in the Philippines.”
- HU00012020067, HU00012120104, HU00012120094.Defense Health Program/CARES Act, NIAID, E. Laing (Associate Investigator). 9/2020-9/2025, “Prospective Assessment of SARS-CoV-2 Seroconversion.”
- HDTRA12010025. DTRA BTRP, E. Laing (Co-I). 7/2020 – 6/2025, “Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa.”
- U01AI151797. NIH, Centers for Research in Emerging Infectious Diseases, E. Laing (Co-I). 02/2020 – 03/2025, “EID-Southeast Asia Research Collaborative Hub.”
- HU00012020067, HU00011920111. Defense Health Program, NIAID, E. Laing (Associate Investigator). 03/2020 – 09/2023, “Epidemiology, Immunology, and Clinical Characteristics of Emerging Infectious Diseases with Pandemic Potential (EpICC-EID).”

B. Positions, Scientific Appointments and Honors

Positions

2021-pres. Assistant Professor, Department of Microbiology and Immunology, School of Medicine, Uniformed Services University, Bethesda, MD.

2021-pres. Joint Appointment, Emerging Infectious Diseases Graduate Program, School of Medicine, Uniformed Services University, Bethesda, MD.

2019-21 Research Assistant Professor, Department of Microbiology and Immunology, Uniformed Services University, Bethesda, MD.

2016-19 Postdoctoral fellow, Henry M. Jackson Foundation, Department of Microbiology, Uniformed Services University, Bethesda, MD.

2010-16 Graduate research student, Department of Microbiology, Uniformed Services University, Bethesda, MD.

Other Experiences and Professional Memberships

2022-pres. Executive committee member, Emerging Infectious Diseases Graduate Program, USUHS

2021-pres. Research center representative, Laboratory Assays Oversight and Quality Working Group, Emerging Infectious Diseases: Southeast Asia Research Collaboration Hub (EID-SEARCH), Centers for Research in Emerging Infectious Diseases (CREID), NIAID, DMID, NIH

2021-pres. Review editor, Frontiers in Virology - Emerging and Reemerging Viruses

2021-pres. Ad hoc reviewer, Frontiers in Immunology, Journal of Clinical Immunology

2021-pres. Steering committee member, JPI/Military Infectious Diseases Research Program, Emerging Infectious Diseases

2019 Ad hoc reviewer, EcoHealthNet 2.0 Program, EcoHealth Alliance

2018 Ad hoc reviewer, Pakistan One-Health Fellowship Program, National Academy of Sciences & Pakistan Academy of Sciences

2014-2019 Member, American Society of Tropical Medicine and Hygiene

2014-2019 Member, American Society of Microbiology

2014-2019 Volunteer, AAAS STEM K-12 Volunteer Program

Mentoring

Postdoctoral fellows Si'Ana A. Coggins, PhD, 2020 - 2022

Graduate students Marana S. Tso, BS, 2021 -
McKenna Roe, BS, 2022 -

Committee member Celeste Huaman, BS, 2021 -
2LT Connor Perry, BS, 2021 -

Honors

2021-2022 Impact Award, USUHS School of Medicine

2021 Outstanding Research Accomplishment/Team/SARS-CoV-2, The EPICC COVID-19 Cohort Team, Military Health System Research Symposium

2020-2021 Impact Award, USUHS School of Medicine

2015-2016 Val G. Hemming Fellowship, Henry M. Jackson Foundation

2015 East Asia and Pacific Summer Institutes Fellowship, National Science Foundation

C. Contributions to Science (†*mentee*, *corresponding)

1. Lyssaviruses and the prototype, rabies virus, remain a public health concern. Beginning with my PhD thesis work, I've researched the virus host-interactions between a rabies-related lyssavirus, Australian bat lyssavirus (ABLV), and its bat host (*Pteropus alecto*). Research has focused on ABLV cellular entry mechanisms, the development of an animal model and ABLV reporters and exploration of novel monoclonal antibodies that neutralize ABLV and other phylogroup I lyssaviruses. Furthermore, comparative bat immunology research was conducted using black flying fox cell lines and ABLV as a model virus/host interaction. Physiological adaptations that accompanied the evolution of flight in bats have been proposed to contribute to the frequent role of bats as asymptomatic hosts of highly pathogenic zoonotic viruses.

Comparatively studying the autophagy pathway in bat cell lines revealed that bat cells had elevated levels of basal autophagy and experienced significantly less cell death when challenged with high virus doses.

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. Virol J. 2013. 11:40. doi: 10.1186/1743-422X-11-40. PMID: 24576301, PMCID: PMC3946599

b. **Laing E.D.***, Sterling S.L., Weir D.L., Beauregard C.R., Smith I.L., Larsen S.E., Wang L-F., Snow A.L., Schaefer B.C., and Broder C.C. Enhanced autophagy contributes to reduced viral infection in black flying fox cells. Viruses. 2019. Mar 14;11(3). pii: E260. doi: 10.3390/v11030260. PMID: 30875748, PMCID: PMC6466025

c. Mastraccio K.E., Huaman C., Warrilow D., Smith G.A., Craig S.B., Weir D.L., **Laing E.D.**, Smith I., Broder C.C. and B.C. Schaefer. Establishment of a longitudinal pre-clinical model of lyssavirus infection. J Virol Methods. 2020 Jul; 281:113882. doi: 10.1016/j.jviromet.2020.113882. Epub 2020 May 12. PMID: 32407866

d. Weir D.L., Coggins S.A., Vu B.K., Coertse J., Yan L., Smith I.L., **Laing E.D.**, Markotter W., Broder C.C., and Schaefer B.C. Isolation and characterization of cross-reactive human monoclonal antibodies that potently neutralize Australian bat lyssavirus variants and other phylogroup 1 lyssaviruses. Viruses. 2021 Mar 1;13(3):391. doi: 10.3390/v13030391. PMID: 33804519; PMCID: PMC8001737.

2. My research experience as a postdoctoral fellow furthered my training in molecular virology techniques. I constructed a recombinant Cedar virus cDNA plasmid and optimized a reverse genetics approach to rescue a recombinant Cedar virus reporter virus, a non-pathogenic *Henipavirus* species. A molecular biology methods chapter detailing recombinant Cedar virus reverse genetics has been submitted and in press. Using this recombinant Cedar virus, we determined that Cedar virus can utilize several non-canonical henipavirus ephrin receptors for cellular entry and explored the structure of the receptor-binding pocket to understand the receptor promiscuity. The non-pathogenic phenotype of CedV creates a potential for CedV to act as a model henipavirus to explore host-pathogen interactions, cellular tropism and factors that determine henipaviral disease pathogenesis. Additionally, I have collaborated on projects detailing henipavirus infection and replication in bat hosts with colleagues at the Rocky Mountain Labs, studying whether specific species of bats are more competent hosts and whether virus-host restriction exists.

a. Amaya M, Broder CC, **Laing ED***. Recombinant Cedar virus: a henipavirus reverse genetics platform. In: Freiberg A.N. and B. Rockx, Nipah Virus: Methods and Protocols, Methods Mol. Biol. (in press)

b. Seifert SN, Letko MC, Bushmaker T, **Laing ED**, Saturday G, Meade-White K, van Doremalen N, Broder CC, Munster VJ. Rousettus aegyptiacus Bats Do Not Support Productive Nipah Virus Replication. J Infect Dis. 2020 May 11;221(Suppl 4):S407-S413. doi: 10.1093/infdis/jiz429. PMID: 31682727; PMCID: PMC7199784.

c. **Laing ED,** Navaratnarajah CK, Cheliout Da Silva S, Petzing SR, Xu Y, Sterling SL, Marsh GA, Wang LF, Amaya M, Nikolov DB, Cattaneo R, Broder CC, Xu K. Structural and functional analyses reveal promiscuous and species specific use of ephrin receptors by Cedar virus. Proc Natl Acad Sci U S A. 2019 Oct 8;116(41):20707-20715. doi: 10.1073/pnas.1911773116. Epub 2019 Sep 23. PMID: 31548390; PMCID: PMC6789926.

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. PMID: 29587789; PMCID: PMC5869790.

3. Bats are increasingly identified as animal reservoirs of emerging zoonotic viruses (e.g. Nipah virus, Ebola virus and SARS-coronavirus). I lead collaborative biosurveillance and research preparedness training including data analysis and interpretations at international partner institutes with lab technicians, field and lab scientists, and masters, doctoral and postdoctoral trainees. Collaborative biosurveillance is presently underway in Thailand (Chulalongkorn University, Bangkok) Malaysia (National Wildlife and Forensic Lab, Universti Purtra Malaysi, National Public Health Lab) via NIH Centers for Research in

Emerging Infectious Diseases, EID-Southeast Asia Research Collaborative Hub. As a collaborator within the DARPA PREEMPT network I supported surveillance for coronaviruses and other priority emerging zoonotic viruses, henipaviruses and filoviruses, in Ghana (Zoological Society of London), Australia (Black Mountain Labs) and Bangladesh (icddr,b). We aim to characterize the geographic distribution of zoonotic filoviruses/henipaviruses/coronaviruses, transmission dynamics in wildlife hosts and generate risk-models for Ebola virus, Nipah virus and SARS-related CoV outbreaks. Results discovered so far suggest a wider geographical footprint of Asiatic filoviruses and have identified several fruit bat species that act as natural reservoirs for these viruses.

a. Paskey AC, Ng JHJ, Rice GK, Chia WN, Philipson CW, Foo RJH, Cer RZ, Long KA, Lueder MR, Lim XF, Frey KG, Hamilton T, Anderson DE, **Laing ED**, Mendenhall IH, Smith GJ, Wang LF, Bishop-Lilly KA. Detection of Recombinant Rousettus Bat Coronavirus GCCDC1 in Lesser Dawn Bats (Eonycteris spelaea) in Singapore. Viruses. 2020 May 14;12(5):539. doi: 10.3390/v12050539. PMID: 32422932; PMCID: PMC7291116.

b. Schulz JE, Seifert SN, Thompson JT, Avanzato V, Sterling SL, Yan L, Letko MC, Matson MJ, Fischer RJ, Tremeau-Bravard A, Seetahal JFR, Ramkissoon V, Foster J, Goldstein T, Anthony SJ, Epstein JH, **Laing ED**, Broder CC, Carrington CVF, Schountz T, Munster VJ. Serological Evidence for Henipa-like and Filo-like Viruses in Trinidad Bats. J Infect Dis. 2020 May 11;221(Suppl 4):S375-S382. doi: 10.1093/infdis/jiz648. PMID: 32034942; PMCID: PMC7213578.

c. Dovih P, **Laing ED**, Chen Y, Low DHW, Ansil BR, Yang X, Shi Z, Broder CC, Smith GJD, Linster M, Ramakrishnan U, Mendenhall IH. Filovirus-reactive antibodies in humans and bats in Northeast India imply zoonotic spillover. PLoS Negl Trop Dis. 2019 Oct 31;13(10):e0007733. doi: 10.1371/journal.pntd.0007733. Erratum in: PLoS Negl Trop Dis. 2021 Nov 16;15(11):e0009836. PMID: 31671094; PMCID: PMC6822707.

d. **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. PMID: 29260678; PMCID: PMC5749470.

4. Responding to the emergence of SARS-CoV-2, our lab developed multiplex serology strategies to identify SARS-CoV-2 antibodies and address research questions related to whether pre-existing antibody memory induced by prior infection with seasonal human coronaviruses effects COVID-19 severity. Our lab's serology efforts supported NIH and DHA collaboratively funded protocols including prospective, longitudinal serological analysis of hospital and community subjects, and health-care workers; and cross-sectional analyses of SARS-CoV-2 infection among deployed military health-care workers.

a. Epsi NJ, Richard SA, Lindholm DA, Mende K, Ganesan A, Huprikar N, Lalani T, Fries AC, Maves RC, Colombo RE, Larson DT, Smith A, Chi SW, Maldonado CJ, Ewers EC, Jones MU, Berjohn CM, Libraty DH, Edwards MS, English C, Rozman JS, Mody RM, Colombo CJ, Samuels EC, Nwachukwu P, Tso MS, Scher AI, Byrne C, Rusiecki J, Simons MP, Tribble D, Broder CC, Agan BK, Burgess TH, **Laing ED**, Pollett SD; EPICC COVID-19 Cohort Study Group. Understanding 'hybrid immunity': comparison and predictors of humoral immune responses to SARS-CoV-2 infection and COVID-19 vaccines. Clin Infect Dis. 2022 May 24:ciac392. doi: 10.1093/cid/ciac392. Epub ahead of print. PMID: 35608504; PMCID: PMC9213853.

b. Lu Z, **Laing ED**, Pena DaMata J, Pohida K, Tso MS, Samuels EC, Epsi NJ, Dorjbal B, Lake C, Richard SA, Maves RC, Lindholm DA, Rozman JS, English C, Huprikar N, Mende K, Colombo RE, Colombo CJ, Broder CC, Ganesan A, Lanteri CA, Agan BK, Tribble D, Simons MP, Dalgard CL, Blair PW, Chenoweth J, Pollett SD, Snow AL, Burgess TH, Malloy AMW; EPICC COVID-19 Cohort Study Group. Durability of SARS-CoV-2-Specific T-Cell Responses at 12 Months Postinfection. J Infect Dis. 2021 Dec 15;224(12):2010-2019. doi: 10.1093/infdis/jiab543. PMID: 34673956; PMCID: PMC8672777.

c. Pollett SD, Richard SA, Fries AC, Simons MP, Mende K, Lalani T, Lee T, Chi S, Mody R, Madar C, Ganesan A, Larson DT, Colombo CJ, Colombo R, Samuels EC, Broder CC, **Laing ED**, Smith DR, Tribble D, Agan BK, Burgess TH. The SARS-CoV-2 mRNA vaccine breakthrough infection phenotype includes significant symptoms, live virus shedding, and viral genetic diversity. Clin Infect Dis. 2021 Jun 12:ciab543. doi: 10.1093/cid/ciab543. Epub ahead of print. PMID: 34117878.

d. Clifton GT, Pati R, Krammer F, **Laing ED**, Broder CC, Mendu DR, Simons MP, Chen HW, Sugiharto VA, Kang AD, Stadlbauer D, Pratt KP, Bandera BC, Fritz DK, Millar EV, Burgess TH, Chung KK. SARS-CoV-2 Infection Risk Among Active Duty Military Members Deployed to a Field Hospital - New York City, April 2020. MMWR Morb Mortal Wkly Rep. 2021 Mar 5;70(9):308-311. doi: 10.15585/mmwr.mm7009a3. PMID: 33661864; PMCID: PMC7948931.

5. In addition to providing serologic assessment of SARS-CoV-2 infection, my research team is actively engaged in examining the durability of COVID-19 vaccine induced humoral immunity. Antibody responses, particularly neutralizing antibodies, are frequently cited as a predictive correlate of protection. With the emergence of variants of concern and waning circulating antibodies, the timing of booster shots remains an important measure for controlling the pandemic. In my lab we evaluate the duration of neutralizing antibodies, durability and breadth of antibody responses against emerging variants of concern, hybrid immune responses, and post-vaccination infections.

a. Wang W, Lusvarghi S, Subramanian R, Epsi NJ, Wang R, Goguet E, Fries AC, Echegaray F, Vassell R, Coggins SA, Richard SA, Lindholm DA, Mende K, Ewers EC, Larson DT, Colombo RE, Colombo CJ, Joseph JO, Rozman JS, Smith A, Lalani T, Berjohn CM, Maves RC, Jones MU, Mody R, Huprikar N, Livezey J, Saunders D, Hollis-Perry M, Wang G, Ganesan A, Simons MP, Broder CC, Tribble DR, **Laing ED**, Agan BK, Burgess TH, Mitre E, Pollett SD, Katzelnick LC, Weiss CD. Antigenic cartography of well-characterized human sera shows SARS-CoV-2 neutralization differences based on infection and vaccination history. Cell Host Microbe. 2022 Dec 14;30(12):1745-1758.e7. doi: 10.1016/j.chom.2022.10.012. Epub 2022 Oct 21. PMID: 36356586; PMCID: PMC9584854.

b. **Laing ED,** Weiss CD, Samuels EC, Coggins SA, Wang W, Wang R, Vassell R, Sterling SL, Tso MS, Conner T, Goguet E, Moser M, Jackson-Thompson BM, Illinik L, Davies J, Ortega O, Parmelee E, Hollis-Perry M, Maiolatesi SE, Wang G, Ramsey KF, Reyes AE, Alcorta Y, Wong MA, Lindrose AR, Duplessis CA, Tribble DR, Malloy AMW, Burgess TH, Pollett SD, Olsen CH, Broder CC, Mitre E. Durability of Antibody Response and Frequency of SARS-CoV-2 Infection 6 Months after COVID-19 Vaccination in Healthcare Workers. Emerg Infect Dis. 2022 Apr;28(4):828-832. doi: 10.3201/eid2804.212037. Epub 2022 Feb 24. PMID: 35203111; PMCID: PMC8962883.

c. Lusvarghi S, Pollett SD, Neerukonda SN, Wang W, Wang R, Vassell R, Epsi NJ, Fries AC, Agan BK, Lindholm DA, Colombo CJ, Mody R, Ewers EC, Lalani T, Ganesan A, Goguet E, Hollis-Perry M, Coggins SA, Simons MP, Katzelnick LC, Wang G, Tribble DR, Bentley L, Eakin AE, Broder CC, Erlandson KJ, **Laing ED**, Burgess TH, Mitre E, Weiss CD. SARS-CoV-2 BA.1 variant is neutralized by vaccine booster-elicited serum but evades most convalescent serum and therapeutic antibodies. Sci Transl Med. 2022 May 18;14(645):eabn8543. doi: 10.1126/scitranslmed.abn8543. Epub 2022 May 18. PMID: 35380448; PMCID: PMC8995032.

d. [†]*Coggins SA*, **Laing ED**, Olsen CH, Goguet E, Moser M, Jackson-Thompson BM, Samuels EC, Pollett SD, Tribble DR, Davies J, Illinik L, Hollis-Perry M, Maiolatesi SE, Duplessis CA, Ramsey KF, Reyes AE, Alcorta Y, Wong MA, Wang G, Ortega O, Parmelee E, Lindrose AR, Snow AL, Malloy AMW, Letizia AG, Ewing D, Powers JH, Schully KL, Burgess TH, Broder CC, Mitre E. Adverse Effects and Antibody Titers in Response to the BNT162b2 mRNA COVID-19 Vaccine in a Prospective Study of Healthcare Workers. Open Forum Infect Dis. 2021 Nov 20;9(1):ofab575. doi: 10.1093/ofid/ofab575. PMID: 35047649; PMCID: PMC8759445.

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: Wacharapluesadee, Supaporn

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

POSITION TITLE: Senior Researcher

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
Chiang Mai University, Thailand	B.S.	02/1991	Medical Technology
Mahidol University, Thailand	M.S.	01/1994	Biochemistry
Chulalongkorn University, Thailand	Ph.D.	03/2006	Biomedical Sciences

A. Personal Statement

I have 29 years in public health research and 20+ years of experience in emerging viral zoonoses. I have managed many internationally funded research projects that involve working with and managing international and local interdisciplinary teams. Most of my research projects are diagnostics development, pathogen discovery, public health surveillance, field surveillance in wild mammals, human behavioral risk surveys, and clinical research. I conduct workshops on the development of novel diagnostic approaches, appropriate sample collection and handling for different pathogens, and viral characterization *in vitro* and *in vivo*. I am a senior researcher at the Thai Red Cross Emerging Infectious Diseases Clinical Center, which conducts research on emerging zoonoses, clinical research, and public health surveillance research. My research background mainly focuses on understanding the process of zoonotic disease emergence, particularly viral zoonoses. This includes identifying the bat origin of Nipah virus, MERS-CoV, and SARS-CoV-2 and pathogenesis and diagnoses of Rabies. My studies on the emergence of novel betacoronaviruses found in Thai bats and Nipah virus have been published. My laboratory has supported the Department of Disease Control (DDC) in diagnosing emerging infectious diseases, communicable diseases, and wastewater surveillance. I am the DDC instructor on the clinical sampling method and consultant for Ebola, MERS, and COVID-19 diagnosis. I am a committee member of the Global Laboratory Leadership Program (GLLP) Thailand, organized by the Department of Medical Sciences. I led the team to diagnose the first human MERS case in 2015 and the first human COVID-19 case outside China in January 2020. I have been the PI on six multidisciplinary research projects that use epidemiology, laboratory, field science, and bioinformatics to diagnose and monitor the emergence of wildlife-origin viral zoonoses, including SARS-CoV, Nipah, and Hendra viruses, Avian influenza, and novel viruses from bats. I am also the Thailand country coordinator for PREDICT project, large contracts from USAID during 2010-2019 involving the successful management of teams of virologists, field biologists, veterinarians, epidemiologists, hospitals, and laboratorians.

1. **Wacharapluesadee S**, Buathong R, Iamsirithawon S, Chaifoo W, Ponpinit T, Ruchisrisarod C, Sonpee C, Katasrila P, Yomrat S, Ghai S, Sirivichayakul S, Okada P, Mekha N, Karnkawinpong O, Uttayamakul S, Vachiraphan A, Plipat T, Hemachudha T. Identification of a Novel Pathogen Using Family-Wide PCR: Initial Confirmation of COVID-19 in Thailand. Front Public Health. 2020 Oct 7;8:555013. doi: 10.3389/fpubh.2020.555013. PMID: 33134237; PMCID: PMC7579402.

2. Buathong R, Chaifoo W, Iamsirithaworn S, **Wacharapluesadee S**, Joyjinda Y, Rodpan A, Ampoot W, Putcharoen O, Paitoonpong L, Suwanpimolkul G, Jantarabenjakul W, Petcharat S, Bunprakob S, Ghai S, Prasithsirikul W, Mungaomklang A, Plipat T, Hemachudha T. Multiple clades of SARS-CoV-2 were introduced to Thailand during the first quarter of 2020. Microbiol Immunol. 2021 Oct;65(10):405-409. doi: 10.1111/1348-0421.12883. Epub 2021 Sep 1. PMID: 33835528; PMCID: PMC8251142.
3. **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. Nat Commun. 2021 Feb 9;12(1):972. doi: 10.1038/s41467-021-21240-1. Erratum in: **Nat Commun**. 2021 Feb 25;12(1):1430.
4. **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 Jul 5;3(1):12. doi: 10.1186/s42522-021-00044-9. PMID: 34218820; PMCID: PMC8255096.

B. Positions, Scientific Appointments, and Honors

Positions and Employment

1994 -97 Biochemical Technician, Department of Entomology, AFRIMS, Thailand
1997 Researcher, Department of Immunology, Chulabhorn Research Institute, Thailand
1997 -00 Medical Technologist, The HIV/AIDS Collaboration Thai-US, Thailand
2000 -16 Laboratory Chief, Neuroscience Centre for Research and Development & WHO Collaborating Centre for Research and Training on Viral Zoonoses, Faculty of Medicine, Chulalongkorn University Hospital, Thai Red Cross Society, Thailand
2016 -21 Deputy Chief of Thai Red Cross Emerging Infectious Diseases Health Science Centre, Faculty of Medicine, Chulalongkorn University Hospital
2021 - Senior Researcher, Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital
Committee member, School of Global Health, Faculty of Medicine, Chulalongkorn University

Other Experience and Professional Membership

2010 -14 PREDICT Thailand Country Coordinator
2014 - Thai Ministry of Public Health (MOPH) Ebola Diagnostic Committee
2015 -19 PREDICT 2 Thailand Country Coordinator
2016 - Steering committee, Bat One Health Research Network, BTRP DTRA
2021 - WHO Scientific Advisory Group for the Origins on Novel Pathogens (SAGO)

C. Contributions to Science

1. **Research on One Health.** One Health is an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals, and ecosystems, as the definition by WHO. Our projects are multidisciplinary studies on human surveillance, wildlife surveillance, wastewater surveillance, and human behavior.
   a. Tangwangvivat R, **Wacharapluesadee S**, Pinyopornpanish P, Petcharat S, Muangnoicharoen H. S, Thippamom N, Phiancharoen C, Hirunpatrawong P, Duangkaewkart P, Chaiden C, Wechsirisan W, Wandee N, Srimuang K, Paitoonpong L, Buathong R, Pawun V, Hinjoy S, Putcharoen O, Iamsirithaworn S. Assessment of SARS-CoV-2 variant wastewater detection strategies in the Bangkok Metropolitan region. **Preprint Research** 2023

b. Keusch GT, Amuasi JH, Anderson DE, Daszak P, Eckerle I, Field H, Koopmans M, Lam SK, Das Neves CG, Peiris M, Perlman S, **Wacharapluesadee S**, Yadana S, Saif L. Pandemic origins and a One Health approach to preparedness and prevention: Solutions based on SARS-CoV-2 and other RNA viruses. **Proc Natl Acad Sci USA**. 2022 Oct 18;119(42):e2202871119. doi: 10.1073/pnas.2202871119. Epub 2022 Oct 10. PMID: 36215506; PMCID: PMC9586299.
c. 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 Infect Dis**. 2022 May 16;22(1):472. doi: 10.1186/s12879-022-07439-7. PMID: 35578171; PMCID: PMC9109443.

2. **Research on Public Health Surveillance in Thailand.** Effective disease control programs rely on effective surveillance and response systems. Our laboratory collaborates with the Department of Diseases Control for active surveillance on syndromic surveillance and outbreak investigation. Both molecular and serology surveillance systems were conducted to identify the cause of the disease.
   a. Plipat T, Buathong R, **Wacharapluesadee S**, Siriarayapon P, Pittayawonganon C, Sangsajja C, Kaewpom T, Petcharat S, Ponpinit T, Jumpasri J, Joyjinda Y, Rodpan A, Ghai S, Jittmittraphap A, Khongwichit S, Smith DR, Corman VM, Drosten C, Hemachudha T (2017). Imported case of Middle East respiratory syndrome coronavirus (MERS-CoV) infection from Oman to Thailand, June 2015. **Euro Surveill** 22(33):pii: 30598.
   b. Okada P, Buathong R, Phuygun S, Thanadachakul T, Parnmen S, Wongboot W, Waicharoen S, **Wacharapluesadee S**, Uttayamakul S, Vachiraphan A, Chittaganpitch M, Mekha N, Janejai N, Iamsirithaworn S, Lee RT, Maurer-Stroh S. Early transmission patterns of coronavirus disease 2019 (COVID-19) in travellers from Wuhan to Thailand, January 2020. **Euro Surveill**. 2020 Feb;25(8):2000097. doi: 10.2807/1560-7917.ES.2020.25.8.2000097. PMID: 32127124; PMCID: PMC7055038.
   c. 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 Feb 12;16(2):e0246864. doi: 10.1371/journal.pone.0246864. PMID: 33577615; PMCID: PMC7880427.
   d. Sangkakam A, Hemachudha P, Saraya AW, Thaweethee-Sukjai B, Cheun-Arom T, Latinne A, Olival KJ, **Wacharapluesadee S**. Detection of influenza virus in rectal swabs of patients admitted in hospital for febrile illnesses in Thailand. **SAGE Open Med**. 2021 Jan 22;9:2050312121989631. doi: 10.1177/2050312121989631. PMID: 33552519; PMCID: PMC7841862.

3. **Research on diagnostic development.** The molecular technique is a gold standard method for pathogen diagnosis and confirmation. The first COVID-19 case outside China in Thailand was primarily detected by family PCR and further confirmed by next-generation sequencing (NGS). The SARS-CoV-2 variants can be identified by the NGS technique or by identifying the mutation markers by MassARRAY technology.
   a. **Wacharapluesadee S**, Kaewpom T, Ampoot W, Ghai S, Khamhang W, Worachotsueptrakun K, Wanthong P, Nopvichai C, Supharatpariyakorn T, Putcharoen O, Paitoonpong L, Suwanpimolkul G, Jantarabenjakul W, Hemachudha P, Krichphiphat A, Buathong R, Plipat T, Hemachudha T. Evaluating the efficiency of specimen pooling for PCR-based detection of COVID-19. **J Med Virol**. 2020 Oct;92(10):2193-2199. doi: 10.1002/jmv.26005. Epub 2020 Jul 21. PMID: 32401343; PMCID: PMC7272832.
   b. **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 R. A, Fernandez S, Putcharoen O. Simultaneous Detection of Omicron and Other SARS-CoV-2 Variants by Multiplex PCR MassARRAY Technology. **Preprint Research** 2023. DOI: https://doi.org/10.21203/rs.3.rs-2482226/v1

c. Joyjinda Y, Rodpan A, Chartpituck P, Suthum K, Yaemsakul S, Cheun-Arom T, Bunprakob S, Olival KJ, Stokes MM, Hemachudha T, **Wacharapluesadee S**. First Complete Genome Sequence of Human Coronavirus HKU1 from a Nonill Bat Guano Miner in Thailand. **Microbiol Resour Announc**. 2019 Feb 7;8(6):e01457-18. doi: 10.1128/MRA.01457-18. PMID: 30746519; PMCID: PMC6368654.

d. Siriyasatien P, **Wacharapluesadee S**, Kraivichian K, Suwanbamrung C, Sutthanont N, Cantos-Barreda A, Phumee A. Development and evaluation of a visible reverse transcription-loop-mediated isothermal amplification (RT-LAMP) for the detection of Asian lineage ZIKV in field-caught mosquitoes. **Acta Trop**. 2022 Dec;236:106691. doi: 10.1016/j.actatropica.2022.106691. Epub 2022 Sep 11. PMID: 36103950.

4. **Research on coronavirus prevalence in Thailand.** Numerous high-impact emerging viruses appear to have bat reservoirs. Our surveillance projects study the diversity of coronavirus (CoV) in bats in Thailand. We have isolated and characterized CoVs from many bat species and detected and sequenced CoV in bat guano collectors. Our surveillance studies continue to analyze the drivers of their emergence and risk factors for spillover.
   a. **Wacharapluesadee S**, Duengkae P, Chaiyes A, Kaewpom T, Rodpan A, Yingsakmongkon S, Petcharat S, Phengsakul P, Maneeorn P, Hemachudha T (2019). Longitudinal study of age-specific pattern of coronavirus infection in Lyle's flying fox (*Pteropus lylei*) in Thailand. **Virol J** 20;15(1):38.
   b. **Wacharapluesadee S**, Duengkae P, Rodparn A, Kaewpom T, Maneeorn P, Kanchanasaka B, Yingsakmongkon S, Sittidetboripat N, Chareesaen C, Khlangsap N, Pidthong A, Leadprathom K, Ghai S, Epstein JH, Daszak P, Olival KJ, Blair PJ, Callahan MV and Hemachudha T (2015). Diversity of Coronavirus in Bats from Eastern Thailand. **Virol J** 12(1):57.
   c. **Wacharapluesadee S**, Sintunawa C, Kaewpom T, Khongnomnan K, Olival KJ, Epstein JH, Rodpan A, Sangsri P, Intarut N, Chindamporn A, Suksawa K, Hemachudha T (2013). Group C betacoronavirus in bat guano fertilizer, Thailand. **Emerg Infect Dis** 19(8).

5. **Research on Nipah virus prevalence in Thai bats.** Nipah virus outbreaks, previously in Thailand's neighboring country, Malaysia, and ongoing in Bangladesh, have high mortality rates. Our surveillance projects study the characterization of Nipah Virus (NiV) in bats in Thailand. In addition, our surveillance studies continue to analyze the drivers of their emergence, understanding their seasonal preferences and risk factors for spillover.
   a. **Wacharapluesadee S**, Samseeneam P, Phermpool M, Kaewpom T, Rodpan A, Maneeorn P, Srongmongkol P, Kanchanasaka B, Hemachudha T (2016). Molecular characterization of Nipah virus from Pteropus hypomelanus in Southern Thailand. **Virol J** 13(1):53
   b. **Wacharapluesadee S**, Jittmittraphap A, Yingsakmongkon S, and Hemachudha T (2016). Molecular Detection of Animal Viral Pathogens. Nipah Virus. **CRC Press**.
   c. **Wacharapluesadee S**, Ngamprasertwong T, Kaewpom T, Kattong P, Rodpana A, Wanghongsa S, Hemachudha T (2013). Genetic characterization of Nipah virus from Thai fruit bats (*Pteropus lylei*). **Asian Biomedicine** 7(6);813-819.
   d. 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 Jun;19(2):175-189. doi: 10.1007/s10393-022-01588-6. Epub 2022 Jun 3. PMID: 35657574.
   e. Breed AC, Meers J, Sendow I, Bossart KN, Barr JA, Smith I, **Wacharapluesadee S**, Wang L, Field HE (2013). The Distribution of Henipaviruses in Southeast Asia and Australasia: Is Wallace's Line a Barrier to Nipah Virus? **PLoS One** 8(4):e61316.

6. **Rabies Neuropathogenesis, diagnosis and management.** The center worked for many years on molecular analyses of rabies, including mutational effects and designing primers to detect the Thai street rabies virus. I regularly organize workshops to teach laboratories in the region how to correctly collect specimens and test for rabies.
    a. Hemachudha T, Ugolini G, Sungkarat W, Laothamatas J, Shuangshoti S, **Wacharapluesadee S** (2013). Human Rabies: neuropathogenesis, diagnosis and management. **Lancet Neurology** 498-513.
    b. Shuangshoti S, Thepa N, Phukpattaranont P, Jittmittraphap A, Intarut N, Tepsumethanon V, **Wacharapluesadee S**, Thorner PS, Hemachudha T (2013). Reduced viral burden in paralytic compared to furious canine rabies is associated with prominent inflammation at the brainstem level. **BMC Vet Res** 14;9(1):31.
    c. Virojanapirom P, Khawplod P, Sawangvaree A, **Wacharapluesadee S**, Hemachudha T, Yamada K, Morimoto K, Nishizono A (2012). Molecular analysis of the mutational effects of Thai street rabies virus with increased virulence in mice after passages in the BHK cell line. **Arch Virol** 157(11):2201-5.
    d. Wilde H, Hemachudha T, **Wacharapluesadee S**, Lumlertdacha B, Tepsumethanon V (2013). Rabies in Asia: The Classical Zoonosis. **Curr Top Microbiol Immunol** 365:185-203.

Co-PI Plan (only needed if applying as Co-PIs):

What each Co-PI will contribute to the proposed research study?

As stated in the Mentoring Plan, the co-PIs, Krongkan Srimuang and Spencer Sterling have different educational and training backgrounds. This Co-PI plan is proposed to fill the gaps in knowledge of each scientist and supports a collaborative working relationship for project success. Moreover, this plan was developed to maximize the strengths of each PI. Overall, the project is separated into two objectives, the proposal of this project will be equally managed by Dr. Srimuang and Mr. Sterling. Dr. Srimuang will be responsible for PBMC isolation, sorting, and characterization. Mr. Sterling will be responsible for the technical serological aspects of the project. Both will work closely with the Research Center Mentor, Dr. Eric Laing, to develop this project, protocol development, and network connections. Dr. Supaporn Wacharapluesade, the Co-mentor, will support the wildlife sampling, samples collection, and molecular aspects within Thailand.

How the Co-PIs will jointly work with the affiliated Research Center?

Dr. Srimuang is currently employed at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University and supports the molecular biology investigations in the EID-SEARCH project. Mr. Sterling is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region. Both Co-PIs will work closely with Dr. Laing, Dr. Wacharapluesadee, and the staff at EcoHealth Alliance to coordinate field sampling, research activities, data analysis, and manuscript production.

How the Co-PIs will jointly manage the proposed study?

This is a great opportunity for both PIs to undergo training in advanced techniques and develop skills in next-generation serology and molecular sequencing. Dr. Srimuang will learn and practice serologic techniques and data analysis from Mr. Sterling. Mr. Sterling will develop skills in cell isolation and molecular sequencing techniques from Dr. Srimuang. Moreover, Mr. Sterling will coordinate planning, sample processing, data analysis, and report preparation for this project with Dr. Srimuang.

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3. Lee, S. H., Kim, K., Kim, J., No, J. S., Park, K., Budhathoki, S., Lee, S. H., Lee, J., Cho, S. H., Cho, S., Lee, G. Y., Hwang, J., Kim, H. C., Klein, T. A., Uhm, C. S., Kim, W. K., & Song, J. W. (2021). Discovery and Genetic Characterization of Novel Paramyxoviruses Related to the Genus Henipavirus in Crocidura Species in the Republic of Korea. Viruses, 13(10), 2020. https://doi.org/10.3390/v13102020
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5. Uwishema, O., Wellington, J., Berjaoui, C., Muoka, K. O., Onyeaka, C. V. P., & Onyeaka, H. (2022). A short communication of Nipah virus outbreak in India: An urgent rising concern. *Annals of medicine and surgery (2012)*, *82*, 104599. https://doi.org/10.1016/j.amsu.2022.104599
6. Bossart, K. N., Tachedjian, M., McEachern, J. A., Crameri, G., Zhu, Z., Dimitrov, D. S., Broder, C. C., & Wang, L. F. (2008). Functional studies of host-specific ephrin-B ligands as Henipavirus receptors. Virology, 372(2), 357–371. https://doi.org/10.1016/j.virol.2007.11.011
7. Middleton, D. J., & Weingartl, H. M. (2012). Henipaviruses in their natural animal hosts. Current topics in microbiology and immunology, 359, 105–121. https://doi.org/10.1007/82_2012_210
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List of abbreviations, acronyms, symbols

AngV: Angavokely virus
BCR: B cell receptor
BSc: Bachelor of Science
BSL2: Biological Safety level 2
BSL3: Biological Safety level 3
cDNA: Complementary DNA
CedV: Cedar virus
Co-PI: Co-principal investigator
CREID: Centers for Research in Emerging Infectious Diseases
DARV: Daeryong virus
DNA: Deoxyribonucleic acid
EHA: EcoHealth Alliance
EID: Emerging Infectious Disease
EID-SEARCH: The Emerging Infectious Disease – South East Asia Research Collaboration Hub
ELISA: Enzyme-linked immunosorbent assay
F: Fusion protein
FACS: Fluorescence-activated cell sorting
FUO: Fever of unknown origin
GAKV: Gamak virus
GhV: Ghana virus
HeV: Hendra virus
HNVs: Henipaviruses
ILI: Influenza-like illness
KCMH: King Chulalongkorn Memorial Hospital
L: large protein
LayV: Langya virus
M: Matrix protein
mAb: Monoclonal antibody
MD: Maryland
MMIA: Multiplex microsphere-based immunoassay
MojV: Mojiang virus
MSc: Master of Science
N: Nucleocapsid
NGS: Next-generation sequencing
NIAID: National Institute of Allergy and Infectious Diseases
NIH: National Institutes of Health
NiV: Nipah virus
P: Phosphoprotein
PBMC: Peripheral blood mononuclear cells
PCR: Polymerase Chain Reaction
PhD: Doctor of Philosophy
PI: Principal investigator
RBP: Receptor-binding protein
RNA: Ribonucleic acid
RT-PCR: Real-time PCR
SARI/ARI: Severe/acute respiratory illness
SARS-CoV-1: Severe acute respiratory syndrome coronavirus 1
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2
SEA: Southeast Asia

Thai-DDC: Department of Disease Control of Thailand
TRC-EIDCC: Thai Red Cross. Emerging Infectious Diseases. Clinical Center
USA: United States
WGS: Whole Genome Sequencing

Facilities and Resources

FACILITIES: Specify the facilities to be used for the conduct of the proposed research.

Laboratory:

The Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC), King Chulalongkorn Memorial Hospital (KCMH) was established by the Committee of the Emerging Infectious Disease Center which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. TRC-EIDCC laboratory facilities include 3 biosafety cabinets (BSL 2, BSL 2+, BSL2+), 3 automated extraction machines, 2 thermocyclers (Proflex), 2 real-time PCR machines (CFX, Bio-rad), 3 Freezers (-80 °C), 2 refrigerators; 4 °C, 2 autoclaves. Our EIDCC uses the Pathogen Asset Control System (PACS) system for sample tracking. Moreover, our Faculty of Medicine, Chulalongkorn University has a central laboratory, the Chula Medical Research Center (Chula MRC), which provide many instruments for medical, masters, and graduate students, Post-doctoral fellows, and researchers at Chulalongkorn University and KCMH. The Chula MRC laboratory facilities are divided into molecular diagnostics (PCR Thermal Cycler, QStudio6 Flex Real-Time PCR, Droplet Digital PCR, GeneXpert, Film array, Illumina MiSeq), immunology facilities (ELISA testing equipment (reader & washer), Luminex, Flow Cytometry), cell sorting machine and a BSL3 laboratory. Moreover, the facility provides general equipment, such as autoclaves, balances, CO2 incubators, Dual Fluorescence Cell Counter, 4 °C refrigerators, -20°C, and -80°C freezers.

Clinical:

KCMH is a 1,000-bed university hospital, in close coordination with the Faculty of Medicine, Chulalongkorn University. The Thai Red Cross Society provides medical treatment, rehabilitation, health promotion and disease prevention services to the general public and the vulnerable. It also provides biopharmaceutical and sterile medicines that are sufficient to meet the needs of patients and safe in accordance with international standards. It serves as a center for providing eye and organ donation services to patients nationwide in line with international standards. It has specialized doctors and public health personnel and also produces specialized nurses. The TRC-EIDCC in the KCMH provides standard medical care to patients with emerging and recurrent diseases and disease spreading prevention.

Animal:

TRC-EIDCC also collaborates with the Department of National Parks, Wildlife and Plant Conservation for detecting the emerging novel pathogens from animals such as bats, rodents, and pangolins in our country.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer, Lee

Computer:

TRC-EIDCC used the service from the Office of Information Technology, Chulalongkorn University that provides the application, network, and software such as Office 365, VPN, WIFI, Microsoft Office, Microsoft Windows, SPSS, Adobe, Zoom Meeting, etc. Moreover, our EIDCC use the Pathogen Asset Control System (PACS) for sample tracking.

Office:

The office and laboratory space are located at the EIDCC at 4th Floor, Jongkolnee Watwong Building and 6th Floor, Aor Por Ror Building, respectively. The applicants will have dedicated desks in both buildings.

Other:

PI Name: Srimuang, Krongkan and Sterling, Spencer, Lee **Foreign Site:** Thailand
CREID CC Grant: 1U01AI151378

Foreign Site Information

If the Pilot Award will have multiple foreign sites, complete this form for EACH research site

Principal Investigator Name:	Krongkan Srimuang
Project Title:	Immune memory bait & capture to identify emerging henipavirus origins
Institution:	King Chulalongkorn Memorial Hospital
Foreign Research Site:	Rama 4 Road, Pathumwan, Bangkok 10330 Thailand
Point of Contact for Research Site:	Krongkan Srimuang Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital Rama 4 Road, Pathumwan, Bangkok 10330 Thailand krongkan.sr@gmail.com

Introduction (3-4 sentences):

Provide a simple description of the overall goals of the project including the work that will be done at the foreign site. Indicate if any of the NIAID grant funds will be sent to the foreign site.

The goal of this project is to identify the origin of zoonotic pathogens by applying serologic and cellular immune tools in humans and animals for early detection and identification of novel pathogens to prevent the next pandemic.

To achieve this, the project will conduct serological surveillance among humans and rodents/shrews at this site, and perform lab analysis collaborating with NIH PREMISE for antibody-mediated bait & capture of Thai MojV-like virus.

$149,937 of grant funds will be sent to the site for these research activities.

Foreign component (3-4 sentences):

Describe the specific role of the foreign site (i.e. conduct critical experiments?, conduct data analyses?, provide consultations?, provide samples?, study human and/or animal populations?, conduct observational or interventional clinical trials?, collect samples and/or data to be brought to the US?).

The site will 1) conduct all field work to collect human and animal samples from the study sites; 2) perform laboratory analysis using collected samples; and 3) conduct data analysis as proposed in the project.

Human Subjects (1 word or 1 sentence per bullet):

- **Parent Study IRB approval**
  - **IRB approval number for parent study:** #894ECOH21b (US); #221/64 (Thailand)
  - **IRB approval date:** 12-05-2021 (US); 08-06-2021 (Thailand)
  - **Human Subject Assurance Number: (FWA)#:** #00001102 (external IRB); #00022431 (EcoHealth Alliance); #00000943 (Chulalongkorn University)
- **Does this study require a modification to the IRB approval of a parent study (Yes or No)?**
  - **Yes**

- **Will existing samples from human subjects will be used: (Yes or No)?**
  - **No**
  - **How many subjects provided the existing samples to be used?** N/A
- **Will human subjects be recruited (Yes or No)**
  - **Yes**
  - **Number of human subjects that will be recruited:** 110

- **Population parameters:**
  - **Gender:** 55 males, 55 females
  - **Age Group:** Age >=18 years who provide informed consent.
  - **Race/Ethnicity:** 110 Asian

- **Sample collection will include:**
  - **Blood:** Yes
  - **Urine:** No
  - **Tissues:** No
  - **Other samples (describe):** Nasopharyngeal swab and throat swab

- **Sample collection will be completed in how many visits:** 2 trips

- **Will samples be de-identified (Yes or No)? If No, describe how they will be protected.**
  - Yes

- **Will local IRB approval be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals being sought or state why local IRB approval is not required.**
  - Yes

- **Will informed consent be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals/consents being sought or state why informed consent is not required.**
  - Yes

- **Will samples be brought back to the US (Yes or No)?**
  - No

- **Will data be brought back to the US (Yes or No)?**
  - Yes, some of the data will be shared with EID-SEARCH partners in the US for relevant analysis.

Animal Subjects (1 word or 1 sentence per bullet):

- **Parent study IACUC approval**
  - **IACUC approval number for parent study:** #G2020-42 (US); #019/2563 (Thailand)
  - **IACUC approval date:** 05-05-2020 (US); 03-12-2020 (Thailand)
    Animal Welfare Assurance Number: D16-00572 (A4059-01)

- **Does this study require a modification to the IACUC approval of a parent study (Yes or No)?**
  - **No**

- **Will existing samples from animal subjects will be used: (Yes or No)?**
  - **No**
  - **How many animal subjects provided the existing samples to be used?** N/A
- **Will vertebrate animals be collected (Yes or No)?**
  - **Yes**

- **Species of animals (e.g. rats, mice, rabbits, monkeys):** Rodents and shrews
- **Animal parameters:**
  - **Total number of animals:** 200 (200 rodents and shrews)
  - **Gender:** 100 males, 100 females
  - **Age range:** 4 - 12 months and elder depending upon species
  - **Lab strain (e.g. Sprague-Dawley rats, Balb/C mice):** None
  - **Wild animals procured in country (e.g. Rhesus monkeys from a reserve):** No

- **What will be done to them or with them and how often?**
  - Free-ranging rodents and shrews will be captured through pit traps and box traps.
  - Anesthesia will be conducted for captive rodents and shrews.
  - Once anesthetized blood will be collected.
  - All actions will only be performed one time for each animal individual.
  - All animals will be released after sampling.

- **What are the follow-ups?**
  - No follow-up relevant as per protocol, since all animals will be released after sampling ups

- **What will be their fate at the end of the experiments – will they be euthanized?**
  - All animals will not be held longer than 6 hours (typically less than 3 hours) during the sampling process and released after sampling.

- **Will in-country clearances be obtained prior to engaging in any animal research (Yes or No)? If No, describe alternate approvals or state why in-country clearances are not required.**
  - Yes
- **Will samples be brought back to the US (Yes or No)?**
  - No
- **Will data be brought back to the US (Yes or No)?**
  - Yes, some of the data will be shared with EID-SEARCH partners in the US for relevant analysis.

27th January 2022

Krongkan Srimuang Ph.D.
Research Scientist
Thai Red Cross Emerging Infectious Diseases Clinical Center
King Chulalongkorn Memorial Hospital
Rama 4 Road
Pathumwan, Bangkok 10330
Thailand

Subject: Letter of Support for CREID Network Pilot Research Program

Dear Dr. Srimuang,

I am writing to express my support for your CREID Network Pilot Research Program proposal titled, "Immune memory bait and capture to identify emerging henipavirus origins." Your preliminary data demonstrating immunoreactivity against Mojiang henipavirus in individuals sampled as part of the Emerging Infectious Diseases Southeast Asia Research Collaborative Hub (EID-SEARCH) center, provides compelling serologic evidence that novel henipaviruses spill over into human populations. Point-of-care diagnostics and nucleic acid-amplification tests rely on our present understanding of medically relevant pathogens. Thus, emergence of novel zoonotic viruses may go undetected. Serological profiling provides us an ability to measure the human infectome through broad-scale peptide arrays or targeted antigen-based detection.

The research activities conducted at the Thai Red Cross Emerging Infectious Diseases Clinical Center are complementary to the focus of the Vaccine Research Center's PREMISE (Pandemic REsponse REpository through Microbial and Immunological Surveillance and Epidemiology) program. PREMISE works closely with intramural and extramural NIH programs and in partnership with a global network of investigators and collaborators to achieve its objectives through 3 core activities: (1) genetic analysis of zoonotic reservoirs and symptomatic humans for pathogen discovery; (2) development of high throughput, multiplexed serologic and cellular assays; and (3) global blood sampling to identify antigen-specific and cross-reactive immune responses to known and previously unknown viruses of pandemic potential. Biological samples including serum and PBMC are obtained from broad-based population cohorts as well as targeted populations at risk from emerging infections. At the same time, sequence data from known and unknown (through virus discovery) pathogens are analyzed to design and express candidate antigenic proteins of interest. These proteins are then used in high throughput multiplexed assays to screen sera for antibody reactivity. Serum reactivity to candidate antigens is followed by further interrogation of the adaptive immune response at the cellular level using corresponding PBMC samples. Proteins used in the serological arrays are conjugated to fluorophores and used as probes to sort antigen-specific B cells by flow cytometry. In addition, the sequence of such candidate antigens provides the template for the design of overlapping peptide pools to assess T cell responses. Further analysis allows for the identification of

neutralizing antibodies specific for the target antigens as well as specific T cell epitopes which may be tested in animal models. Epitope identification informs and guides immunogen design. At the conclusion of this pipeline of assays and analysis, PREMISE delivers the following:

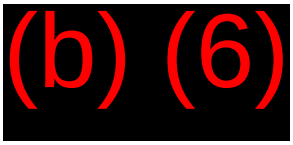
1. reagent and data resources for early detection and diagnosis
2. monoclonal antibodies with therapeutic potential
3. candidate immunogens for further vaccine development

Thus, PREMISE serves as a translational vehicle to integrate serologic and cellular immune discovery, targeting a broad array of pathogens, into product development, and constitutes an anticipatory reagent repository to accelerate the global response to pandemic threats.

As investigators in the Vaccine Research Center at the National Institute of Allergy and Infectious Diseases (NIAID), we are not eligible to be listed as a "co-investigator" on the application but look forward to participating as collaborators. All materials from us will be provided under an approved material transfer or other collaborative agreement. This collaboration is part of our official duties as employees at the NIAID, and no funds from the grant will be used in intramural research, neither will we accept any form of renumeration, whether in the form of salary, honoraria, or travel expenses. We will provide scientific input and mentoring but will not have any duties associated with programmatic stewardship, which will be performed by NIAID extramural program officials. Further, in keeping with the mission of NIAID to promote and facilitate biomedical research and the dissemination of new knowledge, we would supply requested research materials and technical expertise not only to you, but also to other interested and qualified parties for research purposes.

My group has met with Dr. Eric Laing (USU) one of the two mentors for this pilot research program to discuss ways we can collaborate and support your research application. We are enthusiastic about providing the technological and subject-expertise in immunology and translational therapeutic discovery afforded by the PREMISE program that would enhance your knowledge base and broaden your own scientific training.

Sincerely,



Daniel C. Douek

Daniel Douek MD PhD | Chief, Human Immunology Section | Vaccine Research Center
National Institute of Allergy and Infectious Diseases | National Institutes of Health
40 Convent Drive, Bethesda, MD 20892-3022 | Office: +1 301 594 8484

30 January 2023

Dr. Krongkan Srimuang & Mr. Spencer Sterling

Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC),

King Chulalongkorn Memorial Hospital

Object: Thai Red Cross Emerging Infectious Diseases Clinical Center support for your application to the CREID Pilot Research Program, entitled "Immune memory bait & capture to identify emerging henipavirus origins."

Dear Dr. Srimuang & Mr. Sterling,

Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC) is pleased to support your application to the **CREID Pilot Research Program**, entitled "Immune memory bait & capture to identify emerging henipavirus origins." We are fully committed to executing the statement of work to study zoonotic reservoirs for pathogen origins by using serologic and cellular immune discovery in humans and wildlife and their application as a tool for early detection and identification of novel pathogens in pandemic prevention.

The work proposed in this project is a great opportunity for collaboration with our team, it serves to enhance the use of serology and immunology for the detection of novel virus pathogens in Thailand using new technology and techniques. Moreover, the building of capacity and capability in our laboratory and Faculty will enhance the regional capacity, and the Chulalongkorn network will be used to ensure the success and completion of this project.

This letter expresses my strong interest in you pursuing this application and I fully support the project. I look forward to collaborating with you on this project and I wish you success in the CREID Pilot Research Program application.

Sincerely,

Assistant Professor Opass Putcharoen, MD, Msc

Head of Thai Red Cross Emerging Infectious Diseases Clinical Center

King Chulalongkorn Memorial Hospital

EID-SEARCH
Emerging Infectious Diseases
South East Asia Research Collaboration Hub

Dr. Krongkan Srimuang, Mr. Spencer Sterling
Thai Red Cross Emerging Infectious Diseases Clinical Center
King Chulalongkorn Memorial Hospital
1873, Rama 4 Road, Pathumwan
Bangkok, Thailand 10330

Dear Krongkan and Spencer,

This letter conveys a strong interest from the Emerging Infectious Disease-Southeast Asia Research Collaboration Hub (EID-SEARCH) at EcoHealth Alliance to collaborate with you at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital on the proposed project titled "*Immune memory bait & capture to identify emerging henipavirus origins"* in Thailand.

The question of the origin of emerging Henipaviruses (HPVs) was identified from the EID-SEARCH human surveillance when a Mojiang-related virus was identified in a local population highly exposed to bats in Thailand. Faced with the challenges of PCR detection and viral isolation, this project will establish and validate a new serological approach to develop MojV-like virus-specific MAbs and define the antigenic spatial relationships between all known rodent- and bat-borne HPVs, revealing the serologic signature of all related undiscovered HPVs. This will significantly contribute to the objectives of EID-SEARCH to conduct targeted and strategic surveillance to identify emerging pathogens with zoonotic potential at the high-risk human-animal interfaces. The research population and site for this project – bat guano collection – represents one of the key scenarios where zoonotic spillover is mostly concerned that requires further in-depth investigation to identify the reservoir hosts. In addition to the gained knowledge, established methods from this project will be shared and used as standards in zoonotic HPVs surveillance and prevention strategies in Thailand and regionally through collaborative and coordinative cooperation among multiple stakeholders.

I am very excited to work with you both on this project, given your critical roles in the previous surveillance work to identify the question and develop the research idea. This project will bring valuable insights to advance disease surveillance and early warning systems for endemic and emerging infectious diseases in Thailand. Members of EID-SEARCH are committed to working closely with you to develop the research project and support the efforts necessary for the success of this project. You will be invited to join all training conducted by EID-SEARCH and EcoHealth Alliance regarding emerging infectious disease surveillance, relevant statistical analysis, and grant writing to expand your skill set. You'll also be supported to present the findings from this research at international conferences, the CREID Network meetings, and external partners and produce high-quality publications from this research.

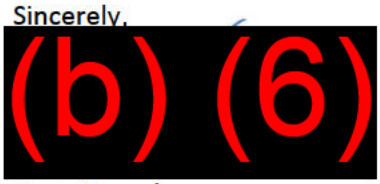
Developing and implementing this project will advance your skills in project management for future research as an independent researcher. Co-Investigators of EID-SEARCH, Dr. Eric Laing from the Uniformed Services University of the Health Sciences and Dr. Supaporn Wacharapluesadee from the Thai Red Cross Emerging Infectious Diseases Clinical Center, have enthusiastically joined the project as your Mentors. Dr. Laing will advise on the study design and provide training on data analysis, and Dr. Wacharapluesadee from your institute will work closely with you to advise on the day-to-day project implementation. Both Mentors will work with you for manuscript writing and provide pertinent and timely career advice to assist in your professional development. They will bring a well-established

EID-SEARCH
Emerging Infectious Diseases
South East Asia Research Collaboration Hub

network of leaders and experts in emerging infectious disease research in Thailand, Southeast Asia, and North America for your network building.

I am confident that this proposed project will bring genuine opportunities for your professional development. And the combined experience in the field and lab and interdisciplinary expertise in serology and public health from the co-PIs will make this a successful and purposeful project to bring public health impacts in a broad region. I look forward to collaborating with you and your team on all phases of this proposed project. I wish you success in the CREID Pilot Research Program application.

Sincerely,



Peter Daszak
Principal Investigator, EID-SEARCH
President, EcoHealth Alliance

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799
www.usuhs.edu

January 27, 2023

Krongkan Srimuang, Ph.D.
Research Scientist
Thai Red Cross Emerging Infectious Diseases Clinical Center
King Chulalongkorn Memorial Hospital
Rama 4 Road
Pathumwan, Bangkok 10330

Subject: Letter of Support for CREID Network Pilot Research Program

Dear Dr. Srimaung,

I am writing to express my enthusiastic support for your CREID Network Pilot Research Program proposal titled, “Immune memory bait & capture to identify emerging henipavirus origins.” In 2018, I led a training and multiplex serology technology transfer to your mentor Dr. Wacharapluesadee’s research group via DTRA-funded project Chulalongkorn Luminex Training and Research Preparedness (HDTRA1-17-C-0019), and was excited to leverage those activities into ongoing research within the EID-SEARCH. Since 2015 I have been developing serological-approaches for biosurveillance of zoonotic viruses in wildlife and human populations, and I am excited to serve as a co-mentor for this pilot program application.

I hope to provide you with the research and career developmental support for the activities outlined in the proposal. The serological data on henipavirus exposure in Thai guano farmers provides compelling evidence that Mojiang-like henipaviruses can cross the species barrier and may be widely distributed in Southeast Asia. The research aims detailed in the proposal will facilitate critical follow-up sampling of this human cohort to identify whether acute infection causes clinical disease, estimate the sero-attack rate of this novel henipavirus, and will apply a cutting-edge serologic and immunologic approach for monoclonal antibody development and novel emergent virus immunogenic characterization. If awarded, a collaboration with VRC PREMISE program scientists will facilitate an important opportunity to broaden your knowledge base of cellular immunology and B cell biology. You have an extensive background in next-generation sequencing application, and through this proposal would gain a considerable understanding of how next-generation sequencing is used for BCR-sequencing, IgG characterization and monoclonal antibody discovery.

Your proposal is an excellent extension of the EID-SEARCH activities and significant training opportunity.

Sincerely,

(b) (6)

Eric D. Laing
Assistant Professor
Department of Microbiology and Immunology
Uniformed Services University

30th January, 2022

Dr. Krongkan Srimuang & Mr. Spencer Sterling

Thai Red Cross Emerging Infectious

Diseases Clinical Centre (TRC-EIDCC),

King Chulalongkorn Memorial Hospital

Dear Dr. Srimuang & Mr. Sterling,

I am writing in support as Research Center Mentor for your application to the CREID Pilot Research Program, entitled "Immune memory bait & capture to identify emerging henipavirus origins."

With my 20 years of experience in conducting surveillance for novel viruses in wildlife, humans, and bats using molecular technology, I will support the growth of the scientists in the CREID Pilot Research Program. The research aims have the potential to significantly enhance our approach to pathogen surveillance at high-risk human-animal interfaces. Given the understanding of pathogen origins by using serologic and cellular immune discovery in humans, and applying these techniques as a tool for early detection and identification of novel pathogens.

As the head laboratory cheif at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, I believe that this is a great opportunity for our Thailand team to train in the characterization of memory B cells from peripheral blood mononuclear cells (PBMC) and enhanced serological surveillance in novel virus pathogens. Moreover, our laboratory has supported the Department of Disease Control (DDC) in diagnosing emerging infectious diseases and communicable diseases such as Ebola, MERS, and COVID-19. I am also a committee member of the Global Laboratory Leadership Program (GLLP) Thailand, organized by the Department of Medical Sciences. With my experience in diagnosing the first human MERS case in Thailand in 2015 and the first human COVID-19 case outside China in January 2020, I will support multidisciplinary projects involving epidemiology, laboratory, field science, and bioinformatics to diagnose and monitor the emergence of wildlife-origin viral zoonoses, including SARS-CoV, Nipah and Hendra viruses, Avian influenza, and novel viruses from bats. I was also the Thailand country coordinator for the PREDICT project, large contracts from USAID during 2010-2019 involving the successful management of teams of virologists, field biologists, veterinarians, epidemiologists, hospitals, and laboratory staff. I will enthusiastically support the development of a strong knowledge base to build capacity and capability in this project and our team in Thailand.

The CREID Network and the Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH) will support scientists in regions around the world where emerging and reemerging infectious disease outbreaks. Many Southeast Asian partnerships in EID-SEARCH have strong potential to enhance innovations in this area.

I believe that Dr. Laing, and I will provide scientific skills, expertise, and networks for this project.

Towards that aim, I am committed to supporting your professional development in the following ways:

1. I will maintain regular bi-weekly meetings for the project progresses.
2. I will support the resources in our country and research team in this project.
3. I will create opportunities for scientists network to ensure the success of research project.
4. I will provide and share advice and technical expertise in specific knowledge for this project.

I'm very much looking forward to working closely with you on this 1-year research project and beyond as you continue to develop your surveillance zoonotic disease research program.

Best regards,

(b) (6)

Supaporn Wacharapluesadee, Ph.D.

Head Laboratory,

Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC),

King Chulalongkorn Memorial Hospital,

Faculty of Medicine,

Chulalongkorn University.

Proposal Cover Sheet

Project Title	Immune memory bait & capture to identify emerging henipavirus origins
Principal Investigator Name	Krongkan Srimuang, Ph.D.
Position/Title	Research Scientist
Department	Thai Red Cross Emerging Infectious Diseases Clinical Center
Institution Name	King Chulalongkorn Memorial Hospital
Street	Rama 4 Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	Krongkan.sr@gmail.com
Phone	+66 (0) 92-453-0808
Country(ies) where work will be conducted	Thailand
Pathogen(s) focus	Mojiang Virus, Henipavirus, Paramyxovirus
Co-Principal Investigator Name (*If applicable*)	Spencer Lee Sterling, MPH
Position/Title	Scientific Project Coordinator
Department	Microbiology and Immunology
Institution Name	Uniformed Services University of the Health Sciences
Street	Jones Bridge Road
City, State, Zip Code	Bethesda, MD 20814
Country	USA
Email	spencer.sterling.ctr@usuhs.edu
Phone	+66 (0) 83-494-5980

Contact information for institution(s) that would financially manage the award *(please note: changing the managing institution upon award may result in substantial funding delays)*	
Contractual Contact, Title	Chula Unisearch
Institution Name	Chulalongkorn University
Street	254 Phyathai Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	unisearch@chula.ac.th
Phone	0 2218 2880
For Co-Principal Investigator (If applicable):	
Contractual Contact, Title	
Institution Name	
Street	
City, State, Zip Code	
Country	
Email	
Phone	

Name of Mentor	Eric Laing, Ph.D.
Mentor Institution	Uniformed Services University of the Health Sciences
Institution Address	Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814
Permanent location of Mentor	4301 Jones Bridge Road, Bethesda, MD 20814
Mentor email	eric.laing@usuhs.edu
Mentor phone	+1 (301) 980-8192
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Name of Mentor	Supaporn Wacharapluesadee, Ph.D.
Mentor Institution	Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital
Institution Address	1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Permanent location of Mentor	4th Floor, Jongkolnee Watwong Building, Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital, 1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Mentor email	spwa@hotmail.com
Mentor phone	+66 (0) 89-920-6292
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Total Budget	***Repeat this table if there are two Co-PIs***
Direct Costs	$138,831
Indirect Costs	$11,106
Proposed Start Date	1 May 2023
Proposed End Date	30 April 2024

Project Abstract (250 words)

Prototypical henipaviruses (Hendra and Nipah virus) are bat-borne zoonotic viruses and cause high mortality. The recent detection of closely related Mojiang and Langya viruses in rats and shrews, and isolation of Langya virus from individuals with acute febrile illness, challenges the dogma that fruit bats are the sole henipavirus reservoirs. The recently expanded *Henipavirus* genus includes two other shrew-borne viruses (Gamak and Daeryong viruses), and the Madagascan fruit bat-borne Angovkeely virus. As part of EID-SEARCH activities, we found serological evidence of infection by a Mojiang-related virus in a community of Thai bat guano collectors who have occupational exposure to microbats and rodents. Identifying the wildlife reservoir of this likely novel virus will assist intervention strategies to reduce spillover risk. However, PCR detection or virus isolation in wildlife is challenging, and new serological approaches may be more successful in identifying reservoir hosts. This project aims to identify novel viruses and zoonotic reservoirs through serologic and cellular immune discovery. We will conduct follow-up human and wildlife shrews and rodents) surveillance in the province where the seropositive humans were identified and apply an expanded serological test and genomic sequencing to detect this novel MojV-like henipavirus. Further, we

plan to isolate anti-MojV monoclonal antibodies (mAb) from peripheral blood mononuclear cells collected from human study participants we have identified as immunoreactive with MojV antigens, and characterize the cross reactive and cross neutralizing potential of these mAbs to LayV.

Project Title **Immune memory bait & capture to identify emerging henipavirus origins**

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Study Personnel

Krongkan Srimuang, Ph.D., is a researcher at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital and supports the Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia (EID-SEARCH) research. Dr. Srimuang is also a medical technologist working to identify, detect and characterize the spill-over risk of high zoonotic potential viruses from wildlife and humans using serology techniques, Next-generation sequencing, and PCR assays to identify novel viruses. Dr. Srimuang also performs the project Smart Sentinel Surveillance System: Emerging Viral Pneumonia and Respiratory tract infections (RTIs) to identify, detect and characterize the novel/known Respiratory Viruses in our country, Thailand by using multiplex real-time PCR, family PCR and Next-generation sequencing of novel viral pathogens. Dr. Srimuang graduated with Ph.D. in Tropical Medicine from the department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University.

Spencer Sterling, MPH, is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region. Mr. Sterling will planning, sample processing, data analysis, and report preparation for this project.

Dr. Eric Laing will act as mentor for Dr. Srimuang and Mr. Sterling under the Centers for Research in Emerging Infectious Diseases (CREID) Pilot Research Program. Dr. Laing is an assistant professor dedicated to biosurveillance and virological research of emergent zoonotic viruses at Uniformed Services University, Bethesda, MD. Part of Dr. Laing's research focus in on the development of serological-based approaches to identify the infectome of wildlife and human communities for the assessment of virus spillovers. Dr. Laing has worked collaboratively with national and international researchers including institutional partners at Duke-NUS, Chulalongkorn University, University of Pretoria, and Rocky Mountain Labs towards these goals. Through these collaborations, Dr. Laing's research group aims to identify known and unknown viruses, the wildlife reservoirs of priority pathogens, the high-risk interfaces for spillover and the biotic/abiotic drivers of virus emergence.

Dr. Supaporn Wacharapluesadee, Ph.D. is the head laboratory at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, a WHO Collaborating Centre for Research and Training on Viral Zoonoses. Dr. Wacharapluesadee research interests include emerging infectious diseases in bats (including Nipah, Rabies, Coronavirus, and novel pathogens) as well as molecular diagnoses and sequencing of viruses. During the past 10 years, Dr. Wacharapluesadee got funding from USAID PREDICT to conduct the surveillance for novel virus in wildlife and human, and from Biological Threat Reduction Program US DTRA to conduct the EID surveillance in bat using the molecular technology and multiplex serology panel (Luminex technology). TRC-EIDCC is responsible for molecular diagnoses services to the hospital, and is the reference laboratory for rabies virus, MERS-CoV, Ebola, Zika, and other infectious diseases diagnoses for Ministry of Public Health, Thailand and laboratory training center on national and regional level. Dr. Wacharapluesadee has served and consulted on several WHO and Thai government committees.

Specific Aims

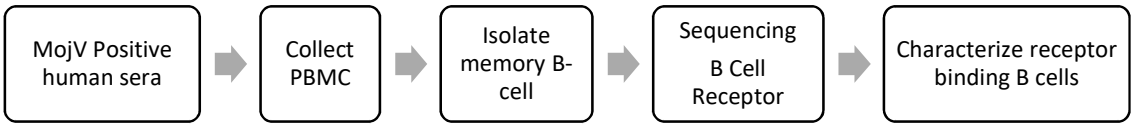
The recent discovery of novel shrew and rodent-borne henipaviruses in East and Southeast Asia has highlighted gaps in our understanding of henipavirus transmission and underscores the importance of surveillance activities in the region. In particular the isolation of Langya virus from acutely ill humans and shrews demonstrated the need to further surveillance and therapeutic development against emergent henipaviruses. Therefore, **this project aims to expand the study of known reservoirs of zoonotic pathogen origin by using genetic and serologic techniques, and to use cellular immune discovery in seropositive humans as a tool to determine henipaviral antigenic relatedness to assist with the functional assessment of current therapeutics as and their effect on these novel viruses.**

In our study, we detected that a high seroprevalence against MojV in human participants near the Khao Chong Phran Non-hunting Area in Ratchaburi province who we identified as representing a high risk population for zoonotic spillover. Yet, no virus sequences were detected. We hypothesize that (i) there is a novel henipavirus present in the local wildlife population that has spilled over into the human population at least once, and (ii) the antibodies from these seropositive people will react with the novel shrew-borne henipaviruses in a way that can inform us on therapeutic approaches for future outbreaks. We will follow-up with persons who had high anti-MojV IgG levels, sample new participants within this community who interact with the local wildlife, and sample shrews and rodents to identify the wildlife source of the MojV-like henipavirus. Additionally, in collaboration with NIH VRC PREMISE, MojV receptor binding protein (RBP) and fusion protein-reactive B cell will be isolated from previously-identified seropositive human participants. MojV-like virus-specific B-cell receptors will be sequences and monoclonal antibodies specific to the infecting MojV-like virus RBP and F protein will be generated and tested for cross reactivity against presently described rodent associated henipavirus envelope glycoproteins, and cross neutralization against Langya virus.

The proposed project has two aims:

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population.

Aim 2. Characterization of monoclonal antibodies from humans with serological profiles consistent with MojV infection.



The proposed project has two objectives:

Objective 1. To use metagenomic and targeted sequencing approaches in human and wildlife populations to detect the Thai MojV-like virus.

Objective 2. To isolate and sequence memory B-cells from humans positive for Mojiang virus in Ratchaburi province, Thailand for antibody discovery.

Study Rationale

Nipah virus (NiV) and henipaviral diseases are among the most virulent of zoonotic diseases. The genus *Henipavirus* is currently composed of nine viruses including the prototypes, NiV and Hendra virus (HeV), and Cedar virus (CedV), Ghana virus (GhV), Mojiang virus (MojV) (McLinton et al., 2017), Langya virus (LayV) (Zhang X et al., 2022), Gamak virus (GAKV), Daeryong virus (DARV) (Lee et al., 2021), and Angavokely virus (AngV) (Madera et al., 2022). HeV was first identified in Australia in 1994 as a cause of respiratory illness and fatal encephalitis in humans and horses. Closely following the discovery of HeV, NiV was identified in 1999 in Malaysia after outbreaks of fatal encephalitis in humans, with swine determined to be the intermediate host. Annual outbreaks of NiV have occurred in Bangladesh and are associated with the consumption of raw date palm sap, and since 19 May 2018 annual outbreaks of NiV associated encephalitis of been recorded in Kerala, India (Uwishema et al., 2022). Both HeV and NiV have a broad host range capable of infecting several mammalian orders, and can enter and infect endothelial cells, vasculature, and the central nervous system leading to severe multisystem organ diseases and high mortality. The broad host and tissue tropism of these viruses has been attributed to the use of the ephrinB2 and ephrinB3 ligands, highly conserved proteins with critical functions in evolutionary development, as virus receptors (Bossart et al., 2008). There are currently no vaccines or therapeutics and NiV and henipaviral diseases remain WHO Blueprint list priority pathogens (Middleton and Weingartl, 2012).

A close relative of HeV, CedV was isolated from Australian bats in 2012 and found to be non-pathogenic, failing to cause any clinical symptoms in ferrets and guinea pig animal models (Marsh GA et al., 2012). The natural reservoirs of HeV, NiV, and CedV are flying foxes (*Pteropus sp.*) (Middleton and Weingartl, 2012). GhV was detected in Ghana in straw-colored fruit bats (*Eidolon helvum*) during routine surveillance in 2015 and was the first henipavirus found outside of the Indo-Pacific region. GhV showed a close phylogenetic relationship with HeV and NiV, and like HeV, NiV, and CedV the GhV RBP can mediate binding to ephrinB2 ligand and mediate cellular fusion (Drexler, J. F., 2009). Recent investigations into Madagascan fruit bats (*Eidolon duprenum*) detected a second African henipavirus, AngV. As neither GhV nor AngV have been isolated or rescued, the pathogenic and zoonotic potential of these African fruit bat associated henipaviruses remains unknown.

Multiple genera of small mammals were tested for viral RNA, and MojV was detected in yellow-breasted rats (R. flavipectus) (Wu Z et al., 2014). The discovery of the MojV genome occurred during follow-up investigations into lethal pneumonia from three miners in 2012 in China, however the pathogenic nature of this virus has only been suggested and no isolates have been discovered or tested in an animal model. Additionally, the novel shrew-borne henipaviruses, GAKV and DARV were found in the Republic of Korea in 2017-2018. (Lee et al., 2021). Most recently, a close relative of MojV, Langya henipavirus (LayV) was recently isolated from febrile patients in eastern China. Subsequently, LayV was identified in shrews (Zhang et al., 2022), increasing the number of Asiatic rodent-associated henipaviruses to four. These recent discoveries are challenging the paradigms surrounding henipaviruses and their associations with bats and may suggest a novel rodent sub-genus of henipaviruses.

The henipaviral genome consists of six structural proteins including nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), receptor-binding protein (RBP), and large (L) protein (approximately length of 18,000 nucleotides) (Luby, S. P., & Gurley, E. S., 2012). In HeV and NiV, slippage sites within the P protein lead to the expression of the V, W, and C proteins. These proteins are virulence factors and are absent in CedV.

Table 1 Summary viruses in Henipavirus

Henipavirus	**Origin (Year)**	**Reservoir**	**Genome (nucleotides)**	**Symptom for human**	**Reference**
Hendra virus (HeV)	Australia (1994)	Fruit bats	18,234	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Nipah virus (NiV)	Malaysia (1999)	Fruit bat	18,246	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Ghana virus (GhV)	Africa (2009)	Fruit bat (*Eidolon helvum)*	18,530	No data	Drexler, J. F., 2009
Cedar virus (CedV)	Australia (2012)	Fruit bat	18,162	Non-pathogenic	Marsh, G. A et al., 2012
Mojiang virus (MojV)	China (2012)	Rodent	18,404	Febrile	Wu Z et al., 2014
Gamak virus (GAKV)	Korea (2017-2018)	Shrew	18,460	No data	Lee et al., 2021
Daeryong virus (DARV)	Korea (2017-2018)	Shrew	19,471	No data	Lee et al., 2021
Langya virus (LayV)	China (2019)	Shrew	18,402	Acute febrile	Zhang et al., 2022
Angavokely virus	Madagascar (2019)	Fruit bat	16,740	No data	Madera et al., 2022

Viruses in the genus *Henipavirus* have a broad reservoir and may cause high case fatality rates following human infection. The discovery and characterization of the novel Henipavirus is a high public health priority.

The greatest bat diversity around the world especially appears to be found in the tropical rain forests of South East Asia. In Thailand, at least 138 species in 11 families and 45 genera of bats have been recorded, such as members of the genus Pteropus, Rhinolophus, and Hipposideros (Soisook et al., 2011). Khao Chong Phran, a cave in Ratchaburi Province, has possibly the largest colony of bats in Thailand. There are estimated to be around 2 million bats in the cave and 95% are insect-eating bats (Department of National Parks, Wildlife and Plant Conservation, Thailand). Moreover, Khao Chong Phran has many community sites, such as cave entrances near communities, guano collection, and tourism sites, which risk zoonotic spillover. Bats are widely considered the animal hosts of zoonotic pathogens such as rhabdoviruses, coronaviruses, paramyxoviruses, and filoviruses, and many of the host species can be found in Thailand.

MojV was associated with a severe pneumonia-like illness killing three of six miners in 2012 in a mineshaft Yunnan, China. Interestingly, this mineshaft was also site where RaTG13, a beta-coronavirus most closely related with SARS-CoV-2, was detected in *Rhinolophid* bats (Rahalkar and Bahulikar, 2020). Identifying the wildlife reservoir of this novel virus will assist intervention strategies to reduce spillover risk.

Research Gaps

As a part of Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH), serological investigations using a multiplex microsphere-based immunoassay (MMIA) have detected 19% (54/284) seroprevalence for MojV in a high-risk cohort, including one person with a high magnitude and durability of anti-MojV IgG across longitudinal samples. Despite this, genomic surveillance has not detected MojV or any novel MojV-like viruses. For this result, we would to further identify and characterize the origin of the Mojiang virus-reactive serum and to explore the novel MojV-like virus in Thailand.

Serological and cellular immunity is an important resource for virus discovery detection by using the specificities and characteristics of memory B cells from peripheral blood mononuclear cells (PBMC). The immune response will recognize and induce antibodies specific to the infecting pathogen. Characterizing the memory B cell response to an unknown pathogen in humans could in the detection of the exposure pathogen in humans. Molecular techniques such as PCR can detect only the genomic material in the body, but memory B cells are long-lived and thus have a much larger window of detection.

Memory B-cell differentiation is characterized by somatic hypermutation in antibody variable region genes (V) and selection of B cells expressing high-affinity variants of this antigen receptor and high sequence similarity in individuals exposed to the same antigen reflect antigen-driven clonal selection and form shared immunological memory between individuals (Yang et al., 2021). Memory B cells are highly abundant in the human spleen, and can proliferate 45% of the total B cell population in this organ (Hauser et al., 2015). Memory B cells can be detected in the Ig variable (IgV) genes in a fraction of IgM+ B cells. In humans, B cells expressing the memory marker CD27+ can be found in the marginal zone of spleen and circulate in the blood. After re-exposure to antigen, memory B cells can quickly proliferate and differentiate into plasma cells. Many studies the antibody diversity and specificity of the naturally paired immunoglobulin heavy and light chains for affinity maturation of B cell receptor (BCR) after virus infection such as SARS-CoV-2 infection (Yang et al., 2021), influenza (Dougan, S et al., 2013), HIV (Moir, S., & Fauci, A. S.,2017), etc. The characterization of memory B cells can be used to develop monoclonal antibodies and produce an effective therapeutic.

Significance and Approach

Southeast Asia is one of the world's highest-risk emerging infectious diseases hotspots. From this region emerged both SARS-CoV-1 and SARS-CoV-2, recurrent outbreaks of novel influenza strains, and the spillover of dangerous viral pathogens such as Nipah virus. The diversity of coronaviruses, henipaviruses, and filoviruses are found in wildlife reservoirs in Southeast Asia and likely due to the high diversity of wildlife, and the demography of the human population brings humans and wildlife into close contact. The spillover of viruses from animals to humans is often misdiagnosed and the clinical symptoms from patients is underestimated during the pandemic. This study provides a surveillance strategy in human and wildlife populations to better understand immunity to novel pathogens and identify the origin of these pathogens to serve as an early warning system to prevent outbreaks of emerging viruses.

This study uses immunology (serologic and cellular) to identify a novel virus that can aide in effective vaccine discovery and development, monoclonal antibody production for prevention and therapy, and generate data resources for early detection and diagnostic assays. Whereas PCR detection or virus

isolation in wildlife is challenging, this new serological approach may be more successful in identifying reservoir hosts.

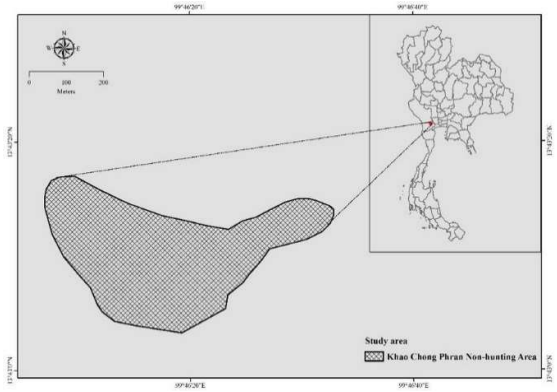
The Indo-Pacific region has a high risk of novel viral emergence. This study provides an opportunity for in-country research capacity building by linking local infectious disease scientists with an international collaborative network that has proven capacity to conduct this work and produce significant findings. Moreover, a global sero-surveillance network for surveillance technology extends virologic and immunologic screening to product discovery. This study will benefit and expand the CREID, EID-SEARCH networks, and the global sero-surveillance network to improve our understanding of immunology to identify novel pathogens and their origin to prevent future pandemics.

Research Methods

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population

Serological profiling provides an indirect measurement of the infecting virus. Despite 19% (54/284) seroprevalence in humans, we have not detected nucleic acid sequences of MojV or MojV-like henipaviruses in this human cohort. Additionally, we intend on expanding our understanding of henipavirus prevalence in this population with longitudinal sampling of the wildlife and human populations in this region.

Figure 1**:** Map of Khao Chong Phran Non-hunting Area, Ratchaburi Province, Thailand (Boonpha N et al., 2019)



Site wildlife samples collection

200 shrews or rodents in and around the Khao Chong Phran cave in Ratchaburi Province will be collected for surveillance targeting the Mojiang-like virus. We plan two sampling trip to collect target shrews and rodents. Due to limited knowledge of the local shrew population, we are unsure as to whether we can collect up to 200 samples and will supplement the collection with rodents, as needed. Samples will be collected based on the following enrollment criteria: random age, random weight, random sex. Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and stored. Oral swab and rectal swab will also collect in 200 shrews or rodents. Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and stored.

Human surveillance

Four of the samples from prior investigations have shown high MojV RBP-reactive antibody titers and will be selected for follow-up sampling. Additionally, 100 human serum samples, nasopharyngeal swab and throat swab will be collected from persons deemed to be in close contact with wildlife, and people who present with or have recent history of undiagnosed acute febrile illness using the following enrollment criteria;

- Adults (≥ 18 years of age) who sign in the informed consent
- Who present with or have recent history of illness without diagnosis, such as severe/acute respiratory illness (SARI/ARI), Influenza-like illness (ILI), fever of unknown origin (FUO), or respiratory tract infection
- Who is the bat guano collector in the cave
- Who lives or works around wildlife habitats
- Who is involved with livestock or wildlife farming
- Who hunts or works with wildlife
- Who has not been previously sampled as part of the EID-SEARCH and PREICT projects

Sample size justification

Human population

$$n = \frac{N}{1 + Ne^2}$$

n = sample size of the study

N = population size for finite populations: 8,150 (data from 2018)

e = error: 10%

$$n = \frac{8{,}150}{1 + (8{,}510 \times 0.1^2)}$$

n = 98.79 (round up to 100 samples)

Wildlife population

We will target a total of 200 individuals of shrews and/or rodents across two trips for this project. This sample size is estimated based on a realistic detection rate and required sampling efforts from previous wildlife surveillance work, given the rodent/shrew population at the study site. Previous publications regarding Henipavirus discovery in small mammals had an average PCR detection prevalence between 20 and 33% (Wu et al., 2014, Lee et al., 2021, and Zhang et al., 2022). We acknowledge the challenge of identifying positive results, and very limited serology research has been done to provide guidance. However, we will make the best use of previous data and experience to conduct sampling at the selected site where human positives have been. We are confident that this targeted sampling and testing strategy offers the best chance to identify positive results and potentially novel henipaviruses.

Targeted Viral Sequencing and Serologic testing

Targeted sequencing

For samples from shrews, rodents, and acute individuals, we will use targeted sequencing and metagenomic approaches in an attempt to detect the Thai MojV-like virus. The nucleic acid from oral swab and rectal swab will do target sequencing in henipavirus Family PCR and Sanger sequencing.

Serological testing

Serum samples will be tested for RBP and envelope fusion protein (F) binding to the known henipavirus species, including the Nipah, Hendra, Cedar, Mojiang, Ghana, Langya, Gamak, and Daerong viruses to further investigate the seroprevalence in this region. Additionally, prior positive samples will be re-tested with the expanded panel to enhance our understanding of the antigenic relationships between the novel shrew-borne henipavirus and the Thai virus.

Data analysis

Sequencing will be analyzed assembling reads in MEGA11. The consensus sequences will compare to the references strains available in the GenBank data-base using the Basic Local Alignment Software Tool (BLAST) (National Center for Bio-technology Information). Serological data will be analyzed using cluster analysis in RStudio.

Aim 2. Characterization of monoclonal antibodies from humans with serological profiles consistent with MojV infection.

In collaboration with NIH VRC PREMISE, MojV RBP and F-reactive B cells will be captured, and the B cell receptor will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific mAbs will be generated from the corresponding sequences and will be assessed for binding potential to LayV RBP and F, as well as their neutralization potential.

Importance: The isolation of these mAbs represents a potential therapeutic for MojV, MojV-like henipas, and LayV, depending on how well these cross react.

Human samples collection site

Human positive serum for MojV was found close to Khao Chong Phran Non-hunting Area in Ratchaburi Province (Figure 2).

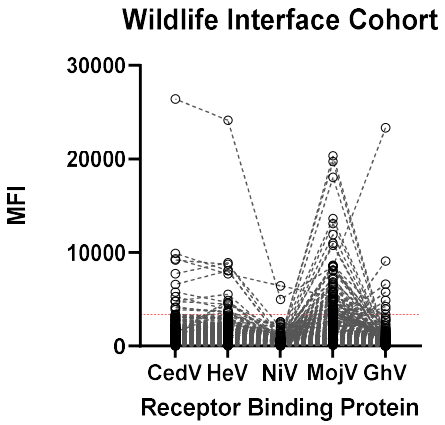


Figure 2: Seroprevalence of henipavirus proteins from a community with a large wildlife interface during 2017-2018. MFI is the median fluorescence intensity for IgG reactivity to each protein. Red-dashed line is at 3,357 MFI, a threshold for seropositivity.

PBMC Sorting and B Cell isolation

Whole blood from MojV-positive people will be collected in individual heparin tubes and processed for PBMC isolation using Density Gradient Centrifugation (SepMate™ PBMC Isolation Tubes). Human PBMCs will be stained with antibodies for positive markers of IgG memory B cells, and antibodies to negatively select and avoid contamination with dead cells, T cells, NK cells or monocytes (live, CD3−,CD14−,CD56−, IgM−, IgA−, CD19+,CD20+,CD27+). Avi-tag for biotinylation and 6xHIS tag for Nickel purification using the HisPur™ Ni-NTA Spin Purification Kit (Thermo Fisher Scientific, Waltham, MA). Single Memory B Cells will be sorted, cells will proliferate, cDNA will synthesized, then Heavy and light chain variable domains will be amplified (Waltari E et al., 2019).

B cell receptor Amplicon Preparation and Recombinant Antibody sorting

Total RNA yields from the PBMC will be determined by absorbance at 260 nm on the NanoDrop™ One (Thermo Fisher Scientific). Total RNA will be used for first strand cDNA synthesis with gene-specific reverse transcription (RT) primers directed to the constant regions. The RT primers for IgG, IgM, IgA, lambda, and kappa will be included. Light chain and heavy chains will be amplified into amplicon. Double-stranded cDNA will be sequenced. After completion of MiSeq sequencing, antibody repertoires will analyze using methods based on the pipeline. Heavy and light-chain sequences will be assembled into an expression vector and transfected into cells for monoclonal antibody expression. Recombinant antibody will be isolated and purified for downstream applications.

Functional assessment

We will first determine the extent to which isolated mAbs can bind binding to native-like MojV RBP and F proteins. We will also characterize the cross-reaction potential of these mAbs will be assessed against GamV, DarV, and LayV RBP and F. Neutralization of viruses remains a critical correlate of protection for most vaccine strategies. However, MojV has not been isolated or rescued, and the virus receptor for MojV has not yet been discovered (Rissanen et al., 2017; Cheliout et al., 2021). Thus, a functional assessment of MojV neutralizing potential is not possible. Though, we will test the cross-neutralization potential of these anti-MojV RBP and F mAbs to neutralize LayV utilizing a plaque reduction neutralization test with EID-SEARCH co-investigators at Duke-NUS. As LayV causes acute illness in human, cross neutralization functionality of these mAbs has therapeutic potential.

Project Timeline

Activity	05/23	06/23	07/23	08/23	09/23	10/23	11/23	12/24	01/24	02/24	03/24	04/24
Mentoring and training activities												
1.Training, literature review												
2.Monthly mentoring meeting												
3.Manuscript writing												
4.EID-SEARCH meeting												
Research activities												
1.IRB amendment												
Aim 1												
2. Human and Wildlife samples collection												
3. Identify Targeted sequencing												
4. Serological testing												
Aim 2												
5. Collect PBMC human sample and isolation memory B-cell for Mojiang virus												
6. PBMC Sorting and B Cell isolation												
7. Determination of envelope glycoprotein epitope binding												

Research Performance Sites

This proposed study will be implemented at the King Chulalongkorn Memorial Hospital (KCMH) collaborating with the Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC). KCMH is a 1,000-bed, university hospital in close coordination with the Faculty of Medicine, Chulalongkorn University. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. It's one of the center hospitals for Emerging Infectious Diseases in Thailand, especially for domestic and international patients.

TRC-EIDCC was established by the Committee of the Emerging Infectious Disease Center, which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. Our clinical center provides standard medical care to patients with emerging and recurrent diseases and disease transmission. Moreover, the center plans preparedness activities, trains medical and government staff on interventions, hosts conferences for medical and public health professionals, creates networks of medical care for patients with emerging and recurrent diseases, and leads research and development projects for medical care and disease control. Apart from medical innovation in emerging and recurrent diseases, the clinical center strives to be the model of infectious diseases clinical services and pandemic control through research activities surrounding infectious diseases and effective disease control measures.

We also collaborate with the Department of National Parks, Wildlife and Plant Conservation for detecting novel pathogens from animals such as bats, rodents, and shrews in our country. We also collaborate with the Department of Disease Control of Thailand (Thai-DDC) to support the Thai government and national policy makers through molecular diagnosis, including the identification of the first case of SARS-CoV-2 infection in Thailand. TRC-EIDCC prides itself on its high performance in research laboratory services and is a part of the national reference laboratory for COVID-19 and other emerging infectious diseases confirmation in Thailand.

CREID Research Center Collaboration

The goals and objectives of the proposed research project, "Immune memory bait & capture to identify emerging henipavirus origins", are fully consistent with the specific aims of EID-SEARCH. EID-SEARCH takes a One Health approach to understanding zoonotic disease emergence, with a focus on viral diversity in wildlife at high-risk human animal interfaces. EID-SEARCH aims to understand the frequency and causes of virus spillover in high-risk communities across Southeast Asia and identify strategies and tools to reduce this risk. As the proposed research focuses on the links between farming practices, wildlife diversity, virus diversity, and cross-species transmission in Thailand, we expect the results of the project to make a significant contribution towards the overall goals of EID-SEARCH.

The proposed project is well-designed, leverages cutting-edge technology, and includes a robust ecological component that corresponds to the overall research strategy of EID-SEARCH. The EID-SEARCH team is highly motivated to support the application of metagenomics to ecological questions and assist in the analysis of the unique dataset that will be generated from this research. With the heightened focus on identifying and limiting risks associated with the complex interactions between humans, wildlife, and domestic mammals that has resulted from the emergence of SARS-CoV-2 from a wet market in China, the proposed research is both timely and likely to yield highly-cited, impactful publications for EID-SEARCH and Thailand partners.

The EID-SEARCH team includes a number of experts in study design, laboratory-based diagnostics, genetic and genomic analyses, and ecological modelling who will contribute to project implementation, data analysis, and manuscript preparation as required. Communication and coordination mechanisms will be set up between the Thailand research team and scientists at the EID-SEARCH research sites for resource sharing, training, knowledge exchange, and mentorship. In particular, EID-SEARCH will facilitate and encourage relationships between our partners in Southeast Asia (based in Malaysia, Singapore, and Thailand) that will facilitate Thailand team professional development and strengthen research capacity in the region. Dr. Eric Laing, the project Mentor, has an established relationship with both Dr. Krongkan and Spencer Sterling, and will act as the liaison between EID-SEARCH and the TRC-EIDCC research team. Further, Dr. Doeuk who is the director of the VRC PREMISE program is enthusiastic about the opportunity to collaborate and apply their rapid assembly, transfection and production of immunoglobulin (RATP-Ig) workflow as a serologic/immunologic based approached for therapeutic and virus discovery (see Letter of Support). An expected outcome of this research project will be pilot data to support a joint funding application in the future.

Mentoring Plan

Goals for Pilot study and Career

Krongan Srimuang: My career goal is to be an expert scientist in either an academic research laboratory or a lecturer in medical science. My long-term research interests include the understanding of priority emerging infectious diseases, genetics, and epigenetics using molecular and serologic techniques. Detection of novel pathogens in their wildlife vectors can help characterize the pathogenicity, virulence, and evolution of these and other closely related pathogens. I believe that this pilot study can provide training in new techniques for the detection, identification, and characterization of novel pathogens that are currently unavailable in Thailand. My goal for the Pilot study is to gain more knowledge and skills for a lifelong career in public health-related research. I wish to expand my skills in novel virus detection using next-generation serologic techniques such as PBMC studies and memory B-cell isolation and sequencing that can contribute to improved surveillance and research capacity at human-animal interfaces. My mentors Dr. Laing and Dr. Wacharapluesadee can provide me with opportunities to develop and apply project skills and improve my scientific writing.

My education has provided me with a strong foundation to expand for this study. I received my undergraduate degree in Medical Technology from Chulalongkorn University which included training in molecular biology, serology, immunology, chemistry, virology, bacteriology, mycology, parasitology, hematology, clinical microscopy, transfusion medicine, and forensic medicine. My interest in molecular technology led me to pursue a Ph.D. at the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, which provided me with training in several molecular techniques including conventional PCR, real-time PCR, Next Generation Sequencing (NGS), etc. The emergence of COVID-19 motivated me to focus on emerging infectious diseases. I am currently completing my post-doctoral fellowship at the Emerging Infectious Diseases Clinical Center (EIDCC), King Chulalongkorn Memorial Hospital with Dr. Wacharpluesadee. Throughout this fellowship, I have continued learning advanced and innovative techniques to detect, identify, and characterize the novel pathogens. Through this pilot project, I will learn about the Peripheral Blood Mononuclear Cells (PBMC) which are a critical part of many subdivisions of medical research including Infectious diseases, Vaccine development, and Immunology. This project aims to study zoonotic reservoirs for by using serologic and cellular immune discovery in humans, and applying this immune knowledge in wildlife for early detection and identification of novel pathogens. This proposed study will expose me to various techniques and immunology topics that I useful in designing and testing new pipelines as technological advances continue to develop. This study will also allow me to develop, manage, and communicate my research.

Spencer Sterling: My overall career goal is to understand the factors that lead to viral zoonosis and to build epidemiological capacity in regions with the highest risk for zoonotic spillover. Through my training and educational experiences, I have learned laboratory and epidemiological techniques that

can assist in the understanding of zoonosis. I wish to continue my education by expanding my repertoire of molecular techniques to include studies of the immune system's response to zoonotic disease in humans and animals, as well as to learn next-generation sequencing techniques. Additionally, I would like to use my experience in epidemiological techniques to train scientists in the Southeast Asian region in order to build local capacity in infectious disease research. Dr. Laing has been a mentor for me throughout my career, and this project would allow for this relationship to expand into new molecular areas, as well as grant writing and project management skills. My professional relationship Dr. Wacharapluesadee with began in 2018 when I participated in a DTRA BTRP funding project that focused on transferring antigen-based multiplex serological technology to her lab group. Currently, I am a Visiting Scientist at TRCEIDCC and I work closely with Dr. Srimuang, and other members of Dr. Wacharapluesadee's research group on EID-SEARCH activities. Additionally, Dr. Wacharapluesadee has decades of experience in infectious disease research in the region and has mentored scientists that have gone on to careers at the Thai Ministry of Public Health, Department of Disease Control, Department of Parks and Wildlife, academia, and the private sector. Both mentors have encouraged Dr. Srimuang and I to apply for this pilot study as well as pursue other funding mechanisms for our research interests. Both mentors will provide for me the ability to independently pursue my research interests in the zoonotic epidemiology and train me on the best practices of scientific communication.

Vertebrate Animals Section Requirements

Animal samples will be collected in Ratchaburi Province for this project under Aim 1 to understand the antigenic relationships among known and unknown henipaviruses. Protocol to perform animal sample collection has been reviewed and approved by the Institutional Animal Care and Use Committee at Tuft University under the EID-SEARCH project (No. G2020-42) and the Institutional Animal Care and Use Committee at Chulalongkorn University (No. 019/2563)

1. Description of Procedures

Rodents (order: *Rodentia*) and Shrew (order: *Scandentia* and *Eulipotyphla*)

- Species: Free-ranging rodents (family *Hystricidae, Muridae, Sciuridae, Spalacidae*) and shrew (tree shrews, shrews, moles) that present at the sites.
- Age & sex: Adults and juveniles, males and females. Neonates will not be sampled.
- Target Number: 200 individuals
- Capture and Restraint: Free-ranging wild mammal species will be captured with metal box traps (Sherman/Tomahawk traps). Traps will be prepared with food, water, padding and shelter, and be checked at least every 12 hours, in the morning and in the afternoon. If adverse weather (extreme heat, rain) is expected or researchers are working in areas where predation is common, traps will be checked more frequently and closed during adverse weather. Veterinary staff will be prepared to deal with a variety of trap injuries, including lacerations, broken teeth, and crush injuries. Depending on the species, individual size, captive or free-ranging status of the individual, manual or chemical restraint and anesthesia (gas or injectable anesthesia) will be applied. Animals will be manually restrained during sampling utilizing insulated bite gloves to prevent injury to the handler and animal. Based upon past field experience (>20 years of animal capture and release in Southeast Asia), we do not expect to trap sick, debilitated, or young animals that would be too young to cope with capture. Sick and debilitated animals tend not to roam widely, reducing their opportunity to enter traps. Additionally, should sick, debilitated, or young animals enter traps, we anticipate that they will not suffer adverse conditions because the traps contain food (the bait) and liquid (water or fruit as a source of water). These individuals would be examined by a veterinarian, rehydrated and if presenting clinical signs treated by a veterinarian, prior to release or sampling then release.
- Sample Collection: Once anesthetized or safely restrained, whole blood will be collected through the appropriate venipuncture site, no more than 1 ml of blood per 100 g (= 10 ml/kg or 1%) of body weight will be collected at any one time. Anesthetized animals will be monitored regularly during recovery until they can no longer be safely handled, at which point they will be confined in a trap or cage. Swabs will be taken from the oropharynx, urogenital tract, and rectum. No destructive sampling is planned, but any individuals that die during capture or sampling will have a necropsy performed with organ samples collected.
- Release: Wild mammals are held for a maximum of thirteen hours depending on trap timing, but typically less than three hours. Captive animals are held for a maximum of two hours but typically less than one hour. At the completion of sampling, animals may be provided with rehydrating

fluids (either subcutaneously if anesthetized or orally if manually handled and accepted). Wild mammals will be released at the site of capture in a location that allows for further recovery or safe escape (e.g., bodies of water, known predatory areas, and human settlements will be avoided). Captive mammals will be released back into their captive setting as is appropriate for the species, either isolated or in social group if it is deemed safe from aggression from enclosure mates while the post-recovery period continues.

2. Justifications

The purpose of this study is to identify zoonotic pathogens through serologic and cellular immune discovery, including developing methods and standards for direct antibody-mediated virus capture in people and wildlife, focusing on rodent/shrew-borne Henipaviruses (HNVs). This will require hands-on fieldwork to collect whole blood and serum samples from rodents and shrews for lab analysis. One technique that avoids the direct capture and sampling of animals is to collect fresh feces or urine, but this will not be able to provide the appropriate samples for serologic analysis, and the research objectives cannot be achieved using an alternative methods (e.g., computational, human, invertebrate, in vitro). Therefore, we believe there are no viable alternatives than the use of live animals.

3. Minimization of Pain and Distress

In every situation, sampling of wildlife will be conducted in the most humane manner while minimizing the impacts on individual animals and their wild populations. In all instances, the fewest number of animals will be sampled that will provide valid information and statistical inference for the pathogen and disease of interest and every effort will be made to minimize stress and discomfort for the animal.

Small mammals may be held for up to 13 hours depending on trap capture timing. Field animal sampling team with zoologists and veterinarians have been well trained and have extensive experience in capture, anesthesia, and sampling of wildlife. In our team's experience, the target species tolerate the described procedure well. Mist nets will be attended continuously during capture periods. This will minimize stress and injury from entanglement.

For rodents and shrews, food, water, padding and shelter will be provided at traps, which will be checked at least every 12 hours. Traps will be checked more frequently or closed if adverse weather (extreme heat, rain) is expected, or researchers are working in areas where predation is common. Veterinary staff will be prepared to deal with a variety of trap injuries, including lacerations, broken teeth, and crush injuries. All animal individuals will be monitored by a veterinarian or experienced field team member during all stages of capture, processing, anesthesia and release. Animals will be kept in a cool place, free from adverse weather conditions and access by non-field team members or other animals while in the pillowcases, trap, or cage for recovery. All recovering individuals will be observed until they are able to effectively ambulate and orient to their surroundings. Animals that are injured during the capture or sampling process will be assessed by an experienced team leader or attending veterinarian, and if the animal is determined to be unlikely to survive if released, it shall be euthanized humanely (see euthanasia section).

4. Method of Euthanasia

In the event that an animal has been injured or is moribund, a determination will be made as to whether it may be treated and released on site by veterinarians or transferred to facility that may treat and rehabilitate the animal, or if, as a last resort, the animal will require euthanasia. Euthanasia methods will vary depending on species; however methods to be used will not deviate from the AVMA "Guidelines for the Euthanasia of Animals" (2020 edition). Any animal that is euthanized using a chemical agent will be disposed of such that it will not be permitted to enter the food supply either through markets or hunting.

Human Subjects Research

1. Risks to the subjects

In this project, we will target populations in one community site who with frequent exposure to bats and other wildlife based on our previous behavioral survey. Subjects will be enrolled voluntarily, and informed consent will be obtained from all participants. Enrolled participants will provide biological samples. Survey data and biological samples will be collected from enrolled participants, and follow-up data collection will be performed among participants whose samples were tested positive.
Biological samples and questionnaire data will be collected from individuals who meet recruitment and inclusion criteria and complete the informed consent process. During data collection, a standardized questionnaire will be administered to all participants. This questionnaire will collect information of demographic background, wildlife contract, travel and daily movement, and unusual SARI/ILI symptoms, and biological specimens will be collected from participants. Both questionnaire and biological data will be analyzed to assess the exposure to coronaviruses and the spread risk among humans. From all participants, a one-time whole blood sample (Max. 15 mL) will be collected during the study period. This sample will allow us to test for historical exposure to bat or rodent-born henipaviruses and collect Peripheral Blood Mononuclear Cells (PBMCs) for those study participants whose serum tests are positive.

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 who are 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, the administration will be conducted privately and confidentially to protect individuals' 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 these risks

2.1 Informed consent and assent

Only consented participants will be enrolled in the study. No research procedures will be undertaken before the participant fully understands the research, agrees to the study procedures, and completes the consent process will be enrolled in the study. Informed consent statements and forms, and project protocols will be translated into the local language of each study site. Research team members involved in this consent process will be required to be fluent in the local language to ensure that the subjects understand the study and all involved procedures.

If participants meet the criteria for enrollment, they will be invited to discuss the details of the study with the research staff. Study staff will review an information sheet and informed consent form with the participant when applicable. Each individual will be provided with a copy of the informed consent form that has been translated into the local language and written with a Flesch–Kincaid readability score equivalent to a 7th-grade reading level or below, to assure that potential participants understand the information being shared. The informed consent form will explain the details of the study, including how and why the individual was selected, the study process and procedures, risks and benefits, financial considerations and the gift of appreciation, confidentiality of data shared, alternatives to participating, and how to obtain more information now or at a later date. The informed consent form will be read in the local language of the site at a location ensuring participant privacy. After which individuals will have as much time as they would like to ask questions and discuss the study with study staff. The study staff will endeavor to ensure that the participant understands the information provided. The study staff will then ask the participant to consider study participation. Participants will have as much time as required to consider the participation.

Those participants who consent to the study will sign and date two copies of the consent form. These form copies will be countersigned and data by the study staff. A copy of the signed consent form must be provided to the subject and the other copy will be kept by study staff. Informed consent paperwork will be kept until the end of the project in a locked box at the local country project office.

2.2 Protection against risks

The potential risks to study participants as a result of study participation are minimal. Collection of venous blood samples and nasal or oropharyngeal swab samples pose minimal risk to subjects. Potential complications associated with venipuncture include pain and/or hematoma at the site of collection. Trained medical professionals and/or clinic staff will monitor the blood collection site and treat any complications according to existing health facility protocols. A potential complication of nasal/ oropharyngeal sampling is minor irritation at the time of collection. Employing trained medical and/or clinic staff to collect blood and swab samples will minimize the potential for complications.

Another risk that this study may pose concerns the information to be gained on henipaviruses newly recognized in the community. We will provide participating communities, hospitals, and clinicians with information and background data on target zoonotic viruses to ensure up-to-date communication of risk. Because of the timeline for diagnostic testing and results interpretation, we are not likely to provide results to participating clinics within a time frame that would be clinically relevant to outbreaks of undiagnosed diseases. Therefore, the information provided by this project will not impact patient management or outlook.

If an individual decides to participate in this research, his/her participation and all information provided by the participant will be strictly confidential, and personal identifying information will not be shared with anyone outside of the study staff. Participants will not be identified or named in any reports or publications. Questionnaire information and all biological samples will be identified by an alphanumeric code, not by the participant's name. 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 study site in locked files cabinets or password-protected devices in areas with access limited to the research staff of this project. Research databases will be secured with password-protected access systems and controlled distribution web-based certificates and will not contain any identifying characteristics about study participants (e.g., name, address, or telephone number). Access to all data will be limited to the staff involved in this study. The health information disclosed by an individual will not be used by or disclosed (released) to another institution. Any surveillance report that is published or shared with partners will not contain any personally identifying information for individual participants.

3. Potential benefits of the research to the subjects and others

There are no measurable benefits to the individual study participants enrolled in this study. There are benefits to the community and regional healthcare providers to help them understand the risk of zoonotic infections among high-risk populations in the regions they work. At the conclusion of the study, we will deliver an educational workshop reporting aggregate study findings that will be open to both study and non-study participants, describing the health benefits of using personal protection equipment (PPE) and handwashing during animal handling activities throughout the day, as well as to share other prevention/interventions that emerge from the research data.

4. Importance of the knowledge to be gained

There are valuable potential benefits to the public from the knowledge to be gained from this study. One key benefit to the community is sharing information and knowledge to better understand the risk of zoonotic spillover events and related health risks, as well as information sharing with communities on practices that could reduce risks, such as the avoidance of particular animal contacts or the need for PPE and extra care when handling wildlife, that may substantially reduce the risk zoonotic pathogen transmission in the community. 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 create or implement public health interventions to disrupt disease emergence and/or spread in an area that is beneficial to all. Additionally, there are valuable benefits to the general public from the knowledge to be gained from this study. Knowledge gained will 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.

5. Country- / institution-specific ethics / IRB regulations addressed

Main research protocol and materials to conduct human subject research in this project have been reviewed and approved by the Health Medical Lab Institutional Review Board, US (No. 894ECOH21b)

and the Institute Review Board of the Faculty of Medicine, Chulalongkorn University, Thailand (No. 211/64). Amendment for PBMCs collection will be made and approved before the start of this project.

Research and Related Other Project Information

1. Are Human Subjects Involved?	Yes	X	No	
1.a. If YES to Human Subjects				
Is the Project Exempt from Federal regulations?	Yes		No	X
If yes, check appropriate exemption number	1		2	
	3		4	
	5		6	
	7		8	
If no, is the IRB review Pending?	Yes	X	No	
IRB Approval Date:				
Human Subject Assurance Number	FWA00000943			

2. Are Vertebrate Animals Used?	Yes	X	No	
2.a. If YES to Vertebrate Animals				
Is the IACUC review Pending?	Yes		No	X
IACUC Approval Date	3 December 2020			
Animal Welfare Assurance Number				

3. Is proprietary/privileged information included in the application?	Yes		No	X

4.a. Does this project have an Actual or Potential	Yes		No	X

impact -- positive or negative -- on the environment?				
4.b. If yes, please explain				
4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed?	Yes		No	
4.d. If yes, please explain				

5. If the research performance site designated, or eligible to be designated, as a historic place?	Yes		No	X
5.a. If yes, please explain				

6. Does this project involve activities outside of the United States or partnership with international collaborators?	Yes	X	No	
6.a. If yes, identify countries	Thailand			
6.b. Optional explanation				

Budget Justification

A. Key Personnel (Total $24,000)

Dr. Krongkan Srimuang, PI (12 months) is a medical research scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital (TRC-EIDCC) who have been leading the laboratory analysis for the EID-SEARCH work in Thailand in the past three years to perform molecular, serological, and next-generation sequencing to identify novel viruses from human and animal samples. Dr. Srimuang will commit 12 months to this project to design, manage, and perform all project activities in the lab and field by supervising a lab technician and working with the field coordinator. Dr. Srimuang will also work closely with the co-PI and Mentors to conduct data analysis and develop manuscript and join EID-SEARCH and CREID meetings to prevent the project findings.

Spencer Sterling, Co-PI (6 months) is the research coordinator of Laing Lab at the Uniformed Services University for Indo-Pacific region and a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC). Sterling will commit 6 months to this project as the co-PI, who will work closely with the PI in the study design, SOP development, sampling process, data analysis of for serological investigations. No salary is required for Sterling.

B. Other Personnel (Total $20,000)

One (1) Laboratory Technician (12 months) will committee 12 months to this project to conduct PCR and serological testing of animal and human samples at TRC-EIDCC, under the supervision of Dr. Srimuang and Sterling. Salaries for 12 months at $1,250 per month is requested ($15,000).

One (1) Field Coordinator (4 months) will committee 4 months to this project, working closely with Dr. Srimuang and Sterling to organize and conduct field sample collection among humans and animals at the study sites, and support relevant financial management. Salaries for 4 months at $1,250 per month is requested ($5,000).

C. Fringe Benefits

No Fringe is requested.

D. Equipment

No equipment is requested.

E. Travel (Total $48,375, Years 1-5)

Domestic Travel ($11,005)
Support for domestic travel for field sampling is requested in the amount of $11,005 per to conduct four (4) trips of sample collection from humans and rodents/shrews.

- The first trip is a one-day trip to collect samples from human participants who were tested positive (n<10), the cost is estimated at van rental for local transportation between Bangkok and the study site ($260) and meals for five (5) field team members at $49 per person ($245), in total of $505 for the first trip.

- The other three (3) field trips will be conducted to collect samples from 100 humans and 200 rodents/shrews, respectively. Each trip is a duration of 6 days and 5 nights. For each person, 5-night stay in hotels at $75.5/night (5 x $75.5 = $378); 3 meals per day and incidental expenses at $49 per day prorated to 75% ($36.75) for first and last day of travel ($36.75 x 2 + $49 x 4 = $270), the total meals and accommodations for each person per trip is $648 ($378+270), cost for five (5) field team members is $3,240. A van will be rented for local transportation between Bangkok and the study site that is estimated at $260 per trip. Total cost for each 6-day trip is $3,500, three trips will be $10,500.

International Travel ($5,023)
Support for international travel for Dr. Srimuang from Bangkok to Washington DC to attend the CREID Annual Meeting and receive lab training at the National Institute of Allergy and Infectious Diseases (NIAID) is requested in the amount of $5,023. Trips are for a duration of 10 days and 9 nights. Travel costs for one trip are estimated using federal per diem rates for hotels, meals, and incidental expenses as follows: nine-night stay in DC area hotel at $286/night (9 x $258 = $2,322); Ten days' meals and incidental expenses at $79 per day prorated to 75% ($59.25) for first and last day of travel ($59.25 x 2 + $79 x 8 = $750.5); Round trip flight between Bangkok to Dulles International Airport is estimated at $1,650; local transportation/taxi between hotel and airport per round trip between Chulalongkorn Hospital to airport is estimated at $40, between hotel and the airport in the US is estimated at $200, and $60 for transportation within the region ($300).

F. Participant Support Costs

No participant support is requested.

G. Other Direct Costs (Total $78,803)

Materials and Supplies ($72,830)
PBMCs isolation ($47,000). We request funds to conduct PBMC isolation from collected sample, costs including the reagents and lab consumable and supplies are estimated at $47 per cell for 1,000 cells.

Multiplex microsphere immunoassay detection ($2,275). Funds are requested to perform serological testing of collected human and animal samples. Costs including the reagents and lab consumable and supplies are estimated at $6.5 per sample for 350 samples.

PCR testing and sequencing ($20,475). Funds are requested to perform serological testing of collected human and animal samples. Costs including the reagents and lab consumable and supplies are estimated at average $58.5 per sample 350 samples, given a 10%~15% positive rate.

Field supplies for PBMC collection and processing ($1,225). Funds are requested to perform PBMCs collection and processing in the field. Costs including the reagents and lab consumable and supplies are estimated at average $3.5 per sample 350 samples.

Field supplies for animal sampling ($1,160). Funds are requested to purchase supplies for animal capture, anesthesia, and sample collection. A total cost of $1,160 is estimated

Field work disposable ($668). Funds are requested to purchase disposable materials (tips, tubes, gloves, masks, googles etc.) for field work.

Consultants Service ($3,000)

One (1) zoologist and one (1) veterinarian will provide consultancy service to this project to help 1) select sites for animal sampling and join the sampling trip to 2) identify species; and 3) provide veterinary care in the sample collection of rodents and shrews. The consultancy fee is estimated at $1,500 per person for the project, in total of $3,000.

Publication Fee ($3,000)

We request $3,000 of publication costs for one peer-review paper generated from the project. The cost is estimated at the reimbursed Open Access fees of $2,900 - $5,000 per manuscript in the past two years for six peer-review papers.

H. Total Direct Costs (Total $138,831)

I. Indirect Costs (Total $11,106)

An indirect cost at 8% is requested.

J. Total Direct and Indirect Costs (Total $149,937)

A total amount of $149,937 is requested.

Co-PI Plan (only needed if applying as Co-PIs):

What each Co-PI will contribute to the proposed research study

As stated in the Mentoring Plan, the co-PIs, Krongkan Srimuang and Spencer Sterling have different educational and training backgrounds. This Co-PI plan is proposed to fill the gaps in knowledge of each scientist and supports a collaborative working relationship for project success. Moreover, this plan was developed to maximize the strengths of each PI. Overall, the project is separated into two objectives, the proposal of this project will be equally managed by Dr. Srimuang and Mr. Sterling. Dr. Srimuang will be responsible for PBMC isolation, sorting, and characterization. Mr. Sterling will be responsible for the technical serological aspects of the project. Both will work closely with the Research Center Mentor, Dr. Eric Laing, to develop this project, protocol development, and network connections. Dr. Supaporn Wacharapluesade, the Co-mentor, will support the wildlife sampling, samples collection, and molecular aspects within Thailand.

How the Co-PIs will jointly work with the affiliated Research Center

Dr. Srimuang is currently employed at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University and supports the molecular biology investigations in the EID-SEARCH project. Mr. Sterling is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region. Both Co-PIs will work closely with Dr. Laing, Dr. Wacharapluesadee, and the staff at EcoHealth Alliance to coordinate field sampling, research activities, data analysis, and manuscript production.

How the Co-PIs will jointly manage the proposed study

This is a great opportunity for both PIs to undergo training in advanced techniques and develop skills in next-generation serology and molecular sequencing. Dr. Srimuang will learn and practice serologic

techniques and data analysis from Mr. Sterling. Mr. Sterling will develop skills in cell isolation and molecular sequencing techniques from Dr. Srimuang. Moreover, Mr. Sterling will coordinate planning, sample processing, data analysis, and report preparation for this project with Dr. Srimuang.

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List of abbreviations, acronyms, symbols

AngV: Angavokely virus
BCR: B cell receptor
BSc: Bachelor of Science
BSL2: Biological Safety level 2
BSL3: Biological Safety level 3
cDNA: Complementary DNA
CedV: Cedar virus
Co-PI: Co-principal investigator
CREID: Centers for Research in Emerging Infectious Diseases
DARV: Daeryong virus
DNA: Deoxyribonucleic acid
EHA: EcoHealth Alliance
EID: Emerging Infectious Disease
EID-SEARCH: The Emerging Infectious Disease – South East Asia Research Collaboration Hub
ELISA: Enzyme-linked immunosorbent assay
F: Fusion protein
FACS: Fluorescence-activated cell sorting
FUO: Fever of unknown origin
GAKV: Gamak virus
GhV: Ghana virus
HeV: Hendra virus
HNVs: Henipaviruses
ILI: Influenza-like illness
KCMH: King Chulalongkorn Memorial Hospital
L: large protein

LayV: Langya virus
M: Matrix protein
mAb: Monoclonal antibody
MD: Maryland
MMIA: Multiplex microsphere-based immunoassay
MojV: Mojiang virus
MSc: Master of Science
N: Nucleocapsid
NGS: Next-generation sequencing
NIAID: National Institute of Allergy and Infectious Diseases
NIH: National Institutes of Health
NiV: Nipah virus
P: Phosphoprotein
PBMC: Peripheral blood mononuclear cells
PCR: Polymerase Chain Reaction
PhD: Doctor of Philosophy
PI: Principal investigator
RBP: Receptor-binding protein
RNA: Ribonucleic acid
RT-PCR: Real-time PCR
SARI/ARI: Severe/acute respiratory illness
SARS-CoV-1: Severe acute respiratory syndrome coronavirus 1
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2
SEA: Southeast Asia
Thai-DDC: Department of Disease Control of Thailand
TRC-EIDCC: Thai Red Cross. Emerging Infectious Diseases. Clinical Center
USA: United States
WGS: Whole Genome Sequencing

Facilities and Resources

FACILITIES: Specify the facilities to be used for the conduct of the proposed research.

Laboratory:

The Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC), King Chulalongkorn Memorial Hospital (KCMH) was established by the Committee of the Emerging Infectious Disease Center which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. TRC-EIDCC laboratory facilities include 3 biosafety cabinets (BSL 2, BSL 2+, BSL2+), 3 automated extraction machines, 2 thermocyclers (Proflex), 2 real-time PCR machines (CFX, Bio-rad), 3 Freezers (-80 °C), 2 refrigerators; 4 °C, 2 autoclaves. Our EIDCC uses the Pathogen Asset Control System (PACS) system for sample tracking. Moreover, our Faculty of Medicine, Chulalongkorn University has a central laboratory, the Chula Medical Research Center (Chula MRC), which provide many instruments for medical, masters, and graduate students, Post-doctoral fellows, and researchers at Chulalongkorn University and KCMH. The Chula MRC laboratory facilities are divided into molecular diagnostics (PCR Thermal Cycler, QStudio6 Flex Real-Time PCR, Droplet Digital PCR, GeneXpert, Film array, Illumina MiSeq), immunology facilities (ELISA testing equipment (reader & washer), Luminex, Flow Cytometry), cell sorting machine and a BSL3 laboratory. Moreover, the facility provides general equipment, such

as autoclaves, balances, CO2 incubators, Dual Fluorescence Cell Counter, 4 °C refrigerators, -20°C, and -80°C freezers.

Clinical:

KCMH is a 1,000-bed university hospital, in close coordination with the Faculty of Medicine, Chulalongkorn University. The Thai Red Cross Society provides medical treatment, rehabilitation, health promotion and disease prevention services to the general public and the vulnerable. It also provides biopharmaceutical and sterile medicines that are sufficient to meet the needs of patients and safe in accordance with international standards. It serves as a center for providing eye and organ donation services to patients nationwide in line with international standards. It has specialized doctors and public health personnel and also produces specialized nurses. The TRC-EIDCC in the KCMH provides standard medical care to patients with emerging and recurrent diseases and disease spreading prevention.

Animal:

TRC-EIDCC also collaborates with the Department of National Parks, Wildlife and Plant Conservation for detecting the emerging novel pathogens from animals such as bats, rodents, and pangolins in our country.

Computer:

TRC-EIDCC used the service from the Office of Information Technology, Chulalongkorn University that provides the application, network, and software such as Office 365, VPN, WIFI, Microsoft Office, Microsoft Windows, SPSS, Adobe, Zoom Meeting, etc. Moreover, our EIDCC use the Pathogen Asset Control System (PACS) for sample tracking.

Office:

The office and laboratory space are located at the EIDCC at 4th Floor, Jongkolnee Watwong Building and 6th Floor, Aor Por Ror Building, respectively. The applicants will have dedicated desks in both buildings.

Other:

PI Name: Srimuang, Krongkan and Sterling, Spencer **Foreign Site: Thailand**
CREID CC Grant: 1U01AI151378

Foreign Site Information

If the Pilot Award will have multiple foreign sites, complete this form for EACH research site

Principal Investigator Name:	Krongkan Srimuang
Project Title:	Immune memory bait & capture to identify emerging henipavirus origins
Institution:	King Chulalongkorn Memorial Hospital
Foreign Research Site:	Rama 4 Road, Pathumwan, Bangkok 10330 Thailand
Point of Contact for Research Site:	Krongkan Srimuang Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital Rama 4 Road, Pathumwan, Bangkok 10330 Thailand krongkan.sr@gmail.com

Introduction (3-4 sentences):

Provide a simple description of the overall goals of the project including the work that will be done at the foreign site. Indicate if any of the NIAID grant funds will be sent to the foreign site.

The goals of this project are to know zoonotic reservoirs for pathogen origin by using serologic and cellular immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

To achieve these goals, the investigator will collaborate with NIH PREMISE for Antibody-mediated bait & capture of Thai MojV-like virus.

$149,937 of grant funds will be sent to the site for these studies.

Foreign component (3-4 sentences):

Describe the specific role of the foreign site (i.e. conduct critical experiments?, conduct data analyses?, provide consultations?, provide samples?, study human and/or animal populations?, conduct observational or interventional clinical trials?, collect samples and/or data to be brought to the US?).

The site will 1) conduct all filed work to collect human and animal samples from the study sites; 2) perform laboratory analysis using collected samples; and 3) conduct data analysis as proposed in the project.

Human Subjects (1 word or 1 sentence per bullet):

- **Parent Study IRB approval**
  - **IRB approval number for parent study:** #894ECOH21b (US); #221/64 (Thailand)
  - **IRB approval date:** 12-05-2021 (US); 08-06-2021 (Thailand)
  - **Human Subject Assurance Number: (FWA)#:** #00001102 (external IRB); #00022431 (EcoHealth Alliance); #00000943 (Chulalongkorn University)
- **Does this study require a modification to the IRB approval of a parent study (Yes or No)?**
  - **Yes**
- **Will existing samples from human subjects will be used: (Yes or No)?**
  - **No**
  - **How many subjects provided the existing samples to be used?** N/A
- **Will human subjects be recruited (Yes or No)**
  - **Yes**
  - **Number of human subjects that will be recruited:** 110
- **Population parameters:**
  - **Gender:** 55 males, 55 females
  - **Age Group:** Age >=18 years who provide informed consent.
  -
  - **Race/Ethnicity:** 110 Asian
- **Sample collection will include:**
  - **Blood:** Yes
  - **Urine:** No
  - **Tissues:** No
  - **Other samples (describe):** Nasopharyngeal swab and throat swab

- **Sample collection will be completed in how many visits:** 2 trips

- **Will samples be de-identified (Yes or No)? If No, describe how they will be protected.**
  - Yes

- **Will local IRB approval be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals being sought or state why local IRB approval is not required.**
  - Yes

- **Will informed consent be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals/consents being sought or state why informed consent is not required.**
  - Yes

- **Will samples be brought back to the US (Yes or No)?**
  - No

- **Will data be brought back to the US (Yes or No)?**
  - Yes, some of the data will be shared with EID-SEARCH partners in the US for relevant analysis.

Animal Subjects (1 word or 1 sentence per bullet):

- **Parent study IACUC approval**
  - **IACUC approval number for parent study:** #G2020-42 (US); #019/2563 (Thailand)
  - **IACUC approval date:** 05-05-2020 (US); 03-12-2020 (Thailand)
    Animal Welfare Assurance Number: D16-00572 (A4059-01)
- **Does this study require a modification to the IACUC approval of a parent study (Yes or No)?**
  - **No**

- **Will existing samples from animal subjects will be used: (Yes or No)?**
  - **No**
  - **How many animal subjects provided the existing samples to be used?** N/A
- **Will vertebrate animals be collected (Yes or No)?**
  - **Yes**

- **Species of animals (e.g. rats, mice, rabbits, monkeys):** Rodents and shrews
- **Animal parameters:**
  - **Total number of animals:** 200 (200 rodents and shrews)
  - **Gender:** 100 males, 100 females
  - **Age range:** 4 - 12 months and elder depending upon species
  - **Lab strain (e.g. Sprague-Dawley rats, Balb/C mice):** None
  - **Wild animals procured in country (e.g. Rhesus monkeys from a reserve):** No

- **What will be done to them or with them and how often?**
  - Free-ranging rodents and shrews will be captured through pit traps and box traps.
  - Anesthesia will be conducted for captive rodents and shrews.
  - Once anesthetized blood will be collected.
  - All action will only be performed one time for each animal individual.
  - All animals will be released after sampling.

- **What are the follow-ups?**
  - No follow-up relevant as per protocol, since all animals will be released after sampling ups

- **What will be their fate at the end of the experiments – will they be euthanized?**
  - All animals will not be held longer than 6 hours (typically less than 3 hours) during the sampling process and released after sampling.

- **Will in-country clearances be obtained prior to engaging in any animal research (Yes or No)? If No, describe alternate approvals or state why in-country clearances are not required.**
  - Yes
- **Will samples be brought back to the US (Yes or No)?**
  - No
- **Will data be brought back to the US (Yes or No)?**
  - Yes, some of the data will be shared with EID-SEARCH partners in the US for relevant analysis.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Proposal Cover Sheet

Project Title	Immune memory bait & capture to identify emerging henipavirus origins
Principal Investigator Name	Krongkan Srimuang, Ph.D.
Position/Title	Research Scientist
Department	Thai Red Cross Emerging Infectious Diseases Clinical Center
Institution Name	King Chulalongkorn Memorial Hospital
Street	Rama 4 Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	Krongkan.sr@gmail.com
Phone	+66 (0) 92-453-0808
Country(ies) where work will be conducted	Thailand
Pathogen(s) focus	Mojiang Virus, Henipavirus, Paramyxovirus
Co-Principal Investigator Name (*If applicable*)	Spencer Sterling, MPH
Position/Title	Scientific Project Coordinator
Department	Microbiology and Immunology
Institution Name	Uniformed Services University of the Health Sciences
Street	Jones Bridge Road
City, State, Zip Code	Bethesda, MD 20814
Country	USA
Email	spencer.sterling.ctr@usuhs.edu
Phone	+66 (0) 83-494-5980

Contact information for institution(s) that would financially manage the award *(please note: changing the managing institution upon award may result in substantial funding delays)*	
Contractual Contact, Title	Chula Unisearch
Institution Name	Chulalongkorn University
Street	254 Phyathai Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	unisearch@chula.ac.th
Phone	0 2218 2880
For Co-Principal Investigator (If applicable):	
Contractual Contact, Title	
Institution Name	
Street	
City, State, Zip Code	
Country	
Email	
Phone	

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Name of Mentor	Eric Laing, Ph.D.
Mentor Institution	Uniformed Services University of the Health Sciences
Institution Address	Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814
Permanent location of Mentor	4301 Jones Bridge Road, Bethesda, MD 20814
Mentor email	eric.laing@usuhs.edu
Mentor phone	+1 (301) 980-8192
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Name of Mentor	Supaporn Wacharapluesadee, Ph.D.
Mentor Institution	Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital
Institution Address	1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Permanent location of Mentor	4th Floor, Jongkolnee Watwong Building, Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital, 1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Mentor email	spwa@hotmail.com
Mentor phone	+66 (0) 89-920-6292
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Total Budget	***Repeat this table if there are two Co-PIs***
Direct Costs	$138,831
Indirect Costs	$11,106
Proposed Start Date	1 May 2023
Proposed End Date	30 April 2024

Project Abstract (250 words)

Prototypical henipaviruses (Hendra and Nipah virus) are bat-borne zoonotic viruses and cause high mortality. The recent detection of closely related Mojiang and Langya viruses in rats and shrews, and isolation of Langya virus from individuals with acute febrile illness, challenges the dogma that fruit bats are the sole henipavirus reservoirs. The recently expanded *Henipavirus* genus includes two other shrew-borne viruses (Gamak and Daeryong viruses), and the Madagascan fruit bat-borne Angovkeely virus. As part of EID-SEARCH activities, we found serological evidence of infection by a Mojiang-related virus in a community of Thai bat guano collectors who have occupational exposure to microbats and rodents. Identifying the wildlife reservoir of this likely novel virus will assist intervention strategies to reduce spillover risk. However, PCR detection or virus isolation in wildlife is challenging, and new serological approaches may be more successful in identifying reservoir hosts. This project aims to identify novel viruses and zoonotic reservoirs through serologic and cellular immune discovery. We will conduct follow-up human and wildlife (bats and rodents) surveillance in the province where the seropositive humans were identified and apply an expanded serological test and genomic sequencing to detect this novel MojV-like henipavirus. Further, we

plan to isolate anti-MojV monoclonal antibodies (mAb) from peripheral blood mononuclear cells collected from human study participants we have identified as immunoreactive with MojV antigens, and characterize the cross reactive and cross neutralizing potential of these mAbs to LayV.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Project Title **Immune memory bait & capture to identify emerging henipavirus origins**

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Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Study Personnel

Krongkan Srimuang, Ph.D., is a researcher at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital and supports the Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia (EID-SEARCH) research. Dr. Srimuang is also a medical technologist working to identify, detect and characterize the spill-over risk of high zoonotic potential viruses from wildlife and humans using serology techniques, Next-generation sequencing, and PCR assays to identify novel viruses. Dr. Srimuang also performs the project Smart Sentinel Surveillance System: Emerging Viral Pneumonia and Respiratory tract infections (RTIs) to identify, detect and characterize the novel/known Respiratory Viruses in our country, Thailand by using multiplex real-time PCR, family PCR and Next-generation sequencing of novel viral pathogens. Dr. Srimuang graduated with Ph.D. in Tropical Medicine from the department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University.

Spencer Sterling, MPH, is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region. Mr. Sterling will planning, sample processing, data analysis, and report preparation for this project.

Dr. Eric Laing will act as mentor for Dr. Srimuang and Mr. Sterling under the Centers for Research in Emerging Infectious Diseases (CREID) Pilot Research Program. Dr. Laing is an assistant professor dedicated to biosurveillance and virological research of emergent zoonotic viruses at Uniformed Services University, Bethesda, MD. Part of Dr. Laing's research focus in on the development of serological-based approaches to identify the infectome of wildlife and human communities for the assessment of virus spillovers. Dr. Laing has worked collaboratively with national and international researchers including institutional partners at Duke-NUS, Chulalongkorn University, University of Pretoria, and Rocky Mountain Labs towards these goals. Through these collaborations, Dr. Laing's research group aims to identify known and unknown viruses, the wildlife reservoirs of priority pathogens, the high-risk interfaces for spillover and the biotic/abiotic drivers of virus emergence.

Dr. Supaporn Wacharapluesadee, Ph.D. is the head laboratory at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, a WHO Collaborating Centre for Research and Training on Viral Zoonoses. Dr. Wacharapluesadee research interests include emerging infectious diseases in bats (including Nipah, Rabies, Coronavirus, and novel pathogens) as well as molecular diagnoses and sequencing of viruses. During the past 10 years, Dr. Wacharapluesadee got funding from USAID PREDICT to conduct the surveillance for novel virus in wildlife and human, and from Biological Threat Reduction Program US DTRA to conduct the EID surveillance in bat using the molecular technology and multiplex serology panel (Luminex technology). TRC-EIDCC is responsible for molecular diagnoses services to the hospital, and is the reference laboratory for rabies virus, MERS-CoV, Ebola, Zika, and other infectious diseases diagnoses for Ministry of Public Health, Thailand and laboratory training center on national and regional level. Dr. Wacharapluesadee has served and consulted on several WHO and Thai government committees.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

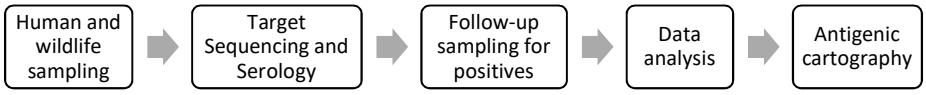
Specific Aims

The recent discovery of novel shrew and rodent-borne henipaviruses in East and Southeast Asia has highlighted gaps in our understanding of henipavirus transmission and underscores the importance of surveillance activities in the region. In particular the isolation of Langya virus from acutely ill humans and shrews demonstrated the need to further surveillance and therapeutic development against emergent henipaviruses. Therefore, **this project aims to expand the study of known reservoirs of zoonotic pathogen origin by using genetic and serologic techniques, and to use cellular immune discovery in seropositive humans ~~to be used~~ as a tool to determine henipaviral antigenic relatedness to assist with the functional assessment of current therapeutics as and their effect on these novel viruses.**

In our study, we detected that a high seroprevalence against MojV in human participants ~~Human populations~~ near the Khao Chong Phran Non-hunting Area in Ratchaburi province who we identified as representing a high risk population for zoonotic spillover. ~~have shown a high seroprevalence to the MojV~~Yet, no virus sequences were detected ~~RBP without confirmed sequencing results~~. We hypothesize that (i) there is a novel henipavirus present in ~~-~~the local wildlife population that has spilled over into the human population at least once, and (ii) the antibodies from these seropositive people will react with the novel shrew-borne henipaviruses in a way that can inform us on therapeutic approaches for future outbreaks. We will follow-up with ~~on~~ persons who had~~ith~~ high anti-MojV ~~RBP~~ IgG level~~stiters~~, sample new ~~p~~participants~~eople~~ within this~~e~~ community ~~population~~ who~~that~~ interact with the local wildlife, and sample bats, shrews, and other rodents to identify the wildlife~~find the~~ source of the MojV-~~reactive antibodies~~like henipavirus. Additionally, in collaboration with NIH VRC PREMISE, MojV receptor binding protein (RBP) and fusion protein~~RBP~~-reactive B cell will be isolated~~receptors will be sequenced~~ from previously-identified seropositive human participants. MojV-like virus-specific B-cell receptors will be sequences and monoclonal ~~M~~antibodies specific to the infecting MojV-like virus RBP and F protein ~~Abs~~ will be generated and tested for cross reactivity against presently described rodent associated henipavirus envelope glycoproteins, and cross neutralization against Langya virus. ~~and used for direct antibody-mediated virus capture in people and wildlife.~~

The proposed project has two aims:

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population.



Aim 2. Characterization of monoclonal antibodies from humans with serological profiles consistent with MojV infection.

The proposed project has three objectives:

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Look at the other successfully awarded applications and how they wrote the specific Aims

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Commented [EL2]: What population?

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Objective 1. To use metagenomic and targeted sequencing approaches in human and wildlife populations to detect the Thai MojV-like virus.

Objective 2. To isolate and sequence memory B-cells from humans positive for Mojiang virus in Ratchaburi province, Thailand for antibody ~~production~~discovery.

~~**Objective 3.** To determine epitope binding and antigenic relationships from henipavirus reactive serum to the known henipaviruses.~~

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Study Rationale

Nipah virus (NiV) and henipaviral diseases are among the most virulent of zoonotic diseases. The genus *Henipavirus* is currently composed of nine viruses including the prototypes, NiV and Hendra virus (HeV), and Cedar virus (CedV), Ghana virus (GhV), Mojiang virus (MojV) (McLinton et al., 2017), Langya virus (LayV) (Zhang X et al., 2022), Gamak virus (GAKV), Daeryong virus (DARV) (Lee et al., 2021), and Angavokely virus (AngV) (Madera et al., 2022). HeV was first identified in Australia in 1994 as a cause of respiratory illness and fatal encephalitis in humans and horses. Closely following the discovery of HeV, NiV was identified in 1999 in Malaysia after outbreaks of fatal encephalitis in humans, with swine determined to be the intermediate host. Annual outbreaks of NiV have occurred in Bangladesh and are associated with the consumption of raw date palm sap, and since 19 May 2018 annual outbreaks of NiV associated encephalitis of been recorded in Kerala, India (Uwishema et al., 2022). Both HeV and NiV have a broad host range capable of infecting several mammalian orders, and can enter and infect endothelial cells, vasculature, and the central nervous system leading to severe multisystem organ diseases and high mortality. The broad host and tissue tropism of these viruses has been attributed to the use of the ephrinB2 and ephrinB3 ligands, highly conserved proteins with critical functions in evolutionary development, as virus receptors (Bossart et al., 2008). There are currently no vaccines or therapeutics and NiV and henipaviral diseases remain WHO Blueprint list priority pathogens (Middleton and Weingartl, 2012).

A close relative of HeV, CedV was isolated from Australian bats in 2012 and found to be non-pathogenic, failing to cause any clinical symptoms in ferrets and guinea pig animal models (Marsh GA et al., 2012). The natural reservoirs of HeV, NiV, and CedV are flying foxes (*Pteropus sp.*) (Middleton and Weingartl, 2012). GhV was detected in Ghana in straw-colored fruit bats (*Eidolon helvum*) during routine surveillance in 2015 and was the first henipavirus found outside of the Indo-Pacific region. GhV showed a close phylogenetic relationship with HeV and NiV, and like HeV, NiV, and CedV the GhV RBP can mediate binding to ephrinB2 ligand and mediate cellular fusion (Drexler, J. F., 2009). Recent investigations into Madagascan fruit bats (*Eidolon duprenum*) detected a second African henipavirus, AngV. As neither GhV nor AngV have been isolated or rescued, the pathogenic and zoonotic potential of these African fruit bat associated henipaviruses remains unknown.

Multiple genera of small mammals were tested for viral RNA, and MojV was detected in yellow-breasted rats (R. flavipectus) (Wu Z et al., 2014). The discovery of the MojV genome occurred during follow-up investigations into lethal pneumonia from three miners in 2012 in China, however the pathogenic nature of this virus has only been suggested and no isolates have been discovered or tested in an animal model. Additionally, the novel shrew-borne henipaviruses, GAKV and DARV were found in the Republic of Korea in 2017-2018. (Lee et al., 2021). Most recently, a close relative of MojV, Langya henipavirus (LayV) was recently isolated from febrile patients in eastern China. Subsequently, LayV was identified in shrews (Zhang et al., 2022), increasing the number of Asiatic rodent-associated henipaviruses to four. These recent discoveries are challenging the paradigms surrounding

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henipaviruses and their associations with bats and may suggest a novel rodent sub-genus of henipaviruses.

The henipaviral genome consists of six structural proteins including nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), receptor-binding protein (RBP), and large (L) protein (approximately length of 18,000 nucleotides) (Luby, S. P., & Gurley, E. S., 2012). In HeV and NiV, slippage sites within the P protein lead to the expression of the V, W, and C proteins. These proteins are virulence factors and are absent in CedV.

Table 1 Summary viruses in Henipavirus

Henipavirus	**Origin (Year)**	**Reservoir**	**Genome (nucleotides)**	**Symptom for human**	**Reference**
Hendra virus (HeV)	Australia (1994)	Fruit bats	18,234	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Nipah virus (NiV)	Malaysia (1999)	Fruit bat	18,246	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Ghana virus (GhV)	Africa (2009)	Fruit bat (*Eidolon helvum)*	18,530	No data	Drexler, J. F., 2009
Cedar virus (CedV)	Australia (2012)	Fruit bat	18,162	Non-pathogenic	Marsh, G. A et al., 2012
Mojiang virus (MojV)	China (2012)	Rodent	18,404	Febrile	Wu Z et al., 2014
Gamak virus (GAKV)	Korea (2017-2018)	Shrew	18,460	No data	Lee et al., 2021
Daeryong virus (DARV)	Korea (2017-2018)	Shrew	19,471	No data	Lee et al., 2021
Langya virus (LayV)	China (2019)	Shrew	18,402	Acute febrile	Zhang et al., 2022
Angavokely virus	Madagascar (2019)	Fruit bat	16,740	No data	Madera et al., 2022

Viruses in the genus *Henipavirus* have a broad reservoir and may cause high case fatality rates following human infection. The discovery and characterization of the novel Henipavirus is a high public health priority.

The greatest bat diversity around the world especially appears to be found in the tropical rain forests of South East Asia. In Thailand, at least 138 species in 11 families and 45 genera of bats have been recorded, such as members of the genus Pteropus, Rhinolophus, and Hipposideros (Soisook et al., 2011). Khao Chong Phran, a cave in Ratchaburi Province, has possibly the largest colony of bats in Thailand. There are estimated to be around 2 million bats in the cave and 95% are insect-eating bats (Department of National Parks, Wildlife and Plant Conservation, Thailand). Moreover, Khao Chong Phran has many community sites, such as cave entrances near communities, guano collection, and tourism sites, which risk zoonotic spillover. Bats are widely considered the animal hosts of zoonotic pathogens such as rhabdoviruses, coronaviruses, paramyxoviruses, and filoviruses, and many of the host species can be found in Thailand.

MojV was associated with a severe pneumonia-like illness killing three of six miners in 2012 in a mineshaft Yunnan, China. Interestingly, this mineshaft was also site where RaTG13, a beta-coronavirus most closely related with SARS-CoV-2, was detected in *Rhinolophid* bats (Rahalkar and Bahulikar, 2020). Identifying the wildlife reservoir of this novel virus will assist intervention strategies to reduce spillover risk.

Research Gaps

As a part of Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH), serological investigations using a multiplex microsphere-based immunoassay (MMIA) have detected 19% (54/284) seroprevalence for MojV in a high-risk cohort, including one person with a high magnitude and durability of anti-MojV IgG across longitudinal samples. Despite this, genomic surveillance has not detected MojV or any novel MojV-like viruses. For this result, we would to further identify and characterize the origin of the Mojiang virus-reactive serum and to explore the novel MojV-like virus in Thailand.

Serological and cellular immunity is an important resource for virus discovery detection by using the specificities and characteristics of memory B cells from peripheral blood mononuclear cells (PBMC). The immune response will recognize and induce antibodies specific to the infecting pathogen. Characterizing the memory B cell response to an unknown pathogen in humans could in the detection of the exposure pathogen in humans. Molecular techniques such as PCR can detect only the genomic material in the body, but memory B cells are long-lived and thus have a much larger window of detection.

Memory B-cell differentiation is characterized by somatic hypermutation in antibody variable region genes (V) and selection of B cells expressing high-affinity variants of this antigen receptor and high sequence similarity in individuals exposed to the same antigen reflect antigen-driven clonal selection and form shared immunological memory between individuals (Yang et al., 2021). Memory B cells are highly abundant in the human spleen, and can proliferate 45% of the total B cell population in this organ (Hauser et al., 2015). Memory B cells can be detected in the Ig variable (IgV) genes in a fraction of IgM+ B cells. In humans, B cells expressing the memory marker CD27+ can be found in the marginal zone of spleen and circulate in the blood. After re-exposure to antigen, memory B cells can quickly proliferate and differentiate into plasma cells. Many studies the antibody diversity and specificity of the naturally paired immunoglobulin heavy and light chains for affinity maturation of B cell receptor (BCR) after virus infection such as SARS-CoV-2 infection (Yang et al., 2021), influenza (Dougan, S et al., 2013), HIV (Moir, S., & Fauci, A. S.,2017), etc. The characterization of memory B cells can be used to develop monoclonal antibodies and produce an effective therapeutic.

Significance and Approach

Southeast Asia is one of the world's highest-risk emerging infectious diseases hotspots. From this region emerged both SARS-CoV-1 and SARS-CoV-2, recurrent outbreaks of novel influenza strains, and the spillover of dangerous viral pathogens such as Nipah virus. The diversity of coronaviruses, henipaviruses, and filoviruses are found in wildlife reservoirs in Southeast Asia and likely due to the high diversity of wildlife, and the demography of the human population brings humans and wildlife into close contact. The spillover of viruses from animals to humans is often misdiagnosed and the

clinical symptoms from patients is underestimated during the pandemic. This study provides a surveillance strategy in human and wildlife populations to better understand immunity to novel pathogens and identify the origin of these pathogens to serve as an early warning system to prevent outbreaks of emerging viruses.

This study uses immunology (serologic and cellular) to identify a novel virus that can aide in effective vaccine discovery and development, monoclonal antibody production for prevention and therapy, and generate data resources for early detection and diagnostic assays. Whereas PCR detection or virus isolation in wildlife is challenging, this new serological approach may be more successful in identifying reservoir hosts.

The Indo-Pacific region has a high risk of novel viral emergence. This study provides an opportunity for in-country research capacity building by linking local infectious disease scientists with an international collaborative network that has proven capacity to conduct this work and produce significant findings. Moreover, a global sero-surveillance network for surveillance technology extends virologic and immunologic screening to product discovery. This study will benefit and expand the CREID, EID-SEARCH networks, and the global sero-surveillance network to improve our understanding of immunology to identify novel pathogens and their origin to prevent future pandemics.

Research Methods

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population

Serological profiling provides an indirect measurement of the infecting virus. Despite 19% (54/284) seroprevalence in humans, we have not detected nucleic acid sequences of MojV or MojV-like henipaviruses in this human cohort. ~~In addition to the translational application of mAbs as therapeutics, we intend to utilize these mAb as non-clinical diagnostics surveillance tools for virus/virus antigen capture.~~ Additionally, we intend on expanding our understanding of henipavirus prevalence in this population with longitudinal sampling of the wildlife and human populations in this region.

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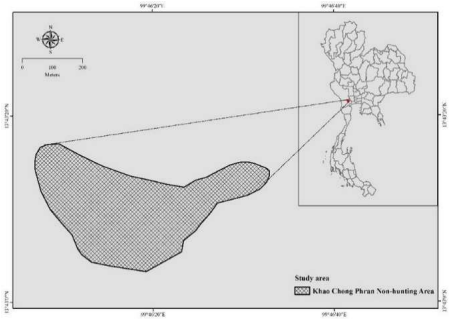

Figure 1: Map of Khao Chong Phran Non-hunting Area, Ratchaburi Province, Thailand (Boonpha N et al., 2019)

Site wildlife samples collection

100 bats and 100 shrews or rodents in and around the Khao Chong Phran cave in Ratchaburi Province will be collected for surveillance targeting the Mojiang-like virus. We plan one sampling trip to collect

bats, and a second trip to target shrews and rodents. Due to limited knowledge of the local shrew population, we are unsure as to whether we can collect 100 samples, and will supplement the collection with rodents, as needed. Samples will be collected based on the following enrollment criteria: random age, random weight, random sex. Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and stored. Oral swab and rectal swab will also collect in 100 bats and 100 shrews or rodents. Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and stored.

Human surveillance

Four of the samples from prior investigations have shown high MojV RBP-reactive antibody titers and will be selected for follow-up sampling. Additionally, 100 human serum samples, nasopharyngeal swab and throat swab will be collected from persons deemed to be in close contact with wildlife, and people who present with or have recent history of undiagnosed acute febrile illness using the following enrollment criteria;

- Adults (≥ 18 years of age) who sign in the informed consent
- Who present with or have recent history of illness without diagnosis, such as severe/acute respiratory illness (SARI/ARI), Influenza-like illness (ILI), fever of unknown origin (FUO), or respiratory tract infection
- Who is the bat guano collector in the cave
- Who lives or works around wildlife habitats
- Who is involved with livestock or wildlife farming
- Who hunts or works with wildlife
- Who has not been previously sampled as part of the EID-SEARCH and PREICT projects

Sample size justification

Human population

$$n = \frac{N}{1 + Ne^2}$$

n = sample size of the study

N = population size for finite populations: 8,150 (data from 2018)

e = error: 10%

$$n = \frac{8{,}150}{1 + (8{,}510 \text{ x } 0.1^2)}$$

n = 98.79 (round up to 100 samples)

Wildlife population

The sample size of 100 of each species follows the guidelines established during the PREDICT project.

Targeted Viral Sequencing and Serologic testing

Targeted sequencing

For samples from shrews, rodents, and acute individuals, we will use targeted sequencing and metagenomic approaches in an attempt to detect the Thai MojV-like virus. The nucleic acid from oral swab and rectal swab will do target sequencing in henipavirus Family PCR and Sanger sequencing.

Serological testing

Serum samples will be tested for RBP and envelope fusion protein (F) binding to the known henipavirus species, including the Nipah, Hendra, Cedar, Mojiang, Ghana, Langya, Gamak, and Daerong viruses to further investigate the seroprevalence in this region. Additionally, prior positive samples will be re-tested with the expanded panel to enhance our understanding of the antigenic relationships between the novel shrew-borne henipavirus and the Thai virus.

Data analysis

Sequencing will be analyzed assembling reads in MEGA11. The consensus sequences will compare to the references strains available in the GenBank data-base using the Basic Local Alignment Software Tool (BLAST) (National Center for Bio-technology Information). Serological data will be analyzed using cluster analysis in RStudio.

Aim 2. Characterization of monoclonal antibodies from humans with serological profiles consistent with MojV infection.

In collaboration with NIH VRC PREMISE, MojV RBP and F-reactive B cells will be captured, and the B cell receptor will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific mAbs will be generated from the corresponding sequences and will be assessed for binding potential to LayV RBP and F, as well as their neutralization potential.

Importance: The isolation of these mAbs represents a potential therapeutic for MojV, MojV-like henipas, and LayV, depending on how well these cross react.

Human samples collection site

Human positive serum for MojV was found close to Khao Chong Phran in Ratchaburi Province, which has the largest colony of bats in Thailand (Figure 2).

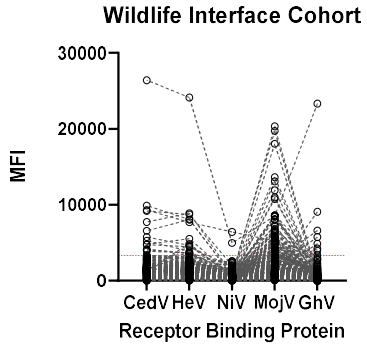

Figure 2: Seroprevalence of henipavirus proteins from a community with a large bat interface during 2017-2018. MFI is the median fluorescence intensity for IgG reactivity to each protein. Red-dashed line is at 3,357 MFI, a threshold for seropositivity.

PBMC Sorting and B Cell isolation

Whole blood from MojV-positive people will be collected in individual heparin tubes and processed for PBMC isolation using Density Gradient Centrifugation (SepMate™ PBMC Isolation Tubes). Human PBMCs will be stained with antibodies for positive markers of IgG memory B cells, and antibodies to negatively select and avoid contamination with dead cells, T cells, NK cells or monocytes (live, CD3−,CD14−,CD56−, IgM−, IgA−, CD19+,CD20+,CD27+). Avi-tag for biotinylation and 6xHIS tag for Nickel purification using the HisPur™ Ni-NTA Spin Purification Kit (Thermo Fisher Scientific, Waltham, MA). Single Memory B Cells will be sorted, cells will proliferate, cDNA will synthesized, then Heavy and light chain variable domains will be amplified (Waltari E et al., 2019).

B cell receptor Amplicon Preparation and Recombinant Antibody sorting

Total RNA yields from the PBMC will be determined by absorbance at 260 nm on the NanoDrop™ One (Thermo Fisher Scientific). Total RNA will be used for first strand cDNA synthesis with gene-specific reverse transcription (RT) primers directed to the constant regions. The RT primers for IgG, IgM, IgA, lambda, and kappa will be included. Light chain and heavy chains will be amplified into amplicon. Double-stranded cDNA will be sequenced. After completion of MiSeq sequencing, antibody repertoires will analyze using methods based on the pipeline. Heavy and light-chain sequences will be assembled into an expression vector and transfected into cells for monoclonal antibody expression. Recombinant antibody will be isolated and purified for downstream applications.

Functional assessment

We will first determine the extent to which isolated mAbs can bind binding to native-like MojV RBP and F proteins. We will also characterize the cross-reaction potential of these mAbs will be assessed against GamV, DarV, and LayV RBP and F. Neutralization of viruses remains a critical correlate of protection for most vaccine strategies. However, MojV has not been isolated or rescued, and the virus receptor for MojV has not yet been discovered (Rissanen et al., 2017; Cheliout et al., 2021). Thus, a functional assessment of MojV neutralizing potential is not possible. Though, we will test the cross-neutralization potential of these anti-MojV RBP and F mAbs to neutralize LayV utilizing a plaque reduction neutralization test with EID-SEARCH co-investigators at Duke-NUS. As LayV causes acute illness in human, cross neutralization functionality of these mAbs has therapeutic potential.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Project Timeline

Activity	05/23	06/23	07/23	08/23	09/23	10/23	11/23	12/24	01/24	02/24	03/24	04/24
Mentoring and training activities												
1.Training, literature review												
2.Monthly mentoring meeting												
3.Manuscript writing												
4.EID-SEARCH meeting												
Research activities												
1.IRB amendment												
Aim 1												
2. Human and Wildlife samples collection												
3. Identify Targeted sequencing												
4. Serological testing												
Aim 2												
5. Collect PBMC human sample and isolation memory B-cell for Mojiang virus												
6. PBMC Sorting and B Cell isolation												
7. Determination of envelope glycoprotein epitope binding												

Research Performance Sites

This proposed study will be implemented at the King Chulalongkorn Memorial Hospital (KCMH) collaborating with the Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC). KCMH is a 1,000-bed, university hospital in close coordination with the Faculty of Medicine, Chulalongkorn University. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. It's one of the center hospitals for Emerging Infectious Diseases in Thailand, especially for domestic and international patients.

TRC-EIDCC was established by the Committee of the Emerging Infectious Disease Center, which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. Our clinical center provides standard medical care to patients with emerging and recurrent diseases and disease transmission. Moreover, the center plans preparedness activities, trains medical and government staff on interventions, hosts conferences for medical and public health professionals, creates networks of medical care for patients with emerging and recurrent diseases, and leads research and development projects for medical care and disease control. Apart from medical innovation in emerging and recurrent diseases, the clinical center strives to be the model of infectious diseases clinical services and pandemic control through research activities surrounding infectious diseases and effective disease control measures.

We also collaborate with the Department of National Parks, Wildlife and Plant Conservation for detecting novel pathogens from animals such as bats, rodents, and shrews in our country. We also collaborate with the Department of Disease Control of Thailand (Thai-DDC) to support the Thai government and national policy makers through molecular diagnosis, including the identification of the first case of SARS-CoV-2 infection in Thailand. TRC-EIDCC prides itself on its high performance in research laboratory services and is a part of the national reference laboratory for COVID-19 and other emerging infectious diseases confirmation in Thailand.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

CREID Research Center Collaboration

The goals and objectives of the proposed research project, "Immune memory bait & capture to identify emerging henipavirus origins", are fully consistent with the specific aims of EID-SEARCH. EID-SEARCH takes a One Health approach to understanding zoonotic disease emergence, with a focus on viral diversity in wildlife at high-risk human animal interfaces. EID-SEARCH aims to understand the frequency and causes of virus spillover in high-risk communities across Southeast Asia and identify strategies and tools to reduce this risk. As the proposed research focuses on the links between farming practices, wildlife diversity, virus diversity, and cross-species transmission in Thailand, we expect the results of the project to make a significant contribution towards the overall goals of EID-SEARCH.

The proposed project is well-designed, leverages cutting-edge technology, and includes a robust ecological component that corresponds to the overall research strategy of EID-SEARCH. The EID-SEARCH team is highly motivated to support the application of metagenomics to ecological questions and assist in the analysis of the unique dataset that will be generated from this research. With the heightened focus on identifying and limiting risks associated with the complex interactions between humans, wildlife, and domestic mammals that has resulted from the emergence of SARS-CoV-2 from a wet market in China, the proposed research is both timely and likely to yield highly-cited, impactful publications for EID-SEARCH and Thailand partners.

The EID-SEARCH team includes a number of experts in study design, laboratory-based diagnostics, genetic and genomic analyses, and ecological modelling who will contribute to project implementation, data analysis, and manuscript preparation as required. Communication and coordination mechanisms will be set up between the Thailand research team and scientists at the EID-SEARCH research sites for resource sharing, training, knowledge exchange, and mentorship. In particular, EID-SEARCH will facilitate and encourage relationships between our partners in Southeast Asia (based in Malaysia, Singapore, and Thailand) that will facilitate Thailand team professional development and strengthen research capacity in the region. Dr. Eric Laing, the project Mentor, has an established relationship with both Dr. Krongkan and Spencer Sterling, and will act as the liaison between EID-SEARCH and the TRC-EIDCC research team. Further, Dr. Doeuk who is the director of the VRC PREMISE program is enthusiastic about the opportunity to collaborate and apply their rapid assembly, transfection and production of immunoglobulin (RATP-Ig) workflow as a serologic/immunologic based approached for therapeutic and virus discovery (see Letter of Support). An expected outcome of this research project will be pilot data to support a joint funding application in the future.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Mentoring Plan

Goals for Pilot study and Career

Krongan Srimuang: My career goal is to be an expert scientist in either an academic research laboratory or a lecturer in medical science. My long-term research interests include the understanding of priority emerging infectious diseases, genetics, and epigenetics using molecular and serologic techniques. Detection of novel pathogens in their wildlife vectors can help characterize the pathogenicity, virulence, and evolution of these and other closely related pathogens. I believe that this pilot study can provide training in new techniques for the detection, identification, and characterization of novel pathogens that are currently unavailable in Thailand. My goal for the Pilot study is to gain more knowledge and skills for a lifelong career in public health-related research. I wish to expand my skills in novel virus detection using next-generation serologic techniques such as PBMC studies and memory B-cell isolation and sequencing that can contribute to improved surveillance and research capacity at human-animal interfaces. My mentors Dr. Laing and Dr. Wacharapluesadee can provide me with opportunities to develop and apply project skills and improve my scientific writing.

My education has provided me with a strong foundation to expand for this study. I received my undergraduate degree in Medical Technology from Chulalongkorn University which included training in molecular biology, serology, immunology, chemistry, virology, bacteriology, mycology, parasitology, hematology, clinical microscopy, transfusion medicine, and forensic medicine. My interest in molecular technology led me to pursue a Ph.D. at the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, which provided me with training in several molecular techniques including conventional PCR, real-time PCR, Next Generation Sequencing (NGS), etc. The emergence of COVID-19 motivated me to focus on emerging infectious diseases. I am currently completing my post-doctoral fellowship at the Emerging Infectious Diseases Clinical Center (EIDCC), King Chulalongkorn Memorial Hospital with Dr. Wacharpluesadee. Throughout this fellowship, I have continued learning advanced and innovative techniques to detect, identify, and characterize the novel pathogens. Through this pilot project, I will learn about the Peripheral Blood Mononuclear Cells (PBMC) which are a critical part of many subdivisions of medical research including Infectious diseases, Vaccine development, and Immunology. This project aims to study zoonotic reservoirs for by using serologic and cellular immune discovery in humans, and applying this immune knowledge in wildlife for early detection and identification of novel pathogens. This proposed study will expose me to various techniques and immunology topics that I useful in designing and testing new pipelines as technological advances continue to develop. This study will also allow me to develop, manage, and communicate my research.

Spencer Sterling: My overall career goal is to understand the factors that lead to viral zoonosis and to build epidemiological capacity in regions with the highest risk for zoonotic spillover. Through my

training and educational experiences, I have learned laboratory and epidemiological techniques that can assist in the understanding of zoonosis. I wish to continue my education by expanding my repertoire of molecular techniques to include studies of the immune system's response to zoonotic disease in humans and animals, as well as to learn next-generation sequencing techniques. Additionally, I would like to use my experience in epidemiological techniques to train scientists in the Southeast Asian region in order to build local capacity in infectious disease research. Dr. Laing has been a mentor for me throughout my career, and this project would allow for this relationship to expand into new molecular areas, as well as grant writing and project management skills. My professional relationship Dr. Wacharapluesadee with began in 2018 when I participated in a DTRA BTRP funding project that focused on transferring antigen-based multiplex serological technology to her lab group. Currently, I am a Visiting Scientist at TRCEIDCC and I work closely with Dr. Srimuang, and other members of Dr. Wacharapluesadee's research group on EID-SEARCH activities. Additionally, Dr. Wacharapluesadee has decades of experience in infectious disease research in the region and has mentored scientists that have gone on to careers at the Thai Ministry of Public Health, Department of Disease Control, Department of Parks and Wildlife, academia, and the private sector. Both mentors have encouraged Dr. Srimuang and I to apply for this pilot study as well as pursue other funding mechanisms for our research interests. Both mentors will provide for me the ability to independently pursue my research interests in the zoonotic epidemiology and train me on the best practices of scientific communication.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Vertebrate Animals Section Requirements

Animal samples will be collected in Ratchaburi Province for this project under Aim 2 to understand the antigenic relationships among known and unknown henipaviruses. Protocol to perform animal sample collection has been reviewed and approved by the Institutional Animal Care and Use Committee at Tuft University under the EID-SEARCH project (No. G2020-42) and the Institutional Animal Care and Use Committee at Chulalongkorn University (No. 019/2563)

1. Description of Procedures

Bats (order *Chiroptera*)

- Species: Free-ranging mega and micro-*Chiroptera* that present at the site (family *Pteropodiae, Rhinopomatidae, Emballonuridae, Craseonycteridae, Megadermatidae, Rhinolophidae, Hipposideridae, Vespertilionidae, Mollossidae*, etc.)
- Age & Sex: Fully flighted adults and juveniles, males and females; neonates dependent on the dam will not be used.
- Target Number: 100 individuals
- Capture and Restraint: Bats will be captured using either a mist net or harp trap. The net system is manned by at least two people during the entire capture period, and bats are removed from the net as soon as they become entangled to minimize stress and prevent injury. In our previous field research experience, a maximum of 20-30 bats can be safely held and processed by a team of three people per trapping period. Duration of trapping will depend on the capture rate. Bats are individually placed into a pillowcase or small cloth bag and hung from a branch or post in a location that is free from access by both predators and people, and is protected from the elements (e.g., rain), until the animal is restrained for sample collection. Bats will be manually restrained during sampling utilizing insulated bite gloves to prevent injury to the handler and bat. The study protocol covers over 69 bat species that are most likely to be captured given their natural occurrence and previous surveillance experience in Southeast Asia. The potential for non-target species to be captured is very low because mist nets are opened at the cave entrance at night, after other species (e.g., birds) have ceased flying and returned to roost. Any non-target species will be rapidly identified, removed from the mist net and released immediately.
- Sample Collection: Depending on the species and size of bat, blood will be collected from smaller insectivorous bats (<50g) using a 27g needle to puncture the brachial artery and a 70ul hematocrit tube to collect the blood. For larger bats (>50g) we will collect blood from either the cephalic vein or from the radial artery or vein using a 25- or 23-gauge needle and 3cc syringe. Blood will be collected in quantities not exceeding 6 µL/g of body mass from each individual. Wing punch biopsies will be collected to confirm host identification using DNA barcoding. Swabs will be taken from the oropharynx, urogenital tract, and rectum. No destructive sampling is planned, but any individuals that die during capture or sampling will have a necropsy performed with organ samples collected and whole body submitted to museums as voucher specimens.

- Release: Bats are held for a maximum of six hours (typically less than three hours), and at the completion of sampling are provided with rehydrating fluids (either subcutaneously or orally as needed) and released in a location that allows for further recovery or safe escape (e.g., bodies of water, known predatory areas, and human settlements will be avoided).

Rodents (order: *Rodentia*) and Shrew (order: *Scandentia* and *Eulipotyphla*)

- Species: Free-ranging rodents (family *Hystricidae, Muridae, Sciuridae, Spalacidae*) and shrew (tree shrews, shrews, moles) that present at the sites.
- Age & sex: Adults and juveniles, males and females. Neonates will not be sampled.
- Target Number: 100 individuals
- Capture and Restraint: Free-ranging wild mammal species will be captured with metal box traps (Sherman/Tomahawk traps). Traps will be prepared with food, water, padding and shelter, and be checked at least every 12 hours, in the morning and in the afternoon. If adverse weather (extreme heat, rain) is expected or researchers are working in areas where predation is common, traps will be checked more frequently and closed during adverse weather. Veterinary staff will be prepared to deal with a variety of trap injuries, including lacerations, broken teeth, and crush injuries. Depending on the species, individual size, captive or free-ranging status of the individual, manual or chemical restraint and anesthesia (gas or injectable anesthesia) will be applied. Animals will be manually restrained during sampling utilizing insulated bite gloves to prevent injury to the handler and animal. Based upon past field experience (>20 years of animal capture and release in Southeast Asia), we do not expect to trap sick, debilitated, or young animals that would be too young to cope with capture. Sick and debilitated animals tend not to roam widely, reducing their opportunity to enter traps. Additionally, should sick, debilitated, or young animals enter traps, we anticipate that they will not suffer adverse conditions because the traps contain food (the bait) and liquid (water or fruit as a source of water). These individuals would be examined by a veterinarian, rehydrated and if presenting clinical signs treated by a veterinarian, prior to release or sampling then release.
- Sample Collection: Once anesthetized or safely restrained, whole blood will be collected through the appropriate venipuncture site, no more than 1 ml of blood per 100 g (= 10 ml/kg or 1%) of body weight will be collected at any one time. Anesthetized animals will be monitored regularly during recovery until they can no longer be safely handled, at which point they will be confined in a trap or cage. Swabs will be taken from the oropharynx, urogenital tract, and rectum. No destructive sampling is planned, but any individuals that die during capture or sampling will have a necropsy performed with organ samples collected.
- Release: Wild mammals are held for a maximum of thirteen hours depending on trap timing, but typically less than three hours. Captive animals are held for a maximum of two hours but typically less than one hour. At the completion of sampling, animals may be provided with rehydrating fluids (either subcutaneously if anesthetized or orally if manually handled and accepted). Wild mammals will be released at the site of capture in a location that allows for further recovery or safe escape (e.g., bodies of water, known predatory areas, and human settlements will be avoided). Captive mammals will be released back into their captive setting as is appropriate for the species, either isolated or in social group if it is deemed safe from aggression from enclosure mates while the post-recovery period continues.

2. Justifications

The purpose of this study is to identify zoonotic pathogens through serologic and cellular immune discovery, including developing methods and standards for direct antibody-mediated virus capture in people and wildlife, focusing on rodent/shrew- and bat-borne HPVs. This will require hands-on fieldwork to collect whole blood and serum samples from bats, rodents, and shrews for lab analysis. One technique that avoids the direct capture and sampling of animals is to collect fresh feces or urine,

but this will not be able to provide the appropriate samples for serologic analysis, and the research objectives cannot be achieved using an alternative methods (e.g., computational, human, invertebrate, in vitro). Therefore, we believe there are no viable alternatives than the use of live animals.

3. Minimization of Pain and Distress

In every situation, sampling of wildlife will be conducted in the most humane manner while minimizing the impacts on individual animals and their wild populations. In all instances, the fewest number of animals will be sampled that will provide valid information and statistical inference for the pathogen and disease of interest and every effort will be made to minimize stress and discomfort for the animal.

Bats will not be held longer than six hours during the sampling process whereas small mammals may be held for up to 13 hours depending on trap capture timing. Field animal sampling team with zoologists and veterinarians have been well trained and have extensive experience in capture, anesthesia, and sampling of wildlife. In our team's experience, the target species tolerate the described procedure well. Mist nets will be attended continuously during capture periods, and bats will be extracted from the net as soon as they become entangled. This will minimize stress and injury from entanglement. Bats will be placed individually in cloth bags and hung from tree branches while awaiting processing and during recovery. The bags are sufficiently porous as to allow for ventilation and are designed for bat capture. The enclosed environment seems to calm the bats, as they do not struggle once inside, but they hang quietly – this is a standard and accepted practice in bat research and the best way to minimize stress to the animal.

For rodents and shrews, food, water, padding and shelter will be provided at traps, which will be checked at least every 12 hours. Traps will be checked more frequently or closed if adverse weather (extreme heat, rain) is expected, or researchers are working in areas where predation is common. Veterinary staff will be prepared to deal with a variety of trap injuries, including lacerations, broken teeth, and crush injuries. All animal individuals will be monitored by a veterinarian or experienced field team member during all stages of capture, processing, anesthesia and release. Animals will be kept in a cool place, free from adverse weather conditions and access by non-field team members or other animals while in the pillowcases, trap, or cage for recovery. All recovering individuals will be observed until they are able to effectively ambulate and orient to their surroundings. Animals that are injured during the capture or sampling process will be assessed by an experienced team leader or attending veterinarian, and if the animal is determined to be unlikely to survive if released, it shall be euthanized humanely (see euthanasia section).

4. Method of Euthanasia

In the event that an animal has been injured or is moribund, a determination will be made as to whether it may be treated and released on site by veterinarians or transferred to facility that may treat and rehabilitate the animal, or if, as a last resort, the animal will require euthanasia. Euthanasia methods will vary depending on species; however methods to be used will not deviate from the AVMA “Guidelines for the Euthanasia of Animals” (2020 edition). Any animal that is euthanized using a chemical agent will be disposed of such that it will not be permitted to enter the food supply either through markets or hunting.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Human Subjects Research

1. Risks to the subjects

In this project, we will target populations in one community site who with frequent exposure to bats and other wildlife based on our previous behavioral survey. Subjects will be enrolled voluntarily, and informed consent will be obtained from all participants, along with assent from all participants aged 12-17. Enrolled participants will provide biological samples. Survey data and biological samples will be collected from enrolled participants, and follow-up data collection will be performed among participants whose samples were tested positive.
Biological samples and questionnaire data will be collected from individuals who meet recruitment and inclusion criteria and complete the informed consent process. During data collection, a standardized questionnaire will be administered to all participants. This questionnaire will collect information of demographic background, wildlife contract, travel and daily movement, and unusual SARI/ILI symptoms, and biological specimens will be collected from participants. Both questionnaire and biological data will be analyzed to assess the exposure to coronaviruses and the spread risk among humans. From all participants, a one-time whole blood sample (Max. 15 mL) will be collected during the study period. This sample will allow us to test for historical exposure to bat or rodent-born henipaviruses and collect Peripheral Blood Mononuclear Cells (PBMCs) for those study participants whose serum tests are positive.

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 who are 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, the administration will be conducted privately and confidentially to protect individuals' 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 these risks

2.1 Informed consent and assent

Only consented participants will be enrolled in the study. No research procedures will be undertaken before the participant fully understands the research, agrees to the study procedures, and completes the consent process will be enrolled in the study. Informed consent statements and forms, and project protocols will be translated into the local language of each study site. Research team members involved in this consent process will be required to be fluent in the local language to ensure that the subjects understand the study and all involved procedures.

If participants meet the criteria for enrollment, they will be invited to discuss the details of the study with the research staff. Study staff will review an information sheet and informed consent form with the participant and the parent or legal guardian when applicable. Each individual will be provided with a copy of the informed consent form that has been translated into the local language and written with a Flesch–Kincaid readability score equivalent to a 7th-grade reading level or below, to assure that potential participants understand the information being shared. The informed consent form will explain the details of the study, including how and why the individual was selected, the study process and procedures, risks and benefits, financial considerations and the gift of appreciation, confidentiality of data shared, alternatives to participating, and how to obtain more information now or at a later date. The informed consent form will be read in the local language of the site at a location ensuring participant privacy. After which individuals will have as much time as they would like to ask questions and discuss the study with study staff. The study staff will endeavor to ensure that the participant understands the information provided. The study staff will then ask the participant/parent or legal guardian to consider study participation. Participants will have as much time as required to consider the participation.

Those participants who consent to the study will sign and date two copies of the consent form. These form copies will be countersigned and data by the study staff. A copy of the signed consent form must be provided to the subject and the other copy will be kept by study staff. Informed consent paperwork will be kept until the end of the project in a locked box at the local country project office.

2.2 Protection against risks

The potential risks to study participants as a result of study participation are minimal. Collection of venous blood samples and nasal or oropharyngeal swab samples pose minimal risk to subjects. Potential complications associated with venipuncture include pain and/or hematoma at the site of collection. Trained medical professionals and/or clinic staff will monitor the blood collection site and treat any complications according to existing health facility protocols. A potential complication of nasal/ oropharyngeal sampling is minor irritation at the time of collection. Employing trained medical and/or clinic staff to collect blood and swab samples will minimize the potential for complications.

Another risk that this study may pose concerns the information to be gained on henipaviruses newly recognized in the community. We will provide participating communities, hospitals, and clinicians with information and background data on target zoonotic viruses to ensure up-to-date communication of risk. Because of the timeline for diagnostic testing and results interpretation, we are not likely to provide results to participating clinics within a time frame that would be clinically relevant to outbreaks of undiagnosed diseases. Therefore, the information provided by this project will not impact patient management or outlook.

If an individual decides to participate in this research, his/her participation and all information provided by the participant will be strictly confidential, and personal identifying information will not be shared with anyone outside of the study staff. Participants will not be identified or named in any reports or publications. Questionnaire information and all biological samples will be identified by an alphanumeric code, not by the participant's name. 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 study site in locked files cabinets or password-protected devices in areas with access limited to the research staff of this project. Research databases will be secured with password-protected access systems and controlled distribution web-based certificates and will not contain any identifying characteristics about study participants (e.g., name, address, or telephone number). Access to all data will be limited to the staff involved in this study. The health information disclosed by an individual will not be used

by or disclosed (released) to another institution. Any surveillance report that is published or shared with partners will not contain any personally identifying information for individual participants.

3. Potential benefits of the research to the subjects and others

There are no measurable benefits to the individual study participants enrolled in this study. There are benefits to the community and regional healthcare providers to help them understand the risk of zoonotic infections among high-risk populations in the regions they work. At the conclusion of the study, we will deliver an educational workshop reporting aggregate study findings that will be open to both study and non-study participants, describing the health benefits of using personal protection equipment (PPE) and handwashing during animal handling activities throughout the day, as well as to share other prevention/interventions that emerge from the research data.

4. Importance of the knowledge to be gained

There are valuable potential benefits to the public from the knowledge to be gained from this study. One key benefit to the community is sharing information and knowledge to better understand the risk of zoonotic spillover events and related health risks, as well as information sharing with communities on practices that could reduce risks, such as the avoidance of particular animal contacts or the need for PPE and extra care when handling wildlife, that may substantially reduce the risk zoonotic pathogen transmission in the community. 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 create or implement public health interventions to disrupt disease emergence and/or spread in an area that is beneficial to all. Additionally, there are valuable benefits to the general public from the knowledge to be gained from this study. Knowledge gained will 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.

5. Country- / institution-specific ethics / IRB regulations addressed

Main research protocol and materials to conduct human subject research in this project have been reviewed and approved by the Health Medical Lab Institutional Review Board, US (No. 894ECOH21b) and the Institute Review Board of the Faculty of Medicine, Chulalongkorn University, Thailand (No. 211/64). Amendment for PBMCs collection will be made and approved before the start of this project.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Research and Related Other Project Information

1. Are Human Subjects Involved?	Yes	X	No													
1.a. If YES to Human Subjects																
Is the Project Exempt from Federal regulations?	Yes		No	X												
If yes, check appropriate exemption number	1		2		3		4		5		6		7		8	
If no, is the IRB review Pending?	Yes	X	No													
IRB Approval Date:																
Human Subject Assurance Number	FWA00000943															

2. Are Vertebrate Animals Used?	Yes	X	No	
2.a. If YES to Vertebrate Animals				
Is the IACUC review Pending?	Yes		No	X
IACUC Approval Date	3 December 2020			
Animal Welfare Assurance Number				

3. Is proprietary/privileged information included in the application?	Yes		No	X

4.a. Does this project have an Actual or Potential impact -- positive or negative -- on the environment?	Yes		No	X
4.b. If yes, please explain				
4.c. If this project has an actual or potential impact on the environment, has an exemption been	Yes		No	

authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed?					
4.d. If yes, please explain					

5. If the research performance site designated, or eligible to be designated, as a historic place?	Yes		No	X
5.a. If yes, please explain				

6. Does this project involve activities outside of the United States or partnership with international collaborators?	Yes	X	No	
6.a. If yes, identify countries	Thailand			
6.b. Optional explanation				

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Budget Justification

A. Key Personnel (Total $24,000)

Dr. Krongkan Srimuang, PI (12 months) is a medical research scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital (TRC-EIDCC) who have been leading the laboratory analysis for the EID-SEARCH work in Thailand in the past three years to perform molecular, serological, and next-generation sequencing to identify novel viruses from human and animal samples. Dr. Srimuang will commit 12 months to this project to design, manage, and perform all project activities in the lab and field by supervising a lab technician and working with the field coordinator. Dr. Srimuang will also work closely with the co-PI and Mentors to conduct data analysis and develop manuscript and join EID-SEARCH and CREID meetings to prevent the project findings.

Spencer Sterling, Co-PI (6 months) is the research coordinator of Laing Lab at the Uniformed Services University for Indo-Pacific region and a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC). Sterling will commit 6 months to this project as the co-PI, who will work closely with the PI in the study design, SOP development, sampling process, data analysis of for serological investigations. No salary is required for Sterling.

B. Other Personnel (Total $20,000)

One (1) Laboratory Technician (12 months) will committee 12 months to this project to conduct PCR and serological testing of animal and human samples at TRC-EIDCC, under the supervision of Dr. Srimuang and Sterling. Salaries for 12 months at $1,250 per month is requested ($15,000).

One (1) Field Coordinator (4 months) will committee 4 months to this project, working closely with Dr. Srimuang and Sterling to organize and conduct field sample collection among humans and animals at the study sites, and support relevant financial management. Salaries for 4 months at $1,250 per month is requested ($5,000).

C. Fringe Benefits

No Fringe is requested.

D. Equipment

No equipment is requested.

E. Travel (Total $48,375, Years 1-5)

Domestic Travel ($11,005)
Support for domestic travel for field sampling is requested in the amount of $11,005 per to conduct four (4) trips of sample collection from humans, bats, and rodents/shrews.

- The first trip is a one-day trip to collect samples from human participants who were tested positive (n<10), the cost is estimated at van rental for local transportation between Bangkok and the study site ($260) and meals for five (5) field team members at $49 per person ($245), in total of $505 for the first trip.

- The other three (3) field trips will be conducted to collect samples from 100 bats, 100 humans, and 100 rodents/shrews, respectively. Each trip is a duration of 6 days and 5 nights. For each person, 5-night stay in hotels at $75.5/night (5 x $75.5 = $378); 3 meals per day and incidental expenses at $49 per day prorated to 75% ($36.75) for first and last day of travel ($36.75 x 2 + $49 x 4 = $270), the total meals and accommodations for each person per trip is $648 ($378+270), cost for five (5) field team members is $3,240. A van will be rented for local transportation between Bangkok and the study site that is estimated at $260 per trip. Total cost for each 6-day trip is $3,500, three trips will be $10,500.

International Travel ($5,023)
Support for international travel for Dr. Srimuang from Bangkok to Washington DC to attend the CREID Annual Meeting and receive lab training at the National Institute of Allergy and Infectious Diseases (NIAID) is requested in the amount of $5,023. Trips are for a duration of 10 days and 9 nights. Travel costs for one trip are estimated using federal per diem rates for hotels, meals, and incidental expenses as follows: nine-night stay in DC area hotel at $286/night (9 x $258 = $2,322); Ten days' meals and incidental expenses at $79 per day prorated to 75% ($59.25) for first and last day of travel ($59.25 x 2 + $79 x 8 = $750.5); Round trip flight between Bangkok to Dulles International Airport is estimated at $1,650; local transportation/taxi between hotel and airport per round trip between Chulalongkorn Hospital to airport is estimated at $40, between hotel and the airport in the US is estimated at $200, and $60 for transportation within the region ($300).

F. Participant Support Costs

No participant support is requested.

G. Other Direct Costs (Total $78,803)

Materials and Supplies ($72,830)
PBMCs isolation ($47,000). We request funds to conduct PBMC isolation from collected sample, costs including the reagents and lab consumable and supplies are estimated at $47 per cell for 1,000 cells.

Multiplex microsphere immunoassay detection ($2,275). Funds are requested to perform serological testing of collected human and animal samples. Costs including the reagents and lab consumable and supplies are estimated at $6.5 per sample for 350 samples.

PCR testing and sequencing ($20,475). Funds are requested to perform serological testing of collected human and animal samples. Costs including the reagents and lab consumable and supplies are estimated at average $58.5 per sample 350 samples, given a 10%~15% positive rate.

Field supplies for PBMC collection and processing ($1,225). Funds are requested to perform PBMCs collection and processing in the field. Costs including the reagents and lab consumable and supplies are estimated at average $3.5 per sample 350 samples.

Field supplies for animal sampling ($1,160). Funds are requested to purchase supplies for animal capture, anesthesia, and sample collection. A total cost of $1,160 is estimated

Field work disposable ($668). Funds are requested to purchase disposable materials (tips, tubes, gloves, masks, googles etc.) for field work.

Consultants Service ($3,000)
One (1) zoologist and one (1) veterinarian will provide consultancy service to this project to help 1) select sites for animal sampling and join the sampling trip to 2) identify species; and 3) provide veterinary care in the sample collection of bats, rodents, and shrews. The consultancy fee is estimated at $1,500 per person for the project, in total of $3,000.

Publication Fee ($3,000)
We request $3,000 of publication costs for one peer-review paper generated from the project. The cost is estimated at the reimbursed Open Access fees of $2,900 - $5,000 per manuscript in the past two years for six peer-review papers.

H. Total Direct Costs (Total $138,831)

I. Indirect Costs (Total $11,106)

An indirect cost at 8% is requested.

J. Total Direct and Indirect Costs (Total $149,937)

A total amount of $149,937 is requested.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Co-PI Plan (only needed if applying as Co-PIs):

What each Co-PI will contribute to the proposed research study

As stated in the Mentoring Plan, the co-PIs, Krongkan Srimuang and Spencer Sterling have different educational and training backgrounds. This Co-PI plan is proposed to fill the gaps in knowledge of each scientist and supports a collaborative working relationship for project success. Moreover, this plan was developed to maximize the strengths of each PI. Overall, the project is separated into two objectives, the proposal of this project will be equally managed by Dr. Srimuang and Mr. Sterling. Dr. Srimuang will be responsible for PBMC isolation, sorting, and characterization. Mr. Sterling will be responsible for the technical serological aspects of the project. Both will work closely with the Research Center Mentor, Dr. Eric Laing, to develop this project, protocol development, and network connections. Dr. Supaporn Wacharapluesade, the Co-mentor, will support the wildlife sampling, samples collection, and molecular aspects within Thailand.

How the Co-PIs will jointly work with the affiliated Research Center

Mr. Sterling is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region.

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How the Co-PIs will jointly manage the proposed study

This is a great opportunity for both PIs to undergo training in advanced techniques and develop skills in next-generation serology and molecular sequencing. Dr. Srimuang will learn and practice serologic techniques and data analysis from Mr. Sterling. Mr. Sterling will develop skills in cell isolation and molecular sequencing techniques from Dr. Srimuang. Moreover, Mr. Sterling will coordinate planning, sample processing, data analysis, and report preparation for this project with Dr. Srimuang.

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List of abbreviations, acronyms, symbols

AngV: Angavokely virus
BCR: B cell receptor
BSc: Bachelor of Science
BSL2: Biological Safety level 2
BSL3: Biological Safety level 3
cDNA: Complementary DNA
CedV: Cedar virus
Co-PI: Co-principal investigator
CREID: Centers for Research in Emerging Infectious Diseases
DARV: Daeryong virus
DNA: Deoxyribonucleic acid
EHA: EcoHealth Alliance
EID: Emerging Infectious Disease
EID-SEARCH: The Emerging Infectious Disease – South East Asia Research Collaboration Hub
ELISA: Enzyme-linked immunosorbent assay
F: Fusion protein
FACS: Fluorescence-activated cell sorting
FUO: Fever of unknown origin
GAKV: Gamak virus
GhV: Ghana virus
HeV: Hendra virus
HPVs: Henipaviruses
ILI: Influenza-like illness
KCMH: King Chulalongkorn Memorial Hospital
L: large protein
LayV: Langya virus
M: Matrix protein
mAb: Monoclonal antibody
MD: Maryland
MMIA: Multiplex microsphere-based immunoassay
MojV: Mojiang virus
MSc: Master of Science
N: Nucleocapsid
NGS: Next-generation sequencing
NIAID: National Institute of Allergy and Infectious Diseases
NIH: National Institutes of Health
NiV: Nipah virus
P: Phosphoprotein
PBMC: Peripheral blood mononuclear cells
PCR: Polymerase Chain Reaction
PhD: Doctor of Philosophy

PI: Principal investigator
RBP: Receptor-binding protein
RNA: Ribonucleic acid
RT-PCR: Real-time PCR
SARI/ARI: Severe/acute respiratory illness
SARS-CoV-1: Severe acute respiratory syndrome coronavirus 1
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2
SEA: Southeast Asia
Thai-DDC: Department of Disease Control of Thailand
TRC-EIDCC: Thai Red Cross. Emerging Infectious Diseases. Clinical Center
USA: United States
WGS: Whole Genome Sequencing

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Facilities and Resources

FACILITIES: Specify the facilities to be used for the conduct of the proposed research.

Laboratory:

The Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC), King Chulalongkorn Memorial Hospital (KCMH) was established by the Committee of the Emerging Infectious Disease Center which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. TRC-EIDCC laboratory facilities include 3 biosafety cabinets (BSL 2, BSL 2+, BSL2+), 3 automated extraction machines, 2 thermocyclers (Proflex), 2 real-time PCR machines (CFX, Bio-rad), 3 Freezers (-80 °C), 2 refrigerators; 4 °C, 2 autoclaves. Our EIDCC uses the Pathogen Asset Control System (PACS) system for sample tracking. Moreover, our Faculty of Medicine, Chulalongkorn University has a central laboratory, the Chula Medical Research Center (Chula MRC), which provide many instruments for medical, masters, and graduate students, Post-doctoral fellows, and researchers at Chulalongkorn University and KCMH. The Chula MRC laboratory facilities are divided into molecular diagnostics (PCR Thermal Cycler, QStudio6 Flex Real-Time PCR, Droplet Digital PCR, GeneXpert, Film array, Illumina MiSeq), immunology facilities (ELISA testing equipment (reader & washer), Luminex, Flow Cytometry), cell sorting machine and a BSL3 laboratory. Moreover, the facility provides general equipment, such as autoclaves, balances, CO2 incubators, Dual Fluorescence Cell Counter, 4 °C refrigerators, -20°C, and -80°C freezers.

Clinical:

KCMH is a 1,000-bed university hospital, in close coordination with the Faculty of Medicine, Chulalongkorn University. The Thai Red Cross Society provides medical treatment, rehabilitation, health promotion and disease prevention services to the general public and the vulnerable. It also provides biopharmaceutical and sterile medicines that are sufficient to meet the needs of patients and safe in accordance with international standards. It serves as a center for providing eye and organ donation services to patients nationwide in line with international standards. It has specialized doctors and public health personnel and also produces specialized nurses. The TRC-EIDCC in the KCMH provides standard medical care to patients with emerging and recurrent diseases and disease spreading prevention.

Animal:

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

TRC-EIDCC also collaborates with the Department of National Parks, Wildlife and Plant Conservation for detecting the emerging novel pathogens from animals such as bats, rodents, and pangolins in our country. Bats are considered protected wildlife in Thailand by Wildlife Conservation, Department of National Parks, Wildlife and Plant Conservation.

Computer:

TRC-EIDCC used the service from the Office of Information Technology, Chulalongkorn University that provides the application, network, and software such as Office 365, VPN, WIFI, Microsoft Office, Microsoft Windows, SPSS, Adobe, Zoom Meeting, etc. Moreover, our EIDCC use the Pathogen Asset Control System (PACS) for sample tracking.

Office:

The office and laboratory space are located at the EIDCC at 4th Floor, Jongkolnee Watwong Building and 6th Floor, Aor Por Ror Building, respectively. The applicants will have dedicated desks in both buildings.

Other:

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

PI Name: Srimuang, Krongkan and Sterling, Spencer **Foreign Site: Thailand**
CREID CC Grant: 1U01AI151378

Foreign Site Information

If the Pilot Award will have multiple foreign sites, complete this form for EACH research site

Principal Investigator Name:	Krongkan Srimuang
Project Title:	Immune memory bait & capture to identify emerging henipavirus origins
Institution:	King Chulalongkorn Memorial Hospital
Foreign Research Site:	Rama 4 Road, Pathumwan, Bangkok 10330 Thailand
Point of Contact for Research Site:	Krongkan Srimuang Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital Rama 4 Road, Pathumwan, Bangkok 10330 Thailand krongkan.sr@gmail.com

Introduction (3-4 sentences):

Provide a simple description of the overall goals of the project including the work that will be done at the foreign site. Indicate if any of the NIAID grant funds will be sent to the foreign site.

The goals of this project are to know zoonotic reservoirs for pathogen origin by using serologic and cellular immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

To achieve these goals, the investigator will collaborate with NIH PREMISE for Antibody-mediated bait & capture of Thai MojV-like virus.

$149,937 of grant funds will be sent to the site for these studies.

Foreign component (3-4 sentences):

Describe the specific role of the foreign site (i.e. conduct critical experiments?, conduct data analyses?, provide consultations?, provide samples?, study human and/or animal populations?, conduct observational or interventional clinical trials?, collect samples and/or data to be brought to the US?).

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

The site will 1) conduct all filed work to collect human and animal samples from the study sites; 2) perform laboratory analysis using collected samples; and 3) conduct data analysis as proposed in the project.

Human Subjects (1 word or 1 sentence per bullet):

- **Parent Study IRB approval**
  - **IRB approval number for parent study:** #894ECOH21b (US); #221/64 (Thailand)
  - **IRB approval date:** 12-05-2021 (US); 08-06-2021 (Thailand)
  - **Human Subject Assurance Number: (FWA)#:** #00001102 (external IRB); #00022431 (EcoHealth Alliance); #00000943 (Chulalongkorn University)
- **Does this study require a modification to the IRB approval of a parent study (Yes or No)?**
  - **Yes**
- **Will existing samples from human subjects will be used: (Yes or No)?**
  - **No**
  - **How many subjects provided the existing samples to be used?** N/A
- **Will human subjects be recruited (Yes or No)**
  - **Yes**
  - **Number of human subjects that will be recruited:** 110
- **Population parameters:**
  - **Gender:** 55 males, 55 females
  - **Age Group:** Age >=18 years who provide informed consent.
  -
  - **Race/Ethnicity:** 110 Asian
- **Sample collection will include:**
  - **Blood:** Yes
  - **Urine:** No
  - **Tissues:** No
  - **Other samples (describe):** Nasopharyngeal swab and throat swab
- **Sample collection will be completed in how many visits:** 2 trips
- **Will samples be de-identified (Yes or No)? If No, describe how they will be protected.**
  - Yes
- **Will local IRB approval be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals being sought or state why local IRB approval is not required.**
  - Yes
- **Will informed consent be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals/consents being sought or state why informed consent is not required.**
  - Yes
- **Will samples be brought back to the US (Yes or No)?**
  - No
- **Will data be brought back to the US (Yes or No)?**

- Yes, some of the data will be shared with EID-SEARCH partners in the US for relevant analysis.

Animal Subjects (1 word or 1 sentence per bullet):

- **Parent study IACUC approval**
  - **IACUC approval number for parent study:** #G2020-42 (US); #019/2563 (Thailand)
  - **IACUC approval date:** 05-05-2020 (US); 03-12-2020 (Thailand)
    Animal Welfare Assurance Number: D16-00572 (A4059-01)
- **Does this study require a modification to the IACUC approval of a parent study (Yes or No)?**
  - **No**

- **Will existing samples from animal subjects will be used: (Yes or No)?**
  - **No**
  - **How many animal subjects provided the existing samples to be used?** N/A
- **Will vertebrate animals be collected (Yes or No)?**
  - **Yes**

- **Species of animals (e.g. rats, mice, rabbits, monkeys):** Bats, rodents, and shrews
- **Animal parameters:**
  - **Total number of animals:** 200 (100 bats, 100 rodents and shrews)
  - **Gender:** 100 males, 100 females
  - **Age range:** 4 - 12 months and elder depending upon species
  - **Lab strain (e.g. Sprague-Dawley rats, Balb/C mice):** None
  - **Wild animals procured in country (e.g. Rhesus monkeys from a reserve):** No

- **What will be done to them or with them and how often?**
  - Free-ranging bats will be captured using either a mist net or harp trap, and manually restrained during sampling; free-ranging rodents and shrews will be captured through pit traps and box traps.
  - Anesthesia will be conducted for captive rodents and shrews.
  - Once anesthetized blood will be collected.
  - All action will only be performed one time for each animal individual.
  - All animals will be released after sampling.

- **What are the follow-ups?**
  - No follow-up relevant as per protocol, since all animals will be released after sampling ups

- **What will be their fate at the end of the experiments – will they be euthanized?**
  - All animals will not be held longer than 6 hours (typically less than 3 hours) during the sampling process and released after sampling.

- **Will in-country clearances be obtained prior to engaging in any animal research (Yes or No)? If No, describe alternate approvals or state why in-country clearances are not required.**
  - Yes
- **Will samples be brought back to the US (Yes or No)?**
  - No
- **Will data be brought back to the US (Yes or No)?**
  - Yes, some of the data will be shared with EID-SEARCH partners in the US for relevant analysis.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Proposal Cover Sheet

Project Title	Immune memory bait & capture to identify emerging henipavirus origins
Principal Investigator Name	Krongkan Srimuang, Ph.D.
Position/Title	Research Scientist
Department	Thai Red Cross Emerging Infectious Diseases Clinical Center
Institution Name	King Chulalongkorn Memorial Hospital
Street	Rama 4 Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	Krongkan.sr@gmail.com
Phone	+66 (0) 92-453-0808
Country(ies) where work will be conducted	Thailand
Pathogen(s) focus	Mojiang Virus, Henipavirus, Paramyxovirus
Co-Principal Investigator Name (*If applicable*)	Spencer Sterling, MPH
Position/Title	Scientific Project Coordinator
Department	Microbiology and Immunology
Institution Name	Uniformed Services University of the Health Sciences
Street	Jones Bridge Road
City, State, Zip Code	Bethesda, MD 20814
Country	USA
Email	spencer.sterling.ctr@usuhs.edu
Phone	+66 (0) 83-494-5980

Contact information for institution(s) that would financially manage the award *(please note: changing the managing institution upon award may result in substantial funding delays)*	
Contractual Contact, Title	Chula Unisearch
Institution Name	Chulalongkorn University
Street	254 Phyathai Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	unisearch@chula.ac.th
Phone	0 2218 2880
For Co-Principal Investigator (If applicable):	
Contractual Contact, Title	
Institution Name	
Street	
City, State, Zip Code	
Country	
Email	
Phone	

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Name of Mentor	Eric Laing, Ph.D.
Mentor Institution	Uniformed Services University of the Health Sciences
Institution Address	Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814
Permanent location of Mentor	4301 Jones Bridge Road, Bethesda, MD 20814
Mentor email	eric.laing@usuhs.edu
Mentor phone	+1 (301) 980-8192
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Name of Mentor	Supaporn Wacharapluesadee, Ph.D.
Mentor Institution	Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital
Institution Address	1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Permanent location of Mentor	4th Floor, Jongkolnee Watwong Building, Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital, 1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Mentor email	spwa@hotmail.com
Mentor phone	+66 (0) 89-920-6292
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Total Budget	***Repeat this table if there are two Co-PIs***
Direct Costs	$138,831
Indirect Costs	$11,106
Proposed Start Date	1 May 2023
Proposed End Date	30 April 2024

Project Abstract (250 words)

Prototypical henipaviruses (Hendra and Nipah virus) are bat-borne zoonotic viruses and cause high mortality. The recent detection of closely related Mojiang and Langya viruses in rats and shrews, and isolation of Langya virus from individuals with acute febrile illness, challenges the dogma that fruit bats are the sole henipavirus reservoirs. The recently expanded *Henipavirus* genus includes two other shrew-borne viruses (Gamak and Daeryong viruses), and the Madagascan fruit bat-borne Angovkeely virus. As part of EID-SEARCH activities, we found serological evidence of infection by a Mojiang-related virus in a community of Thai bat guano collectors who have occupational exposure to microbats and rodents. Identifying the wildlife reservoir of this likely novel virus will assist intervention strategies to reduce spillover risk. However, PCR detection or virus isolation in wildlife is challenging, and new serological approaches may be more successful in identifying reservoir hosts. This project aims to identify novel viruses and zoonotic reservoirs through serologic and cellular immune discovery. We will conduct follow-up human and wildlife (bats and rodents) surveillance in the province where the seropositive humans were identified and apply an expanded serological test and genomic sequencing to detect this novel MojV-like henipavirus. Further, we

plan to isolate anti-MojV monoclonal antibodies (mAb) from peripheral blood mononuclear cells collected from human study participants we have identified as immunoreactive with MojV antigens, and characterize the cross reactive and cross neutralizing potential of these mAbs to LayV.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Project Title **Immune memory bait & capture to identify emerging henipavirus origins**

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Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Study Personnel

Krongkan Srimuang, Ph.D., is a researcher at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital and supports the Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia (EID-SEARCH) research. Dr. Srimuang is also a medical technologist working to identify, detect and characterize the spill-over risk of high zoonotic potential viruses from wildlife and humans using serology techniques, Next-generation sequencing, and PCR assays to identify novel viruses. Dr. Srimuang also performs the project Smart Sentinel Surveillance System: Emerging Viral Pneumonia and Respiratory tract infections (RTIs) to identify, detect and characterize the novel/known Respiratory Viruses in our country, Thailand by using multiplex real-time PCR, family PCR and Next-generation sequencing of novel viral pathogens. Dr. Srimuang graduated with Ph.D. in Tropical Medicine from the department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University.

Spencer Sterling, MPH, is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region. Mr. Sterling will planning, sample processing, data analysis, and report preparation for this project.

Dr. Eric Laing will act as mentor for Dr. Srimuang and Mr. Sterling under the Centers for Research in Emerging Infectious Diseases (CREID) Pilot Research Program. Dr. Laing is an assistant professor dedicated to biosurveillance and virological research of emergent zoonotic viruses at Uniformed Services University, Bethesda, MD. Part of Dr. Laing's research focus in on the development of serological-based approaches to identify the infectome of wildlife and human communities for the assessment of virus spillovers. Dr. Laing has worked collaboratively with national and international researchers including institutional partners at Duke-NUS, Chulalongkorn University, University of Pretoria, and Rocky Mountain Labs towards these goals. Through these collaborations, Dr. Laing's research group aims to identify known and unknown viruses, the wildlife reservoirs of priority pathogens, the high-risk interfaces for spillover and the biotic/abiotic drivers of virus emergence.

Dr. Supaporn Wacharapluesadee, Ph.D. is the head laboratory at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, a WHO Collaborating Centre for Research and Training on Viral Zoonoses. Dr. Wacharapluesadee research interests include emerging infectious diseases in bats (including Nipah, Rabies, Coronavirus, and novel pathogens) as well as molecular diagnoses and sequencing of viruses. During the past 10 years, Dr. Wacharapluesadee got funding from USAID PREDICT to conduct the surveillance for novel virus in wildlife and human, and from Biological Threat Reduction Program US DTRA to conduct the EID surveillance in bat using the molecular technology and multiplex serology panel (Luminex technology). TRC-EIDCC is responsible for molecular diagnoses services to the hospital, and is the reference laboratory for rabies virus, MERS-CoV, Ebola, Zika, and other infectious diseases diagnoses for Ministry of Public Health, Thailand and laboratory training center on national and regional level. Dr. Wacharapluesadee has served and consulted on several WHO and Thai government committees.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

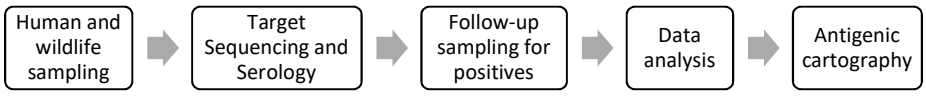
Specific Aims

The recent discovery of novel shrew and rodent-borne henipaviruses in East and Southeast Asia has highlighted gaps in our understanding of henipavirus transmission and underscores the importance of surveillance activities in the region. Therefore, **this project aims to expand the study of known reservoirs of zoonotic pathogen origin by using genetic and serologic techniques, and to use cellular immune discovery in seropositive humans to be used as a tool to determine henipaviral antigenic relatedness to assist with the functional assessment of current therapeutics as and their effect on these novel viruses.**

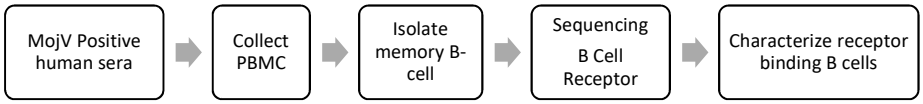
Human populations near the Khao Chong Phran Non-hunting Area in Ratchaburi province have shown a high seroprevalence to the MojV RBP without confirmed sequencing results. We hypothesize that (i) there is a novel henipavirus present in the local wildlife population that has spilled over into the human population at least once, and (ii) the antibodies from these seropositive people will react with the novel shrew-borne henipaviruses in a way that can inform us on therapeutic approaches for future outbreaks. We will follow-up on persons with high MojV RBP IgG titers, sample new people within the population that interact with the local wildlife, and sample bats, shrews, and rodents to find the source of the MojV-reactive antibodies. Additionally, in collaboration with NIH PREMISE, MojV RBP-reactive B cell receptors will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific MAbs will be generated and used for direct antibody-mediated virus capture in people and wildlife.

The proposed project has two aims:

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population.



Aim 2. Characterization of monoclonal antibodies from humans with serological profiles consistent with MojV infection.



The proposed project has three objectives:

Objective 1. To use metagenomic and targeted sequencing approaches in human and wildlife populations to detect the Thai MojV-like virus.

Objective 2. To isolate and sequence memory B-cells from humans positive for Mojiang virus in Ratchaburi province, Thailand for antibody production.

Objective 3. To determine epitope binding and antigenic relationships from henipavirus-reactive serum to the known henipaviruses.

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Look at the other successfully awarded applications and how they wrote the specific Aims

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Study Rationale

Nipah virus (NiV) and henipaviral diseases are among the most virulent of zoonotic diseases. The genus *Henipavirus* is currently composed of nine viruses including the prototypes, NiV and Hendra virus (HeV), and Cedar virus (CedV), Ghana virus (GhV), Mojiang virus (MojV) (McLinton et al., 2017), Langya virus (LayV) (Zhang X et al., 2022), Gamak virus (GAKV), Daeryong virus (DARV) (Lee et al., 2021), and Angavokely virus (AngV) (Madera et al., 2022). HeV was first identified in Australia in 1994 as a cause of respiratory illness and fatal encephalitis in humans and horses. Closely following the discovery of HeV, NiV was identified in 1999 in Malaysia after outbreaks of fatal encephalitis in humans, with swine determined to be the intermediate host. Annual outbreaks of NiV have occurred in Bangladesh and are associated with the consumption of raw date palm sap, and since 19 May 2018 annual outbreaks of NiV associated encephalitis of been recorded in Kerala, India (Uwishema et al., 2022). Both HeV and NiV have a broad host range capable of infecting several mammalian orders, and can enter and infect endothelial cells, vasculature, and the central nervous system leading to severe multisystem organ diseases and high mortality. The broad host and tissue tropism of these viruses has been attributed to the use of the ephrinB2 and ephrinB3 ligands, highly conserved proteins with critical functions in evolutionary development, as virus receptors (Bossart et al., 2008). There are currently no vaccines or therapeutics and NiV and henipaviral diseases remain WHO Blueprint list priority pathogens (Middleton and Weingartl, 2012).

A close relative of HeV, CedV was isolated from Australian bats in 2012 and found to be non-pathogenic, failing to cause any clinical symptoms in ferrets and guinea pig animal models (Marsh GA et al., 2012). The natural reservoirs of HeV, NiV, and CedV are flying foxes (*Pteropus sp.*) (Middleton and Weingartl, 2012). GhV was detected in Ghana in straw-colored fruit bats (*Eidolon helvum*) during routine surveillance in 2015 and was the first henipavirus found outside of the Indo-Pacific region. GhV showed a close phylogenetic relationship with HeV and NiV, and like HeV, NiV, and CedV the GhV RBP can mediate binding to ephrinB2 ligand and mediate cellular fusion (Drexler, J. F., 2009). Recent investigations into Madagascan fruit bats (*Eidolon duprenum*) detected a second African henipavirus, AngV. As neither GhV nor AngV have been isolated or rescued, the pathogenic and zoonotic potential of these African fruit bat associated henipaviruses remains unknown.

Multiple genera of small mammals were tested for viral RNA, and MojV was detected in yellow-breasted rats (R. flavipectus) (Wu Z et al., 2014). The discovery of the MojV genome occurred during follow-up investigations into lethal pneumonia from three miners in 2012 in China, however the pathogenic nature of this virus has only been suggested and no isolates have been discovered or tested in an animal model. Additionally, the novel shrew-borne henipaviruses, GAKV and DARV were found in the Republic of Korea in 2017-2018. (Lee et al., 2021). Most recently, a close relative of MojV, Langya henipavirus (LayV) was recently isolated from febrile patients in eastern China. Subsequently, LayV was identified in shrews (Zhang et al., 2022), increasing the number of Asiatic rodent-associated henipaviruses to four. These recent discoveries are challenging the paradigms surrounding henipaviruses and their associations with bats and may suggest a novel rodent sub-genus of henipaviruses.

Commented [EL2]: Abbreviations already used above

The henipaviral genome consists of six structural proteins including nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), receptor-binding protein (RBP), and large (L) protein (approximately length of 18,000 nucleotides) (Luby, S. P., & Gurley, E. S., 2012). In HeV and NiV, slippage sites within the P protein lead to the expression of the V, W, and C proteins. These proteins are virulence factors and are absent in CedV.

Table 1 Summary viruses in Henipavirus

Henipavirus	**Origin (Year)**	**Reservoir**	**Genome (nucleotides)**	**Symptom for human**	**Reference**
Hendra virus (HeV)	Australia (1994)	Fruit bats	18,234	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Nipah virus (NiV)	Malaysia (1999)	Fruit bat	18,246	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Ghana virus (GhV)	Africa (2009)	Fruit bat (*Eidolon helvum)*	18,530	No data	Drexler, J. F., 2009
Cedar virus (CedV)	Australia~~n~~ (2012)	Fruit bat	18,162	Non-pathogenic	Marsh, G. A et al., 2012
Mojiang virus (MojV)	China (2012)	Rodent	18,404	Febrile	Wu Z et al., 2014
Gamak virus (GAKV)	Korea (2017-2018)	Shrew	18,460	No data	Lee et al., 2021
Daeryong virus (DARV)	Korea (2017-2018)	Shrew	19,471	No data	Lee et al., 2021
Langya virus (LayV)	China (2019)	Shrew	18,402	Acute febrile	Zhang et al., 2022
Angavokely virus	Madagascar (2019)	Fruit bat	16,740	No data	Madera et al., 2022

Viruses in the genus *Henipavirus* have a broad reservoir and may cause high case fatality rates following human infection. The discovery and characterization of the novel Henipavirus is a high public health priority.

The greatest bat diversity around the world especially appears to be found in the tropical rain forests of South East Asia. In Thailand, at least 138 species in 11 families and 45 genera of bats have been recorded, such as members of the genus Pteropus, Rhinolophus, and Hipposideros (Soisook et al., 2011). Khao Chong Phran, a cave in Ratchaburi Province, has possibly the largest colony of bats in Thailand. There are estimated to be around 2 million bats in the cave and 95% are insect-eating bats (Department of National Parks, Wildlife and Plant Conservation, Thailand). Moreover, Khao Chong Phran has many community sites, such as cave entrances near communities, guano collection, and tourism sites, which risk zoonotic spillover. Bats are widely considered the animal hosts of zoonotic pathogens such as rhabdoviruses, coronaviruses, paramyxoviruses, and filoviruses, and many of the host species can be found in Thailand.

MojV was associated with a severe pneumonia-like illness killing three of six miners in 2012 in a mineshaft Yunnan, China. Interestingly, this mineshaft was also site where RaTG13, a beta-coronavirus most closely related with SARS-CoV-2, was detected in *Rhinolophid* bats (Rahalkar and Bahulikar, 2020). Identifying the wildlife reservoir of this novel virus will assist intervention strategies to reduce spillover risk.

Research Gaps

As a part of Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH), serological investigations using a multiplex microsphere-based immunoassay (MMIA) have detected 19% (54/284) seroprevalence for MojV in a high-risk cohort, including one person with a high magnitude and durability of anti-MojV IgG across longitudinal samples. Despite this, genomic surveillance has not detected MojV or any novel MojV-like viruses. For this result, we would to further identify and characterize the origin of the Mojiang virus-reactive serum and to explore the novel MojV-like virus in Thailand.

Serological and cellular immunity is an important resource for virus discovery detection by using the specificities and characteristics of memory B cells from peripheral blood mononuclear cells (PBMC). The immune response will recognize and induce antibodies specific to the infecting pathogen. Characterizing the memory B cell response to an unknown pathogen in humans could in the detection of the exposure pathogen in humans. Molecular techniques such as PCR can detect only the genomic material in the body, but memory B cells are long-lived and thus have a much larger window of detection.

Memory B-cell differentiation is characterized by somatic hypermutation in antibody variable region genes (V) and selection of B cells expressing high-affinity variants of this antigen receptor and high sequence similarity in individuals exposed to the same antigen reflect antigen-driven clonal selection and form shared immunological memory between individuals (Yang et al., 2021). Memory B cells are highly abundant in the human spleen, and can proliferate 45% of the total B cell population in this organ (Hauser et al., 2015). Memory B cells can be detected in the Ig variable (IgV) genes in a fraction of IgM+ B cells. In humans, B cells expressing the memory marker CD27+ can be found in the marginal zone of spleen and circulate in the blood. After re-exposure to antigen, memory B cells can quickly proliferate and differentiate into plasma cells. Many studies the antibody diversity and specificity of the naturally paired immunoglobulin heavy and light chains for affinity maturation of B cell receptor (BCR) after virus infection such as SARS-CoV-2 infection (Yang et al., 2021), influenza (Dougan, S et al., 2013), HIV (Moir, S., & Fauci, A. S.,2017), etc. The characterization of memory B cells can be used to develop monoclonal antibodies and produce an effective therapeutic.

Significance and Approach

Southeast Asia is one of the world's highest-risk emerging infectious diseases hotspots. From this region emerged both SARS-CoV-1 and SARS-CoV-2, recurrent outbreaks of novel influenza strains, and the spillover of dangerous viral pathogens such as Nipah virus. The diversity of coronaviruses, henipaviruses, and filoviruses are found in wildlife reservoirs in Southeast Asia and likely due to the high diversity of wildlife, and the demography of the human population brings humans and wildlife into close contact. The spillover of viruses from animals to humans is often misdiagnosed and the clinical symptoms from patients is underestimated during the pandemic. This study provides a surveillance strategy in human and wildlife populations to better understand immunity to novel pathogens and identify the origin of these pathogens to serve as an early warning system to prevent outbreaks of emerging viruses.

This study uses immunology (serologic and cellular) to identify a novel virus that can aide in effective vaccine discovery and development, monoclonal antibody production for prevention and therapy, and generate data resources for early detection and diagnostic assays. Whereas PCR detection or virus

isolation in wildlife is challenging, this new serological approach may be more successful in identifying reservoir hosts.

The Indo-Pacific region has a high risk of novel viral emergence. This study provides an opportunity for in-country research capacity building by linking local infectious disease scientists with an international collaborative network that has proven capacity to conduct this work and produce significant findings. Moreover, a global sero-surveillance network for surveillance technology extends virologic and immunologic screening to product discovery. This study will benefit and expand the CREID, EID-SEARCH networks, and the global sero-surveillance network to improve our understanding of immunology to identify novel pathogens and their origin to prevent future pandemics.

Research Methods

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population

Serological profiling provides an indirect measurement of the infecting virus. Despite 19% (54/284) seroprevalence in humans, we have not detected nucleic acid sequences of MojV or MojV-like henipaviruses in this human cohort. ~~In addition to the translational application of mAbs as therapeutics, we intend to utilize these mAb as non-clinical diagnostics surveillance tools for virus/virus antigen capture.~~ Additionally, we intend on expanding our understanding of henipavirus prevalence in this population with longitudinal sampling of the wildlife and human populations in this region.

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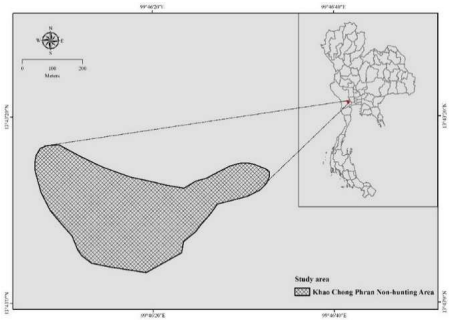


Figure 1: Map of Khao Chong Phran Non-hunting Area, Ratchaburi Province, Thailand (Boonpha N et al., 2019)

Site wildlife samples collection

100 bats and 100 shrews or rodents in and around the Khao Chong Phran cave in Ratchaburi Province will be collected for surveillance targeting the Mojiang-like virus. We plan one sampling trip to collect bats, and a second trip to target shrews and rodents. Due to limited knowledge of the local shrew population, we are unsure as to whether we can collect 100 samples, and will supplement the collection with rodents, as needed. Samples will be collected based on the following enrollment criteria: random age, random weight, random sex. Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and stored. Oral swab and rectal swab will also collect in 100 bats and 100 shrews or rodents. Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and stored.

Human surveillance

Four of the samples from prior investigations have shown high MojV RBP-reactive antibody titers and will be selected for follow-up sampling. Additionally, 100 human serum samples, nasopharyngeal swab and throat swab will be collected from persons deemed to be in close contact with wildlife, and people who present with or have recent history of undiagnosed acute febrile illness using the following enrollment criteria;

- Adults (≥ 18 years of age) who sign in the informed consent
- Who present with or have recent history of illness without diagnosis, such as severe/acute respiratory illness (SARI/ARI), Influenza-like illness (ILI), fever of unknown origin (FUO), or respiratory tract infection
- Who is the bat guano collector in the cave
- Who lives or works around wildlife habitats
- Who is involved with livestock or wildlife farming
- Who hunts or works with wildlife
- Who has not been previously sampled as part of the EID-SEARCH and PREICT projects

Sample size justification

Human population

$$\mathrm{n} = \frac{\mathrm{N}}{1 + \mathrm{N}e^2}$$

n = sample size of the study

N = population size for finite populations: 8,150 (data from 2018)

e = error: 10%

$$\mathrm{n} = \frac{8{,}150}{1 + (8{,}510 \text{ x } 0.1^2)}$$

n = 98.79 (round up to 100 samples)

Wildlife population

The sample size of 100 of each species follows the guidelines established during the PREDICT project.

Targeted Viral Sequencing and Serologic testing

Targeted sequencing

For samples from shrews, rodents, and acute individuals, we will use targeted sequencing and metagenomic approaches in an attempt to detect the Thai MojV-like virus. The nucleic acid from oral swab and rectal swab will do target sequencing in henipavirus Family PCR and Sanger sequencing.

Serological testing

Serum samples will be tested for RBP and envelope fusion protein (F) binding to the known henipavirus species, including the Nipah, Hendra, Cedar, Mojiang, Ghana, Langya, Gamak, and Daerong viruses to further investigate the seroprevalence in this region. Additionally, prior positive samples will be re-tested with the expanded panel to enhance our understanding of the antigenic relationships between the novel shrew-borne henipavirus and the Thai virus.

Data analysis

Sequencing will be analyzed assembling reads in MEGA11. The consensus sequences will compare to the references strains available in the GenBank data-base using the Basic Local Alignment Software Tool (BLAST) (National Center for Bio-technology Information). Serological data will be analyzed using cluster analysis in RStudio.

Aim 2. Characterization of monoclonal antibodies from humans with serological profiles consistent with MojV infection.

In collaboration with NIH VRC PREMISE, MojV RBP and F-reactive B cells will be captured, and the B cell receptor will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific mAbs will be generated from the corresponding sequences and will be assessed for binding potential to LayV RBP and F, as well as their neutralization potential.

Importance: The isolation of these mAbs represents a potential therapeutic for MojV, MojV-like henipas, and LayV, depending on how well these cross react.

Human samples collection site

Human positive serum for MojV was found close to Khao Chong Phran in Ratchaburi Province, which has the largest colony of bats in Thailand (Figure 2).

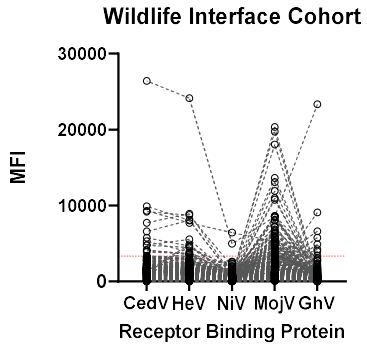


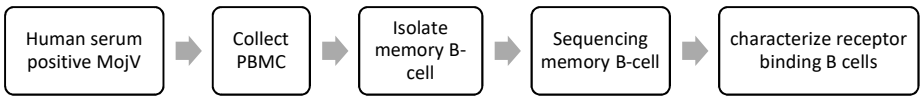
Figure 2: Seroprevalence of henipavirus proteins from a community with a large bat interface during 2017-2018. MFI is the median fluorescence intensity for IgG reactivity to each protein. Red-dashed line is at 3,357 MFI, a threshold for seropositivity.

PBMC Sorting and B Cell isolation

Whole blood from MojV-positive people will be collected in individual heparin tubes and processed for PBMC isolation using Density Gradient Centrifugation (SepMate™ PBMC Isolation Tubes). Human PBMCs will be stained with antibodies for positive markers of IgG memory B cells, and antibodies to negatively select and avoid contamination with dead cells, T cells, NK cells or monocytes (live, CD3−,CD14−,CD56−, IgM−, IgA−, CD19+,CD20+,CD27+). Avi-tag for biotinylation and 6xHIS tag for Nickel purification using the HisPur™ Ni-NTA Spin Purification Kit (Thermo Fisher Scientific, Waltham, MA). Single Memory B Cells will be sorted, cells will proliferate, cDNA will synthesized, then Heavy and light chain variable domains will be amplified (Waltari E et al., 2019).

B cell receptor Amplicon Preparation and Recombinant Antibody sorting

Total RNA yields from the PBMC will be determined by absorbance at 260 nm on the NanoDrop™ One (Thermo Fisher Scientific). Total RNA will be used for first strand cDNA synthesis with gene-specific reverse transcription (RT) primers directed to the constant regions. The RT primers for IgG, IgM, IgA, lambda, and kappa will be included. Light chain and heavy chains will be amplified into amplicon. Double-stranded cDNA will be sequenced. After completion of MiSeq sequencing, antibody repertoires will analyze using methods based on the pipeline. Heavy and light-chain sequences will be assembled into an expression vector and transfected into cells for monoclonal antibody expression. Recombinant antibody will be isolated and purified for downstream applications.



Functional assessment

We will first determine the extent to which isolated mAbs can bind binding to native-like MojV RBP and F proteins. We will also characterize the cross-reaction potential of these mAbs will be assessed against GamV, DarV, and LayV RBP and F. Neutralization of viruses remains a critical correlate of protection for most vaccine strategies. However, MojV has not been isolated or rescued, and the virus receptor for MojV has not yet been discovered (Rissanen et al., 2017; Cheliout et al., 2021). Thus, a functional assessment of MojV neutralizing potential is not possible. Though, we will test the cross-neutralization potential of these anti-MojV RBP and F mAbs to neutralize LayV utilizing a plaque reduction neutralization test with EID-SEARCH co-investigators at Duke-NUS. As LayV causes acute illness in human, cross neutralization functionality of these mAbs has therapeutic potential.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Project Timeline

Activity	05/23	06/23	07/23	08/23	09/23	10/23	11/23	12/24	01/24	02/24	03/24	04/24
Mentoring and training activities												
1.Training, literature review												
2.Monthly mentoring meeting												
3.Manuscript writing												
4.EID-SEARCH meeting												
Research activities												
1.IRB amendment												
Aim 1												
2. Human and Wildlife samples collection												
3. Identify Targeted sequencing												
4. Serological testing												
Aim 2												
5. Collect PBMC human sample and isolation memory B-cell for Mojiang virus												
6. PBMC Sorting and B Cell isolation												
7. Determination of envelope glycoprotein epitope binding												

Research Performance Sites

This proposed study will be implemented at the King Chulalongkorn Memorial Hospital (KCMH) collaborating with the Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC). KCMH is a 1,000-bed, university hospital in close coordination with the Faculty of Medicine, Chulalongkorn University. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. It's one of the center hospitals for Emerging Infectious Diseases in Thailand, especially for domestic and international patients.

TRC-EIDCC was established by the Committee of the Emerging Infectious Disease Center, which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. Our clinical center provides standard medical care to patients with emerging and recurrent diseases and disease transmission. Moreover, the center plans preparedness activities, trains medical and government staff on interventions, hosts conferences for medical and public health professionals, creates networks of medical care for patients with emerging and recurrent diseases, and leads research and development projects for medical care and disease control. Apart from medical innovation in emerging and recurrent diseases, the clinical center strives to be the model of infectious diseases clinical services and pandemic control through research activities surrounding infectious diseases and effective disease control measures.

We also collaborate with the Department of National Parks, Wildlife and Plant Conservation for detecting novel pathogens from animals such as bats, rodents, and shrews in our country. We also collaborate with the Department of Disease Control of Thailand (Thai-DDC) to support the Thai government and national policy makers through molecular diagnosis, including the identification of the first case of SARS-CoV-2 infection in Thailand. TRC-EIDCC prides itself on its high performance in research laboratory services and is a part of the national reference laboratory for COVID-19 and other emerging infectious diseases confirmation in Thailand.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

CREID Research Center Collaboration

The goals and objectives of the proposed research project, "Immune memory bait & capture to identify emerging henipavirus origins", are fully consistent with the specific aims of EID-SEARCH. EID-SEARCH takes a One Health approach to understanding zoonotic disease emergence, with a focus on viral diversity in wildlife at high-risk human animal interfaces. EID-SEARCH aims to understand the frequency and causes of virus spillover in high-risk communities across Southeast Asia and identify strategies and tools to reduce this risk. As the proposed research focuses on the links between farming practices, wildlife diversity, virus diversity, and cross-species transmission in Thailand, we expect the results of the project to make a significant contribution towards the overall goals of EID-SEARCH.

The proposed project is well-designed, leverages cutting-edge technology, and includes a robust ecological component that corresponds to the overall research strategy of EID-SEARCH. The EID-SEARCH team is highly motivated to support the application of metagenomics to ecological questions and assist in the analysis of the unique dataset that will be generated from this research. With the heightened focus on identifying and limiting risks associated with the complex interactions between humans, wildlife, and domestic mammals that has resulted from the emergence of SARS-CoV-2 from a wet market in China, the proposed research is both timely and likely to yield highly-cited, impactful publications for EID-SEARCH and Thailand partners.

The EID-SEARCH team includes a number of experts in study design, laboratory-based diagnostics, genetic and genomic analyses, and ecological modelling who will contribute to project implementation, data analysis, and manuscript preparation as required. Communication and coordination mechanisms will be set up between the Thailand research team and scientists at the EID-SEARCH research sites for resource sharing, training, knowledge exchange, and mentorship. In particular, EID-SEARCH will facilitate and encourage relationships between our partners in Southeast Asia (based in Malaysia, Singapore, and Thailand) that will facilitate Thailand team professional development and strengthen research capacity in the region. Dr. Eric Laing, the project Mentor, has an established relationship with both Dr. Krongkan and Spencer Sterling, and will act as the liaison between EID-SEARCH and the TRC-EIDCC research team. Further, Dr. Doeuk who is the director of the VRC PREMISE program is enthusiastic about the opportunity to collaborate and apply their rapid assembly, transfection and production of immunoglobulin (RATP-Ig) workflow as a serologic/immunologic based approached for therapeutic and virus discovery (see Letter of Support). An expected outcome of this research project will be pilot data to support a joint funding application in the future.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Mentoring Plan

Goals for Pilot study and Career

Krongan Srimuang: My career goal is to be an expert scientist in either an academic research laboratory or a lecturer in medical science. My long-term research interests include the understanding of priority emerging infectious diseases, genetics, and epigenetics using molecular and serologic techniques. Detection of novel pathogens in their wildlife vectors can help characterize the pathogenicity, virulence, and evolution of these and other closely related pathogens. I believe that this pilot study can provide training in new techniques for the detection, identification, and characterization of novel pathogens that are currently unavailable in Thailand. My goal for the Pilot study is to gain more knowledge and skills for a lifelong career in public health-related research. I wish to expand my skills in novel virus detection using next-generation serologic techniques such as PBMC studies and memory B-cell isolation and sequencing that can contribute to improved surveillance and research capacity at human-animal interfaces. My mentors Dr. Laing and Dr. Wacharapluesadee can provide me with opportunities to develop and apply project skills and improve my scientific writing.

My education has provided me with a strong foundation to expand for this study. I received my undergraduate degree in Medical Technology from Chulalongkorn University which included training in molecular biology, serology, immunology, chemistry, virology, bacteriology, mycology, parasitology, hematology, clinical microscopy, transfusion medicine, and forensic medicine. My interest in molecular technology led me to pursue a Ph.D. at the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, which provided me with training in several molecular techniques including conventional PCR, real-time PCR, Next Generation Sequencing (NGS), etc. The emergence of COVID-19 motivated me to focus on emerging infectious diseases. I am currently completing my post-doctoral fellowship at the Emerging Infectious Diseases Clinical Center (EIDCC), King Chulalongkorn Memorial Hospital with Dr. Wacharpluesadee. Throughout this fellowship, I have continued learning advanced and innovative techniques to detect, identify, and characterize the novel pathogens. Through this pilot project, I will learn about the Peripheral Blood Mononuclear Cells (PBMC) which are a critical part of many subdivisions of medical research including Infectious diseases, Vaccine development, and Immunology. This project aims to study zoonotic reservoirs for by using serologic and cellular immune discovery in humans, and applying this immune knowledge in wildlife for early detection and identification of novel pathogens. This proposed study will expose me to various techniques and immunology topics that I useful in designing and testing new pipelines as technological advances continue to develop. This study will also allow me to develop, manage, and communicate my research.

Spencer Sterling: My overall career goal is to understand the factors that lead to viral zoonosis and to build epidemiological capacity in regions with the highest risk for zoonotic spillover. Through my training and educational experiences, I have learned laboratory and epidemiological techniques that can assist in the understanding of zoonosis. I wish to continue my education by expanding my repertoire of molecular techniques to include studies of the immune system's response to zoonotic disease in humans and animals, as well as to learn next-generation sequencing techniques. Additionally, I would like to use my experience in epidemiological techniques to train scientists in the Southeast Asian region in order to build local capacity in infectious disease research. Dr. Laing has been a mentor for me throughout my career, and this project would allow for this relationship to expand into new molecular areas, as well as grant writing and project management skills. My professional relationship Dr. Wacharapluesadee with began in 2018 when I participated in a DTRA

BTRP funding project that focused on transferring antigen-based multiplex serological technology to her lab group. Currently, I am a Visiting Scientist at TRCEIDCC and I work closely with Dr. Srimuang, and other members of Dr. Wacharapluesadee's research group on EID-SEARCH activities. Additionally, Dr. Wacharapluesadee has decades of experience in infectious disease research in the region and has mentored scientists that have gone on to careers at the Thai Ministry of Public Health, Department of Disease Control, Department of Parks and Wildlife, academia, and the private sector. Both mentors have encouraged Dr. Srimuang and I to apply for this pilot study as well as pursue other funding mechanisms for our research interests. Both mentors will provide for me the ability to independently pursue my research interests in the zoonotic epidemiology and train me on the best practices of scientific communication.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Vertebrate Animals Section Requirements

Animal samples will be collected in Ratchaburi Province for this project under Aim 2 to understand the antigenic relationships among known and unknown henipaviruses. Protocol to perform animal sample collection has been reviewed and approved by the Institutional Animal Care and Use Committee at Tuft University under the EID-SEARCH project (No. G2020-42) and the Institutional Animal Care and Use Committee at Chulalongkorn University (No. 019/2563)

1. Description of Procedures

Bats (order *Chiroptera*)

- Species: Free-ranging mega and micro-*Chiroptera* that present at the site (family *Pteropodiae, Rhinopomatidae, Emballonuridae, Craseonycteridae, Megadermatidae, Rhinolophidae, Hipposideridae, Vespertilionidae, Mollossidae*, etc.)
- Age & Sex: Fully flighted adults and juveniles, males and females; neonates dependent on the dam will not be used.
- Target Number: 100 individuals
- Capture and Restraint: Bats will be captured using either a mist net or harp trap. The net system is manned by at least two people during the entire capture period, and bats are removed from the net as soon as they become entangled to minimize stress and prevent injury. In our previous field research experience, a maximum of 20-30 bats can be safely held and processed by a team of three people per trapping period. Duration of trapping will depend on the capture rate. Bats are individually placed into a pillowcase or small cloth bag and hung from a branch or post in a location that is free from access by both predators and people, and is protected from the elements (e.g., rain), until the animal is restrained for sample collection. Bats will be manually restrained during sampling utilizing insulated bite gloves to prevent injury to the handler and bat. The study protocol covers over 69 bat species that are most likely to be captured given their natural occurrence and previous surveillance experience in Southeast Asia. The potential for non-target species to be captured is very low because mist nets are opened at the cave entrance at night, after other species (e.g., birds) have ceased flying and returned to roost. Any non-target species will be rapidly identified, removed from the mist net and released immediately.
- Sample Collection: Depending on the species and size of bat, blood will be collected from smaller insectivorous bats (<50g) using a 27g needle to puncture the brachial artery and a 70ul hematocrit tube to collect the blood. For larger bats (>50g) we will collect blood from either the cephalic vein or from the radial artery or vein using a 25- or 23-gauge needle and 3cc syringe. Blood will be collected in quantities not exceeding 6 µL/g of body mass from each individual. Wing punch biopsies will be collected to confirm host identification using DNA barcoding. Swabs will be taken from the oropharynx, urogenital tract, and rectum. No destructive sampling is planned, but any individuals that die during capture or sampling will have a necropsy performed with organ samples collected and whole body submitted to museums as voucher specimens.
- Release: Bats are held for a maximum of six hours (typically less than three hours), and at the completion of sampling are provided with rehydrating fluids (either subcutaneously or orally as needed) and released in a location that allows for further recovery or safe escape (e.g., bodies of water, known predatory areas, and human settlements will be avoided).

Rodents (order: *Rodentia*) and Shrew (order: *Scandentia* and *Eulipotyphla*)

- Species: Free-ranging rodents (family *Hystricidae, Muridae, Sciuridae, Spalacidae*) and shrew (tree shrews, shrews, moles) that present at the sites.
- Age & sex: Adults and juveniles, males and females. Neonates will not be sampled.
- Target Number: 100 individuals

- Capture and Restraint: Free-ranging wild mammal species will be captured with metal box traps (Sherman/Tomahawk traps). Traps will be prepared with food, water, padding and shelter, and be checked at least every 12 hours, in the morning and in the afternoon. If adverse weather (extreme heat, rain) is expected or researchers are working in areas where predation is common, traps will be checked more frequently and closed during adverse weather. Veterinary staff will be prepared to deal with a variety of trap injuries, including lacerations, broken teeth, and crush injuries. Depending on the species, individual size, captive or free-ranging status of the individual, manual or chemical restraint and anesthesia (gas or injectable anesthesia) will be applied. Animals will be manually restrained during sampling utilizing insulated bite gloves to prevent injury to the handler and animal. Based upon past field experience (>20 years of animal capture and release in Southeast Asia), we do not expect to trap sick, debilitated, or young animals that would be too young to cope with capture. Sick and debilitated animals tend not to roam widely, reducing their opportunity to enter traps. Additionally, should sick, debilitated, or young animals enter traps, we anticipate that they will not suffer adverse conditions because the traps contain food (the bait) and liquid (water or fruit as a source of water). These individuals would be examined by a veterinarian, rehydrated and if presenting clinical signs treated by a veterinarian, prior to release or sampling then release.
- Sample Collection: Once anesthetized or safely restrained, whole blood will be collected through the appropriate venipuncture site, no more than 1 ml of blood per 100 g (= 10 ml/kg or 1%) of body weight will be collected at any one time. Anesthetized animals will be monitored regularly during recovery until they can no longer be safely handled, at which point they will be confined in a trap or cage. Swabs will be taken from the oropharynx, urogenital tract, and rectum. No destructive sampling is planned, but any individuals that die during capture or sampling will have a necropsy performed with organ samples collected.
- Release: Wild mammals are held for a maximum of thirteen hours depending on trap timing, but typically less than three hours. Captive animals are held for a maximum of two hours but typically less than one hour. At the completion of sampling, animals may be provided with rehydrating fluids (either subcutaneously if anesthetized or orally if manually handled and accepted). Wild mammals will be released at the site of capture in a location that allows for further recovery or safe escape (e.g., bodies of water, known predatory areas, and human settlements will be avoided). Captive mammals will be released back into their captive setting as is appropriate for the species, either isolated or in social group if it is deemed safe from aggression from enclosure mates while the post-recovery period continues.

2. Justifications

The purpose of this study is to identify zoonotic pathogens through serologic and cellular immune discovery, including developing methods and standards for direct antibody-mediated virus capture in people and wildlife, focusing on rodent/shrew- and bat-borne HPVs. This will require hands-on fieldwork to collect whole blood and serum samples from bats, rodents, and shrews for lab analysis. One technique that avoids the direct capture and sampling of animals is to collect fresh feces or urine, but this will not be able to provide the appropriate samples for serologic analysis, and the research objectives cannot be achieved using an alternative methods (e.g., computational, human, invertebrate, in vitro). Therefore, we believe there are no viable alternatives than the use of live animals.

3. Minimization of Pain and Distress

In every situation, sampling of wildlife will be conducted in the most humane manner while minimizing the impacts on individual animals and their wild populations. In all instances, the fewest number of animals will be sampled that will provide valid information and statistical inference for the pathogen and disease of interest and every effort will be made to minimize stress and discomfort for the animal.

Bats will not be held longer than six hours during the sampling process whereas small mammals may be held for up to 13 hours depending on trap capture timing. Field animal sampling team with zoologists and veterinarians have been well trained and have extensive experience in capture, anesthesia, and sampling of wildlife. In our team's experience, the target species tolerate the described procedure well. Mist nets will be attended continuously during capture periods, and bats will be extracted from the net as soon as they become entangled. This will minimize stress and injury from entanglement. Bats will be placed individually in cloth bags and hung from tree branches while awaiting processing and during recovery. The bags are sufficiently porous as to allow for ventilation and are designed for bat capture. The enclosed environment seems to calm the bats, as they do not struggle once inside, but they hang quietly – this is a standard and accepted practice in bat research and the best way to minimize stress to the animal.

For rodents and shrews, food, water, padding and shelter will be provided at traps, which will be checked at least every 12 hours. Traps will be checked more frequently or closed if adverse weather (extreme heat, rain) is expected, or researchers are working in areas where predation is common. Veterinary staff will be prepared to deal with a variety of trap injuries, including lacerations, broken teeth, and crush injuries. All animal individuals will be monitored by a veterinarian or experienced field team member during all stages of capture, processing, anesthesia and release. Animals will be kept in a cool place, free from adverse weather conditions and access by non-field team members or other animals while in the pillowcases, trap, or cage for recovery. All recovering individuals will be observed until they are able to effectively ambulate and orient to their surroundings. Animals that are injured during the capture or sampling process will be assessed by an experienced team leader or attending veterinarian, and if the animal is determined to be unlikely to survive if released, it shall be euthanized humanely (see euthanasia section).

4. Method of Euthanasia

In the event that an animal has been injured or is moribund, a determination will be made as to whether it may be treated and released on site by veterinarians or transferred to facility that may treat and rehabilitate the animal, or if, as a last resort, the animal will require euthanasia. Euthanasia methods will vary depending on species; however methods to be used will not deviate from the AVMA “Guidelines for the Euthanasia of Animals” (2020 edition). Any animal that is euthanized using a chemical agent will be disposed of such that it will not be permitted to enter the food supply either through markets or hunting.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Human Subjects Research

1. Risks to the subjects

In this project, we will target populations in one community site who with frequent exposure to bats and other wildlife based on our previous behavioral survey. Subjects will be enrolled voluntarily, and informed consent will be obtained from all participants, along with assent from all participants aged 12-17. Enrolled participants will provide biological samples. Survey data and biological samples will be collected from enrolled participants, and follow-up data collection will be performed among participants whose samples were tested positive.
Biological samples and questionnaire data will be collected from individuals who meet recruitment and inclusion criteria and complete the informed consent process. During data collection, a standardized questionnaire will be administered to all participants. This questionnaire will collect information of demographic background, wildlife contract, travel and daily movement, and unusual SARI/ILI symptoms, and biological specimens will be collected from participants. Both questionnaire and biological data will be analyzed to assess the exposure to coronaviruses and the spread risk among humans. From all participants, a one-time whole blood sample (Max. 15 mL) will be collected during the study period. This sample will allow us to test for historical exposure to bat or rodent-born henipaviruses and collect Peripheral Blood Mononuclear Cells (PBMCs) for those study participants whose serum tests are positive.

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 who are 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, the administration will be conducted privately and confidentially to protect individuals' 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 these risks

2.1 Informed consent and assent

Only consented participants will be enrolled in the study. No research procedures will be undertaken before the participant fully understands the research, agrees to the study procedures, and completes the consent process will be enrolled in the study. Informed consent statements and forms, and project protocols will be translated into the local language of each study site. Research team members involved in this consent process will be required to be fluent in the local language to ensure that the subjects understand the study and all involved procedures.

If participants meet the criteria for enrollment, they will be invited to discuss the details of the study with the research staff. Study staff will review an information sheet and informed consent form with the participant and the parent or legal guardian when applicable. Each individual will be provided with a copy of the informed consent form that has been translated into the local language and written with a Flesch–Kincaid readability score equivalent to a 7th-grade reading level or below, to assure that potential participants understand the information being shared. The informed consent form will explain the details of the study, including how and why the individual was selected, the study process and procedures, risks and benefits, financial considerations and the gift of appreciation, confidentiality of data shared, alternatives to participating, and how to obtain more information now or at a later

date. The informed consent form will be read in the local language of the site at a location ensuring participant privacy. After which individuals will have as much time as they would like to ask questions and discuss the study with study staff. The study staff will endeavor to ensure that the participant understands the information provided. The study staff will then ask the participant/parent or legal guardian to consider study participation. Participants will have as much time as required to consider the participation.

Those participants who consent to the study will sign and date two copies of the consent form. These form copies will be countersigned and data by the study staff. A copy of the signed consent form must be provided to the subject and the other copy will be kept by study staff. Informed consent paperwork will be kept until the end of the project in a locked box at the local country project office.

2.2 Protection against risks

The potential risks to study participants as a result of study participation are minimal. Collection of venous blood samples and nasal or oropharyngeal swab samples pose minimal risk to subjects. Potential complications associated with venipuncture include pain and/or hematoma at the site of collection. Trained medical professionals and/or clinic staff will monitor the blood collection site and treat any complications according to existing health facility protocols. A potential complication of nasal/ oropharyngeal sampling is minor irritation at the time of collection. Employing trained medical and/or clinic staff to collect blood and swab samples will minimize the potential for complications.

Another risk that this study may pose concerns the information to be gained on henipaviruses newly recognized in the community. We will provide participating communities, hospitals, and clinicians with information and background data on target zoonotic viruses to ensure up-to-date communication of risk. Because of the timeline for diagnostic testing and results interpretation, we are not likely to provide results to participating clinics within a time frame that would be clinically relevant to outbreaks of undiagnosed diseases. Therefore, the information provided by this project will not impact patient management or outlook.

If an individual decides to participate in this research, his/her participation and all information provided by the participant will be strictly confidential, and personal identifying information will not be shared with anyone outside of the study staff. Participants will not be identified or named in any reports or publications. Questionnaire information and all biological samples will be identified by an alphanumeric code, not by the participant's name. 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 study site in locked files cabinets or password-protected devices in areas with access limited to the research staff of this project. Research databases will be secured with password-protected access systems and controlled distribution web-based certificates and will not contain any identifying characteristics about study participants (e.g., name, address, or telephone number). Access to all data will be limited to the staff involved in this study. The health information disclosed by an individual will not be used by or disclosed (released) to another institution. Any surveillance report that is published or shared with partners will not contain any personally identifying information for individual participants.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

3. Potential benefits of the research to the subjects and others

There are no measurable benefits to the individual study participants enrolled in this study. There are benefits to the community and regional healthcare providers to help them understand the risk of zoonotic infections among high-risk populations in the regions they work. At the conclusion of the study, we will deliver an educational workshop reporting aggregate study findings that will be open to both study and non-study participants, describing the health benefits of using personal protection equipment (PPE) and handwashing during animal handling activities throughout the day, as well as to share other prevention/interventions that emerge from the research data.

4. Importance of the knowledge to be gained

There are valuable potential benefits to the public from the knowledge to be gained from this study. One key benefit to the community is sharing information and knowledge to better understand the risk of zoonotic spillover events and related health risks, as well as information sharing with communities on practices that could reduce risks, such as the avoidance of particular animal contacts or the need for PPE and extra care when handling wildlife, that may substantially reduce the risk zoonotic pathogen transmission in the community. 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 create or implement public health interventions to disrupt disease emergence and/or spread in an area that is beneficial to all. Additionally, there are valuable benefits to the general public from the knowledge to be gained from this study. Knowledge gained will 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.

5. Country- / institution-specific ethics / IRB regulations addressed

Main research protocol and materials to conduct human subject research in this project have been reviewed and approved by the Health Medical Lab Institutional Review Board, US (No. 894ECOH21b) and the Institute Review Board of the Faculty of Medicine, Chulalongkorn University, Thailand (No. 211/64). Amendment for PBMCs collection will be made and approved before the start of this project.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Research and Related Other Project Information

1. Are Human Subjects Involved?	Yes	X	No	
1.a. If YES to Human Subjects				
Is the Project Exempt from Federal regulations?	Yes		No	X
If yes, check appropriate exemption number	1		2	
	3		4	
	5		6	
	7		8	
If no, is the IRB review Pending?	Yes	X	No	
IRB Approval Date:				
Human Subject Assurance Number	FWA00000943			

2. Are Vertebrate Animals Used?	Yes	X	No	
2.a. If YES to Vertebrate Animals				
Is the IACUC review Pending?	Yes		No	X
IACUC Approval Date	3 December 2020			
Animal Welfare Assurance Number				

3. Is proprietary/privileged information included in the application?	Yes		No	X

4.a. Does this project have an Actual or Potential impact -- positive or negative -- on the environment?	Yes		No	X
4.b. If yes, please explain				
4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed?	Yes		No	
4.d. If yes, please explain				

5. If the research performance site designated, or eligible to	Yes		No	X

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

be designated, as a historic place?				
5.a. If yes, please explain				

6. Does this project involve activities outside of the United States or partnership with international collaborators?	Yes	X	No	
6.a. If yes, identify countries	Thailand			
6.b. Optional explanation				

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Budget Justification

A. Key Personnel (Total $24,000)

Dr. Krongkan Srimuang, PI (12 months) is a medical research scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital (TRC-EIDCC) who have been leading the laboratory analysis for the EID-SEARCH work in Thailand in the past three years to perform molecular, serological, and next-generation sequencing to identify novel viruses from human and animal samples. Dr. Srimuang will commit 12 months to this project to design, manage, and perform all project activities in the lab and field by supervising a lab technician and working with the field coordinator. Dr. Srimuang will also work closely with the co-PI and Mentors to conduct data analysis and develop manuscript and join EID-SEARCH and CREID meetings to prevent the project findings.

Spencer Sterling, Co-PI (6 months) is the research coordinator of Laing Lab at the Uniformed Services University for Indo-Pacific region and a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC). Sterling will commit 6 months to this project as the co-PI, who will work closely with the PI in the study design, SOP development, sampling process, data analysis of for serological investigations. No salary is required for Sterling.

B. Other Personnel (Total $20,000)

One (1) Laboratory Technician (12 months) will committee 12 months to this project to conduct PCR and serological testing of animal and human samples at TRC-EIDCC, under the supervision of Dr. Srimuang and Sterling. Salaries for 12 months at $1,250 per month is requested ($15,000).

One (1) Field Coordinator (4 months) will committee 4 months to this project, working closely with Dr. Srimuang and Sterling to organize and conduct field sample collection among humans and animals at the study sites, and support relevant financial management. Salaries for 4 months at $1,250 per month is requested ($5,000).

C. Fringe Benefits

No Fringe is requested.

D. Equipment

No equipment is requested.

E. Travel (Total $48,375, Years 1-5)

Domestic Travel ($11,005)
Support for domestic travel for field sampling is requested in the amount of $11,005 per to conduct four (4) trips of sample collection from humans, bats, and rodents/shrews.

- The first trip is a one-day trip to collect samples from human participants who were tested positive (n<10), the cost is estimated at van rental for local transportation between Bangkok and the study site ($260) and meals for five (5) field team members at $49 per person ($245), in total of $505 for the first trip.

- The other three (3) field trips will be conducted to collect samples from 100 bats, 100 humans, and 100 rodents/shrews, respectively. Each trip is a duration of 6 days and 5 nights. For each person, 5-night stay in hotels at $75.5/night (5 x $75.5 = $378); 3 meals per day and incidental expenses at $49 per day prorated to 75% ($36.75) for first and last day of travel ($36.75 x 2 + $49 x 4 = $270), the total meals and accommodations for each person per trip is $648 ($378+270), cost for five (5) field team members is $3,240. A van will be rented for local transportation between Bangkok and the study site that is estimated at $260 per trip. Total cost for each 6-day trip is $3,500, three trips will be $10,500.

International Travel ($5,023)
Support for international travel for Dr. Srimuang from Bangkok to Washington DC to attend the CREID Annual Meeting and receive lab training at the National Institute of Allergy and Infectious Diseases (NIAID) is requested in the amount of $5,023. Trips are for a duration of 10 days and 9 nights. Travel costs for one trip are estimated using federal per diem rates for hotels, meals, and incidental expenses as follows: nine-night stay in DC area hotel at $286/night (9 x $258 = $2,322); Ten days' meals and incidental expenses at $79 per day prorated to 75% ($59.25) for first and last day of travel ($59.25 x 2 + $79 x 8 = $750.5); Round trip flight between Bangkok to Dulles International Airport is estimated at $1,650; local transportation/taxi between hotel and airport per round trip between Chulalongkorn Hospital to airport is estimated at $40, between hotel and the airport in the US is estimated at $200, and $60 for transportation within the region ($300).

F. Participant Support Costs

No participant support is requested.

G. Other Direct Costs (Total $78,803)

Materials and Supplies ($72,830)
PBMCs isolation ($47,000). We request funds to conduct PBMC isolation from collected sample, costs including the reagents and lab consumable and supplies are estimated at $47 per cell for 1,000 cells.

Multiplex microsphere immunoassay detection ($2,275). Funds are requested to perform serological testing of collected human and animal samples. Costs including the reagents and lab consumable and supplies are estimated at $6.5 per sample for 350 samples.

PCR testing and sequencing ($20,475). Funds are requested to perform serological testing of collected human and animal samples. Costs including the reagents and lab consumable and supplies are estimated at average $58.5 per sample 350 samples, given a 10%~15% positive rate.

Field supplies for PBMC collection and processing ($1,225). Funds are requested to perform PBMCs collection and processing in the field. Costs including the reagents and lab consumable and supplies are estimated at average $3.5 per sample 350 samples.

Field supplies for animal sampling ($1,160). Funds are requested to purchase supplies for animal capture, anesthesia, and sample collection. A total cost of $1,160 is estimated

Field work disposable ($668). Funds are requested to purchase disposable materials (tips, tubes, gloves, masks, googles etc.) for field work.

Consultants Service ($3,000)
One (1) zoologist and one (1) veterinarian will provide consultancy service to this project to help 1) select sites for animal sampling and join the sampling trip to 2) identify species; and 3) provide veterinary care in the sample collection of bats, rodents, and shrews. The consultancy fee is estimated at $1,500 per person for the project, in total of $3,000.

Publication Fee ($3,000)
We request $3,000 of publication costs for one peer-review paper generated from the project. The cost is estimated at the reimbursed Open Access fees of $2,900 - $5,000 per manuscript in the past two years for six peer-review papers.

H. Total Direct Costs (Total $138,831)

I. Indirect Costs (Total $11,106)

An indirect cost at 8% is requested.

J. Total Direct and Indirect Costs (Total $149,937)

A total amount of $149,937 is requested.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Co-PI Plan (only needed if applying as Co-PIs):

What each Co-PI will contribute to the proposed research study

As stated in the Mentoring Plan, the co-PIs, Krongkan Srimuang and Spencer Sterling have different educational and training backgrounds. This Co-PI plan is proposed to fill the gaps in knowledge of each scientist and supports a collaborative working relationship for project success. Moreover, this plan was developed to maximize the strengths of each PI. Overall, the project is separated into two objectives, the proposal of this project will be equally managed by Dr. Srimuang and Mr. Sterling. Dr. Srimuang will be responsible for PBMC isolation, sorting, and characterization. Mr. Sterling will be responsible for the technical serological aspects of the project. Both will work closely with the Research Center Mentor, Dr. Eric Laing, to develop this project, protocol development, and network connections. Dr. Supaporn Wacharapluesade, the Co-mentor, will support the wildlife sampling, samples collection, and molecular aspects within Thailand.

How the Co-PIs will jointly work with the affiliated Research Center

Mr. Sterling is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region.

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How the Co-PIs will jointly manage the proposed study

This is a great opportunity for both PIs to undergo training in advanced techniques and develop skills in next-generation serology and molecular sequencing. Dr. Srimuang will learn and practice serologic techniques and data analysis from Mr. Sterling. Mr. Sterling will develop skills in cell isolation and molecular sequencing techniques from Dr. Srimuang. Moreover, Mr. Sterling will coordinate planning, sample processing, data analysis, and report preparation for this project with Dr. Srimuang.

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Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

List of abbreviations, acronyms, symbols

AngV: Angavokely virus
BCR: B cell receptor
BSc: Bachelor of Science
BSL2: Biological Safety level 2
BSL3: Biological Safety level 3
cDNA: Complementary DNA
CedV: Cedar virus
Co-PI: Co-principal investigator
CREID: Centers for Research in Emerging Infectious Diseases
DARV: Daeryong virus
DNA: Deoxyribonucleic acid
EHA: EcoHealth Alliance
EID: Emerging Infectious Disease
EID-SEARCH: The Emerging Infectious Disease – South East Asia Research Collaboration Hub
ELISA: Enzyme-linked immunosorbent assay
F: Fusion protein
FACS: Fluorescence-activated cell sorting
FUO: Fever of unknown origin
GAKV: Gamak virus
GhV: Ghana virus
HeV: Hendra virus
HPVs: Henipaviruses
ILI: Influenza-like illness
KCMH: King Chulalongkorn Memorial Hospital
L: large protein
LayV: Langya virus
M: Matrix protein
mAb: Monoclonal antibody
MD: Maryland
MMIA: Multiplex microsphere-based immunoassay
MojV: Mojiang virus
MSc: Master of Science
N: Nucleocapsid
NGS: Next-generation sequencing
NIAID: National Institute of Allergy and Infectious Diseases
NIH: National Institutes of Health
NiV: Nipah virus
P: Phosphoprotein
PBMC: Peripheral blood mononuclear cells
PCR: Polymerase Chain Reaction
PhD: Doctor of Philosophy
PI: Principal investigator
RBP: Receptor-binding protein
RNA: Ribonucleic acid
RT-PCR: Real-time PCR
SARI/ARI: Severe/acute respiratory illness
SARS-CoV-1: Severe acute respiratory syndrome coronavirus 1
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2
SEA: Southeast Asia
Thai-DDC: Department of Disease Control of Thailand

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

TRC-EIDCC: Thai Red Cross. Emerging Infectious Diseases. Clinical Center
USA: United States
WGS: Whole Genome Sequencing

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Facilities and Resources

FACILITIES: Specify the facilities to be used for the conduct of the proposed research.

Laboratory:

The Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC), King Chulalongkorn Memorial Hospital (KCMH) was established by the Committee of the Emerging Infectious Disease Center which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. TRC-EIDCC laboratory facilities include 3 biosafety cabinets (BSL 2, BSL 2+, BSL2+), 3 automated extraction machines, 2 thermocyclers (Proflex), 2 real-time PCR machines (CFX, Bio-rad), 3 Freezers (-80 °C), 2 refrigerators; 4 °C, 2 autoclaves. Our EIDCC uses the Pathogen Asset Control System (PACS) system for sample tracking. Moreover, our Faculty of Medicine, Chulalongkorn University has a central laboratory, the Chula Medical Research Center (Chula MRC), which provide many instruments for medical, masters, and graduate students, Post-doctoral fellows, and researchers at Chulalongkorn University and KCMH. The Chula MRC laboratory facilities are divided into molecular diagnostics (PCR Thermal Cycler, QStudio6 Flex Real-Time PCR, Droplet Digital PCR, GeneXpert, Film array, Illumina MiSeq), immunology facilities (ELISA testing equipment (reader & washer), Luminex, Flow Cytometry), cell sorting machine and a BSL3 laboratory. Moreover, the facility provides general equipment, such as autoclaves, balances, CO2 incubators, Dual Fluorescence Cell Counter, 4 °C refrigerators, -20°C, and -80°C freezers.

Clinical:

KCMH is a 1,000-bed university hospital, in close coordination with the Faculty of Medicine, Chulalongkorn University. The Thai Red Cross Society provides medical treatment, rehabilitation, health promotion and disease prevention services to the general public and the vulnerable. It also provides biopharmaceutical and sterile medicines that are sufficient to meet the needs of patients and safe in accordance with international standards. It serves as a center for providing eye and organ donation services to patients nationwide in line with international standards. It has specialized doctors and public health personnel and also produces specialized nurses. The TRC-EIDCC in the KCMH provides standard medical care to patients with emerging and recurrent diseases and disease spreading prevention.

Animal:

TRC-EIDCC also collaborates with the Department of National Parks, Wildlife and Plant Conservation for detecting the emerging novel pathogens from animals such as bats, rodents, and pangolins in our country. Bats are considered protected wildlife in Thailand by Wildlife Conservation, Department of National Parks, Wildlife and Plant Conservation.

Computer:

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

TRC-EIDCC used the service from the Office of Information Technology, Chulalongkorn University that provides the application, network, and software such as Office 365, VPN, WIFI, Microsoft Office, Microsoft Windows, SPSS, Adobe, Zoom Meeting, etc. Moreover, our EIDCC use the Pathogen Asset Control System (PACS) for sample tracking.

Office:

The office and laboratory space are located at the EIDCC at 4th Floor, Jongkolnee Watwong Building and 6th Floor, Aor Por Ror Building, respectively. The applicants will have dedicated desks in both buildings.

Other:

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

PI Name: Srimuang, Krongkan and Sterling, Spencer **Foreign Site: Thailand**
CREID CC Grant: 1U01AI151378

Foreign Site Information

If the Pilot Award will have multiple foreign sites, complete this form for EACH research site

Principal Investigator Name:	Krongkan Srimuang
Project Title:	Immune memory bait & capture to identify emerging henipavirus origins
Institution:	King Chulalongkorn Memorial Hospital
Foreign Research Site:	Rama 4 Road, Pathumwan, Bangkok 10330 Thailand
Point of Contact for Research Site:	Krongkan Srimuang Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital Rama 4 Road, Pathumwan, Bangkok 10330 Thailand krongkan.sr@gmail.com

Introduction (3-4 sentences):

Provide a simple description of the overall goals of the project including the work that will be done at the foreign site. Indicate if any of the NIAID grant funds will be sent to the foreign site.

The goals of this project are to know zoonotic reservoirs for pathogen origin by using serologic and cellular immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

To achieve these goals, the investigator will collaborate with NIH PREMISE for Antibody-mediated bait & capture of Thai MojV-like virus.

$149,937 of grant funds will be sent to the site for these studies.

Foreign component (3-4 sentences):

Describe the specific role of the foreign site (i.e. conduct critical experiments?, conduct data analyses?, provide consultations?, provide samples?, study human and/or animal populations?, conduct observational or interventional clinical trials?, collect samples and/or data to be brought to the US?).

The site will 1) conduct all filed work to collect human and animal samples from the study sites; 2) perform laboratory analysis using collected samples; and 3) conduct data analysis as proposed in the project.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Human Subjects (1 word or 1 sentence per bullet):

- **Parent Study IRB approval**
  - **IRB approval number for parent study:** #894ECOH21b (US); #221/64 (Thailand)
  - **IRB approval date:** 12-05-2021 (US); 08-06-2021 (Thailand)
  - **Human Subject Assurance Number: (FWA)#:** #00001102 (external IRB); #00022431 (EcoHealth Alliance); #00000943 (Chulalongkorn University)
- **Does this study require a modification to the IRB approval of a parent study (Yes or No)?**
  - **Yes**
- **Will existing samples from human subjects will be used: (Yes or No)?**
  - **No**
  - **How many subjects provided the existing samples to be used?** N/A
- **Will human subjects be recruited (Yes or No)**
  - **Yes**
  - **Number of human subjects that will be recruited:** 110
- **Population parameters:**
  - **Gender:** 55 males, 55 females
  - **Age Group:** Age >=18 years who provide informed consent.
  -
  - **Race/Ethnicity:** 110 Asian
- **Sample collection will include:**
  - **Blood:** Yes
  - **Urine:** No
  - **Tissues:** No
  - **Other samples (describe):** Nasopharyngeal swab and throat swab
- **Sample collection will be completed in how many visits:** 2 trips
- **Will samples be de-identified (Yes or No)? If No, describe how they will be protected.**
  - Yes
- **Will local IRB approval be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals being sought or state why local IRB approval is not required.**
  - Yes
- **Will informed consent be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals/consents being sought or state why informed consent is not required.**
  - Yes
- **Will samples be brought back to the US (Yes or No)?**
  - No
- **Will data be brought back to the US (Yes or No)?**
  - Yes, some of the data will be shared with EID-SEARCH partners in the US for relevant analysis.

Animal Subjects (1 word or 1 sentence per bullet):

- **Parent study IACUC approval**
  - **IACUC approval number for parent study:** #G2020-42 (US); #019/2563 (Thailand)
  - **IACUC approval date:** 05-05-2020 (US); 03-12-2020 (Thailand)
    Animal Welfare Assurance Number: D16-00572 (A4059-01)

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

- **Does this study require a modification to the IACUC approval of a parent study (Yes or No)?**
  - **No**

- **Will existing samples from animal subjects will be used: (Yes or No)?**
  - **No**
  - **How many animal subjects provided the existing samples to be used?** N/A
- **Will vertebrate animals be collected (Yes or No)?**
  - **Yes**

- **Species of animals (e.g. rats, mice, rabbits, monkeys):** Bats, rodents, and shrews
- **Animal parameters:**
  - **Total number of animals:** 200 (100 bats, 100 rodents and shrews)
  - **Gender:** 100 males, 100 females
  - **Age range:** 4 - 12 months and elder depending upon species
  - **Lab strain (e.g. Sprague-Dawley rats, Balb/C mice):** None
  - **Wild animals procured in country (e.g. Rhesus monkeys from a reserve):** No

- **What will be done to them or with them and how often?**
  - Free-ranging bats will be captured using either a mist net or harp trap, and manually restrained during sampling; free-ranging rodents and shrews will be captured through pit traps and box traps.
  - Anesthesia will be conducted for captive rodents and shrews.
  - Once anesthetized blood will be collected.
  - All action will only be performed one time for each animal individual.
  - All animals will be released after sampling.

- **What are the follow-ups?**
  - No follow-up relevant as per protocol, since all animals will be released after sampling ups

- **What will be their fate at the end of the experiments – will they be euthanized?**
  - All animals will not be held longer than 6 hours (typically less than 3 hours) during the sampling process and released after sampling.

- **Will in-country clearances be obtained prior to engaging in any animal research (Yes or No)? If No, describe alternate approvals or state why in-country clearances are not required.**
  - Yes
- **Will samples be brought back to the US (Yes or No)?**
  - No
- **Will data be brought back to the US (Yes or No)?**
  - Yes, some of the data will be shared with EID-SEARCH partners in the US for relevant analysis.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Proposal Cover Sheet

Project Title	Immune memory bait & capture to identify emerging henipavirus origins
Principal Investigator Name	Krongkan Srimuang, Ph.D.
Position/Title	Research Scientist
Department	Thai Red Cross Emerging Infectious Diseases Clinical Center
Institution Name	King Chulalongkorn Memorial Hospital
Street	Rama 4 Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	Krongkan.sr@gmail.com
Phone	+66 (0) 92-453-0808
Country(ies) where work will be conducted	Thailand
Pathogen(s) focus	Mojiang Virus, Henipavirus, Paramyxovirus
Co-Principal Investigator Name (*If applicable*)	Spencer Sterling, MPH
Position/Title	Scientific Project Coordinator
Department	Microbiology and Immunology
Institution Name	Uniformed Services University of the Health Sciences
Street	Jones Bridge Road
City, State, Zip Code	Bethesda, MD 20814
Country	USA
Email	spencer.sterling.ctr@usuhs.edu
Phone	+66 (0) 83-494-5980

Contact information for institution(s) that would financially manage the award *(please note: changing the managing institution upon award may result in substantial funding delays)*	
Contractual Contact, Title	Chula Unisearch
Institution Name	Chulalongkorn University
Street	254 Phyathai Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	unisearch@chula.ac.th
Phone	0 2218 2880
For Co-Principal Investigator (If applicable):	
Contractual Contact, Title	
Institution Name	
Street	
City, State, Zip Code	
Country	
Email	
Phone	

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Name of Mentor	Eric Laing, Ph.D.
Mentor Institution	Uniformed Services University of the Health Sciences
Institution Address	Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814
Permanent location of Mentor	4301 Jones Bridge Road, Bethesda, MD 20814
Mentor email	eric.laing@usuhs.edu
Mentor phone	+1 (301) 980-8192
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Name of Mentor	Supaporn Wacharapluesadee, Ph.D.
Mentor Institution	Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital
Institution Address	1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Permanent location of Mentor	4th Floor, Jongkolnee Watwong Building, Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital, 1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Mentor email	spwa@hotmail.com
Mentor phone	+66 (0) 89-920-6292
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Total Budget	***Repeat this table if there are two Co-PIs***
Direct Costs	$138,831
Indirect Costs	$11,106
Proposed Start Date	1 May 2023
Proposed End Date	30 April 2024

Project Abstract (250 words)

Prototypical henipaviruses (Hendra and Nipah virus) are bat-borne zoonotic viruses and~~,~~ cause high mortality. The recent detection of closely-related Mojiang and Langya viruses in rats and shrews, and isolation of Langya virus from individuals with acute febrile illness, challenges the dogma that fruit bats are the ~~key~~ sole henipavirus reservoirs. The recently expanded *Henipavirus* genus includes two other shrew-borne viruses (Gamak and Daeryong viruses), and the Madagascan fruit bat-borne Angovkeely virus. As part of EID-SEARCH activities, we found serological evidence of infection by a Mojiang-related virus in a community of Thai bat guano collectors who have occupational exposure to microbats and rodents. Identifying the wildlife reservoir of this likely novel virus will assist intervention strategies to reduce spillover risk. However, PCR detection or virus isolation in wildlife is challenging, and new serological approaches may be more successful in identifying reservoir hosts. This project aims to identify novel viruses and zoonotic reservoirs through serologic and cellular immune discovery. We will conduct follow-up human and wildlife (bats and rodents) surveillance in the province where the seropositive humans were identified, and apply an expanded serological test and genomic sequencing to detect this novel MojV-like henipavirus.~~.~~ Further, we

plan to isolate anti-MojV monoclonal antibodies (mAb) from peripheral blood mononuclear cells collected from human study participants we have identified as immunoreactive with MojV antigens, and characterize the cross reactive and cross neutralizing potential of these mAbs to ~~MojV and~~ LayV~~. envelope glycoproteins. In addition to our antigen-based approach, we will utilize the mAbs for direct antibody-mediated virus capture of this novel henipavirus from human and wildlife samples, and downstream genomic sequencing.~~

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Project Title **Immune memory bait & capture to identify emerging henipavirus origins**

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Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Study Personnel

Krongkan Srimuang, Ph.D., is a researcher at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital and supports the Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia (EID-SEARCH) research. Dr. Srimuang is also a medical technologist working to identify, detect and characterize the spill-over risk of high zoonotic potential viruses from wildlife and humans using serology techniques, Next-generation sequencing, and PCR assays to identify novel viruses. Dr. Srimuang also performs the project Smart Sentinel Surveillance System: Emerging Viral Pneumonia and Respiratory tract infections (RTIs) to identify, detect and characterize the novel/known Respiratory Viruses in our country, Thailand by using multiplex real-time PCR, family PCR and Next-generation sequencing of novel viral pathogens. Dr. Srimuang graduated with Ph.D. in Tropical Medicine from the department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University.

Spencer Sterling, MPH, is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region. Mr. Sterling will planning, sample processing, data analysis, and report preparation for this project.

Dr. Eric Laing will act as m~~M~~entor for Dr. Srimuang and Mr.Sterling under the Centers for Research in Emerging Infectious Diseases (CREID) Pilot Research Program. Dr. Laing is an assistant professor dedicated to biosurveillance and virological research of emergent zoonotic viruses at Uniformed Services University, Bethesda, MD. Part of Dr. Laing's research focus in on the development ~~study to develop~~ of serological-based approaches to identify the infectome of wildlife and human communities for the assessment of virus spillovers. Dr. Laing has~~ve~~ worked collaboratively with national and international researchers including institutional partners at Duke-NUS, Chulalongkorn University, University of Pretoria, and Rocky Mountain Labs towards these goals. Through~~Across all of~~ these collaborations, Dr. Laing's research group aims ~~the aimed~~ to identify known and unknown viruses, the wildlife reservoirs of priority pathogens, the high-risk interfaces for spillover and the biotic/abiotic drivers of virus emergence.

Dr. Supaporn Wacharapluesadee, Ph.D. is the head laboratory at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, a WHO Collaborating Centre for Research and Training on Viral Zoonoses. Dr. Wacharapluesadee research interests include emerging infectious diseases in bats (including Nipah, Rabies, Coronavirus, and novel pathogens) as well as molecular diagnoses and sequencing of viruses. During the past 10 years, Dr. Wacharapluesadee got funding from USAID PREDICT to conduct the surveillance for novel virus in wildlife and human, and from Biological Threat Reduction Program US DTRA to conduct the EID surveillance in bat using the molecular technology and multiplex serology panel (Luminex technology). TRC-EIDCC is responsible for molecular diagnoses services to the hospital, and is the reference laboratory for rabies virus, MERS-CoV, Ebola, Zika, and other infectious diseases diagnoses for Ministry of Public Health, Thailand and laboratory training center on national and regional level. Dr. Wacharapluesadee has served and consulted on several WHO and Thai government committees.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Specific Aims

Commented [EL1]: Revise

Look at the other successfully awarded applications and how they wrote the specific Aims

This project aims to study zoonotic reservoirs for pathogen origin by using serologic and cellular immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

The hypothesis

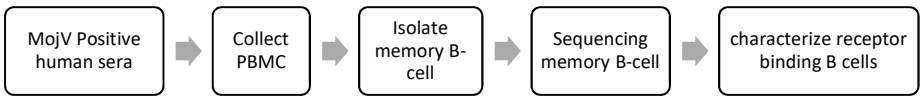
1. Can the memory B-cell immune response be used as a tool for the discovery of novel henipaviruses?
2. Can the spatial relationships between henipaviruses receptor binding proteins be generated that would allow for an understanding of antigenic evolution and functional characterization?

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population.

Human populations near the Khao Chong Phran Non-hunting Area in Ratchaburi province have shown a high seroprevalence to the MojV RBP without confirmed sequencing results. We will follow-up on persons with high MojV RBP IgG titers, sample new people within the population that interact with the local wildlife, and sample shrews and rodents to find the source of the MojV-reactive antibodies.

Aim 2. Antibody-mediated bait & capture of the Thai MojV-like virus

In collaboration with NIH PREMISE, MojV RBP-reactive B cell receptors will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific MAbs will be generated and used for direct antibody-mediated virus capture in people and wildlife.



Objective 1. To use metagenomic and targeted sequencing approaches in human and wildlife populations to detect the Thai MojV-like virus.

Objective 2. To isolate and sequence memory B-cells from humans positive for Mojiang virus in Ratchaburi province, Thailand for antibody production.

Objective 3. To determine epitope binding and antigenic relationships from henipavirus-reactive serum to the known henipaviruses.

Study Rationale

Nipah virus (NiV) and henipaviral diseases are among the most virulent of zoonotic diseases. The genus *Henipavirus* is currently composed of nine viruses including the prototypes, NiV and Hendra virus (HeV), and Cedar virus (CedV), Ghana virus (GhV), Mojiang virus (MojV) (McLinton et al., 2017), Langya virus (LayV) (Zhang X et al., 2022), Gamak virus (GAKV), Daeryong virus (DARV) (Lee et al., 2021), and Angavokely virus (AngV) (Madera et al., 2022). HeV was first identified in Australia in 1994 as a cause of respiratory illness and fatal encephalitis in humans and horses. Closely following the discovery of HeV, NiV was identified in 1999 in Malaysia after outbreaks of fatal encephalitis in humans, with swine determined to be the intermediate host. Annual outbreaks of NiV have occurred in Bangladesh and are associated with the consumption of raw date palm sap, and since 19 May 2018 annual outbreaks of NiV associated encephalitis of been recorded in Kerala, India (Uwishema et al., 2022). Both HeV and NiV have a broad host range capable of infecting several mammalian orders, and can enter and infect endothelial cells, vasculature, and the central nervous system leading to severe multisystem organ diseases and high mortality. The broad host and tissue tropism of these viruses has been attributed to the use of the ephrinB2 and ephrinB3 ligands, highly conserved proteins with critical functions in evolutionary development, as virus receptors (Bossart et al., 2008). There are currently no vaccines or therapeutics and NiV and henipaviral diseases remain WHO Blueprint list priority pathogens (Middleton and Weingartl, 2012).

A close relative of HeV, CedV was isolated from Australian bats in 2012 and found to be non-pathogenic, failing to cause any clinical symptoms in ferrets and guinea pig animal models (Marsh GA et al., 2012). The natural reservoirs of HeV, NiV, and CedV are flying foxes (*Pteropus sp.*) (Middleton and Weingartl, 2012). GhV was detected in Ghana in straw-colored fruit bats (*Eidolon helvum*) during routine surveillance in 2015 and was the first henipavirus found outside of the Indo-Pacific region. GhV showed a close phylogenetic relationship with HeV and NiV, and like HeV, NiV, and CedV the GhV RBP can mediate binding to ephrinB2 ligand and mediate cellular fusion (Drexler, J. F., 2009). Recent investigations into Madagascan fruit bats (*Eidolon duprenum*) detected a second African henipavirus, AngV. As neither GhV nor AngV have been isolated or rescued, the pathogenic and zoonotic potential of these African fruit bat associated henipaviruses remains unknown.

Multiple genera~~us~~ of small mammals were tested for viral RNA, and MojV was detected in yellow-breasted rats (R. flavipectus) (Wu Z et al., 2014). The discovery of the MojV genome occurred during follow-up investigations into lethal pneumonia from three miners in 2012 in China, however the pathogenic nature of this virus has only been suggested and no isolates have been discovered or tested in an animal model. Additionally, the novel shrew-borne henipaviruses, Gamak virus (GAKV), and Daeryong virus (DARV) were found in the Republic of Korea in 2017-2018. (Lee et al., 2021). Most recently, a close relative of MojV, Langya henipavirus (LayV) was recently isolated from febrile patients in eastern China. Subsequently, LayV was identified in shrews (Zhang et al., 2022), increasing the number of Asiatic rodent-associated henipaviruses to four. These recent discoveries are challenging the paradigms surrounding henipaviruses and their associations with bats and may suggest a novel rodent sub-genus of henipaviruses.

Commented [EL2]: Abbreviations already used above

The h~~H~~enipiral~~us~~ genome consists of six structural proteins including nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), receptor-binding protein (RBP), and large (L) protein (approximately length of 18,000 nucleotides) (Luby, S. P., & Gurley, E. S., 2012). In HeV and

NiV, slippage sites within the P protein lead to the expression of the V, W, and C proteins. These proteins are virulence factors and are absent in CedV.

Table 1 Summary viruses in Henipavirus

Henipavirus	**Origin (Year)**	**Reservoir**	**Genome (nucleotides)**	**Symptom for human**	**Reference**
Hendra virus (HeV)	Australia (1994)	Fruit bats	18,234	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Nipah virus (NiV)	Malaysia (1999)	Fruit bat	18,246	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Ghana virus (GhV)	Africa (2009)	Fruit bat (*Eidolon helvum)*	18,530	No data	Drexler, J. F., 2009
Cedar virus (CedV)	Australian (2012)	Fruit bat	18,162	Non-pathogenic	Marsh, G. A et al., 2012
Mojiang virus (MojV)	China (2012)	Rodent	18,404	Febrile	Wu Z et al., 2014
Gamak virus (GAKV)	Korea (2017-2018)	Shrew	18,460	No data	Lee et al., 2021
Daeryong virus (DARV)	Korea (2017-2018)	Shrew	19,471	No data	Lee et al., 2021
Langya virus (LayV)	China (2019)	Shrew	18,402	Acute febrile	Zhang et al., 2022
Angavokely virus	Madagascar (2019)	Fruit bat	16,740	No data	Madera et al., 2022

Viruses in the genus *Henipavirus* have a broad reservoir and may cause high case fatality rates following human infection. The discovery and characterization of the novel Henipavirus is a high public health priority.

The greatest bat diversity around the world especially appears to be found in the tropical rain forests of South East Asia. In Thailand, at least 138 species in 11 families and 45 genera of bats have been recorded, such as members of the genus Pteropus, Rhinolophus, and Hipposideros (Soisook et al., 2011). Khao Chong Phran, a cave in Ratchaburi Province, has possibly the largest colony of bats in Thailand. There are estimated to be around 2 million bats in the cave and 95% are insect-eating bats (Department of National Parks, Wildlife and Plant Conservation, Thailand). Moreover, Khao Chong Phran has many community sites, such as cave entrances near communities, guano collection, and tourism sites, which risk zoonotic spillover. Bats are widely considered the animal hosts of zoonotic pathogens such as rhabdoviruses, coronaviruses, paramyxoviruses, and filoviruses, and many of the host species can be found in Thailand.

MojV was associated with a severe pneumonia-like illness killing three of six miners in 2012 in a mineshaft Yunnan, China. Interestingly, this mineshaft was also site where RaTG13, a beta-coronavirus most closely related with SARS-CoV-2, was detected in *Rhinolophid* bats (Rahalkar and Bahulikar, 2020). Identifying the wildlife reservoir of this novel virus will assist intervention strategies to reduce spillover risk.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Research Gaps

As a part of Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH), serological investigations using a multiplex microsphere-based immunoassay (MMIA) have detected 19% (54/284) seroprevalence ~~four~~ for ~~members of a high-risk cohort with~~ MojV~~-reactive serum~~ a high-risk cohort, including one person with a high magnitude and durability of anti-MojV IgG across longitudinal samples. Despite this, genomic surveillance has not detected MojV or any novel MojV-like viruses. For this result, we would to further identify and characterize the origin of the Mojiang virus-reactive serum and to explore the novel MojV-like virus in Thailand.

Serological and cellular immunity is an important resource for virus discovery detection by using the specificities and characteristics of memory B cells from peripheral blood mononuclear cells (PBMC). The immune response will recognize and induce antibodies specific to the infecting pathogen. Characterizing the memory B cell response to an unknown pathogen in humans could in the detection of the exposure pathogen in humans. Molecular techniques such as PCR can detect only the genomic material in the body, but memory B cells are long-lived and thus have a much larger window of detection.

Memory B-cell differentiation is characterized by somatic hypermutation in antibody variable region genes (V) and selection of B cells expressing high-affinity variants of this antigen receptor and high sequence similarity in individuals exposed to the same antigen reflect antigen-driven clonal selection and form shared immunological memory between individuals (Yang et al., 2021). Memory B cells are highly abundant in the human spleen, and can proliferate 45% of the total B cell population in this organ (Hauser et al., 2015). Memory B cells can be detected in the Ig variable (IgV) genes in a fraction of IgM+ B cells. In humans, B cells expressing the memory marker CD27+ can be found in the marginal zone of spleen and circulate in the blood. After re-exposure to antigen, memory B cells can quickly proliferate and differentiate into plasma cells. Many studies the antibody diversity and specificity of the naturally paired immunoglobulin heavy and light chains for affinity maturation of B cell receptor (BCR) after virus infection such as SARS-CoV-2 infection (Yang et al., 2021), influenza (Dougan, S et al., 2013), HIV (Moir, S., & Fauci, A. S.,2017), etc. The characterization of memory B cells can be used to develop monoclonal antibodies and produce an effective ~~theraputic~~therapeutic.

Significance and Approach

Southeast Asia is one of the world's highest-risk emerging infectious diseases hotspots. From this region emerged both SARS-CoV-1 and SARS-CoV-2, recurrent outbreaks of novel influenza strains, and the spillover of dangerous viral pathogens such as Nipah virus. The diversity of coronaviruses, henipaviruses, and filoviruses are found in wildlife reservoirs in Southeast Asia and likely ~~is~~ due to the high diversity of wildlife, and the demography of the human population brings humans and wildlife into close contact. The spillover of viruses from animals to humans is often misdiagnosed and the clinical symptoms from patients is underestimated during the pandemic. This study provides a surveillance strategy in human and wildlife populations to better understand immunity to novel pathogens and identify the origin of these pathogens to serve as an early warning system to prevent outbreaks of emerging viruses.

This study uses immunology (serologic and cellular) to identify a novel virus that can aide in effective vaccine discovery and development, monoclonal antibody production for prevention and therapy, and generate data resources for early detection and diagnostic assays. Whereas PCR detection or virus isolation in wildlife is challenging, this new serological approach may be more successful in identifying reservoir hosts.

The Indo-Pacific region has a high risk of novel viral emergence. This study provides an opportunity for in-country research capacity building by linking local infectious disease scientists with an international collaborative network that has proven capacity to conduct this work and produce significant findings. Moreover, a global sero-surveillance network for surveillance technology extends virologic and immunologic screening to product discovery. This study will benefit and expand the CREID, EID-SEARCH networks, and the global sero-surveillance network to improve our understanding of immunology to identify novel pathogens and their origin to prevent future pandemics.

Research Methods

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population

Serological profiling provides an indirect measurement of the infecting virus. Despite 19% (54/284) seroprevalence in humans, we have not detected nucleic acid sequences of MojV or MojV-like henipaviruses in this human cohort. In addition to the translational application of mAbs as therapeutics, we intend to utilize these mAb as non-clinical diagnostics surveillance tools for virus/virus antigen capture. Additionally, we intend on expanding our understanding of henipavirus prevalence in this population with longitudinal sampling of the wildlife and human populations in this region.

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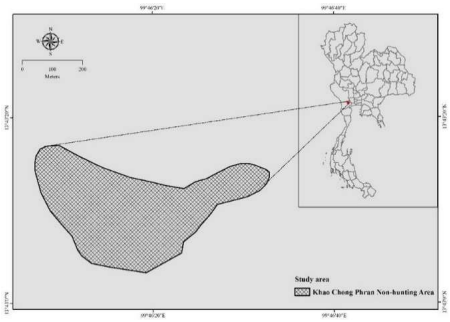


Figure 1: Map of Khao Chong Phran Non-hunting Area, Ratchaburi Province, Thailand (Boonpha N et al., 2019)

Site wildlife samples collection

100 bats and 100 shrews or rodents in and around the Khao Chong Phran cave in Ratchaburi Province will be collected for surveillance targeting the Mojiang-like virus. We plan one sampling trip to collect bats, and a second trip to target shrews and rodents. Due to limited knowledge of the local shrew population, we are unsure as to whether we can collect 100 samples, and will supplement the collection with rodents, as needed. Samples will be collected based on the following enrollment criteria: random age, random weight, random sex. Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and

stored. Oral swab and rectal swab will also collect in 100 bats and 100 shrews or rodents. Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and stored.

Human surveillance

Four of the samples from prior investigations have shown high MojV RBP-reactive antibody titers and will be selected for follow-up sampling. Additionally, 100 human serum samples, nasopharyngeal swab and throat swab will be collected from persons deemed to be in close contact with wildlife, and people who present with or have recent history of undiagnosed acute febrile illness using the following enrollment criteria;

- Adults (≥ 18 years of age) who sign in the informed consent
- Who present with or have recent history of illness without diagnosis, such as severe/acute respiratory illness (SARI/ARI), Influenza-like illness (ILI), fever of unknown origin (FUO), or respiratory tract infection
- Who is the bat guano collector in the cave
- Who lives or works around wildlife habitats
- Who is involved with livestock or wildlife farming
- Who hunts or works with wildlife
- Who has not been previously sampled as part of the EID-SEARCH and PREICT projects

Sample size justification

Human population

$$n = \frac{N}{1 + Ne^2}$$

n = sample size of the study

N = population size for finite populations: 8,150 (data from 2018)

e = error: 10%

$$n = \frac{8{,}150}{1 + (8{,}510 \text{ x } 0.1^2)}$$

n = 98.79 (round up to 100 samples)

Wildlife population

The sample size of 100 of each species follows the guidelines established during the PREDICT project.

Targeted Viral Sequencing and Serologic testing

Targeted sequencing

For samples from shrews, rodents, and acute individuals, we will use targeted sequencing and metagenomic approaches in an attempt to detect the Thai MojV-like virus. The nucleic acid from oral swab and rectal swab will do target sequencing in henipavirus Family PCR and Sanger sequencing.

Serological testing

Serum samples will be tested for RBP and envelope fusion protein (F) binding to the known henipavirus species, including the Nipah, Hendra, Cedar, Mojiang, Ghana, Langya, Gamak, and Daerong viruses to

further investigate the seroprevalence in this region. Additionally, prior positive samples will be re-tested with the expanded panel to enhance our understanding of the antigenic relationships between the novel shrew-borne henipavirus and the Thai virus.

Data analysis

Sequencing will be analyzed assembling reads in MEGA11. The consensus sequences will compare to the references strains available in the GenBank data-base using the Basic Local Alignment Software Tool (BLAST) (National Center for Bio-technology Information). Serological data will be analyzed using cluster analysis in RStudio.

Aim 2. Characterization of monoclonal antibodies from humans with serological profiles consistent with MojV infection.

In collaboration with NIH VRC PREMISE, MojV RBP and F-reactive B cells will be captured, and the B cell receptor will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific mAbs will be generated from the corresponding sequences and will be assessed for binding potential to LayV RBP and F, as well as their neutralization potential.

Importance: The isolation of these mAbs represents a potential therapeutic for MojV, MojV-like henipas, and LayV, depending on how well these cross react.

Human samples collection site

Human positive serum for MojV was found close to Khao Chong Phran in Ratchaburi Province, which has the largest colony of bats in Thailand (Figure 2).

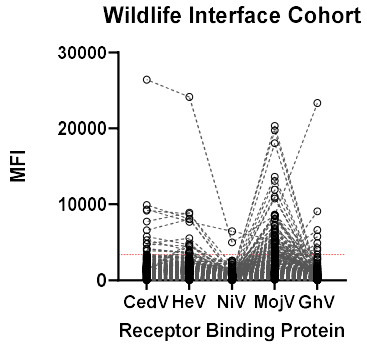


Figure 2: Seroprevalence of henipavirus proteins from a community with a large bat interface during 2017-2018. MFI is the median fluorescence intensity for IgG reactivity to each protein. Red-dashed line is at 3,357 MFI, a threshold for seropositivity.~~the assay cutoff.~~

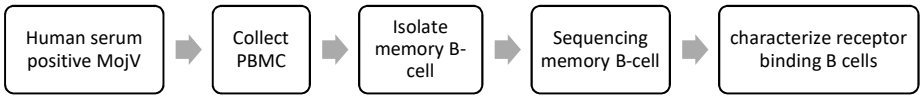
PBMC Sorting and B Cell isolation

Whole blood from MojV-positive people will be collected in individual heparin tubes and processed for PBMC isolation using Density Gradient Centrifugation (SepMate™ PBMC Isolation Tubes). Human PBMCs will be stained with antibodies for positive markers of IgG memory B cells, and antibodies to negatively select and avoid contamination with dead cells, T cells, NK cells or monocytes (live, CD3−,CD14−,CD56−, IgM−, IgA−, CD19+,CD20+,CD27+). Avi-tag for biotinylation and 6xHIS tag for

Nickel purification using the HisPur™ Ni-NTA Spin Purification Kit (Thermo Fisher Scientific, Waltham, MA). Single Memory B Cells will be sorted, cells will proliferate, cDNA will synthesized, then Heavy and light chain variable domains will be amplified (Waltari E et al., 2019).

B cell receptor Amplicon Preparation and Recombinant Antibody sorting

Total RNA yields from the PBMC will be determined by absorbance at 260 nm on the NanoDrop™ One (Thermo Fisher Scientific). Total RNA will be used for first strand cDNA synthesis with gene-specific reverse transcription (RT) primers directed to the constant regions. The RT primers for IgG, IgM, IgA, lambda, and kappa will be included. Light chain and heavy chains will be amplified into amplicon. Double-stranded cDNA will be sequenced. After completion of MiSeq sequencing, antibody repertoires will analyze using methods based on the pipeline. Heavy and light-chain sequences will be assembled into an expression vector and transfected into cells for monoclonal antibody expression. Recombinant antibody will be isolated and purified for downstream applications.



Functional assessment

We will first determine the extent to which ~~Isolated~~isolated mAbs can bind ~~will be assessed for~~ binding to native-like MojV RBP and F proteins. We will also characterize ~~through multiplex binding serology. Further,~~ the cross reaction potential of these mAbs will be assessed against GamV, DarV, and LayV RBP and F. Neutralization of viruses remains a critical correlate of protection for most vaccine strategies. However, MojV has not been isolated or rescued, and the virus receptor for MojV has not yet been discovered (Rissanen et al., 2017; Cheliout et al., 2021). Thus, a functional assessment of MojV ~~cross-~~neutralizing potential is not possible. ~~Next~~Though, we will test the cross neutralization potential of these anti-MojV RBP and F mAbs to neutralize LayV utilizing a plaque reduction neutralization test with EID-SEARCH co-investigators at Duke-NUS.~~(Duke NUS/LinFa should be able to do this, if they have LayV and are propagating it, they can use cell-cell fusion/CPE in a straight PRNT).~~ As LayV causes acute illness in human, cross neutralization functionality of these mAbs has therapeutic potential.

~~**Determination of envelope glycoprotein epitope binding**~~

~~Isolated mAbs will be assessed for binding to MojV RBP and F proteins through multiplex binding serology. Further, the cross reaction potential of these mAbs will be assessed against GamV, DarV, and LayV RBP and F. MojV has not been isolated or rescued, and the virus receptor for MojV has not yet been discovered (Rissanen et al., 2017; Cheliout et al., 2021). Thus, a functional assessment of cross-neutralizing potential is not possible. However, through cyro-electron microscopy we can investigate binding epitopes between these mAbs and MojV and LayV envelope glycoproteins. Epitope identification can then be used to make predictions about neutralizing potential through deductions about the location of henipavirus RBP receptor-binding pockets.~~

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Project Timeline

Activity	05/23	06/23	07/23	08/23	09/23	10/23	11/23	12/24	01/24	02/24	03/24	04/24
Mentoring and training activities												
1.Training, literature review												
2.Monthly mentoring meeting												
3.Manuscript writing												
4.EID-SEARCH meeting												
Research activities												
1.IRB amendment												
Aim 1												
2. Human and Wildlife samples collection												
3. Identify Targeted sequencing												
4. Serological testing												
Aim 2												
5. Collect PBMC human sample and isolation memory B-cell for Mojiang virus												

6. PBMC Sorting and B Cell isolation												
7. Determination of envelope glycoprotein epitope binding												

Research Performance Sites

This proposed study will be implemented at the King Chulalongkorn Memorial Hospital (KCMH) collaborating with the Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC). KCMH is a 1,000-bed, university hospital in close coordination with the Faculty of Medicine, Chulalongkorn University. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. It's one of the center hospitals for Emerging Infectious Diseases in Thailand, especially for domestic and international patients.

TRC-EIDCC was established by the Committee of the Emerging Infectious Disease Center, which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. Our clinical center provides standard medical care to patients with emerging and recurrent diseases and disease transmission. Moreover, the center plans preparedness activities, trains medical and government staff on interventions, hosts conferences for medical and public health professionals, creates networks of medical care for patients with emerging and recurrent diseases, and leads research and development projects for medical care and disease control. Apart from medical innovation in emerging and recurrent diseases, the clinical center strives to be the model of infectious diseases clinical services and pandemic control through research activities surrounding infectious diseases and effective disease control measures.

-We also collaborate with the Department of National Parks, Wildlife and Plant Conservation for detecting novel pathogens from animals such as bats, rodents, and shrews in our country. We also collaborate with the Department of Disease Control of Thailand (Thai-DDC) to support the Thai government and national policy makers through molecular diagnosis, including the identification of the first case of SARS-CoV-2 infection in Thailand. TRC-EIDCC prides itself on its high performance in research laboratory services and is a part of the national reference laboratory for COVID-19 and other emerging infectious diseases confirmation in Thailand.

CREID Research Center Collaboration

The goals and objectives of the proposed research project, "Immune memory bait & capture to identify emerging henipavirus origins", are fully consistent with the specific aims of EID-SEARCH. EID-SEARCH takes a One Health approach to understanding zoonotic disease emergence, with a focus on viral diversity in wildlife at high-risk human animal interfaces. EID-SEARCH aims to understand the frequency and causes of virus spillover in high-risk communities across Southeast Asia and identify strategies and tools to reduce this risk. As the proposed research focuses on the links between farming practices, wildlife diversity, virus diversity, and cross-species transmission in Thailand, we expect the results of the project to make a significant contribution towards the overall goals of EID-SEARCH.

The proposed project is well-designed, leverages cutting-edge technology, and includes a robust ecological component that corresponds to the overall research strategy of EID-SEARCH. The EID-SEARCH team is highly motivated to support the application of metagenomics to ecological questions and assist in the analysis of the unique dataset that will be generated from this research. With the heightened focus on identifying and limiting risks associated with the complex interactions between humans, wildlife, and domestic mammals that has resulted from the emergence of SARS-CoV-2 from a wet market in China, the proposed research is both timely and likely to yield highly-cited, impactful publications for EID-SEARCH and Thailand partners.

The EID-SEARCH team includes a number of experts in study design, laboratory-based diagnostics, genetic and genomic analyses, and ecological modelling who will contribute to project implementation, data analysis, and manuscript preparation as required. Communication and coordination mechanisms will be set up between the Thailand research team and scientists at the EID-SEARCH research sites for resource sharing, training, knowledge exchange, and mentorship. In particular, EID-SEARCH will facilitate and encourage relationships between our partners in Southeast Asia (based in Malaysia, Singapore, and Thailand) that will facilitate Thailand team professional development and strengthen research capacity in the region. Dr. Eric Laing, the project Mentor, has an established relationship with both Dr. Krongkan and Spencer Sterling, and will act as the liaison between EID-SEARCH and the TRC-EIDCC research team. Further, Dr. Doeuk who is the ~~director~~director of the VRC PREMISE program is ~~enthuasiatic~~enthusiastic about the opportunity to collaborate and apply their rapid assembly, transfection and production of immunoglobulin (RATP-Ig) workflow as a serologic/immunologic based approached for therapeutic and virus discovery (see

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Letter of Support). An expected outcome of this research project will be pilot data to support a joint funding application in the future.

Mentoring Plan

Goals for Pilot study and Career

Krongan Srimuang: My career goal is to be an expert scientist in either an academic research laboratory or a lecturer in medical science. My long-term research interests include the understanding of priority emerging infectious diseases, genetics, and epigenetics using molecular and serologic techniques. Detection of novel pathogens in their wildlife vectors can help characterize the pathogenicity, virulence, and evolution of these and other closely related pathogens. I believe that this pilot study can provide training in new techniques for the detection, identification, and characterization of novel pathogens that are currently unavailable in Thailand. My goal for the Pilot study is to gain more knowledge and skills for a lifelong career in public health-related research. I wish to expand my skills in novel virus detection using next-generation serologic techniques such as PBMC studies and memory B-cell isolation and sequencing that can contribute to improved surveillance and research capacity at human-animal interfaces. My mentors Dr. Laing and Dr. Wacharapluesadee can provide me with opportunities to develop and apply project skills and improve my scientific writing.

My education has provided me with a strong foundation to expand for this study. I received my undergraduate degree in Medical Technology from Chulalongkorn University which included training in molecular biology, serology, immunology, chemistry, virology, bacteriology, mycology, parasitology, hematology, clinical microscopy, transfusion medicine, and forensic medicine. My interest in molecular technology led me to pursue a Ph.D. at the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, which provided me with training in several molecular techniques including conventional PCR, real-time PCR, Next Generation Sequencing (NGS), etc. The emergence of COVID-19 motivated me to focus on emerging infectious diseases. I am currently completing my post-doctoral fellowship at the Emerging Infectious Diseases Clinical Center (EIDCC), King Chulalongkorn Memorial Hospital with Dr. Wacharpluesadee. Throughout this fellowship, I have continued learning advanced and innovative techniques to detect, identify, and characterize the novel pathogens. Through this pilot project, I will learn about the Peripheral Blood Mononuclear Cells (PBMC) which are a critical part of many subdivisions of medical research including Infectious diseases, Vaccine development, and Immunology. This project aims to study zoonotic reservoirs for by using serologic and cellular immune discovery in humans, and applying this immune

knowledge in wildlife for early detection and identification of novel pathogens. This proposed study will expose me to various techniques and immunology topics that I useful in designing and testing new pipelines as technological advances continue to develop. This study will also allow me to develop, manage, and communicate my research.

Spencer Sterling: My overall career goal is to understand the factors that lead to viral zoonosis and to build epidemiological capacity in regions with the highest risk for zoonotic spillover. Through my training and educational experiences, I have learned laboratory and epidemiological techniques that can assist in the understanding of zoonosis. I wish to continue my education by expanding my repertoire of molecular techniques to include studies of the immune system's response to zoonotic disease in humans and animals, as well as to learn next-generation sequencing techniques. Additionally, I would like to use my experience in epidemiological techniques to train scientists in the Southeast Asian region in order to build local capacity in infectious disease research. Dr. Laing has been a mentor for me throughout my career, and this project would allow for this relationship to expand into new molecular areas, as well as ~~grant writing~~grant writing and project management skills. My professional relationship Dr. Wacharapluesadee with began in 2018 when I participated in a DTRA BTRP funding project that focused on transferring antigen-based multiplex serological technology to her lab group. Currently, I am a Visiting Scientist at TRCEIDCC and I work closely with Dr. Srimuang, and other members of Dr. Wacharapluesadee's research group on EID-SEARCH activities. Additionally, Dr. Wacharapluesadee has decades of experience in infectious disease research in the region and has mentored scientists that have gone on to careers at the Thai Ministry of Public Health, Department of Disease Control, Department of Parks and Wildlife, academia, and the private sector. Both mentors have encouraged Dr. Srimuang and I to apply for this pilot study as well as pursue other funding mechanisms for our research interests. Both mentors ~~will provide~~will provide for me the ability to independently pursue my research interests in the zoonotic epidemiology and train me on the best practices of scientific communication.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Vertebrate Animals Section Requirements

Animal samples will be collected in Ratchaburi Province for this project under Aim 2 to understand the antigenic relationships among known and unknown henipaviruses. Protocol to perform animal sample collection has been reviewed and approved by the Institutional Animal Care and Use Committee at Tuft University under the EID-SEARCH project (No. G2020-42) and the Institutional Animal Care and Use Committee at Chulalongkorn University (No. 019/2563)

1. Description of Procedures

Bats (order *Chiroptera*)

- Species: Free-ranging mega and micro-*Chiroptera* that present at the site (family *Pteropodiae, Rhinopomatidae, Emballonuridae, Craseonycteridae, Megadermatidae, Rhinolophidae, Hipposideridae, Vespertilionidae, Mollossidae*, etc.)
- Age & Sex: Fully flighted adults and juveniles, males and females; neonates dependent on the dam will not be used.
- Target Number: 100 individuals
- Capture and Restraint: Bats will be captured using either a mist net or harp trap. The net system is manned by at least two people during the entire capture period, and bats are removed from the net as soon as they become entangled to minimize stress and prevent injury. In our previous field research experience, a maximum of 20-30 bats can be safely held and processed by a team of three people per trapping period. Duration of trapping will depend on the capture rate. Bats are individually placed into a pillowcase or small cloth bag and hung from a branch or post in a location that is free from access by both predators and people, and is protected from the elements (e.g., rain), until the animal is restrained for sample collection. Bats will be manually restrained during sampling utilizing insulated bite gloves to prevent injury to the handler and bat. The study protocol covers over 69 bat species that are most likely to be captured given their natural occurrence and previous surveillance experience in Southeast Asia. The potential for non-target species to be captured is very low because mist nets are opened at the cave entrance at night, after other species (e.g., birds) have ceased flying and returned to roost. Any non-target species will be rapidly identified, removed from the mist net and released immediately.
- Sample Collection: Depending on the species and size of bat, blood will be collected from smaller insectivorous bats (<50g) using a 27g needle to puncture the brachial artery and a 70ul hematocrit

tube to collect the blood. For larger bats (>50g) we will collect blood from either the cephalic vein or from the radial artery or vein using a 25- or 23-gauge needle and 3cc syringe. Blood will be collected in quantities not exceeding 6 µL/g of body mass from each individual. Wing punch biopsies will be collected to confirm host identification using DNA barcoding. Swabs will be taken from the oropharynx, urogenital tract, and rectum. No destructive sampling is planned, but any individuals that die during capture or sampling will have a necropsy performed with organ samples collected and whole body submitted to museums as voucher specimens.

- Release: Bats are held for a maximum of six hours (typically less than three hours), and at the completion of sampling are provided with rehydrating fluids (either subcutaneously or orally as needed) and released in a location that allows for further recovery or safe escape (e.g., bodies of water, known predatory areas, and human settlements will be avoided).

Rodents (order: *Rodentia*) and Shrew (order: *Scandentia* and *Eulipotyphla*)

- Species: Free-ranging rodents (family *Hystricidae, Muridae, Sciuridae, Spalacidae*) and shrew (tree shrews, shrews, moles) that present at the sites.
- Age & sex: Adults and juveniles, males and females. Neonates will not be sampled.
- Target Number: 100 individuals
- Capture and Restraint: Free-ranging wild mammal species will be captured with metal box traps (Sherman/Tomahawk traps). Traps will be prepared with food, water, padding and shelter, and be checked at least every 12 hours, in the morning and in the afternoon. If adverse weather (extreme heat, rain) is expected or researchers are working in areas where predation is common, traps will be checked more frequently and closed during adverse weather. Veterinary staff will be prepared to deal with a variety of trap injuries, including lacerations, broken teeth, and crush injuries. Depending on the species, individual size, captive or free-ranging status of the individual, manual or chemical restraint and anesthesia (gas or injectable anesthesia) will be applied. Animals will be manually restrained during sampling utilizing insulated bite gloves to prevent injury to the handler and animal. Based upon past field experience (>20 years of animal capture and release in Southeast Asia), we do not expect to trap sick, debilitated, or young animals that would be too young to cope with capture. Sick and debilitated animals tend not to roam widely, reducing their opportunity to enter traps. Additionally, should sick, debilitated, or young animals enter traps, we anticipate that they will not suffer adverse conditions because the traps contain food (the bait) and liquid (water or fruit as a source of water). These individuals would be examined by a veterinarian, rehydrated and if presenting clinical signs treated by a veterinarian, prior to release or sampling then release.
- Sample Collection: Once anesthetized or safely restrained, whole blood will be collected through the appropriate venipuncture site, no more than 1 ml of blood per 100 g (= 10 ml/kg or 1%) of body weight will be collected at any one time. Anesthetized animals will be monitored regularly during recovery until they can no longer be safely handled, at which point they will be confined in a trap or cage. Swabs will be taken from the oropharynx, urogenital tract, and rectum. No destructive sampling is planned, but any individuals that die during capture or sampling will have a necropsy performed with organ samples collected.
- Release: Wild mammals are held for a maximum of thirteen hours depending on trap timing, but typically less than three hours. Captive animals are held for a maximum of two hours but typically less than one hour. At the completion of sampling, animals may be provided with rehydrating fluids (either subcutaneously if anesthetized or orally if manually handled and accepted). Wild mammals will be released at the site of capture in a location that allows for further recovery or safe escape (e.g., bodies of water, known predatory areas, and human settlements will be avoided). Captive mammals will be released back into their captive setting as is appropriate for the species, either isolated or in social group if it is deemed safe from aggression from enclosure mates while the post-recovery period continues.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

2. Justifications

The purpose of this study is to identify zoonotic pathogens through serologic and cellular immune discovery, including developing methods and standards for direct antibody-mediated virus capture in people and wildlife, focusing on rodent/shrew- and bat-borne HPVs. This will require hands-on fieldwork to collect whole blood and serum samples from bats, rodents, and shrews for lab analysis. One technique that avoids the direct capture and sampling of animals is to collect fresh feces or urine, but this will not be able to provide the appropriate samples for serologic analysis, and the research objectives cannot be achieved using an alternative methods (e.g., computational, human, invertebrate, in vitro). Therefore, we believe there are no viable alternatives than the use of live animals.

3. Minimization of Pain and Distress

In every situation, sampling of wildlife will be conducted in the most humane manner while minimizing the impacts on individual animals and their wild populations. In all instances, the fewest number of animals will be sampled that will provide valid information and statistical inference for the pathogen and disease of interest and every effort will be made to minimize stress and discomfort for the animal.

Bats will not be held longer than six hours during the sampling process whereas small mammals may be held for up to 13 hours depending on trap capture timing. Field animal sampling team with zoologists and veterinarians have been well trained and have extensive experience in capture, anesthesia, and sampling of wildlife. In our team's experience, the target species tolerate the described procedure well. Mist nets will be attended continuously during capture periods, and bats will be extracted from the net as soon as they become entangled. This will minimize stress and injury from entanglement. Bats will be placed individually in cloth bags and hung from tree branches while awaiting processing and during recovery. The bags are sufficiently porous as to allow for ventilation and are designed for bat capture. The enclosed environment seems to calm the bats, as they do not struggle once inside, but they hang quietly – this is a standard and accepted practice in bat research and the best way to minimize stress to the animal.

For rodents and shrews, food, water, padding and shelter will be provided at traps, which will be checked at least every 12 hours. Traps will be checked more frequently or closed if adverse weather (extreme heat, rain) is expected, or researchers are working in areas where predation is common. Veterinary staff will be prepared to deal with a variety of trap injuries, including lacerations, broken teeth, and crush injuries. All animal individuals will be monitored by a veterinarian or experienced field team member during all stages of capture, processing, anesthesia and release. Animals will be kept in a cool place, free from adverse weather conditions and access by non-field team members or other animals while in the pillowcases, trap, or cage for recovery. All recovering individuals will be observed until they are able to effectively ambulate and orient to their surroundings. Animals that are injured during the capture or sampling process will be assessed by an experienced team leader or attending veterinarian, and if the animal is determined to be unlikely to survive if released, it shall be euthanized humanely (see euthanasia section).

4. Method of Euthanasia

In the event that an animal has been injured or is moribund, a determination will be made as to whether it may be treated and released on site by veterinarians or transferred to facility that may treat and rehabilitate the animal, or if, as a last resort, the animal will require euthanasia. Euthanasia methods will vary depending on species; however methods to be used will not deviate from the AVMA "Guidelines for the Euthanasia of Animals" (2020 edition). Any animal that is euthanized using a chemical agent will be disposed of such that it will not be permitted to enter the food supply either through markets or hunting.

Human Subjects Research

1. Risks to the subjects

In this project, we will target populations in one community site who with frequent exposure to bats and other wildlife based on our previous behavioral survey. Subjects will be enrolled voluntarily, and informed consent will be obtained from all participants, along with assent from all participants aged 12-17. Enrolled participants will provide biological samples. Survey data and biological samples will be collected from enrolled participants, and follow-up data collection will be performed among participants whose samples were tested positive.
Biological samples and questionnaire data will be collected from individuals who meet recruitment and inclusion criteria and complete the informed consent process. During data collection, a standardized questionnaire will be administered to all participants. This questionnaire will collect information of demographic background, wildlife contract, travel and daily movement, and unusual SARI/ILI symptoms, and biological specimens will be collected from participants. Both questionnaire and biological data will be analyzed to assess the exposure to coronaviruses and the spread risk among humans. From all participants, a one-time whole blood sample (Max. 15 mL) will be collected during the study period. This sample will allow us to test for historical exposure to bat or rodent-born henipaviruses and collect Peripheral Blood Mononuclear Cells (PBMCs) for those study participants whose serum tests are positive.

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 who are 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, the administration will be conducted privately and confidentially to protect individuals' 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 these risks

2.1 Informed consent and assent

Only consented participants will be enrolled in the study. No research procedures will be undertaken before the participant fully understands the research, agrees to the study procedures, and completes the consent process will be enrolled in the study. Informed consent statements and forms, and project protocols will be translated into the local language of each study site. Research team members involved in this consent process will be required to be fluent in the local language to ensure that the subjects understand the study and all involved procedures.

If participants meet the criteria for enrollment, they will be invited to discuss the details of the study with the research staff. Study staff will review an information sheet and informed consent form with the participant and the parent or legal guardian when applicable. Each individual will be provided with a copy of the informed consent form that has been translated into the local language and written with a Flesch–Kincaid readability score equivalent to a 7th-grade reading level or below, to assure that potential participants understand the information being shared. The informed consent form will explain the details of the study, including how and why the individual was selected, the study process and procedures, risks and benefits, financial considerations and the gift of appreciation, confidentiality of data shared, alternatives to participating, and how to obtain more information now or at a later date. The informed consent form will be read in the local language of the site at a location ensuring participant privacy. After which individuals will have as much time as they would like to ask questions and discuss the study with study staff. The study staff will endeavor to ensure that the participant understands the information provided. The study staff will then ask the participant/parent or legal guardian to consider study participation. Participants will have as much time as required to consider the participation.

Those participants who consent to the study will sign and date two copies of the consent form. These form copies will be countersigned and data by the study staff. A copy of the signed consent form must be provided to the subject and the other copy will be kept by study staff. Informed consent paperwork will be kept until the end of the project in a locked box at the local country project office.

2.2 Protection against risks

The potential risks to study participants as a result of study participation are minimal. Collection of venous blood samples and nasal or oropharyngeal swab samples pose minimal risk to subjects. Potential complications associated with venipuncture include pain and/or hematoma at the site of collection. Trained medical professionals and/or clinic staff will monitor the blood collection site and treat any complications according to existing health facility protocols. A potential complication of nasal/ oropharyngeal sampling is minor irritation at the time of collection. Employing trained medical and/or clinic staff to collect blood and swab samples will minimize the potential for complications.

Another risk that this study may pose concerns the information to be gained on henipaviruses newly recognized in the community. We will provide participating communities, hospitals, and clinicians with information and background data on target zoonotic viruses to ensure up-to-date communication of risk. Because of the timeline for diagnostic testing and results interpretation, we are not likely to provide results to participating clinics within a time frame that would be clinically relevant to outbreaks of undiagnosed diseases. Therefore, the information provided by this project will not impact patient management or outlook.

If an individual decides to participate in this research, his/her participation and all information provided by the participant will be strictly confidential, and personal identifying information will not be shared with anyone outside of the study staff. Participants will not be identified or named in any reports or publications. Questionnaire information and all biological samples will be identified by an

alphanumeric code, not by the participant's name. 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 study site in locked files cabinets or password-protected devices in areas with access limited to the research staff of this project. Research databases will be secured with password-protected access systems and controlled distribution web-based certificates and will not contain any identifying characteristics about study participants (e.g., name, address, or telephone number). Access to all data will be limited to the staff involved in this study. The health information disclosed by an individual will not be used by or disclosed (released) to another institution. Any surveillance report that is published or shared with partners will not contain any personally identifying information for individual participants.

3. Potential benefits of the research to the subjects and others

There are no measurable benefits to the individual study participants enrolled in this study. There are benefits to the community and regional healthcare providers to help them understand the risk of zoonotic infections among high-risk populations in the regions they work. At the conclusion of the study, we will deliver an educational workshop reporting aggregate study findings that will be open to both study and non-study participants, describing the health benefits of using personal protection equipment (PPE) and handwashing during animal handling activities throughout the day, as well as to share other prevention/interventions that emerge from the research data.

4. Importance of the knowledge to be gained

There are valuable potential benefits to the public from the knowledge to be gained from this study. One key benefit to the community is sharing information and knowledge to better understand the risk of zoonotic spillover events and related health risks, as well as information sharing with communities on practices that could reduce risks, such as the avoidance of particular animal contacts or the need for PPE and extra care when handling wildlife, that may substantially reduce the risk zoonotic pathogen transmission in the community. 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 create or implement public health interventions to disrupt disease emergence and/or spread in an area that is beneficial to all. Additionally, there are valuable benefits to the general public from the knowledge to be gained from this study. Knowledge gained will 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.

5. Country- / institution-specific ethics / IRB regulations addressed

Main research protocol and materials to conduct human subject research in this project have been reviewed and approved by the Health Medical Lab Institutional Review Board, US (No. 894ECOH21b)

and the Institute Review Board of the Faculty of Medicine, Chulalongkorn University, Thailand (No. 211/64). Amendment for PBMCs collection will be made and approved before the start of this project.

Research and Related Other Project Information

1. Are Human Subjects Involved?	Yes	X	No	
1.a. If YES to Human Subjects				
Is the Project Exempt from Federal regulations?	Yes		No	X
If yes, check appropriate exemption number	1		2	
	3		4	
	5		6	
	7		8	
If no, is the IRB review Pending?	Yes	X	No	
IRB Approval Date:				
Human Subject Assurance Number	FWA00000943			

2. Are Vertebrate Animals Used?	Yes	X	No	
2.a. If YES to Vertebrate Animals				
Is the IACUC review Pending?	Yes		No	X
IACUC Approval Date	3 December 2020			
Animal Welfare Assurance Number				

3. Is proprietary/privileged information included in the application?	Yes		No	X

4.a. Does this project have an Actual or Potential	Yes		No	X

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

impact -- positive or negative -- on the environment?				
4.b. If yes, please explain				
4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed?	Yes		No	
4.d. If yes, please explain				

5. If the research performance site designated, or eligible to be designated, as a historic place?	Yes		No	X
5.a. If yes, please explain				

6. Does this project involve activities outside of the United States or partnership with international collaborators?	Yes	X	No	
6.a. If yes, identify countries	Thailand			
6.b. Optional explanation				

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Budget Justification

A. Key Personnel (Total $24,000)

Dr. Krongkan Srimuang, PI (12 months) is a medical research scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital (TRC-EIDCC) who have been leading the laboratory analysis for the EID-SEARCH work in Thailand in the past three years to perform molecular, serological, and next-generation sequencing to identify novel viruses from human and animal samples. Dr. Srimuang will commit 12 months to this project to design, manage, and perform all project activities in the lab and field by supervising a lab technician and working with the field coordinator. Dr. Srimuang will also work closely with the co-PI and Mentors to conduct data analysis and develop manuscript and join EID-SEARCH and CREID meetings to prevent the project findings.

Spencer Sterling, Co-PI (6 months) is the research coordinator of Laing Lab at the Uniformed Services University for Indo-Pacific region and a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC). Sterling will commit 6 months to this project as the co-PI, who will work closely with the PI in the study design, SOP development, sampling process, data analysis of for serological investigations. No salary is required for Sterling.

B. Other Personnel (Total $20,000)

One (1) Laboratory Technician (12 months) will committee 12 months to this project to conduct PCR and serological testing of animal and human samples at TRC-EIDCC, under the supervision of Dr. Srimuang and Sterling. Salaries for 12 months at $1,250 per month is requested ($15,000).

One (1) Field Coordinator (4 months) will committee 4 months to this project, working closely with Dr. Srimuang and Sterling to organize and conduct field sample collection among humans and animals at the study sites, and support relevant financial management. Salaries for 4 months at $1,250 per month is requested ($5,000).

C. Fringe Benefits

No Fringe is requested.

D. Equipment

No equipment is requested.

E. Travel (Total $48,375, Years 1-5)

Domestic Travel ($11,005)
Support for domestic travel for field sampling is requested in the amount of $11,005 per to conduct four (4) trips of sample collection from humans, bats, and rodents/shrews.

- The first trip is a one-day trip to collect samples from human participants who were tested positive (n<10), the cost is estimated at van rental for local transportation between Bangkok and the study site ($260) and meals for five (5) field team members at $49 per person ($245), in total of $505 for the first trip.

- The other three (3) field trips will be conducted to collect samples from 100 bats, 100 humans, and 100 rodents/shrews, respectively. Each trip is a duration of 6 days and 5 nights. For each person, 5-night stay in hotels at $75.5/night (5 x $75.5 = $378); 3 meals per day and incidental expenses at $49 per day prorated to 75% ($36.75) for first and last day of travel ($36.75 x 2 + $49 x 4 = $270), the total meals and accommodations for each person per trip is $648 ($378+270), cost for five (5) field team members is $3,240. A van will be rented for local transportation between Bangkok and the study site that is estimated at $260 per trip. Total cost for each 6-day trip is $3,500, three trips will be $10,500.

International Travel ($5,023)
Support for international travel for Dr. Srimuang from Bangkok to Washington DC to attend the CREID Annual Meeting and receive lab training at the National Institute of Allergy and Infectious Diseases (NIAID) is requested in the amount of $5,023. Trips are for a duration of 10 days and 9 nights. Travel costs for one trip are estimated using federal per diem rates for hotels, meals, and incidental expenses as follows: nine-night stay in DC area hotel at $286/night (9 x $258 = $2,322); Ten days' meals and incidental expenses at $79 per day prorated to 75% ($59.25) for first and last day of travel ($59.25 x 2 + $79 x 8 = $750.5); Round trip flight between Bangkok to Dulles International Airport is estimated at $1,650; local transportation/taxi between hotel and airport per round trip between Chulalongkorn Hospital to airport is estimated at $40, between hotel and the airport in the US is estimated at $200, and $60 for transportation within the region ($300).

F. Participant Support Costs

No participant support is requested.

G. Other Direct Costs (Total $78,803)

Materials and Supplies ($72,830)
PBMCs isolation ($47,000). We request funds to conduct PBMC isolation from collected sample, costs including the reagents and lab consumable and supplies are estimated at $47 per cell for 1,000 cells.

Multiplex microsphere immunoassay detection ($2,275). Funds are requested to perform serological testing of collected human and animal samples. Costs including the reagents and lab consumable and supplies are estimated at $6.5 per sample for 350 samples.

PCR testing and sequencing ($20,475). Funds are requested to perform serological testing of collected human and animal samples. Costs including the reagents and lab consumable and supplies are estimated at average $58.5 per sample 350 samples, given a 10%~15% positive rate.

Field supplies for PBMC collection and processing ($1,225). Funds are requested to perform PBMCs collection and processing in the field. Costs including the reagents and lab consumable and supplies are estimated at average $3.5 per sample 350 samples.

Field supplies for animal sampling ($1,160). Funds are requested to purchase supplies for animal capture, anesthesia, and sample collection. A total cost of $1,160 is estimated

Field work disposable ($668). Funds are requested to purchase disposable materials (tips, tubes, gloves, masks, googles etc.) for field work.

Consultants Service ($3,000)
One (1) zoologist and one (1) veterinarian will provide consultancy service to this project to help 1) select sites for animal sampling and join the sampling trip to 2) identify species; and 3) provide veterinary care in the sample collection of bats, rodents, and shrews. The consultancy fee is estimated at $1,500 per person for the project, in total of $3,000.

Publication Fee ($3,000)
We request $3,000 of publication costs for one peer-review paper generated from the project. The cost is estimated at the reimbursed Open Access fees of $2,900 - $5,000 per manuscript in the past two years for six peer-review papers.

H. Total Direct Costs (Total $138,831)

I. Indirect Costs (Total $11,106)

An indirect cost at 8% is requested.

J. Total Direct and Indirect Costs (Total $149,937)

A total amount of $149,937 is requested.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Co-PI Plan (only needed if applying as Co-PIs):

What each Co-PI will contribute to the proposed research study

As stated in the Mentoring Plan, the co-PIs, Krongkan Srimuang and Spencer Sterling have different educational and training backgrounds. This Co-PI plan is proposed to fill the gaps in knowledge of each scientist and supports a collaborative working relationship for project success. Moreover, this plan was developed to maximize the strengths of each PI. Overall, the project is separated into two objectives, the proposal of this project will be equally managed by Dr. Srimuang and Mr. Sterling. Dr. Srimuang will be responsible for PBMC isolation, sorting, and characterization. Mr. Sterling will be responsible for the technical serological aspects of the project. Both will work closely with the Research Center Mentor, Dr. Eric Laing, to develop this project, protocol development, and network connections. Dr. Supaporn Wacharapluesade, the Co-mentor, will support the wildlife sampling, samples collection, and molecular aspects within Thailand.

How the Co-PIs will jointly work with the affiliated Research Center

Mr. Sterling is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region.

Commented [EL4]: Need something for KIO

How the Co-PIs will jointly manage the proposed study

This is a great opportunity for both PIs to undergo training in advanced techniques and develop skills in next-generation serology and molecular sequencing. Dr. Srimuang will learn and practice serologic techniques and data analysis from Mr. Sterling. Mr. Sterling will develop skills in cell isolation and molecular sequencing techniques from Dr. Srimuang. Moreover, Mr. Sterling will coordinate planning, sample processing, data analysis, and report preparation for this project with Dr. Srimuang.

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List of abbreviations, acronyms, symbols

AngV: Angavokely virus
BCR: B cell receptor
BSc: Bachelor of Science
BSL2: Biological Safety level 2
BSL3: Biological Safety level 3
cDNA: Complementary DNA
CedV: Cedar virus
Co-PI: Co-principal investigator
CREID: Centers for Research in Emerging Infectious Diseases
DARV: Daeryong virus
DNA: Deoxyribonucleic acid
EHA: EcoHealth Alliance
EID: Emerging Infectious Disease
EID-SEARCH: The Emerging Infectious Disease – South East Asia Research Collaboration Hub
ELISA: Enzyme-linked immunosorbent assay
F: Fusion protein
FACS: Fluorescence-activated cell sorting
FUO: Fever of unknown origin
GAKV: Gamak virus
GhV: Ghana virus
HeV: Hendra virus
HPVs: Henipaviruses
ILI: Influenza-like illness
KCMH: King Chulalongkorn Memorial Hospital
L: large protein
LayV: Langya virus
M: Matrix protein
mAb: Monoclonal antibody
MD: Maryland
MMIA: Multiplex microsphere-based immunoassay
MojV: Mojiang virus

MSc: Master of Science
N: Nucleocapsid
NGS: Next-generation sequencing
NIAID: National Institute of Allergy and Infectious Diseases
NIH: National Institutes of Health
NiV: Nipah virus
P: Phosphoprotein
PBMC: Peripheral blood mononuclear cells
PCR: Polymerase Chain Reaction
PhD: Doctor of Philosophy
PI: Principal investigator
RBP: Receptor-binding protein
RNA: Ribonucleic acid
RT-PCR: Real-time PCR
SARI/ARI: Severe/acute respiratory illness
SARS-CoV-1: Severe acute respiratory syndrome coronavirus 1
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2
SEA: Southeast Asia
Thai-DDC: Department of Disease Control of Thailand
TRC-EIDCC: Thai Red Cross. Emerging Infectious Diseases. Clinical Center
USA: United States
WGS: Whole Genome Sequencing

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Facilities and Resources

FACILITIES: Specify the facilities to be used for the conduct of the proposed research.

Laboratory:

The Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC), King Chulalongkorn Memorial Hospital (KCMH) was established by the Committee of the Emerging Infectious Disease Center which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. TRC-EIDCC laboratory facilities include 3 biosafety cabinets (BSL 2, BSL 2+, BSL2+), 3 automated extraction machines, 2 thermocyclers (Proflex), 2 real-time PCR machines (CFX, Bio-rad), 3 Freezers (-80 °C), 2 refrigerators; 4 °C, 2 autoclaves. Our EIDCC uses the Pathogen Asset Control System (PACS) system for sample tracking. Moreover, our Faculty of Medicine, Chulalongkorn University has a central laboratory, the Chula Medical Research Center (Chula MRC), which provide many instruments for medical, masters, and graduate students, Post-doctoral fellows, and researchers at Chulalongkorn University and KCMH. The Chula MRC laboratory facilities are divided into molecular diagnostics (PCR Thermal Cycler, QStudio6 Flex Real-Time PCR, Droplet Digital PCR, GeneXpert, Film array, Illumina MiSeq), immunology facilities (ELISA testing equipment (reader & washer), Luminex, Flow Cytometry), cell sorting machine and a BSL3 laboratory. Moreover, the facility provides general equipment, such as autoclaves, balances, CO2 incubators, Dual Fluorescence Cell Counter, 4 °C refrigerators, -20°C, and -80°C freezers.

Clinical:

KCMH is a 1,000-bed university hospital, in close coordination with the Faculty of Medicine, Chulalongkorn University. The Thai Red Cross Society provides medical treatment, rehabilitation, health promotion and disease prevention services to the general public and the vulnerable. It also provides biopharmaceutical and sterile medicines that are sufficient to meet the needs of patients

and safe in accordance with international standards. It serves as a center for providing eye and organ donation services to patients nationwide in line with international standards. It has specialized doctors and public health personnel and also produces specialized nurses. The TRC-EIDCC in the KCMH provides standard medical care to patients with emerging and recurrent diseases and disease spreading prevention.

Animal:

TRC-EIDCC also collaborates with the Department of National Parks, Wildlife and Plant Conservation for detecting the emerging novel pathogens from animals such as bats, rodents, and pangolins in our country. Bats are considered protected wildlife in Thailand by Wildlife Conservation, Department of National Parks, Wildlife and Plant Conservation.

Computer:

TRC-EIDCC used the service from the Office of Information Technology, Chulalongkorn University that provides the application, network, and software such as Office 365, VPN, WIFI, Microsoft Office, Microsoft Windows, SPSS, Adobe, Zoom Meeting, etc. Moreover, our EIDCC use the Pathogen Asset Control System (PACS) for sample tracking.

Office:

The office and laboratory space are located at the EIDCC at 4th Floor, Jongkolnee Watwong Building and 6th Floor, Aor Por Ror Building, respectively. The applicants will have dedicated desks in both buildings.

Other:

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

PI Name: Srimuang, Krongkan and Sterling, Spencer **Foreign Site:** Thailand
CREID CC Grant: 1U01AI151378

Foreign Site Information

If the Pilot Award will have multiple foreign sites, complete this form for EACH research site

Principal Investigator Name:	Krongkan Srimuang
Project Title:	Immune memory bait & capture to identify emerging henipavirus origins
Institution:	King Chulalongkorn Memorial Hospital
Foreign Research Site:	Rama 4 Road, Pathumwan, Bangkok 10330 Thailand
Point of Contact for Research Site:	Krongkan Srimuang Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital Rama 4 Road, Pathumwan, Bangkok 10330 Thailand krongkan.sr@gmail.com

Introduction (3-4 sentences):

Provide a simple description of the overall goals of the project including the work that will be done at the foreign site. Indicate if any of the NIAID grant funds will be sent to the foreign site.

The goals of this project are to know zoonotic reservoirs for pathogen origin by using serologic and cellular immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

To achieve these goals, the investigator will collaborate with NIH PREMISE for Antibody-mediated bait & capture of Thai MojV-like virus.

$149,937 of grant funds will be sent to the site for these studies.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Foreign component (3-4 sentences):

Describe the specific role of the foreign site (i.e. conduct critical experiments?, conduct data analyses?, provide consultations?, provide samples?, study human and/or animal populations?, conduct observational or interventional clinical trials?, collect samples and/or data to be brought to the US?).

The site will 1) conduct all filed work to collect human and animal samples from the study sites; 2) perform laboratory analysis using collected samples; and 3) conduct data analysis as proposed in the project.

Human Subjects (1 word or 1 sentence per bullet):

- **Parent Study IRB approval**
  - **IRB approval number for parent study:** #894ECOH21b (US); #221/64 (Thailand)
  - **IRB approval date:** 12-05-2021 (US); 08-06-2021 (Thailand)
  - **Human Subject Assurance Number: (FWA)#:** #00001102 (external IRB); #00022431 (EcoHealth Alliance); #00000943 (Chulalongkorn University)
- **Does this study require a modification to the IRB approval of a parent study (Yes or No)?**
  - **Yes**
- **Will existing samples from human subjects will be used: (Yes or No)?**
  - **No**
  - **How many subjects provided the existing samples to be used?** N/A
- **Will human subjects be recruited (Yes or No)**
  - **Yes**
  - **Number of human subjects that will be recruited:** 110
- **Population parameters:**
  - **Gender:** 55 males, 55 females
  - **Age Group:** Age >=18 years who provide informed consent.
  -
  - **Race/Ethnicity:** 110 Asian
- **Sample collection will include:**
  - **Blood:** Yes
  - **Urine:** No
  - **Tissues:** No
  - **Other samples (describe):** Nasopharyngeal swab and throat swab
- **Sample collection will be completed in how many visits:** 2 trips
- **Will samples be de-identified (Yes or No)? If No, describe how they will be protected.**
  - Yes
- **Will local IRB approval be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals being sought or state why local IRB approval is not required.**
  - Yes

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

- **Will informed consent be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals/consents being sought or state why informed consent is not required.**
  - Yes

- **Will samples be brought back to the US (Yes or No)?**
  - No

- **Will data be brought back to the US (Yes or No)?**
  - Yes, some of the data will be shared with EID-SEARCH partners in the US for relevant analysis.

Animal Subjects (1 word or 1 sentence per bullet):

- **Parent study IACUC approval**
  - **IACUC approval number for parent study:** #G2020-42 (US); #019/2563 (Thailand)
  - **IACUC approval date:** 05-05-2020 (US); 03-12-2020 (Thailand)
    Animal Welfare Assurance Number: D16-00572 (A4059-01)
- **Does this study require a modification to the IACUC approval of a parent study (Yes or No)?**
  - **No**

- **Will existing samples from animal subjects will be used: (Yes or No)?**
  - **No**
  - **How many animal subjects provided the existing samples to be used?** N/A
- **Will vertebrate animals be collected (Yes or No)?**
  - **Yes**

- **Species of animals (e.g. rats, mice, rabbits, monkeys):** Bats, rodents, and shrews
- **Animal parameters:**
  - **Total number of animals:** 200 (100 bats, 100 rodents and shrews)
  - **Gender:** 100 males, 100 females
  - **Age range:** 4 - 12 months and elder depending upon species
  - **Lab strain (e.g. Sprague-Dawley rats, Balb/C mice):** None
  - **Wild animals procured in country (e.g. Rhesus monkeys from a reserve):** No

- **What will be done to them or with them and how often?**
  - Free-ranging bats will be captured using either a mist net or harp trap, and manually restrained during sampling; free-ranging rodents and shrews will be captured through pit traps and box traps.
  - Anesthesia will be conducted for captive rodents and shrews.
  - Once anesthetized blood will be collected.
  - All action will only be performed one time for each animal individual.
  - All animals will be released after sampling.

- **What are the follow-ups?**
  - No follow-up relevant as per protocol, since all animals will be released after sampling ups

- **What will be their fate at the end of the experiments – will they be euthanized?**
  - All animals will not be held longer than 6 hours (typically less than 3 hours) during the sampling process and released after sampling.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

- **Will in-country clearances be obtained prior to engaging in any animal research (Yes or No)? If No, describe alternate approvals or state why in-country clearances are not required.**
  - Yes
- **Will samples be brought back to the US (Yes or No)?**
  - No
- **Will data be brought back to the US (Yes or No)?**
  - Yes, some of the data will be shared with EID-SEARCH partners in the US for relevant analysis.

Proposal Cover Sheet

Project Title	Immune memory bait & capture to identify emerging henipavirus origins
Principal Investigator Name	Krongkan Srimuang, Ph.D.
Position/Title	Research Scientist
Department	Thai Red Cross Emerging Infectious Diseases Clinical Center
Institution Name	King Chulalongkorn Memorial Hospital
Street	Rama 4 Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	Krongkan.sr@gmail.com
Phone	+66 (0) 92-453-0808
Country(ies) where work will be conducted	Thailand
Pathogen(s) focus	Mojiang Virus, Henipavirus, Paramyxovirus
Co-Principal Investigator Name (*If applicable*)	Spencer Sterling, MPH
Position/Title	Scientific Project Coordinator
Department	Microbiology and Immunology
Institution Name	Uniformed Services University of the Health Sciences
Street	Jones Bridge Road
City, State, Zip Code	Bethesda, MD 20814
Country	USA
Email	spencer.sterling.ctr@usuhs.edu
Phone	+66 (0) 83-494-5980

Contact information for institution(s) that would financially manage the award *(please note: changing the managing institution upon award may result in substantial funding delays)*	
Contractual Contact, Title	Chula Unisearch
Institution Name	Chulalongkorn University
Street	254 Phyathai Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	unisearch@chula.ac.th
Phone	0 2218 2880
For Co-Principal Investigator (If applicable):	
Contractual Contact, Title	
Institution Name	
Street	
City, State, Zip Code	
Country	
Email	
Phone	

Name of Mentor	Eric Laing, Ph.D.
Mentor Institution	Uniformed Services University of the Health Sciences
Institution Address	Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814
Permanent location of Mentor	4301 Jones Bridge Road, Bethesda, MD 20814
Mentor email	eric.laing@usuhs.edu
Mentor phone	+1 (301) 980-8192
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Name of Mentor	Supaporn Wacharapluesadee, Ph.D.
Mentor Institution	Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital
Institution Address	1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Permanent location of Mentor	4th Floor, Jongkolnee Watwong Building, Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital, 1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Mentor email	spwa@hotmail.com
Mentor phone	+66 (0) 89-920-6292
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Total Budget	***Repeat this table if there are two Co-PIs***
Direct Costs	$138,831
Indirect Costs	$11,106
Proposed Start Date	1 May 2023
Proposed End Date	30 April 2024

Project Abstract (250 words)

Prototypical henipaviruses (Hendra and Nipah virus) are bat-borne zoonotic viruses, cause high mortality. The recent detection of closely-related Mojiang and Langya viruses in rats and shrews, and isolation of Langya virus from individuals with acute febrile illness, challenges the dogma that fruit bats are the key henipavirus reservoirs. The recently expanded *Henipavirus* genus includes two other shrew-borne viruses (Gamak and Daeryong viruses), and the Madagascan fruit bat-borne Angovkeely virus. As part of EID-SEARCH activities, we found serological evidence of infection by a Mojiang-related virus in a community of Thai bat guano collectors who have occupational exposure to microbats and rodents. Identifying the wildlife reservoir of this likely novel virus will assist intervention strategies to reduce spillover risk. However, PCR detection or virus isolation in wildlife is challenging, and new serological approaches may be more successful in identifying reservoir hosts. This project aims to identify novel viruses and zoonotic reservoirs through serologic and cellular immune discovery. We will conduct follow-up human and wildlife (bats and rodents) surveillance in the province where the seropositive humans were identified. Further, we plan to isolate anti-MojV monoclonal antibodies (mAb) from peripheral blood mononuclear cells collected

from human study participants we have identified as immunoreactive with MojV antigens, and characterize the cross neutralizing potential of these mAbs to MojV and LayV envelope glycoproteins. In addition to our antigen-based approach, we will utilize the mAbs for direct antibody-mediated virus capture of this novel henipavirus from human and wildlife samples, and downstream genomic sequencing.

Project Title **Immune memory bait & capture to identify emerging henipavirus origins**

Table of Contents

Study Personnel

Krongkan Srimuang, Ph.D., is a researcher at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital and supports the Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia (EID-SEARCH) research. Dr. Srimuang is also a medical technologist working to identify, detect and characterize the spill-over risk of high zoonotic potential viruses from wildlife and humans using serology techniques, Next-generation sequencing, and PCR assays to identify novel viruses. Dr. Srimuang also performs the project Smart Sentinel Surveillance System: Emerging Viral Pneumonia and Respiratory tract infections (RTIs) to identify, detect and characterize the novel/known Respiratory Viruses in our country, Thailand by using multiplex real-time PCR, family PCR and Next-generation sequencing of novel viral pathogens. Dr. Srimuang graduated with Ph.D. in Tropical Medicine from the department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University.

Spencer Sterling, MPH, is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region. Mr. Sterling will planning, sample processing, data analysis, and report preparation for this project.

Dr. Eric Laing will act as Mentor for Dr. Srimuang and Mr.Sterling under the Centers for Research in Emerging Infectious Diseases (CREID) Pilot Research Program. Dr.Laing is an assistant professor dedicated to biosurveillance and virological research of emergent zoonotic viruses at Uniformed Services University, Bethesda, MD. Dr.Laing study to develop serological-based approaches to identify the infectome of wildlife and human communities for the assessment of virus spillovers. Dr.Laing have worked collaboratively with national and international researchers including institutional partners at Duke-NUS, Chulalongkorn University, University of Pretoria, and Rocky Mountain Labs towards these goals. Across all of these collaborations, the aimed to identify known and unknown viruses, the wildlife reservoirs of priority pathogens, the high-risk interfaces for spillover and the biotic/abiotic drivers of virus emergence.

Dr. Supaporn Wacharapluesadee, Ph.D. is the head laboratory at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, a WHO Collaborating Centre for Research and Training on Viral Zoonoses. Dr. Wacharapluesadee research interests include emerging infectious diseases in bats (including Nipah, Rabies, Coronavirus, and novel pathogens) as well as molecular diagnoses and sequencing of viruses. During the past 10 years, Dr. Wacharapluesadee got funding from USAID PREDICT to conduct the surveillance for novel virus in wildlife and human, and from Biological Threat Reduction Program US DTRA to conduct the EID surveillance in bat using the molecular technology and multiplex serology panel (Luminex technology). TRC-EIDCC is responsible for molecular diagnoses services to the hospital, and is the reference laboratory for rabies virus, MERS-CoV, Ebola, Zika, and other infectious diseases diagnoses for Ministry of Public Health, Thailand and laboratory training center on national and regional level. Dr. Wacharapluesadee has served and consulted on several WHO and Thai government committees.

Specific Aims

This project aims to study zoonotic reservoirs for pathogen origin by using serologic and cellular immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

The hypothesis

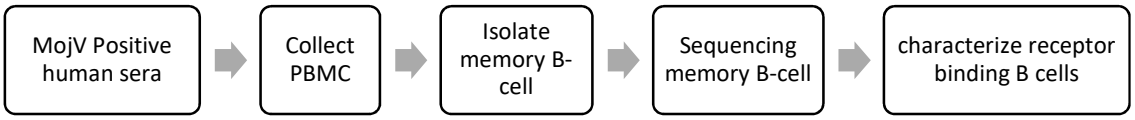
1. Can the memory B-cell immune response be used as a tool for the discovery of novel henipaviruses?
2. Can the spatial relationships between henipaviruses receptor binding proteins be generated that would allow for an understanding of antigenic evolution and functional characterization?

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population.

Human populations near the Khao Chong Phran Non-hunting Area in Ratchaburi province have shown a high seroprevalence to the MojV RBP without confirmed sequencing results. We will follow-up on persons with high MojV RBP IgG titers, sample new people within the population that interact with the local wildlife, and sample shrews and rodents to find the source of the MojV-reactive antibodies.

Aim 2. Antibody-mediated bait & capture of the Thai MojV-like virus

In collaboration with NIH PREMISE, MojV RBP-reactive B cell receptors will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific MAbs will be generated and used for direct antibody-mediated virus capture in people and wildlife.



Objective 1. To use metagenomic and targeted sequencing approaches in human and wildlife populations to detect the Thai MojV-like virus.

Objective 2. To isolate and sequence memory B-cells from humans positive for Mojiang virus in Ratchaburi province, Thailand for antibody production.

Objective 3. To determine epitope binding and antigenic relationships from henipavirus-reactive serum to the known henipaviruses.

Study Rationale

Nipah virus (NiV) and henipaviral diseases are among the most virulent of zoonotic diseases. The genus *Henipavirus* is currently composed of nine viruses including the prototypes, NiV and Hendra virus (HeV), and Cedar virus (CedV), Ghana virus (GhV), Mojiang virus (MojV) (McLinton et al., 2017), Langya virus (LayV) (Zhang X et al., 2022), Gamak virus (GAKV), Daeryong virus (DARV) (Lee et al., 2021), and Angavokely virus (AngV) (Madera et al., 2022). HeV was first identified in Australia in 1994 as a cause of respiratory illness and fatal encephalitis in humans and horses. Closely following the discovery of HeV, NiV was identified in 1999 in Malaysia after outbreaks of fatal encephalitis in humans, with swine determined to be the intermediate host. Annual outbreaks of NiV have occurred in Bangladesh and are associated with the consumption of raw date palm sap, and since 19 May 2018 annual outbreaks of NiV associated encephalitis of been recorded in Kerala, India (Uwishema et al., 2022). Both HeV and NiV have a broad host range capable of infecting several mammalian orders, and can enter and infect endothelial cells, vasculature, and the central nervous system leading to severe multisystem organ diseases and high mortality. The broad host and tissue tropism of these viruses has been attributed to the use of the ephrinB2 and ephrinB3 ligands, highly conserved proteins with critical functions in evolutionary development, as virus receptors (Bossart et al., 2008). There are currently no vaccines or therapeutics and NiV and henipaviral diseases remain WHO Blueprint list priority pathogens (Middleton and Weingartl, 2012).

A close relative of HeV, CedV was isolated from Australian bats in 2012 and found to be non-pathogenic, failing to cause any clinical symptoms in ferrets and guinea pig animal models (Marsh GA et al., 2012). The natural reservoirs of HeV, NiV, and CedV are flying foxes (*Pteropus sp.*) (Middleton and Weingartl, 2012). GhV was detected in Ghana in straw-colored fruit bats (*Eidolon helvum*) during routine surveillance in 2015 and was the first henipavirus found outside of the Indo-Pacific region. GhV showed a close phylogenetic relationship with HeV and NiV, and like HeV, NiV, and CedV the GhV RBP can mediate binding to ephrinB2 ligand and mediate cellular fusion (Drexler, J. F., 2009). Recent investigations into Madagascan fruit bats (*Eidolon duprenum*) detected a second African henipavirus, AngV. As neither GhV nor AngV have been isolated or rescued, the pathogenic and zoonotic potential of these African fruit bat associated henipaviruses remains unknown.

Multiple genus of small mammals were tested for viral RNA, and MojV was detected in yellow-breasted rats (R. flavipectus) (Wu Z et al., 2014). The discovery of the MojV genome occurred during follow-up investigations into lethal pneumonia from three miners in 2012 in China, however the pathogenic nature of this virus has only been suggested and no isolates have been discovered or tested in an animal model. Additionally, the novel shrew-borne henipaviruses, Gamak virus (GAKV), and Daeryong virus (DARV) were found in the Republic of Korea in 2017-2018. (Lee et al., 2021). Most recently, a close relative of MojV, Langya henipavirus (LayV) was recently isolated from febrile patients in eastern China. Subsequently, LayV was identified in shrews (Zhang et al., 2022), increasing the number of Asiatic rodent-associated henipaviruses to four. These recent discoveries are challenging the paradigms surrounding henipaviruses and their associations with bats and may suggest a novel rodent sub-genus of henipaviruses.

The Henipavirus genome consists of six structural proteins including nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), receptor-binding protein (RBP), and large (L) protein (approximately length of 18,000 nucleotides) (Luby, S. P., & Gurley, E. S., 2012). In HeV and NiV, slippage sites within the P protein lead to the expression of the V, W, and C proteins. These proteins are virulence factors and are absent in CedV.

Table 1 Summary viruses in Henipavirus

Henipavirus	**Origin (Year)**	**Reservoir**	**Genome (nucleotides)**	**Symptom for human**	**Reference**
Hendra virus (HeV)	Australia (1994)	Fruit bats	18,234	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Nipah virus (NiV)	Malaysia (1999)	Fruit bat	18,246	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Ghana virus (GhV)	Africa (2009)	Fruit bat (*Eidolon helvum)*	18,530	No data	Drexler, J. F., 2009
Cedar virus (CedV)	Australian (2012)	Fruit bat	18,162	Non-pathogenic	Marsh, G. A et al., 2012
Mojiang virus (MojV)	China (2012)	Rodent	18,404	Febrile	Wu Z et al., 2014
Gamak virus (GAKV)	Korea (2017-2018)	Shrew	18,460	No data	Lee et al., 2021
Daeryong virus (DARV)	Korea (2017-2018)	Shrew	19,471	No data	Lee et al., 2021
Langya virus (LayV)	China (2019)	Shrew	18,402	Acute febrile	Zhang et al., 2022
Angavokely virus	Madagascar (2019)	Fruit bat	16,740	No data	Madera et al., 2022

Viruses in the genus *Henipavirus* have a broad reservoir and may cause high case fatality rates following human infection. The discovery and characterization of the novel Henipavirus is a high public health priority.

The greatest bat diversity around the world especially appears to be found in the tropical rain forests of South East Asia. In Thailand, at least 138 species in 11 families and 45 genera of bats have been recorded, such as members of the genus Pteropus, Rhinolophus, and Hipposideros (Soisook et al., 2011). Khao Chong Phran, a cave in Ratchaburi Province, has possibly the largest colony of bats in Thailand. There are estimated to be around 2 million bats in the cave and 95% are insect-eating bats (Department of National Parks, Wildlife and Plant Conservation, Thailand). Moreover, Khao Chong Phran has many community sites, such as cave entrances near communities, guano collection, and tourism sites, which risk zoonotic spillover. Bats are widely considered the animal hosts of zoonotic pathogens such as rhabdoviruses, coronaviruses, paramyxoviruses, and filoviruses, and many of the host species can be found in Thailand.

MojV was associated with a severe pneumonia-like illness killing three of six miners in 2012 in a mineshaft Yunnan, China. Interestingly, this mineshaft was also site where RaTG13, a beta-coronavirus most closely related with SARS-CoV-2, was detected in *Rhinolophid* bats (Rahalkar and Bahulikar, 2020). Identifying the wildlife reservoir of this novel virus will assist intervention strategies to reduce spillover risk.

Research Gaps

As a part of Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH), serological investigations using a multiplex microsphere-based immunoassay (MMIA) have detected four members of a high-risk cohort with MojV-reactive serum, including one person with a high magnitude and durability of anti-MojV IgG across longitudinal samples. Despite this, genomic surveillance has not detected MojV or any novel MojV-like viruses. For this result, we would to further identify and characterize the origin of the Mojiang virus-reactive serum and to explore the novel MojV-like virus in Thailand.

Serological and cellular immunity is an important resource for virus discovery detection by using the specificities and characteristics of memory B cells from peripheral blood mononuclear cells (PBMC). The immune response will recognize and induce antibodies specific to the infecting pathogen. Characterizing the memory B cell response to an unknown pathogen in humans could in the detection of the exposure pathogen in humans. Molecular techniques such as PCR can detect only the genomic material in the body, but memory B cells are long-lived and thus have a much larger window of detection.

Memory B-cell differentiation is characterized by somatic hypermutation in antibody variable region genes (V) and selection of B cells expressing high-affinity variants of this antigen receptor and high sequence similarity in individuals exposed to the same antigen reflect antigen-driven clonal selection and form shared immunological memory between individuals (Yang et al., 2021). Memory B cells are highly abundant in the human spleen, and can proliferate 45% of the total B cell population in this organ (Hauser et al., 2015). Memory B cells can be detected in the Ig variable (IgV) genes in a fraction of IgM+ B cells. In humans, B cells expressing the memory marker CD27+ can be found in the marginal zone of spleen and circulate in the blood. After re-exposure to antigen, memory B cells can quickly proliferate and differentiate into plasma cells. Many studies the antibody diversity and specificity of the naturally paired immunoglobulin heavy and light chains for affinity maturation of B cell receptor (BCR) after virus infection such as SARS-CoV-2 infection (Yang et al., 2021), influenza (Dougan, S et al., 2013), HIV (Moir, S., & Fauci, A. S.,2017), etc. The characterization of memory B cells can be used to develop monoclonal antibodies and produce an effective theraputic.

Significance and Approach

Southeast Asia is one of the world's highest-risk emerging infectious diseases hotspots. From this region emerged both SARS-CoV-1 and SARS-CoV-2, recurrent outbreaks of novel influenza strains, and the spillover of dangerous viral pathogens such as Nipah virus. The diversity of coronaviruses, henipaviruses, and filoviruses are found in wildlife reservoirs in Southeast Asia is due to the high diversity of wildlife, and the demography of the human population brings humans and wildlife into close contact. The spillover of viruses from animals to humans is often misdiagnosed and the clinical symptoms from patients is underestimated during the pandemic. This study provides a surveillance strategy in human and wildlife populations to better understand immunity to novel pathogens and identify the origin of these pathogens to serve as an early warning system to prevent outbreaks of emerging viruses.

This study uses immunology to identify a novel virus that can aide in effective vaccine discovery and development, monoclonal antibody production for prevention and therapy, and generate data resources for early detection and diagnostic assays. Whereas PCR detection or virus isolation in

wildlife is challenging, this new serological approach may be more successful in identifying reservoir hosts.

The Indo-Pacific region has a high risk of novel viral emergence. This study provides an opportunity for in-country research capacity building by linking local infectious disease scientists with an international collaborative network that has proven capacity to conduct this work and produce significant findings. Moreover, a global sero-surveillance network for surveillance technology extends virologic and immunologic screening to product discovery. This study will benefit and expand the CREID, EID-SEARCH networks, and the global sero-surveillance network to improve our understanding of immunology to identify novel pathogens and their origin to prevent future pandemics.

Research Methods

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population

Serological profiling provides an indirect measurement of the infecting virus. Despite 19% (54/284) seroprevalence in humans, we have not detected nucleic acid sequences of MojV or MojV-like henipaviruses in this human cohort. In addition to the translational application of mAbs as therapeutics, we intend to utilize these mAb as non-clinical diagnostics surveillance tools for virus/virus antigen capture. Additionally, we intend on expanding our understanding of henipavirus prevalence in this population with longitudinal sampling of the wildlife and human populations in this region.

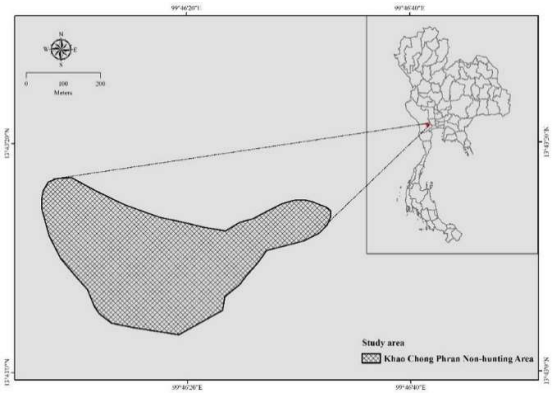


Figure 1**:** Map of Khao Chong Phran Non-hunting Area, Ratchaburi Province, Thailand (Boonpha N et al., 2019)

Site wildlife samples collection

100 bats and 100 shrews or rodents in and around the Khao Chong Phran cave in Ratchaburi Province will be collected for surveillance targeting the Mojiang-like virus. We plan one sampling trip to collect bats, and a second trip to target shrews and rodents. Due to limited knowledge of the local shrew population, we are unsure as to whether we can collect 100 samples, and will supplement the collection with rodents, as needed. Samples will be collected based on the following enrollment criteria: random age, random weight, random sex. Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and stored. Oral swab and rectal swab will also collect in 100 bats and 100 shrews or rodents. Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and stored.

Human surveillance

Four of the samples from prior investigations have shown high MojV RBP-reactive antibody titers and will be selected for follow-up sampling. Additionally, 100 human serum samples, nasopharyngeal swab and throat swab will be collected from persons deemed to be in close contact with wildlife, and people who present with or have recent history of undiagnosed acute febrile illness using the following enrollment criteria;

- Adults (≥ 18 years of age) who sign in the informed consent
- Who present with or have recent history of illness without diagnosis, such as severe/acute respiratory illness (SARI/ARI), Influenza-like illness (ILI), fever of unknown origin (FUO), or respiratory tract infection
- Who is the bat guano collector in the cave
- Who lives or works around wildlife habitats
- Who is involved with livestock or wildlife farming
- Who hunts or works with wildlife
- Who has not been previously sampled as part of the EID-SEARCH and PREICT projects

Sample size justification

Human population

$$\mathrm{n} = \frac{\mathrm{N}}{1 + \mathrm{N}e^2}$$

n = sample size of the study

N = population size for finite populations: 8,150 (data from 2018)

e = error: 10%

$$\mathrm{n} = \frac{8{,}150}{1 + (8{,}510 \times 0.1^2)}$$

n = 98.79 (round up to 100 samples)

Wildlife population

The sample size of 100 of each species follows the guidelines established during the PREDICT project.

Targeted Viral Sequencing and Serologic testing

Targeted sequencing

For samples from shrews, rodents, and acute individuals, we will use targeted sequencing and metagenomic approaches in an attempt to detect the Thai MojV-like virus. The nucleic acid from oral swab and rectal swab will do target sequencing in henipavirus Family PCR and Sanger sequencing.

Serological testing

Serum samples will be tested for RBP binding to the known henipavirus species, including the Nipah, Hendra, Cedar, Mojiang, Ghana, Langya, Gamak, and Daerong viruses to further investigate the seroprevalence in this region. Additionally, prior positive samples will be re-tested with the expanded panel to enhance our understanding of the antigenic relationships between the novel shrew-borne henipavirus and the Thai virus.

Data analysis

Sequencing will be analyzed assembling reads in MEGA11. The consensus sequences will compare to the references strains available in the GenBank data-base using the Basic Local Alignment Software Tool (BLAST) (National Center for Bio-technology Information). Serological data will be analyzed using cluster analysis in RStudio.

Aim 2. Characterization of monoclonal antibodies from humans with serological profiles consistent with MojV infection.

In collaboration with NIH VRC PREMISE, MojV RBP and F-reactive B cells will be captured, and the B cell receptor will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific mAbs will be generated from the corresponding sequences and will be assessed for binding potential to LayV RBP and F, as well as their neutralization potential.

Importance: The isolation of these mAbs represents a potential therapeutic for MojV, MojV-like henipas, and LayV, depending on how well these cross react.

Human samples collection site

Human positive serum for MojV was found close to Khao Chong Phran in Ratchaburi Province, which has the largest colony of bats in Thailand (Figure 2).

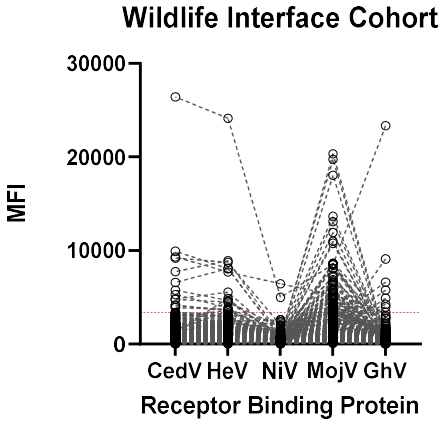


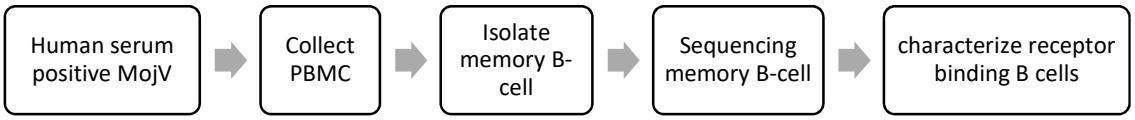
Figure 2: Seroprevalence of henipavirus proteins from a community with a large bat interface during 2017-2018. MFI is the median fluorescence intensity for IgG reactivity to each protein. Red-dashed line is at 3,357 MFI, the assay cutoff.

PBMC Sorting and B Cell isolation

Whole blood from MojV-positive people will be collected in individual heparin tubes and processed for PBMC isolation using Density Gradient Centrifugation (SepMate™ PBMC Isolation Tubes). Human PBMCs will be stained with antibodies for positive markers of IgG memory B cells, and antibodies to negatively select and avoid contamination with dead cells, T cells, NK cells or monocytes (live, CD3−,CD14−,CD56−, IgM−, IgA−, CD19+,CD20+,CD27+). Avi-tag for biotinylation and 6xHIS tag for Nickel purification using the HisPur™ Ni-NTA Spin Purification Kit (Thermo Fisher Scientific, Waltham, MA). Single Memory B Cells will be sorted, cells will proliferate, cDNA will synthesized, then Heavy and light chain variable domains will be amplified (Waltari E et al., 2019).

B cell receptor Amplicon Preparation and Recombinant Antibody sorting

Total RNA yields from the PBMC will be determined by absorbance at 260 nm on the NanoDrop™ One (Thermo Fisher Scientific). Total RNA will be used for first strand cDNA synthesis with gene-specific reverse transcription (RT) primers directed to the constant regions. The RT primers for IgG, IgM, IgA, lambda, and kappa will be included. Light chain and heavy chains will be amplified into amplicon. Double-stranded cDNA will be sequenced. After completion of MiSeq sequencing, antibody repertoires will analyze using methods based on the pipeline. Heavy and light-chain sequences will be assembled into an expression vector and transfected into cells for monoclonal antibody expression. Recombinant antibody will be isolated and purified for downstream applications.



Functional assessment

Neutralization of viruses remains a critical correlate of protection for most vaccine strategies. Next, we will test the cross neutralization potential of these anti-MojV RBP and F mAbs to neutralize LayV (Duke NUS/LinFa should be able to do this, if they have LayV and are propagating it, they can use cell-cell fusion/CPE in a straight PRNT).

Determination of envelope glycoprotein epitope binding

Isolated mAbs will be assessed for binding to MojV RBP and F proteins through multiplex binding serology. Further, the cross reaction potential of these mAbs will be assessed against GamV, DarV, and LayV RBP and F. MojV has not been isolated or rescued, and the virus receptor for MojV has not yet been discovered (Rissanen et al., 2017; Cheliout et al., 2021). Thus, a functional assessment of cross neutralizing potential is not possible. However, through cyro-electron microscopy we can investigate binding epitopes between these mAbs and MojV and LayV envelope glycoproteins. Epitope identification can then be used to make predictions about neutralizing potential through deductions about the location of henipavirus RBP receptor-binding pockets.

Project Timeline

Activity	05/23	06/23	07/23	08/23	09/23	10/23	11/23	12/24	01/24	02/24	03/24	04/24
Mentoring and training activities												
1.Training, literature review												
2.Monthly mentoring meeting												
3.Manuscript writing												
4.EID-SEARCH meeting												
Research activities												
1.IRB amendment												
Aim 1												
2. Human and Wildlife samples collection												
3. Identify Targeted sequencing												
4. Serological testing												
Aim 2												
5. Collect PBMC human sample and isolation memory B-cell for Mojiang virus												
6. PBMC Sorting and B Cell isolation												
7. Determination of envelope glycoprotein epitope binding												

Research Performance Sites

This proposed study will be implemented at the King Chulalongkorn Memorial Hospital (KCMH) collaborating with the Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC). KCMH is a 1,000-bed, university hospital in close coordination with the Faculty of Medicine, Chulalongkorn University. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. It's one of the center hospitals for Emerging Infectious Diseases in Thailand, especially for domestic and international patients.

TRC-EIDCC was established by the Committee of the Emerging Infectious Disease Center, which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. Our clinical center provides standard medical care to patients with emerging and recurrent diseases and disease transmission. Moreover, the center plans preparedness activities, trains medical and government staff on interventions, hosts conferences for medical and public health professionals, creates networks of medical care for patients with emerging and recurrent diseases, and leads research and development projects for medical care and disease control. Apart from medical innovation in emerging and recurrent diseases, the clinical center strives to be the model of infectious diseases clinical services and pandemic control through research activities surrounding infectious diseases and effective disease control measures.

We also collaborate with the Department of National Parks, Wildlife and Plant Conservation for detecting novel pathogens from animals such as bats, rodents, and shrews in our country. We also collaborate with the Department of Disease Control of Thailand (Thai-DDC) to support the Thai government and national policy makers through molecular diagnosis, including the identification of the first case of SARS-CoV-2 infection in Thailand. TRC-EIDCC prides itself on its high performance in research laboratory services and is a part of the national reference laboratory for COVID-19 and other emerging infectious diseases confirmation in Thailand.

CREID Research Center Collaboration

The goals and objectives of the proposed research project, "Immune memory bait & capture to identify emerging henipavirus origins", are fully consistent with the specific aims of EID-SEARCH. EID-SEARCH takes a One Health approach to understanding zoonotic disease emergence, with a focus on viral diversity in wildlife at high-risk human animal interfaces. EID-SEARCH aims to understand the frequency and causes of virus spillover in high-risk communities across Southeast Asia and identify strategies and tools to reduce this risk. As the proposed research focuses on the links between farming practices, wildlife diversity, virus diversity, and cross-species transmission in Thailand, we expect the results of the project to make a significant contribution towards the overall goals of EID-SEARCH.

The proposed project is well-designed, leverages cutting-edge technology, and includes a robust ecological component that corresponds to the overall research strategy of EID-SEARCH. The EID-SEARCH team is highly motivated to support the application of metagenomics to ecological questions and assist in the analysis of the unique dataset that will be generated from this research. With the heightened focus on identifying and limiting risks associated with the complex interactions between humans, wildlife, and domestic mammals that has resulted from the emergence of SARS-CoV-2 from a wet market in China, the proposed research is both timely and likely to yield highly-cited, impactful publications for EID-SEARCH and Thailand partners.

The EID-SEARCH team includes a number of experts in study design, laboratory-based diagnostics, genetic and genomic analyses, and ecological modelling who will contribute to project implementation, data analysis, and manuscript preparation as required. Communication and coordination mechanisms will be set up between the Thailand research team and scientists at the EID-SEARCH research sites for resource sharing, training, knowledge exchange, and mentorship. In particular, EID-SEARCH will facilitate and encourage relationships between our partners in Southeast Asia (based in Malaysia, Singapore, and Thailand) that will facilitate Thailand team professional development and strengthen research capacity in the region. Dr. Eric Laing, the project Mentor, has an established relationship with both Dr. Krongkan and Spencer Sterling, and will act as the liaison between EID-SEARCH and the TRC-EIDCC research team. Further, Dr. Doeuk who is the directior of the VRC PREMISE program is enthuasiatic about the opportunity to collaborate and apply their rapid assembly, transfection and production of immunoglobulin (RATP-Ig) workflow as a serologic/immunologic based approached for therapeutic and virus discovery (see Letter of Support). An expected outcome of this research project will be pilot data to support a joint funding application in the future.

Mentoring Plan

Goals for Pilot study and Career

Krongan Srimuang: My career goal is to be an expert scientist in either an academic research laboratory or a lecturer in medical science. My long-term research interests include the understanding of priority emerging infectious diseases, genetics, and epigenetics using molecular and serologic techniques. Detection of novel pathogens in their wildlife vectors can help characterize the pathogenicity, virulence, and evolution of these and other closely related pathogens. I believe that this pilot study can provide training in new techniques for the detection, identification, and characterization of novel pathogens that are currently unavailable in Thailand. My goal for the Pilot study is to gain more knowledge and skills for a lifelong career in public health-related research. I wish to expand my skills in novel virus detection using next-generation serologic techniques such as PBMC studies and memory B-cell isolation and sequencing that can contribute to improved surveillance and research capacity at human-animal interfaces. My mentors Dr. Laing and Dr. Wacharapluesadee can provide me with opportunities to develop and apply project skills and improve my scientific writing.

My education has provided me with a strong foundation to expand for this study. I received my undergraduate degree in Medical Technology from Chulalongkorn University which included training in molecular biology, serology, immunology, chemistry, virology, bacteriology, mycology, parasitology, hematology, clinical microscopy, transfusion medicine, and forensic medicine. My interest in molecular technology led me to pursue a Ph.D. at the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, which provided me with training in several molecular techniques including conventional PCR, real-time PCR, Next Generation Sequencing (NGS), etc. The emergence of COVID-19 motivated me to focus on emerging infectious diseases. I am currently completing my post-doctoral fellowship at the Emerging Infectious Diseases Clinical Center (EIDCC), King Chulalongkorn Memorial Hospital with Dr. Wacharpluesadee. Throughout this fellowship, I have continued learning advanced and innovative techniques to detect, identify, and characterize the novel pathogens. Through this pilot project, I will learn about the Peripheral Blood Mononuclear Cells (PBMC) which are a critical part of many subdivisions of medical research including Infectious diseases, Vaccine development, and Immunology. This project aims to study zoonotic reservoirs for by using serologic and cellular immune discovery in humans, and applying this immune knowledge in wildlife for early detection and identification of novel pathogens. This proposed study will expose me to various techniques and immunology topics that I useful in designing and testing new pipelines as technological advances continue to develop. This study will also allow me to develop, manage, and communicate my research.

Spencer Sterling: My overall career goal is to understand the factors that lead to viral zoonosis and to build epidemiological capacity in regions with the highest risk for zoonotic spillover. Through my training and educational experiences, I have learned laboratory and epidemiological techniques that can assist in the understanding of zoonosis. I wish to continue my education by expanding my repertoire of molecular techniques to include studies of the immune system's response to zoonotic disease in humans and animals, as well as to learn next-generation sequencing techniques. Additionally, I would like to use my experience in epidemiological techniques to train scientists in the Southeast Asian region in order to build local capacity in infectious disease research. Dr. Laing has been a mentor for me throughout my career, and this project would allow for this relationship to expand into new molecular areas, as well as grant writing and project management skills. My professional relationship Dr. Wacharapluesadee with began in 2018 when I participated in a DTRA

BTRP funding project that focused on transferring antigen-based multiplex serological technology to her lab group. Currently, I am a Visiting Scientist at TRCEIDCC and I work closely with Dr. Srimuang, and other members of Dr. Wacharapluesadee's research group on EID-SEARCH activities. Additionally, Dr. Wacharapluesadee has decades of experience in infectious disease research in the region and has mentored scientists that have gone on to careers at the Thai Ministry of Public Health, Department of Disease Control, Department of Parks and Wildlife, academia, and the private sector. Both mentors have encouraged Dr. Srimuang and I to apply for this pilot study as well as pursue other funding mechanisms for our research interests. Both mentors will provide for me the ability to independently pursue my research interests in the zoonotic epidemiology and train me on the best practices of scientific communication.

Vertebrate Animals Section Requirements

Animal samples will be collected in Ratchaburi Province for this project under Aim 2 to understand the antigenic relationships among known and unknown henipaviruses. Protocol to perform animal sample collection has been reviewed and approved by the Institutional Animal Care and Use Committee at Tuft University under the EID-SEARCH project (No. G2020-42) and the Institutional Animal Care and Use Committee at Chulalongkorn University (No. 019/2563)

1. Description of Procedures

Bats (order *Chiroptera*)

- Species: Free-ranging mega and micro-*Chiroptera* that present at the site (family *Pteropodiae, Rhinopomatidae, Emballonuridae, Craseonycteridae, Megadermatidae, Rhinolophidae, Hipposideridae, Vespertilionidae, Mollossidae*, etc.)
- Age & Sex: Fully flighted adults and juveniles, males and females; neonates dependent on the dam will not be used.
- Target Number: 100 individuals
- Capture and Restraint: Bats will be captured using either a mist net or harp trap. The net system is manned by at least two people during the entire capture period, and bats are removed from the net as soon as they become entangled to minimize stress and prevent injury. In our previous field research experience, a maximum of 20-30 bats can be safely held and processed by a team of three people per trapping period. Duration of trapping will depend on the capture rate. Bats are individually placed into a pillowcase or small cloth bag and hung from a branch or post in a location that is free from access by both predators and people, and is protected from the elements (e.g., rain), until the animal is restrained for sample collection. Bats will be manually restrained during sampling utilizing insulated bite gloves to prevent injury to the handler and bat. The study protocol covers over 69 bat species that are most likely to be captured given their natural occurrence and previous surveillance experience in Southeast Asia. The potential for non-target species to be captured is very low because mist nets are opened at the cave entrance at night, after other species (e.g., birds) have ceased flying and returned to roost. Any non-target species will be rapidly identified, removed from the mist net and released immediately.
- Sample Collection: Depending on the species and size of bat, blood will be collected from smaller insectivorous bats (<50g) using a 27g needle to puncture the brachial artery and a 70ul hematocrit tube to collect the blood. For larger bats (>50g) we will collect blood from either the cephalic vein or from the radial artery or vein using a 25- or 23-gauge needle and 3cc syringe. Blood will be collected in quantities not exceeding 6 µL/g of body mass from each individual. Wing punch biopsies will be collected to confirm host identification using DNA barcoding. Swabs will be taken from the oropharynx, urogenital tract, and rectum. No destructive sampling is planned, but any individuals that die during capture or sampling will have a necropsy performed with organ samples collected and whole body submitted to museums as voucher specimens.
- Release: Bats are held for a maximum of six hours (typically less than three hours), and at the completion of sampling are provided with rehydrating fluids (either subcutaneously or orally as needed) and released in a location that allows for further recovery or safe escape (e.g., bodies of water, known predatory areas, and human settlements will be avoided).

Rodents (order: *Rodentia*) and Shrew (order: *Scandentia* and *Eulipotyphla*)

- Species: Free-ranging rodents (family *Hystricidae, Muridae, Sciuridae, Spalacidae*) and shrew (tree shrews, shrews, moles) that present at the sites.
- Age & sex: Adults and juveniles, males and females. Neonates will not be sampled.
- Target Number: 100 individuals

- Capture and Restraint: Free-ranging wild mammal species will be captured with metal box traps (Sherman/Tomahawk traps). Traps will be prepared with food, water, padding and shelter, and be checked at least every 12 hours, in the morning and in the afternoon. If adverse weather (extreme heat, rain) is expected or researchers are working in areas where predation is common, traps will be checked more frequently and closed during adverse weather. Veterinary staff will be prepared to deal with a variety of trap injuries, including lacerations, broken teeth, and crush injuries. Depending on the species, individual size, captive or free-ranging status of the individual, manual or chemical restraint and anesthesia (gas or injectable anesthesia) will be applied. Animals will be manually restrained during sampling utilizing insulated bite gloves to prevent injury to the handler and animal. Based upon past field experience (>20 years of animal capture and release in Southeast Asia), we do not expect to trap sick, debilitated, or young animals that would be too young to cope with capture. Sick and debilitated animals tend not to roam widely, reducing their opportunity to enter traps. Additionally, should sick, debilitated, or young animals enter traps, we anticipate that they will not suffer adverse conditions because the traps contain food (the bait) and liquid (water or fruit as a source of water). These individuals would be examined by a veterinarian, rehydrated and if presenting clinical signs treated by a veterinarian, prior to release or sampling then release.
- Sample Collection: Once anesthetized or safely restrained, whole blood will be collected through the appropriate venipuncture site, no more than 1 ml of blood per 100 g (= 10 ml/kg or 1%) of body weight will be collected at any one time. Anesthetized animals will be monitored regularly during recovery until they can no longer be safely handled, at which point they will be confined in a trap or cage. Swabs will be taken from the oropharynx, urogenital tract, and rectum. No destructive sampling is planned, but any individuals that die during capture or sampling will have a necropsy performed with organ samples collected.
- Release: Wild mammals are held for a maximum of thirteen hours depending on trap timing, but typically less than three hours. Captive animals are held for a maximum of two hours but typically less than one hour. At the completion of sampling, animals may be provided with rehydrating fluids (either subcutaneously if anesthetized or orally if manually handled and accepted). Wild mammals will be released at the site of capture in a location that allows for further recovery or safe escape (e.g., bodies of water, known predatory areas, and human settlements will be avoided). Captive mammals will be released back into their captive setting as is appropriate for the species, either isolated or in social group if it is deemed safe from aggression from enclosure mates while the post-recovery period continues.

2. Justifications

The purpose of this study is to identify zoonotic pathogens through serologic and cellular immune discovery, including developing methods and standards for direct antibody-mediated virus capture in people and wildlife, focusing on rodent/shrew- and bat-borne HPVs. This will require hands-on fieldwork to collect whole blood and serum samples from bats, rodents, and shrews for lab analysis. One technique that avoids the direct capture and sampling of animals is to collect fresh feces or urine, but this will not be able to provide the appropriate samples for serologic analysis, and the research objectives cannot be achieved using an alternative methods (e.g., computational, human, invertebrate, in vitro). Therefore, we believe there are no viable alternatives than the use of live animals.

3. Minimization of Pain and Distress

In every situation, sampling of wildlife will be conducted in the most humane manner while minimizing the impacts on individual animals and their wild populations. In all instances, the fewest number of animals will be sampled that will provide valid information and statistical inference for the pathogen and disease of interest and every effort will be made to minimize stress and discomfort for the animal.

Bats will not be held longer than six hours during the sampling process whereas small mammals may be held for up to 13 hours depending on trap capture timing. Field animal sampling team with zoologists and veterinarians have been well trained and have extensive experience in capture, anesthesia, and sampling of wildlife. In our team's experience, the target species tolerate the described procedure well. Mist nets will be attended continuously during capture periods, and bats will be extracted from the net as soon as they become entangled. This will minimize stress and injury from entanglement. Bats will be placed individually in cloth bags and hung from tree branches while awaiting processing and during recovery. The bags are sufficiently porous as to allow for ventilation and are designed for bat capture. The enclosed environment seems to calm the bats, as they do not struggle once inside, but they hang quietly – this is a standard and accepted practice in bat research and the best way to minimize stress to the animal.

For rodents and shrews, food, water, padding and shelter will be provided at traps, which will be checked at least every 12 hours. Traps will be checked more frequently or closed if adverse weather (extreme heat, rain) is expected, or researchers are working in areas where predation is common. Veterinary staff will be prepared to deal with a variety of trap injuries, including lacerations, broken teeth, and crush injuries. All animal individuals will be monitored by a veterinarian or experienced field team member during all stages of capture, processing, anesthesia and release. Animals will be kept in a cool place, free from adverse weather conditions and access by non-field team members or other animals while in the pillowcases, trap, or cage for recovery. All recovering individuals will be observed until they are able to effectively ambulate and orient to their surroundings. Animals that are injured during the capture or sampling process will be assessed by an experienced team leader or attending veterinarian, and if the animal is determined to be unlikely to survive if released, it shall be euthanized humanely (see euthanasia section).

4. Method of Euthanasia

In the event that an animal has been injured or is moribund, a determination will be made as to whether it may be treated and released on site by veterinarians or transferred to facility that may treat and rehabilitate the animal, or if, as a last resort, the animal will require euthanasia. Euthanasia methods will vary depending on species; however methods to be used will not deviate from the AVMA “Guidelines for the Euthanasia of Animals” (2020 edition). Any animal that is euthanized using a chemical agent will be disposed of such that it will not be permitted to enter the food supply either through markets or hunting.

Human Subjects Research

1. Risks to the subjects

In this project, we will target populations in one community site who with frequent exposure to bats and other wildlife based on our previous behavioral survey. Subjects will be enrolled voluntarily, and informed consent will be obtained from all participants, along with assent from all participants aged 12-17. Enrolled participants will provide biological samples. Survey data and biological samples will be collected from enrolled participants, and follow-up data collection will be performed among participants whose samples were tested positive.
Biological samples and questionnaire data will be collected from individuals who meet recruitment and inclusion criteria and complete the informed consent process. During data collection, a standardized questionnaire will be administered to all participants. This questionnaire will collect information of demographic background, wildlife contract, travel and daily movement, and unusual SARI/ILI symptoms, and biological specimens will be collected from participants. Both questionnaire and biological data will be analyzed to assess the exposure to coronaviruses and the spread risk among humans. From all participants, a one-time whole blood sample (Max. 15 mL) will be collected during the study period. This sample will allow us to test for historical exposure to bat or rodent-born henipaviruses and collect Peripheral Blood Mononuclear Cells (PBMCs) for those study participants whose serum tests are positive.

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 who are 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, the administration will be conducted privately and confidentially to protect individuals' 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 these risks

2.1 Informed consent and assent

Only consented participants will be enrolled in the study. No research procedures will be undertaken before the participant fully understands the research, agrees to the study procedures, and completes the consent process will be enrolled in the study. Informed consent statements and forms, and project protocols will be translated into the local language of each study site. Research team members involved in this consent process will be required to be fluent in the local language to ensure that the subjects understand the study and all involved procedures.

If participants meet the criteria for enrollment, they will be invited to discuss the details of the study with the research staff. Study staff will review an information sheet and informed consent form with the participant and the parent or legal guardian when applicable. Each individual will be provided with a copy of the informed consent form that has been translated into the local language and written with a Flesch–Kincaid readability score equivalent to a 7th-grade reading level or below, to assure that potential participants understand the information being shared. The informed consent form will explain the details of the study, including how and why the individual was selected, the study process and procedures, risks and benefits, financial considerations and the gift of appreciation, confidentiality of data shared, alternatives to participating, and how to obtain more information now or at a later

date. The informed consent form will be read in the local language of the site at a location ensuring participant privacy. After which individuals will have as much time as they would like to ask questions and discuss the study with study staff. The study staff will endeavor to ensure that the participant understands the information provided. The study staff will then ask the participant/parent or legal guardian to consider study participation. Participants will have as much time as required to consider the participation.

Those participants who consent to the study will sign and date two copies of the consent form. These form copies will be countersigned and data by the study staff. A copy of the signed consent form must be provided to the subject and the other copy will be kept by study staff. Informed consent paperwork will be kept until the end of the project in a locked box at the local country project office.

2.2 Protection against risks

The potential risks to study participants as a result of study participation are minimal. Collection of venous blood samples and nasal or oropharyngeal swab samples pose minimal risk to subjects. Potential complications associated with venipuncture include pain and/or hematoma at the site of collection. Trained medical professionals and/or clinic staff will monitor the blood collection site and treat any complications according to existing health facility protocols. A potential complication of nasal/ oropharyngeal sampling is minor irritation at the time of collection. Employing trained medical and/or clinic staff to collect blood and swab samples will minimize the potential for complications.

Another risk that this study may pose concerns the information to be gained on henipaviruses newly recognized in the community. We will provide participating communities, hospitals, and clinicians with information and background data on target zoonotic viruses to ensure up-to-date communication of risk. Because of the timeline for diagnostic testing and results interpretation, we are not likely to provide results to participating clinics within a time frame that would be clinically relevant to outbreaks of undiagnosed diseases. Therefore, the information provided by this project will not impact patient management or outlook.

If an individual decides to participate in this research, his/her participation and all information provided by the participant will be strictly confidential, and personal identifying information will not be shared with anyone outside of the study staff. Participants will not be identified or named in any reports or publications. Questionnaire information and all biological samples will be identified by an alphanumeric code, not by the participant's name. 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 study site in locked files cabinets or password-protected devices in areas with access limited to the research staff of this project. Research databases will be secured with password-protected access systems and controlled distribution web-based certificates and will not contain any identifying characteristics about study participants (e.g., name, address, or telephone number). Access to all data will be limited to the staff involved in this study. The health information disclosed by an individual will not be used by or disclosed (released) to another institution. Any surveillance report that is published or shared with partners will not contain any personally identifying information for individual participants.

3. Potential benefits of the research to the subjects and others

There are no measurable benefits to the individual study participants enrolled in this study. There are benefits to the community and regional healthcare providers to help them understand the risk of zoonotic infections among high-risk populations in the regions they work. At the conclusion of the study, we will deliver an educational workshop reporting aggregate study findings that will be open to both study and non-study participants, describing the health benefits of using personal protection equipment (PPE) and handwashing during animal handling activities throughout the day, as well as to share other prevention/interventions that emerge from the research data.

4. Importance of the knowledge to be gained

There are valuable potential benefits to the public from the knowledge to be gained from this study. One key benefit to the community is sharing information and knowledge to better understand the risk of zoonotic spillover events and related health risks, as well as information sharing with communities on practices that could reduce risks, such as the avoidance of particular animal contacts or the need for PPE and extra care when handling wildlife, that may substantially reduce the risk zoonotic pathogen transmission in the community. 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 create or implement public health interventions to disrupt disease emergence and/or spread in an area that is beneficial to all. Additionally, there are valuable benefits to the general public from the knowledge to be gained from this study. Knowledge gained will 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.

5. Country- / institution-specific ethics / IRB regulations addressed

Main research protocol and materials to conduct human subject research in this project have been reviewed and approved by the Health Medical Lab Institutional Review Board, US (No. 894ECOH21b) and the Institute Review Board of the Faculty of Medicine, Chulalongkorn University, Thailand (No. 211/64). Amendment for PBMCs collection will be made and approved before the start of this project.

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

Research and Related Other Project Information

1. Are Human Subjects Involved?	Yes	X	No	
1.a. If YES to Human Subjects				
Is the Project Exempt from Federal regulations?	Yes		No	X
If yes, check appropriate exemption number	1		2		3		4		5		6		7		8	
If no, is the IRB review Pending?	Yes	X	No	
IRB Approval Date:				
Human Subject Assurance Number	FWA00000943			

2. Are Vertebrate Animals Used?	Yes	X	No	
2.a. If YES to Vertebrate Animals				
Is the IACUC review Pending?	Yes		No	X
IACUC Approval Date	3 December 2020			
Animal Welfare Assurance Number				

3. Is proprietary/privileged information included in the application?	Yes		No	X

4.a. Does this project have an Actual or Potential impact -- positive or negative -- on the environment?	Yes		No	X
4.b. If yes, please explain				
4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed?	Yes		No	
4.d. If yes, please explain				

5. If the research performance site designated, or eligible to	Yes		No	X

be designated, as a historic place?				
5.a. If yes, please explain				

6. Does this project involve activities outside of the United States or partnership with international collaborators?	Yes	X	No	
6.a. If yes, identify countries	Thailand			
6.b. Optional explanation				

Budget Justification

A. Key Personnel (Total $24,000)

Dr. Krongkan Srimuang, PI (12 months) is a medical research scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital (TRC-EIDCC) who have been leading the laboratory analysis for the EID-SEARCH work in Thailand in the past three years to perform molecular, serological, and next-generation sequencing to identify novel viruses from human and animal samples. Dr. Srimuang will commit 12 months to this project to design, manage, and perform all project activities in the lab and field by supervising a lab technician and working with the field coordinator. Dr. Srimuang will also work closely with the co-PI and Mentors to conduct data analysis and develop manuscript and join EID-SEARCH and CREID meetings to prevent the project findings.

Spencer Sterling, Co-PI (6 months) is the research coordinator of Laing Lab at the Uniformed Services University for Indo-Pacific region and a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC). Sterling will commit 6 months to this project as the co-PI, who will work closely with the PI in the study design, SOP development, sampling process, data analysis of for serological investigations. No salary is required for Sterling.

B. Other Personnel (Total $20,000)

One (1) Laboratory Technician (12 months) will committee 12 months to this project to conduct PCR and serological testing of animal and human samples at TRC-EIDCC, under the supervision of Dr. Srimuang and Sterling. Salaries for 12 months at $1,250 per month is requested ($15,000).

One (1) Field Coordinator (4 months) will committee 4 months to this project, working closely with Dr. Srimuang and Sterling to organize and conduct field sample collection among humans and animals at the study sites, and support relevant financial management. Salaries for 4 months at $1,250 per month is requested ($5,000).

C. Fringe Benefits

No Fringe is requested.

D. Equipment

No equipment is requested.

E. Travel (Total $48,375, Years 1-5)

Domestic Travel ($11,005)
Support for domestic travel for field sampling is requested in the amount of $11,005 per to conduct four (4) trips of sample collection from humans, bats, and rodents/shrews.

- The first trip is a one-day trip to collect samples from human participants who were tested positive (n<10), the cost is estimated at van rental for local transportation between Bangkok and the study site ($260) and meals for five (5) field team members at $49 per person ($245), in total of $505 for the first trip.

- The other three (3) field trips will be conducted to collect samples from 100 bats, 100 humans, and 100 rodents/shrews, respectively. Each trip is a duration of 6 days and 5 nights. For each person, 5-night stay in hotels at $75.5/night (5 x $75.5 = $378); 3 meals per day and incidental expenses at $49 per day prorated to 75% ($36.75) for first and last day of travel ($36.75 x 2 + $49 x 4 = $270), the total meals and accommodations for each person per trip is $648 ($378+270), cost for five (5) field team members is $3,240. A van will be rented for local transportation between Bangkok and the study site that is estimated at $260 per trip. Total cost for each 6-day trip is $3,500, three trips will be $10,500.

International Travel ($5,023)

Support for international travel for Dr. Srimuang from Bangkok to Washington DC to attend the CREID Annual Meeting and receive lab training at the National Institute of Allergy and Infectious Diseases (NIAID) is requested in the amount of $5,023. Trips are for a duration of 10 days and 9 nights. Travel costs for one trip are estimated using federal per diem rates for hotels, meals, and incidental expenses as follows: nine-night stay in DC area hotel at $286/night (9 x $258 = $2,322); Ten days' meals and incidental expenses at $79 per day prorated to 75% ($59.25) for first and last day of travel ($59.25 x 2 + $79 x 8 = $750.5); Round trip flight between Bangkok to Dulles International Airport is estimated at $1,650; local transportation/taxi between hotel and airport per round trip between Chulalongkorn Hospital to airport is estimated at $40, between hotel and the airport in the US is estimated at $200, and $60 for transportation within the region ($300).

F. Participant Support Costs

No participant support is requested.

G. Other Direct Costs (Total $78,803)

Materials and Supplies ($72,830)

PBMCs isolation ($47,000). We request funds to conduct PBMC isolation from collected sample, costs including the reagents and lab consumable and supplies are estimated at $47 per cell for 1,000 cells.

Multiplex microsphere immunoassay detection ($2,275). Funds are requested to perform serological testing of collected human and animal samples. Costs including the reagents and lab consumable and supplies are estimated at $6.5 per sample for 350 samples.

PCR testing and sequencing ($20,475). Funds are requested to perform serological testing of collected human and animal samples. Costs including the reagents and lab consumable and supplies are estimated at average $58.5 per sample 350 samples, given a 10%~15% positive rate.

Field supplies for PBMC collection and processing ($1,225). Funds are requested to perform PBMCs collection and processing in the field. Costs including the reagents and lab consumable and supplies are estimated at average $3.5 per sample 350 samples.

Field supplies for animal sampling ($1,160). Funds are requested to purchase supplies for animal capture, anesthesia, and sample collection. A total cost of $1,160 is estimated

Field work disposable ($668). Funds are requested to purchase disposable materials (tips, tubes, gloves, masks, googles etc.) for field work.

Consultants Service ($3,000)

One (1) zoologist and one (1) veterinarian will provide consultancy service to this project to help 1) select sites for animal sampling and join the sampling trip to 2) identify species; and 3) provide veterinary care in the sample collection of bats, rodents, and shrews. The consultancy fee is estimated at $1,500 per person for the project, in total of $3,000.

Publication Fee ($3,000)

We request $3,000 of publication costs for one peer-review paper generated from the project. The cost is estimated at the reimbursed Open Access fees of $2,900 - $5,000 per manuscript in the past two years for six peer-review papers.

H. Total Direct Costs (Total $138,831)

I. Indirect Costs (Total $11,106)

An indirect cost at 8% is requested.

J. Total Direct and Indirect Costs (Total $149,937)

A total amount of $149,937 is requested.

Co-PI Plan (only needed if applying as Co-PIs):

What each Co-PI will contribute to the proposed research study

As stated in the Mentoring Plan, the co-PIs, Krongkan Srimuang and Spencer Sterling have different educational and training backgrounds. This Co-PI plan is proposed to fill the gaps in knowledge of each scientist and supports a collaborative working relationship for project success. Moreover, this plan was developed to maximize the strengths of each PI. Overall, the project is separated into two objectives, the proposal of this project will be equally managed by Dr. Srimuang and Mr. Sterling. Dr. Srimuang will be responsible for PBMC isolation, sorting, and characterization. Mr. Sterling will be responsible for the technical serological aspects of the project. Both will work closely with the Research Center Mentor, Dr. Eric Laing, to develop this project, protocol development, and network connections. Dr. Supaporn Wacharapluesade, the Co-mentor, will support the wildlife sampling, samples collection, and molecular aspects within Thailand.

How the Co-PIs will jointly work with the affiliated Research Center

Mr. Sterling is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region.

How the Co-PIs will jointly manage the proposed study

This is a great opportunity for both PIs to undergo training in advanced techniques and develop skills in next-generation serology and molecular sequencing. Dr. Srimuang will learn and practice serologic techniques and data analysis from Mr. Sterling. Mr. Sterling will develop skills in cell isolation and molecular sequencing techniques from Dr. Srimuang. Moreover, Mr. Sterling will coordinate planning, sample processing, data analysis, and report preparation for this project with Dr. Srimuang.

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List of abbreviations, acronyms, symbols

AngV: Angavokely virus
BCR: B cell receptor
BSc: Bachelor of Science
BSL2: Biological Safety level 2
BSL3: Biological Safety level 3
cDNA: Complementary DNA
CedV: Cedar virus
Co-PI: Co-principal investigator
CREID: Centers for Research in Emerging Infectious Diseases
DARV: Daeryong virus
DNA: Deoxyribonucleic acid
EHA: EcoHealth Alliance
EID: Emerging Infectious Disease
EID-SEARCH: The Emerging Infectious Disease – South East Asia Research Collaboration Hub
ELISA: Enzyme-linked immunosorbent assay
F: Fusion protein
FACS: Fluorescence-activated cell sorting
FUO: Fever of unknown origin
GAKV: Gamak virus
GhV: Ghana virus
HeV: Hendra virus
HPVs: Henipaviruses
ILI: Influenza-like illness
KCMH: King Chulalongkorn Memorial Hospital
L: large protein
LayV: Langya virus
M: Matrix protein
mAb: Monoclonal antibody
MD: Maryland
MMIA: Multiplex microsphere-based immunoassay
MojV: Mojiang virus
MSc: Master of Science
N: Nucleocapsid
NGS: Next-generation sequencing
NIAID: National Institute of Allergy and Infectious Diseases
NIH: National Institutes of Health
NiV: Nipah virus
P: Phosphoprotein
PBMC: Peripheral blood mononuclear cells
PCR: Polymerase Chain Reaction
PhD: Doctor of Philosophy
PI: Principal investigator
RBP: Receptor-binding protein
RNA: Ribonucleic acid
RT-PCR: Real-time PCR
SARI/ARI: Severe/acute respiratory illness
SARS-CoV-1: Severe acute respiratory syndrome coronavirus 1
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2
SEA: Southeast Asia
Thai-DDC: Department of Disease Control of Thailand

TRC-EIDCC: Thai Red Cross. Emerging Infectious Diseases. Clinical Center
USA: United States
WGS: Whole Genome Sequencing

Facilities and Resources

FACILITIES: Specify the facilities to be used for the conduct of the proposed research.

Laboratory:

The Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC), King Chulalongkorn Memorial Hospital (KCMH) was established by the Committee of the Emerging Infectious Disease Center which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. TRC-EIDCC laboratory facilities include 3 biosafety cabinets (BSL 2, BSL 2+, BSL2+), 3 automated extraction machines, 2 thermocyclers (Proflex), 2 real-time PCR machines (CFX, Bio-rad), 3 Freezers (-80 °C), 2 refrigerators; 4 °C, 2 autoclaves. Our EIDCC uses the Pathogen Asset Control System (PACS) system for sample tracking. Moreover, our Faculty of Medicine, Chulalongkorn University has a central laboratory, the Chula Medical Research Center (Chula MRC), which provide many instruments for medical, masters, and graduate students, Post-doctoral fellows, and researchers at Chulalongkorn University and KCMH. The Chula MRC laboratory facilities are divided into molecular diagnostics (PCR Thermal Cycler, QStudio6 Flex Real-Time PCR, Droplet Digital PCR, GeneXpert, Film array, Illumina MiSeq), immunology facilities (ELISA testing equipment (reader & washer), Luminex, Flow Cytometry), cell sorting machine and a BSL3 laboratory. Moreover, the facility provides general equipment, such as autoclaves, balances, CO2 incubators, Dual Fluorescence Cell Counter, 4 °C refrigerators, -20°C, and -80°C freezers.

Clinical:

KCMH is a 1,000-bed university hospital, in close coordination with the Faculty of Medicine, Chulalongkorn University. The Thai Red Cross Society provides medical treatment, rehabilitation, health promotion and disease prevention services to the general public and the vulnerable. It also provides biopharmaceutical and sterile medicines that are sufficient to meet the needs of patients and safe in accordance with international standards. It serves as a center for providing eye and organ donation services to patients nationwide in line with international standards. It has specialized doctors and public health personnel and also produces specialized nurses. The TRC-EIDCC in the KCMH provides standard medical care to patients with emerging and recurrent diseases and disease spreading prevention.

Animal:

TRC-EIDCC also collaborates with the Department of National Parks, Wildlife and Plant Conservation for detecting the emerging novel pathogens from animals such as bats, rodents, and pangolins in our country. Bats are considered protected wildlife in Thailand by Wildlife Conservation, Department of National Parks, Wildlife and Plant Conservation.

Computer:

Applicant(s) Name (Last, first, middle): Srimuang, Krongkan and Sterling, Spencer

TRC-EIDCC used the service from the Office of Information Technology, Chulalongkorn University that provides the application, network, and software such as Office 365, VPN, WIFI, Microsoft Office, Microsoft Windows, SPSS, Adobe, Zoom Meeting, etc. Moreover, our EIDCC use the Pathogen Asset Control System (PACS) for sample tracking.

Office:

The office and laboratory space are located at the EIDCC at 4th Floor, Jongkolnee Watwong Building and 6th Floor, Aor Por Ror Building, respectively. The applicants will have dedicated desks in both buildings.

Other:

PI Name: Srimuang, Krongkan and Sterling, Spencer **Foreign Site:** Thailand
CREID CC Grant: 1U01AI151378

Foreign Site Information

If the Pilot Award will have multiple foreign sites, complete this form for EACH research site

Principal Investigator Name:	Krongkan Srimuang
Project Title:	Immune memory bait & capture to identify emerging henipavirus origins
Institution:	King Chulalongkorn Memorial Hospital
Foreign Research Site:	Rama 4 Road, Pathumwan, Bangkok 10330 Thailand
Point of Contact for Research Site:	Krongkan Srimuang Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital Rama 4 Road, Pathumwan, Bangkok 10330 Thailand krongkan.sr@gmail.com

Introduction (3-4 sentences):

Provide a simple description of the overall goals of the project including the work that will be done at the foreign site. Indicate if any of the NIAID grant funds will be sent to the foreign site.

The goals of this project are to know zoonotic reservoirs for pathogen origin by using serologic and cellular immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

To achieve these goals, the investigator will collaborate with NIH PREMISE for Antibody-mediated bait & capture of Thai MojV-like virus.

$149,937 of grant funds will be sent to the site for these studies.

Foreign component (3-4 sentences):

Describe the specific role of the foreign site (i.e. conduct critical experiments?, conduct data analyses?, provide consultations?, provide samples?, study human and/or animal populations?, conduct observational or interventional clinical trials?, collect samples and/or data to be brought to the US?).

The site will 1) conduct all filed work to collect human and animal samples from the study sites; 2) perform laboratory analysis using collected samples; and 3) conduct data analysis as proposed in the project.

Human Subjects (1 word or 1 sentence per bullet):

- **Parent Study IRB approval**
  - **IRB approval number for parent study:** #894ECOH21b (US); #221/64 (Thailand)
  - **IRB approval date:** 12-05-2021 (US); 08-06-2021 (Thailand)
  - **Human Subject Assurance Number: (FWA)#:** #00001102 (external IRB); #00022431 (EcoHealth Alliance); #00000943 (Chulalongkorn University)
- **Does this study require a modification to the IRB approval of a parent study (Yes or No)?**
  - **Yes**
- **Will existing samples from human subjects will be used: (Yes or No)?**
  - **No**
  - **How many subjects provided the existing samples to be used?** N/A
- **Will human subjects be recruited (Yes or No)**
  - **Yes**
  - **Number of human subjects that will be recruited:** 110
- **Population parameters:**
  - **Gender:** 55 males, 55 females
  - **Age Group:** Age >=18 years who provide informed consent.
  -
  - **Race/Ethnicity:** 110 Asian
- **Sample collection will include:**
  - **Blood:** Yes
  - **Urine:** No
  - **Tissues:** No
  - **Other samples (describe):** Nasopharyngeal swab and throat swab
- **Sample collection will be completed in how many visits:** 2 trips
- **Will samples be de-identified (Yes or No)? If No, describe how they will be protected.**
  - Yes
- **Will local IRB approval be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals being sought or state why local IRB approval is not required.**
  - Yes
- **Will informed consent be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals/consents being sought or state why informed consent is not required.**
  - Yes
- **Will samples be brought back to the US (Yes or No)?**
  - No
- **Will data be brought back to the US (Yes or No)?**
  - Yes, some of the data will be shared with EID-SEARCH partners in the US for relevant analysis.

Animal Subjects (1 word or 1 sentence per bullet):

- **Parent study IACUC approval**
  - **IACUC approval number for parent study:** #G2020-42 (US); #019/2563 (Thailand)
  - **IACUC approval date:** 05-05-2020 (US); 03-12-2020 (Thailand)
    Animal Welfare Assurance Number: D16-00572 (A4059-01)

- **Does this study require a modification to the IACUC approval of a parent study (Yes or No)?**
  - **No**

- **Will existing samples from animal subjects will be used: (Yes or No)?**
  - **No**
  - **How many animal subjects provided the existing samples to be used?** N/A
- **Will vertebrate animals be collected (Yes or No)?**
  - **Yes**

- **Species of animals (e.g. rats, mice, rabbits, monkeys):** Bats, rodents, and shrews
- **Animal parameters:**
  - **Total number of animals:** 200 (100 bats, 100 rodents and shrews)
  - **Gender:** 100 males, 100 females
  - **Age range:** 4 - 12 months and elder depending upon species
  - **Lab strain (e.g. Sprague-Dawley rats, Balb/C mice):** None
  - **Wild animals procured in country (e.g. Rhesus monkeys from a reserve):** No

- **What will be done to them or with them and how often?**
  - Free-ranging bats will be captured using either a mist net or harp trap, and manually restrained during sampling; free-ranging rodents and shrews will be captured through pit traps and box traps.
  - Anesthesia will be conducted for captive rodents and shrews.
  - Once anesthetized blood will be collected.
  - All action will only be performed one time for each animal individual.
  - All animals will be released after sampling.

- **What are the follow-ups?**
  - No follow-up relevant as per protocol, since all animals will be released after sampling ups

- **What will be their fate at the end of the experiments – will they be euthanized?**
  - All animals will not be held longer than 6 hours (typically less than 3 hours) during the sampling process and released after sampling.

- **Will in-country clearances be obtained prior to engaging in any animal research (Yes or No)? If No, describe alternate approvals or state why in-country clearances are not required.**
  - Yes
- **Will samples be brought back to the US (Yes or No)?**
  - No
- **Will data be brought back to the US (Yes or No)?**
  - Yes, some of the data will be shared with EID-SEARCH partners in the US for relevant analysis.

Proposal Cover Sheet

Project Title	Immune memory bait & capture to identify emerging henipavirus origins
Principal Investigator Name	Krongkan Srimuang, Ph.D.
Position/Title	Research Scientist
Department	Thai Red Cross Emerging Infectious Diseases Clinical Center
Institution Name	King Chulalongkorn Memorial Hospital
Street	Rama 4 Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	Krongkan.sr@gmail.com
Phone	+66 (0) 92-453-0808
Country(ies) where work will be conducted	Thailand
Pathogen(s) focus	Mojiang Virus, Henipavirus, Paramyxovirus
Co-Principal Investigator Name (*If applicable*)	Spencer Sterling, MPH
Position/Title	Scientific Project Coordinator
Department	Microbiology and Immunology
Institution Name	Uniformed Services University of the Health Sciences
Street	Jones Bridge Road
City, State, Zip Code	Bethesda, MD 20814
Country	USA
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Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Name of Mentor	Supaporn Wacharapluesadee, Ph.D.
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Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Total Budget	***Repeat this table if there are two Co-PIs***
Direct Costs	$138,831
Indirect Costs	$11,106
Proposed Start Date	1 May 2023
Proposed End Date	30 April 2024

Project Abstract (250 words)

Prototypical henipaviruses (Hendra and Nipah virus) are bat-borne zoonotic viruses, cause high mortality. The recent detection of closely-related Mojiang and Langya viruses in rats and shrews, and isolation of Langya virus from individuals with acute febrile illness, challenges the dogma that fruit bats are the key henipavirus reservoirs. The recently expanded *Henipavirus* genus includes two other shrew-borne viruses (Gamak and Daeryong viruses), and the Madagascan fruit bat-borne Angovkeely virus. As part of EID-SEARCH activities, we found serological evidence of infection by a Mojiang-related virus in a community of Thai bat guano collectors who have occupational exposure to microbats and rodents. Identifying the wildlife reservoir of this likely novel virus will assist intervention strategies to reduce spillover risk. However, PCR detection or virus isolation in wildlife is challenging, and new serological approaches may be more successful in identifying reservoir hosts. This project aims to identify novel viruses and zoonotic reservoirs through serologic and cellular immune discovery. We will conduct

follow-up human and wildlife (bats and rodents) surveillance in the province where the seropositive humans were identified. Further, we plan to isolate anti-MojV monoclonal antibodies (mAb) from peripheral blood mononuclear cells collected from human study participants we have identified as immunoreactive with MojV antigens, and characterize the cross neutralizing potential of these mAbs to MojV and LayV envelope glycoproteins. In addition to our antigen-based approach, we will utilize the mAbs for direct antibody-mediated virus capture of this novel henipavirus from human and wildlife samples, and downstream genomic sequencing.

Project Title **Immune memory bait & capture to identify emerging henipavirus origins**

Table of Contents

Study Personnel

Krongkan Srimuang, Ph.D., is a researcher at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital and supports the Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia (EID-SEARCH) research. Dr. Srimuang is also a medical technologist working to identify, detect and characterize the spill-over risk of high zoonotic potential viruses from wildlife and humans using serology techniques, Next-generation sequencing, and PCR assays to identify novel viruses. Dr. Srimuang also performs the project Smart Sentinel Surveillance System: Emerging Viral Pneumonia and Respiratory tract infections (RTIs) to identify, detect and characterize the novel/known Respiratory Viruses in our country, Thailand by using multiplex real-time PCR, family PCR and Next-generation sequencing of novel viral pathogens. Dr. Srimuang graduated with Ph.D. in Tropical Medicine from the department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University.

Spencer Sterling, MPH, is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region. Mr. Sterling will planning, sample processing, data analysis, and report preparation for this project.

Dr. Eric Laing will act as Mentor for Dr. Srimuang and Mr.Sterling under the Centers for Research in Emerging Infectious Diseases (CREID) Pilot Research Program. Dr.Laing is an assistant professor dedicated to biosurveillance and virological research of emergent zoonotic viruses at Uniformed Services University, Bethesda, MD. Dr.Laing study to develop serological-based approaches to identify the infectome of wildlife and human communities for the assessment of virus spillovers. Dr.Laing have worked collaboratively with national and international researchers including institutional partners at Duke-NUS, Chulalongkorn University, University of Pretoria, and Rocky Mountain Labs towards these goals. Across all of these collaborations, the aimed to identify known and unknown viruses, the wildlife reservoirs of priority pathogens, the high-risk interfaces for spillover and the biotic/abiotic drivers of virus emergence.

Dr. Supaporn Wacharapluesadee, Ph.D. is the head laboratory at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, a WHO Collaborating Centre for Research and Training on Viral Zoonoses. Dr. Wacharapluesadee research interests include emerging infectious diseases in bats (including Nipah, Rabies, Coronavirus, and novel pathogens) as well as molecular diagnoses and sequencing of viruses. During the past 10 years, Dr. Wacharapluesadee got funding from USAID PREDICT to conduct the surveillance for novel virus in wildlife and human, and from Biological Threat Reduction Program US DTRA to conduct the EID surveillance in bat using the molecular technology and multiplex serology panel (Luminex technology). TRC-EIDCC is responsible for molecular diagnoses services to the hospital, and is the reference laboratory for rabies virus, MERS-CoV, Ebola, Zika, and other infectious diseases diagnoses for Ministry of Public Health, Thailand and laboratory training center on national and regional level. Dr. Wacharapluesadee has served and consulted on several WHO and Thai government committees.

Specific Aims

This project aims to study zoonotic reservoirs for pathogen origin by using serologic and cellular immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

The hypothesis

1. Can the memory B-cell immune response be used as a tool for the discovery of novel henipaviruses?
2. Can the spatial relationships between henipaviruses receptor binding proteins be generated that would allow for an understanding of antigenic evolution and functional characterization?

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population.

Human populations near the Khao Chong Phran Non-hunting Area in Ratchaburi province have shown a high seroprevalence to the MojV RBP without confirmed sequencing results. We will follow-up on persons with high MojV RBP IgG titers, sample new people within the population that interact with the local wildlife, and sample shrews and rodents to find the source of the MojV-reactive antibodies.

Aim 2. Antibody-mediated bait & capture of the Thai MojV-like virus

In collaboration with NIH PREMISE, MojV RBP-reactive B cell receptors will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific MAbs will be generated and used for direct antibody-mediated virus capture in people and wildlife.

Objective 1. To use metagenomic and targeted sequencing approaches in human and wildlife populations to detect the Thai MojV-like virus.

Objective 2. To isolate and sequence memory B-cells from humans positive for Mojiang virus in Ratchaburi province, Thailand for antibody production.

Objective 3. To determine epitope binding and antigenic relationships from henipavirus-reactive serum to the known henipaviruses.

Study Rationale

Nipah virus (NiV) and henipaviral diseases are among the most virulent of zoonotic diseases. The genus *Henipavirus* is currently composed of nine viruses including the prototypes, NiV and Hendra virus (HeV), and Cedar virus (CedV), Ghana virus (GhV), Mojiang virus (MojV) (McLinton et al., 2017), Langya virus (LayV) (Zhang X et al., 2022), Gamak virus (GAKV), Daeryong virus (DARV) (Lee et al., 2021), and Angavokely virus (AngV) (Madera et al., 2022). HeV was first identified in Australia in 1994 as a cause of respiratory illness and fatal encephalitis in humans and horses. Closely following the discovery of HeV, NiV was identified in 1999 in Malaysia after outbreaks of fatal encephalitis in humans, with swine determined to be the intermediate host. Annual outbreaks of NiV have occurred in Bangladesh and are associated with the consumption of raw date palm sap, and since 19 May 2018 annual outbreaks of NiV associated encephalitis of been recorded in Kerala, India (Uwishema et al., 2022). Both HeV and NiV have a broad host range capable of infecting several mammalian orders, and can enter and infect endothelial cells, vasculature, and the central nervous system leading to severe multisystem organ diseases and high mortality. The broad host and tissue tropism of these viruses has been attributed to the use of the ephrinB2 and ephrinB3 ligands, highly conserved proteins with critical functions in evolutionary development, as virus receptors (Bossart et al., 2008). There are currently no vaccines or therapeutics and NiV and henipaviral diseases remain WHO Blueprint list priority pathogens (Middleton and Weingartl, 2012).

A close relative of HeV, CedV was isolated from Australian bats in 2012 and found to be non-pathogenic, failing to cause any clinical symptoms in ferrets and guinea pig animal models (Marsh GA et al., 2012). The natural reservoirs of HeV, NiV, and CedV are flying foxes (*Pteropus sp.*) (Middleton and Weingartl, 2012). GhV was detected in Ghana in straw-colored fruit bats (*Eidolon helvum*) during routine surveillance in 2015 and was the first henipavirus found outside of the Indo-Pacific region. GhV showed a close phylogenetic relationship with HeV and NiV, and like HeV, NiV, and CedV the GhV RBP can mediate binding to ephrinB2 ligand and mediate cellular fusion (Drexler, J. F., 2009). Recent investigations into Madagascan fruit bats (*Eidolon duprenum*) detected a second African henipavirus, AngV. As neither GhV nor AngV have been isolated or rescued, the pathogenic and zoonotic potential of these African fruit bat associated henipaviruses remains unknown.

Multiple genus of small mammals were tested for viral RNA, and MojV was detected in yellow-breasted rats (R. flavipectus) (Wu Z et al., 2014). The discovery of the MojV genome occurred during follow-up investigations into lethal pneumonia from three miners in 2012 in China, however the pathogenic nature of this virus has only been suggested and no isolates have been discovered or tested in an animal model. Additionally, the novel shrew-borne henipaviruses, Gamak virus (GAKV), and Daeryong virus (DARV) were found in the Republic of Korea in 2017-2018. (Lee et al., 2021). Most recently, a close relative of MojV, Langya henipavirus (LayV) was recently isolated from febrile patients in eastern China. Subsequently, LayV was identified in shrews (Zhang et al., 2022), increasing the number of Asiatic rodent-associated henipaviruses to four. These recent discoveries are challenging the paradigms surrounding henipaviruses and their associations with bats and may suggest a novel rodent sub-genus of henipaviruses.

The Henipavirus genome consists of six structural proteins including nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), receptor-binding protein (RBP), and large (L) protein (approximately length of 18,000 nucleotides) (Luby, S. P., & Gurley, E. S., 2012). In HeV and NiV, slippage sites within the P protein lead to the expression of the V, W, and C proteins. These proteins are virulence factors and are absent in CedV.

Table 1 Summary viruses in Henipavirus

Henipavirus	**Origin (Year)**	**Reservoir**	**Genome (nucleotides)**	**Symptom for human**	**Reference**
Hendra virus (HeV)	Australia (1994)	Fruit bats	18,234	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Nipah virus (NiV)	Malaysia (1999)	Fruit bat	18,246	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Ghana virus (GhV)	Africa (2009)	Fruit bat (*Eidolon helvum)*	18,530	No data	Drexler, J. F., 2009
Cedar virus (CedV)	Australian (2012)	Fruit bat	18,162	Non-pathogenic	Marsh, G. A et al., 2012
Mojiang virus (MojV)	China (2012)	Rodent	18,404	Febrile	Wu Z et al., 2014
Gamak virus (GAKV)	Korea (2017-2018)	Shrew	18,460	No data	Lee et al., 2021
Daeryong virus (DARV)	Korea (2017-2018)	Shrew	19,471	No data	Lee et al., 2021
Langya virus (LayV)	China (2019)	Shrew	18,402	Acute febrile	Zhang et al., 2022
Angavokely virus	Madagascar (2019)	Fruit bat	16,740	No data	Madera et al., 2022

Viruses in the genus *Henipavirus* have a broad reservoir and may cause high case fatality rates following human infection. The discovery and characterization of the novel Henipavirus is a high public health priority.

The greatest bat diversity around the world especially appears to be found in the tropical rain forests of South East Asia. In Thailand, at least 138 species in 11 families and 45 genera of bats have been recorded, such as members of the genus Pteropus, Rhinolophus, and Hipposideros (Soisook et al., 2011). Khao Chong Phran, a cave in Ratchaburi Province, has possibly the largest colony of bats in Thailand. There are estimated to be around 2 million bats in the cave and 95% are insect-eating bats (Department of National Parks, Wildlife and Plant Conservation, Thailand). Moreover, Khao Chong Phran has many community sites, such as cave entrances near communities, guano collection, and tourism sites, which risk zoonotic spillover. Bats are widely considered the animal hosts of zoonotic pathogens such as rhabdoviruses, coronaviruses, paramyxoviruses, and filoviruses, and many of the host species can be found in Thailand.

MojV was associated with a severe pneumonia-like illness killing three of six miners in 2012 in a mineshaft Yunnan, China. Interestingly, this mineshaft was also site where RaTG13, a beta-coronavirus most closely related with SARS-CoV-2, was detected in *Rhinolophid* bats (Rahalkar and Bahulikar, 2020). Identifying the wildlife reservoir of this novel virus will assist intervention strategies to reduce spillover risk.

Research Gaps

As a part of Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH), serological investigations using a multiplex microsphere-based immunoassay (MMIA) have detected four members of a high-risk cohort with MojV-reactive serum, including one person with a high magnitude and durability of anti-MojV IgG across longitudinal samples. Despite this, genomic surveillance has not detected MojV or any novel MojV-like viruses. For this result, we would to further identify and characterize the origin of the Mojiang virus-reactive serum and to explore the novel MojV-like virus in Thailand.

Serological and cellular immunity is an important resource for virus discovery detection by using the specificities and characteristics of memory B cells from peripheral blood mononuclear cells (PBMC). The immune response will recognize and induce antibodies specific to the infecting pathogen. Characterizing the memory B cell response to an unknown pathogen in humans could in the detection of the exposure pathogen in humans. Molecular techniques such as PCR can detect only the genomic material in the body, but memory B cells are long-lived and thus have a much larger window of detection.

Memory B-cell differentiation is characterized by somatic hypermutation in antibody variable region genes (V) and selection of B cells expressing high-affinity variants of this antigen receptor and high sequence similarity in individuals exposed to the same antigen reflect antigen-driven clonal selection and form shared immunological memory between individuals (Yang et al., 2021). Memory B cells are highly abundant in the human spleen, and can proliferate 45% of the total B cell population in this organ (Hauser et al., 2015). Memory B cells can be detected in the Ig variable (IgV) genes in a fraction of IgM+ B cells. In humans, B cells expressing the memory marker CD27+ can be found in the marginal zone of spleen and circulate in the blood. After re-exposure to antigen, memory B cells can quickly proliferate and differentiate into plasma cells. Many studies the antibody diversity and specificity of the naturally paired immunoglobulin heavy and light chains for affinity maturation of B cell receptor (BCR) after virus infection such as SARS-CoV-2 infection (Yang et al., 2021), influenza (Dougan, S et al., 2013), HIV (Moir, S., & Fauci, A. S.,2017), etc. The characterization of memory B cells can be used to develop monoclonal antibodies and produce an effective theraputic.

Significance and Approach

Southeast Asia is one of the world's highest-risk emerging infectious diseases hotspots. From this region emerged both SARS-CoV-1 and SARS-CoV-2, recurrent outbreaks of novel influenza strains, and the spillover of dangerous viral pathogens such as Nipah virus. The diversity of coronaviruses, henipaviruses, and filoviruses are found in wildlife reservoirs in Southeast Asia is due to the high diversity of wildlife, and the demography of the human population brings humans and wildlife into close contact. The spillover of viruses from animals to humans is often misdiagnosed and the clinical symptoms from patients is underestimated during the pandemic. This study provides a surveillance strategy in human and wildlife populations to better understand immunity to novel pathogens and identify the origin of these pathogens to serve as an early warning system to prevent outbreaks of emerging viruses.

This study uses immunology to identify a novel virus that can aide in effective vaccine discovery and development, monoclonal antibody production for prevention and therapy, and generate data resources for early detection and diagnostic assays. Whereas PCR detection or virus isolation in

wildlife is challenging, this new serological approach may be more successful in identifying reservoir hosts.

The Indo-Pacific region has a high risk of novel viral emergence. This study provides an opportunity for in-country research capacity building by linking local infectious disease scientists with an international collaborative network that has proven capacity to conduct this work and produce significant findings. Moreover, a global sero-surveillance network for surveillance technology extends virologic and immunologic screening to product discovery. This study will benefit and expand the CREID, EID-SEARCH networks, and the global sero-surveillance network to improve our understanding of immunology to identify novel pathogens and their origin to prevent future pandemics.

Research Methods

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population

Serological profiling provides an indirect measurement of the infecting virus. Despite 19% (54/284) seroprevalence in humans, we have not detected nucleic acid sequences of MojV or MojV-like henipaviruses in this human cohort. In addition to the translational application of mAbs as therapeutics, we intend to utilize these mAb as non-clinical diagnostics surveillance tools for virus/virus antigen capture. Additionally, we intend on expanding our understanding of henipavirus prevalence in this population with longitudinal sampling of the wildlife and human populations in this region.

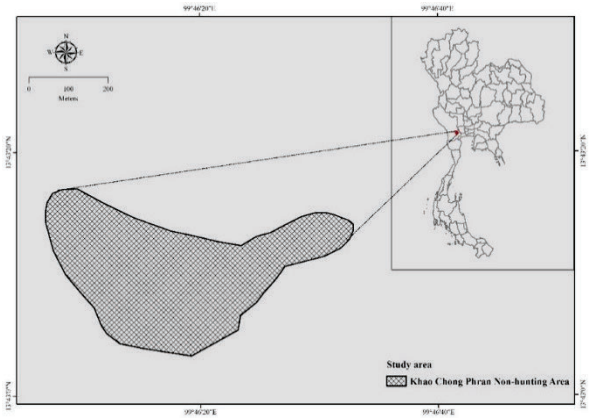

Figure 1**:** Map of Khao Chong Phran Non-hunting Area, Ratchaburi Province, Thailand (Boonpha N et al., 2019)

Site wildlife samples collection

100 bats and 100 shrews or rodents in and around the Khao Chong Phran cave in Ratchaburi Province will be collected for surveillance targeting the Mojiang-like virus. We plan one sampling trip to collect bats, and a second trip to target shrews and rodents. Due to limited knowledge of the local shrew population, we are unsure as to whether we can collect 100 samples, and will supplement the collection with rodents, as needed. Samples will be collected based on the following enrollment criteria: random age, random weight, random sex. Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and stored. Oral swab and rectal swab will also collect in 100 bats and 100 shrews or rodents. Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and stored.

Human surveillance

Four of the samples from prior investigations have shown high MojV RBP-reactive antibody titers and will be selected for follow-up sampling. Additionally, 100 human serum samples, nasopharyngeal swab and throat swab will be collected from persons deemed to be in close contact with wildlife, and people who present with or have recent history of undiagnosed acute febrile illness using the following enrollment criteria;

-
- Who present with or have recent history of illness without diagnosis, such as severe/acute respiratory illness (SARI/ARI), Influenza-like illness (ILI), fever of unknown origin (FUO), or respiratory tract infection
- Who is the bat guano collector in the cave
- Who lives or works around wildlife habitats
- Who is involved with livestock or wildlife farming
- Who hunts or works with wildlife
- Who has not been previously sampled as part of the EID-SEARCH and PREICT projects

Sample size justification

Human population

$$\mathrm{n} = \frac{\mathrm{N}}{1 + \mathrm{N}e^2}$$

n = sample size of the study

N = population size for finite populations: 8,150 (data from 2018)

e = error: 10%

$$\mathrm{n} = \frac{8{,}150}{1 + (8{,}510 \times 0.1^2)}$$

n = 98.79 (round up to 100 samples)

Wildlife population

The sample size of 100 of each species follows the guidelines established during the PREDICT project.

Targeted Viral Sequencing and Serologic testing

Targeted sequencing

For samples from shrews, rodents, and acute individuals, we will use targeted sequencing and metagenomic approaches in an attempt to detect the Thai MojV-like virus. The nucleic acid from oral swab and rectal swab will do target sequencing in henipavirus Family PCR and Sanger sequencing.

Serological testing

Serum samples will be tested for RBP binding to the known henipavirus species, including the Nipah, Hendra, Cedar, Mojiang, Ghana, Langya, Gamak, and Daerong viruses to further investigate the seroprevalence in this region. Additionally, prior positive samples will be re-tested with the expanded panel to enhance our understanding of the antigenic relationships between the novel shrew-borne henipavirus and the Thai virus.

Data analysis

Sequencing will be analyzed assembling reads in MEGA11. The consensus sequences will compare to the references strains available in the GenBank data-base using the Basic Local Alignment Software Tool (BLAST) (National Center for Bio-technology Information). Serological data will be analyzed using cluster analysis in RStudio.

Aim 2. Characterization of monoclonal antibodies from humans with serological profiles consistent with MojV infection.

In collaboration with NIH VRC PREMISE, MojV RBP and F-reactive B cells will be captured, and the B cell receptor will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific mAbs will be generated from the corresponding sequences and will be assessed for binding potential to LayV RBP and F, as well as their neutralization potential.

Importance: The isolation of these mAbs represents a potential therapeutic for MojV, MojV-like henipas, and LayV, depending on how well these cross react.

Human samples collection site

Human positive serum for MojV was found close to Khao Chong Phran in Ratchaburi Province, which has the largest colony of bats in Thailand (Figure 2).

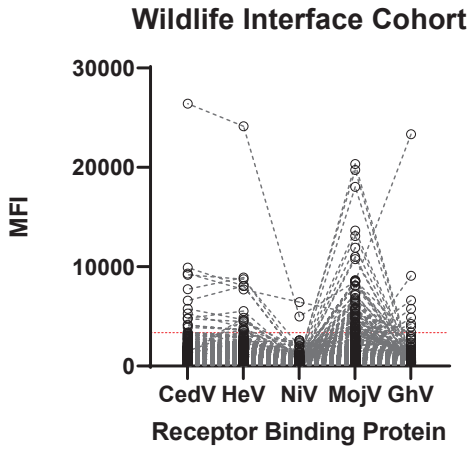

Figure 2: Seroprevalence of henipavirus proteins from a community with a large bat interface during 2017-2018. MFI is the median fluorescence intensity for IgG reactivity to each protein. Red-dashed line is at 3,357 MFI, the assay cutoff.

PBMC Sorting and B Cell isolation

Whole blood from MojV-positive people will be collected in individual heparin tubes and processed for PBMC isolation using Density Gradient Centrifugation (SepMate™ PBMC Isolation Tubes). Human PBMCs will be stained with antibodies for positive markers of IgG memory B cells, and antibodies to negatively select and avoid contamination with dead cells, T cells, NK cells or monocytes (live, 4 5 7 -tag for biotinylation and 6xHIS tag for Nickel purification using the HisPur™ Ni-NTA Spin Purification Kit (Thermo Fisher Scientific, Waltham, MA). Single Memory B Cells will be sorted, cells will proliferate, cDNA will synthesized, then Heavy and light chain variable domains will be amplified (Waltari E et al., 2019).

B cell receptor Amplicon Preparation and Recombinant Antibody sorting

Total RNA yields from the PBMC will be determined by absorbance at 260 nm on the NanoDrop™ One (Thermo Fisher Scientific). Total RNA will be used for first strand cDNA synthesis with gene-specific reverse transcription (RT) primers directed to the constant regions. The RT primers for IgG, IgM, IgA, lambda, and kappa will be included. Light chain and heavy chains will be amplified into amplicon. Double-stranded cDNA will be sequenced. After completion of MiSeq sequencing, antibody repertoires will analyze using methods based on the pipeline. Heavy and light-chain sequences will be assembled into an expression vector and transfected into cells for monoclonal antibody expression. Recombinant antibody will be isolated and purified for downstream applications.

Functional assessment

Neutralization of viruses remains a critical correlate of protection for most vaccine strategies. Next, we will test the cross neutralization potential of these anti-MojV RBP and F mAbs to neutralize LayV (Duke NUS/LinFa should be able to do this, if they have LayV and are propagating it, they can use cell-cell fusion/CPE in a straight PRNT).

Determination of envelope glycoprotein epitope binding

Isolated mAbs will be assessed for binding to MojV RBP and F proteins through multiplex binding serology. Further, the cross reaction potential of these mAbs will be assessed against GamV, DarV, and LayV RBP and F. MojV has not been isolated or rescued, and the virus receptor for MojV has not yet been discovered (Rissanen et al., 2017; Cheliout et al., 2021). Thus, a functional assessment of cross neutralizing potential is not possible. However, through cyro-electron microscopy we can investigate binding epitopes between these mAbs and MojV and LayV envelope glycoproteins. Epitope identification can then be used to make predictions about neutralizing potential through deductions about the location of henipavirus RBP receptor-binding pockets.

Project Timeline

Activity	05/23	06/23	07/23	08/23	09/23	10/23	11/23	12/24	01/24	02/24	03/24	04/24
Mentoring and training activities												
1.Training, literature review												
2.Monthly mentoring meeting												
3.Manuscript writing												
4.EID-SEARCH meeting												
Research activities												
1.IRB amendment												
Aim 1												
2. Human and Wildlife samples collection												
3. Identify **Targeted sequencing**												
4. Serological testing												
Aim 2												
5. Collect PBMC human sample and isolation memory B-cell for Mojiang virus												
6. PBMC Sorting and B Cell isolation												
7. Determination of envelope glycoprotein epitope binding												

Research Performance Sites

This proposed study will be implemented at the King Chulalongkorn Memorial Hospital (KCMH) collaborating with the Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC). KCMH is a 1,000-bed, university hospital in close coordination with the Faculty of Medicine, Chulalongkorn University. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. It's one of the center hospitals for Emerging Infectious Diseases in Thailand, especially for domestic and international patients.

TRC-EIDCC was established by the Committee of the Emerging Infectious Disease Center, which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. Our clinical center provides standard medical care to patients with emerging and recurrent diseases and disease transmission. Moreover, the center plans preparedness activities, trains medical and government staff on interventions, hosts conferences for medical and public health professionals, creates networks of medical care for patients with emerging and recurrent diseases, and leads research and development projects for medical care and disease control. Apart from medical innovation in emerging and recurrent diseases, the clinical center strives to be the model of infectious diseases clinical services and pandemic control through research activities surrounding infectious diseases and effective disease control measures.

We also collaborate with the Department of National Parks, Wildlife and Plant Conservation for detecting novel pathogens from animals such as bats, rodents, and shrews in our country. We also collaborate with the Department of Disease Control of Thailand (Thai-DDC) to support the Thai government and national policy makers through molecular diagnosis, including the identification of the first case of SARS-CoV-2 infection in Thailand. TRC-EIDCC prides itself on its high performance in research laboratory services and is a part of the national reference laboratory for COVID-19 and other emerging infectious diseases confirmation in Thailand.

CREID Research Center Collaboration

The goals and objectives of the proposed research project, "Immune memory bait & capture to identify emerging henipavirus origins", are fully consistent with the specific aims of EID-SEARCH. EID-SEARCH takes a One Health approach to understanding zoonotic disease emergence, with a focus on viral diversity in wildlife at high-risk human animal interfaces. EID-SEARCH aims to understand the frequency and causes of virus spillover in high-risk communities across Southeast Asia and identify strategies and tools to reduce this risk. As the proposed research focuses on the links between farming practices, wildlife diversity, virus diversity, and cross-species transmission in Thailand, we expect the results of the project to make a significant contribution towards the overall goals of EID-SEARCH.

The proposed project is well-designed, leverages cutting-edge technology, and includes a robust ecological component that corresponds to the overall research strategy of EID-SEARCH. The EID-SEARCH team is highly motivated to support the application of metagenomics to ecological questions and assist in the analysis of the unique dataset that will be generated from this research. With the heightened focus on identifying and limiting risks associated with the complex interactions between humans, wildlife, and domestic mammals that has resulted from the emergence of SARS-CoV-2 from a wet market in China, the proposed research is both timely and likely to yield highly-cited, impactful publications for EID-SEARCH and Thailand partners.

The EID-SEARCH team includes a number of experts in study design, laboratory-based diagnostics, genetic and genomic analyses, and ecological modelling who will contribute to project implementation, data analysis, and manuscript preparation as required. Communication and coordination mechanisms will be set up between the Thailand research team and scientists at the EID-SEARCH research sites for resource sharing, training, knowledge exchange, and mentorship. In particular, EID-SEARCH will facilitate and encourage relationships between our partners in Southeast Asia (based in Malaysia, Singapore, and Thailand) that will facilitate Thailand team professional development and strengthen research capacity in the region. Dr. Eric Laing, the project Mentor, has an established relationship with both Dr. Krongkan and Spencer Sterling, and will act as the liaison between EID-SEARCH and the TRC-EIDCC research team. Further, Dr. Doeuk who is the directior of the VRC PREMISE program is enthuasiatic about the opportunity to collaborate and apply their rapid assembly, transfection and production of immunoglobulin (RATP-Ig) workflow as a serologic/immunologic based approached for therapeutic and virus discovery (see Letter of Support). An expected outcome of this research project will be pilot data to support a joint funding application in the future.

Mentoring Plan

Goals for Pilot study and Career

Krongan Srimuang: My career goal is to be an expert scientist in either an academic research laboratory or a lecturer in medical science. My long-term research interests include the understanding of priority emerging infectious diseases, genetics, and epigenetics using molecular and serologic techniques. Detection of novel pathogens in their wildlife vectors can help characterize the pathogenicity, virulence, and evolution of these and other closely related pathogens. I believe that this pilot study can provide training in new techniques for the detection, identification, and characterization of novel pathogens that are currently unavailable in Thailand. My goal for the Pilot study is to gain more knowledge and skills for a lifelong career in public health-related research. I wish to expand my skills in novel virus detection using next-generation serologic techniques such as PBMC studies and memory B-cell isolation and sequencing that can contribute to improved surveillance and research capacity at human-animal interfaces. My mentors Dr. Laing and Dr. Wacharapluesadee can provide me with opportunities to develop and apply project skills and improve my scientific writing.

My education has provided me with a strong foundation to expand for this study. I received my undergraduate degree in Medical Technology from Chulalongkorn University which included training in molecular biology, serology, immunology, chemistry, virology, bacteriology, mycology, parasitology, hematology, clinical microscopy, transfusion medicine, and forensic medicine. My interest in molecular technology led me to pursue a Ph.D. at the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, which provided me with training in several molecular techniques including conventional PCR, real-time PCR, Next Generation Sequencing (NGS), etc. The emergence of COVID-19 motivated me to focus on emerging infectious diseases. I am currently completing my post-doctoral fellowship at the Emerging Infectious Diseases Clinical Center (EIDCC), King Chulalongkorn Memorial Hospital with Dr. Wacharpluesadee. Throughout this fellowship, I have continued learning advanced and innovative techniques to detect, identify, and characterize the novel pathogens. Through this pilot project, I will learn about the Peripheral Blood Mononuclear Cells (PBMC) which are a critical part of many subdivisions of medical research including Infectious diseases, Vaccine development, and Immunology. This project aims to study zoonotic reservoirs for by using serologic and cellular immune discovery in humans, and applying this immune knowledge in wildlife for early detection and identification of novel pathogens. This proposed study will expose me to various techniques and immunology topics that I useful in designing and testing new pipelines as technological advances continue to develop. This study will also allow me to develop, manage, and communicate my research.

Spencer Sterling: My overall career goal is to understand the factors that lead to viral zoonosis and to build epidemiological capacity in regions with the highest risk for zoonotic spillover. Through my training and educational experiences, I have learned laboratory and epidemiological techniques that can assist in the understanding of zoonosis. I wish to continue my education by expanding my repertoire of molecular techniques to include studies of the immune system's response to zoonotic disease in humans and animals, as well as to learn next-generation sequencing techniques. Additionally, I would like to use my experience in epidemiological techniques to train scientists in the Southeast Asian region in order to build local capacity in infectious disease research. Dr. Laing has been a mentor for me throughout my career, and this project would allow for this relationship to expand into new molecular areas, as well as grant

writing and project management skills. My professional relationship Dr. Wacharapluesadee with began in 2018 when I participated in a DTRA BTRP funding project that focused on transferring antigen-based multiplex serological technology to her lab group. Currently, I am a Visiting Scientist at TRCEIDCC and I work closely with Dr. Srimuang, and other members of Dr. Wacharapluesadee's research group on EID-SEARCH activities. Additionally, Dr. Wacharapluesadee has decades of experience in infectious disease research in the region and has mentored scientists that have gone on to careers at the Thai Ministry of Public Health, Department of Disease Control, Department of Parks and Wildlife, academia, and the private sector. Both mentors have encouraged Dr. Srimuang and I to apply for this pilot study as well as pursue other funding mechanisms for our research interests. Both mentors will provide for me the ability to independently pursue my research interests in the zoonotic epidemiology and train me on the best practices of scientific communication.

Vertebrate Animals Section Requirements

Animal samples will be collected in Ratchaburi Province for this project under Aim 2 to understand the antigenic relationships among known and unknown henipaviruses. Protocol to perform animal sample collection has been reviewed and approved by the Institutional Animal Care and Use Committee at Tuft University under the EID-SEARCH project (No. G2020-42) and the Institutional Animal Care and Use Committee at Chulalongkorn University (No. 019/2563)

1. Description of Procedures

Bats (order *Chiroptera*)

- Species: Free-ranging mega and micro-*Chiroptera* that present at the site (family *Pteropodiae, Rhinopomatidae, Emballonuridae, Craseonycteridae, Megadermatidae, Rhinolophidae, Hipposideridae, Vespertilionidae, Mollossidae*, etc.)
- Age & Sex: Fully flighted adults and juveniles, males and females; neonates dependent on the dam will not be used.
- Target Number: 100 individuals
- Capture and Restraint: Bats will be captured using either a mist net or harp trap. The net system is manned by at least two people during the entire capture period, and bats are removed from the net as soon as they become entangled to minimize stress and prevent injury. In our previous field research experience, a maximum of 20-30 bats can be safely held and processed by a team of three people per trapping period. Duration of trapping will depend on the capture rate. Bats are individually placed into a pillowcase or small cloth bag and hung from a branch or post in a location that is free from access by both predators and people, and is protected from the elements (e.g., rain), until the animal is restrained for sample collection. Bats will be manually restrained during sampling utilizing insulated bite gloves to prevent injury to the handler and bat. The study protocol covers over 69 bat species that are most likely to be captured given their natural occurrence and previous surveillance experience in Southeast Asia. The potential for non-target species to be captured is very low because mist nets are opened at the cave entrance at night, after other species (e.g., birds) have ceased flying and returned to roost. Any non-target species will be rapidly identified, removed from the mist net and released immediately.
- Sample Collection: Depending on the species and size of bat, blood will be collected from smaller insectivorous bats (<50g) using a 27g needle to puncture the brachial artery and a 70ul hematocrit tube to collect the blood. For larger bats (>50g) we will collect blood from either the cephalic vein or from the radial artery or vein using a 25- or 23-gauge needle and 3cc syringe. Blood will be collected

  collected to confirm host identification using DNA barcoding. Swabs will be taken from the oropharynx, urogenital tract, and rectum. No destructive sampling is planned, but any individuals that die during capture or sampling will have a necropsy performed with organ samples collected and whole body submitted to museums as voucher specimens.
- Release: Bats are held for a maximum of six hours (typically less than three hours), and at the completion of sampling are provided with rehydrating fluids (either subcutaneously or orally as needed) and released in a location that allows for further recovery or safe escape (e.g., bodies of water, known predatory areas, and human settlements will be avoided).

Rodents (order: *Rodentia*) and Shrew (order: *Scandentia* and *Eulipotyphla*)

- Species: Free-ranging rodents (family *Hystricidae, Muridae, Sciuridae, Spalacidae*) and shrew (tree shrews, shrews, moles) that present at the sites.
- Age & sex: Adults and juveniles, males and females. Neonates will not be sampled.
- Target Number: 100 individuals
- Capture and Restraint: Free-ranging wild mammal species will be captured with metal box traps (Sherman/Tomahawk traps). Traps will be prepared with food, water, padding and shelter, and be checked at least every 12 hours, in the morning and in the afternoon. If adverse weather (extreme heat, rain) is expected or researchers are working in areas where predation is common, traps will be checked more frequently and closed during adverse weather. Veterinary staff will be prepared to deal with a variety of trap injuries, including lacerations, broken teeth, and crush injuries. Depending on the species, individual size, captive or free-ranging status of the individual, manual or chemical restraint and anesthesia (gas or injectable anesthesia) will be applied. Animals will be manually restrained during sampling utilizing insulated bite gloves to prevent injury to the handler and animal. Based upon past field experience (>20 years of animal capture and release in Southeast Asia), we do not expect to trap sick, debilitated, or young animals that would be too young to cope with capture. Sick and debilitated animals tend not to roam widely, reducing their opportunity to enter traps. Additionally, should sick, debilitated, or young animals enter traps, we anticipate that they will not suffer adverse conditions because the traps contain food (the bait) and liquid (water or fruit as a source of water). These individuals would be examined by a veterinarian, rehydrated and if presenting clinical signs treated by a veterinarian, prior to release or sampling then release.
- Sample Collection: Once anesthetized or safely restrained, whole blood will be collected through the appropriate venipuncture site, no more than 1 ml of blood per 100 g (= 10 ml/kg or 1%) of body weight will be collected at any one time. Anesthetized animals will be monitored regularly during recovery until they can no longer be safely handled, at which point they will be confined in a trap or cage. Swabs will be taken from the oropharynx, urogenital tract, and rectum. No destructive sampling is planned, but any individuals that die during capture or sampling will have a necropsy performed with organ samples collected.
- Release: Wild mammals are held for a maximum of thirteen hours depending on trap timing, but typically less than three hours. Captive animals are held for a maximum of two hours but typically less than one hour. At the completion of sampling, animals may be provided with rehydrating fluids (either subcutaneously if anesthetized or orally if manually handled and accepted). Wild mammals will be released at the site of capture in a location that allows for further recovery or safe escape (e.g., bodies of water, known predatory areas, and human settlements will be avoided). Captive mammals will be released back into their captive setting as is appropriate for the species, either isolated or in social group if it is deemed safe from aggression from enclosure mates while the post-recovery period continues.

2. Justifications

The purpose of this study is to identify zoonotic pathogens through serologic and cellular immune discovery, including developing methods and standards for direct antibody-mediated virus capture in people and wildlife, focusing on rodent/shrew- and bat-borne HPVs. This will require hands-on fieldwork to collect whole blood and serum samples from bats, rodents, and shrews for lab analysis. One technique that avoids the direct capture and sampling of animals is to collect fresh feces or urine, but this will not be able to provide the appropriate samples for serologic analysis, and the research objectives cannot be

achieved using an alternative methods (e.g., computational, human, invertebrate, in vitro). Therefore, we believe there are no viable alternatives than the use of live animals.

3. Minimization of Pain and Distress

In every situation, sampling of wildlife will be conducted in the most humane manner while minimizing the impacts on individual animals and their wild populations. In all instances, the fewest number of animals will be sampled that will provide valid information and statistical inference for the pathogen and disease of interest and every effort will be made to minimize stress and discomfort for the animal.

Bats will not be held longer than six hours during the sampling process whereas small mammals may be held for up to 13 hours depending on trap capture timing. Field animal sampling team with zoologists and veterinarians have been well trained and have extensive experience in capture, anesthesia, and sampling of wildlife. In our team's experience, the target species tolerate the described procedure well. Mist nets will be attended continuously during capture periods, and bats will be extracted from the net as soon as they become entangled. This will minimize stress and injury from entanglement. Bats will be placed individually in cloth bags and hung from tree branches while awaiting processing and during recovery. The bags are sufficiently porous as to allow for ventilation and are designed for bat capture. The enclosed environment seems to calm the bats, as they do not struggle once inside, but they hang quietly – this is a standard and accepted practice in bat research and the best way to minimize stress to the animal.

For rodents and shrews, food, water, padding and shelter will be provided at traps, which will be checked at least every 12 hours. Traps will be checked more frequently or closed if adverse weather (extreme heat, rain) is expected, or researchers are working in areas where predation is common. Veterinary staff will be prepared to deal with a variety of trap injuries, including lacerations, broken teeth, and crush injuries. All animal individuals will be monitored by a veterinarian or experienced field team member during all stages of capture, processing, anesthesia and release. Animals will be kept in a cool place, free from adverse weather conditions and access by non-field team members or other animals while in the pillowcases, trap, or cage for recovery. All recovering individuals will be observed until they are able to effectively ambulate and orient to their surroundings. Animals that are injured during the capture or sampling process will be assessed by an experienced team leader or attending veterinarian, and if the animal is determined to be unlikely to survive if released, it shall be euthanized humanely (see euthanasia section).

4. Method of Euthanasia

In the event that an animal has been injured or is moribund, a determination will be made as to whether it may be treated and released on site by veterinarians or transferred to facility that may treat and rehabilitate the animal, or if, as a last resort, the animal will require euthanasia. Euthanasia methods will will not deviate from the AVMA “Guidelines for the Euthanasia of Animals” (2020 edition). Any animal that is euthanized using a chemical agent will be disposed of such that it will not be permitted to enter the food supply either through markets or hunting.

Human Subjects Research

1. Risks to the subjects

In this project, we will target populations in one community site who with frequent exposure to bats and other wildlife based on our previous behavioral survey. Subjects will be enrolled voluntarily, and informed consent will be obtained from all participants, along with assent from all participants aged 12-17. Enrolled participants will provide biological samples. Survey data and biological samples will be collected from enrolled participants, and follow-up data collection will be performed among participants whose samples were tested positive.
Biological samples and questionnaire data will be collected from individuals who meet recruitment and inclusion criteria and complete the informed consent process. During data collection, a standardized questionnaire will be administered to all participants. This questionnaire will collect information of demographic background, wildlife contract, travel and daily movement, and unusual SARI/ILI symptoms, and biological specimens will be collected from participants. Both questionnaire and biological data will be analyzed to assess the exposure to coronaviruses and the spread risk among humans. From all participants, a one-time whole blood sample (Max. 15 mL) will be collected during the study period. This sample will allow us to test for historical exposure to bat or rodent-born henipaviruses and collect Peripheral Blood Mononuclear Cells (PBMCs) for those study participants whose serum tests are positive.

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 who are 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, the administration will be conducted privately and confidentially to protect individuals' 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 these risks

2.1 Informed consent and assent

Only consented participants will be enrolled in the study. No research procedures will be undertaken before the participant fully understands the research, agrees to the study procedures, and completes the consent process will be enrolled in the study. Informed consent statements and forms, and project protocols will be translated into the local language of each study site. Research team members involved in this consent process will be required to be fluent in the local language to ensure that the subjects understand the study and all involved procedures.

If participants meet the criteria for enrollment, they will be invited to discuss the details of the study with the research staff. Study staff will review an information sheet and informed consent form with the participant and the parent or legal guardian when applicable. Each individual will be provided with a copy of the informed consent form that has been translated into the local language and written with a Flesch–Kincaid readability score equivalent to a 7th-grade reading level or below, to assure that potential participants understand the information being shared. The informed consent form will explain the details

of the study, including how and why the individual was selected, the study process and procedures, risks and benefits, financial considerations and the gift of appreciation, confidentiality of data shared, alternatives to participating, and how to obtain more information now or at a later date. The informed consent form will be read in the local language of the site at a location ensuring participant privacy. After which individuals will have as much time as they would like to ask questions and discuss the study with study staff. The study staff will endeavor to ensure that the participant understands the information provided. The study staff will then ask the participant/parent or legal guardian to consider study participation. Participants will have as much time as required to consider the participation.

Those participants who consent to the study will sign and date two copies of the consent form. These form copies will be countersigned and data by the study staff. A copy of the signed consent form must be provided to the subject and the other copy will be kept by study staff. Informed consent paperwork will be kept until the end of the project in a locked box at the local country project office.

2.2 Protection against risks

The potential risks to study participants as a result of study participation are minimal. Collection of venous blood samples and nasal or oropharyngeal swab samples pose minimal risk to subjects. Potential complications associated with venipuncture include pain and/or hematoma at the site of collection. Trained medical professionals and/or clinic staff will monitor the blood collection site and treat any complications according to existing health facility protocols. A potential complication of nasal/ oropharyngeal sampling is minor irritation at the time of collection. Employing trained medical and/or clinic staff to collect blood and swab samples will minimize the potential for complications.

Another risk that this study may pose concerns the information to be gained on henipaviruses newly recognized in the community. We will provide participating communities, hospitals, and clinicians with information and background data on target zoonotic viruses to ensure up-to-date communication of risk. Because of the timeline for diagnostic testing and results interpretation, we are not likely to provide results to participating clinics within a time frame that would be clinically relevant to outbreaks of undiagnosed diseases. Therefore, the information provided by this project will not impact patient management or outlook.

If an individual decides to participate in this research, his/her participation and all information provided by the participant will be strictly confidential, and personal identifying information will not be shared with anyone outside of the study staff. Participants will not be identified or named in any reports or publications. Questionnaire information and all biological samples will be identified by an alphanumeric code, not by the participant's name. 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 study site in locked files cabinets or password-protected devices in areas with access limited to the research staff of this project. Research databases will be secured with password-protected access systems and controlled distribution web-based certificates and will not contain any identifying characteristics about study participants (e.g., name, address, or telephone number). Access to all data will be limited to the staff involved in this study. The health information disclosed by an individual will not be used by or disclosed (released) to another institution. Any surveillance report that is published or shared with partners will not contain any personally identifying information for individual participants.

3. Potential benefits of the research to the subjects and others

There are no measurable benefits to the individual study participants enrolled in this study. There are benefits to the community and regional healthcare providers to help them understand the risk of zoonotic infections among high-risk populations in the regions they work. At the conclusion of the study, we will deliver an educational workshop reporting aggregate study findings that will be open to both study and non-study participants, describing the health benefits of using personal protection equipment (PPE) and handwashing during animal handling activities throughout the day, as well as to share other prevention/interventions that emerge from the research data.

4. Importance of the knowledge to be gained

There are valuable potential benefits to the public from the knowledge to be gained from this study. One key benefit to the community is sharing information and knowledge to better understand the risk of zoonotic spillover events and related health risks, as well as information sharing with communities on practices that could reduce risks, such as the avoidance of particular animal contacts or the need for PPE and extra care when handling wildlife, that may substantially reduce the risk zoonotic pathogen transmission in the community. 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 create or implement public health interventions to disrupt disease emergence and/or spread in an area that is beneficial to all. Additionally, there are valuable benefits to the general public from the knowledge to be gained from this study. Knowledge gained will 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.

5. Country- / institution-specific ethics / IRB regulations addressed

Main research protocol and materials to conduct human subject research in this project have been reviewed and approved by the Health Medical Lab Institutional Review Board, US (No. 894ECOH21b) and the Institute Review Board of the Faculty of Medicine, Chulalongkorn University, Thailand (No. 211/64). Amendment for PBMCs collection will be made and approved before the start of this project.

Research and Related Other Project Information

1. Are Human Subjects Involved?	Yes	X	No													
1.a. If YES to Human Subjects																
Is the Project Exempt from Federal regulations?	Yes		No	X												
If yes, check appropriate exemption number	1		2		3		4		5		6		7		8	
If no, is the IRB review Pending?	Yes	X	No													
IRB Approval Date:																
Human Subject Assurance Number	FWA00000943															

2. Are Vertebrate Animals Used?	Yes	X	No	
2.a. If YES to Vertebrate Animals				
Is the IACUC review Pending?	Yes		No	X
IACUC Approval Date	3 December 2020			
Animal Welfare Assurance Number				

3. Is proprietary/privileged information included in the application?	Yes		No	X

4.a. Does this project have an Actual or Potential impact -- positive or negative -- on the environment?	Yes		No	X
4.b. If yes, please explain				
4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed?	Yes		No	
4.d. If yes, please explain				

5. If the research performance site designated, or eligible to be designated, as a historic place?	Yes		No	X
5.a. If yes, please explain				

6. Does this project involve activities outside of the United States or partnership with international collaborators?	Yes	X	No	
6.a. If yes, identify countries	Thailand			
6.b. Optional explanation				

Budget Justification

A. Key Personnel (Total $24,000)

Dr. Krongkan Srimuang, PI (12 months) is a medical research scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital (TRC-EIDCC) who have been leading the laboratory analysis for the EID-SEARCH work in Thailand in the past three years to perform molecular, serological, and next-generation sequencing to identify novel viruses from human and animal samples. Dr. Srimuang will commit 12 months to this project to design, manage, and perform all project activities in the lab and field by supervising a lab technician and working with the field coordinator. Dr. Srimuang will also work closely with the co-PI and Mentors to conduct data analysis and develop manuscript and join EID-SEARCH and CREID meetings to prevent the project findings.

Spencer Sterling, Co-PI (6 months) is the research coordinator of Laing Lab at the Uniformed Services University for Indo-Pacific region and a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC). Sterling will commit 6 months to this project as the co-PI, who will work closely with the PI in the study design, SOP development, sampling process, data analysis of for serological investigations. No salary is required for Sterling.

B. Other Personnel (Total $20,000)

One (1) Laboratory Technician (12 months) will committee 12 months to this project to conduct PCR and serological testing of animal and human samples at TRC-EIDCC, under the supervision of Dr. Srimuang and Sterling. Salaries for 12 months at $1,250 per month is requested ($15,000).

One (1) Field Coordinator (4 months) will committee 4 months to this project, working closely with Dr. Srimuang and Sterling to organize and conduct field sample collection among humans and animals at the study sites, and support relevant financial management. Salaries for 4 months at $1,250 per month is requested ($5,000).

C. Fringe Benefits

No Fringe is requested.

D. Equipment

No equipment is requested.

E. Travel (Total $48,375, Years 1-5)

Domestic Travel ($11,005)
Support for domestic travel for field sampling is requested in the amount of $11,005 per to conduct four (4) trips of sample collection from humans, bats, and rodents/shrews.

- The first trip is a one-day trip to collect samples from human participants who were tested positive (n<10), the cost is estimated at van rental for local transportation between Bangkok and the study site ($260) and meals for five (5) field team members at $49 per person ($245), in total of $505 for the first trip.

- The other three (3) field trips will be conducted to collect samples from 100 bats, 100 humans, and 100 rodents/shrews, respectively. Each trip is a duration of 6 days and 5 nights. For each person, 5-night stay in hotels at $75.5/night (5 x $75.5 = $378); 3 meals per day and incidental expenses at $49 per day prorated to 75% ($36.75) for first and last day of travel ($36.75 x 2 + $49 x 4 = $270), the total meals and accommodations for each person per trip is $648 ($378+270), cost for five (5) field team members is $3,240. A van will be rented for local transportation between Bangkok and the study site that is estimated at $260 per trip. Total cost for each 6-day trip is $3,500, three trips will be $10,500.

International Travel ($5,023)

Support for international travel for Dr. Srimuang from Bangkok to Washington DC to attend the CREID Annual Meeting and receive lab training at the National Institute of Allergy and Infectious Diseases (NIAID) is requested in the amount of $5,023. Trips are for a duration of 10 days and 9 nights. Travel costs for one trip are estimated using federal per diem rates for hotels, meals, and incidental expenses as follows: nine-night stay in DC area hotel at $286/night (9 x $258 = $2,322); Ten days' meals and incidental expenses at $79 per day prorated to 75% ($59.25) for first and last day of travel ($59.25 x 2 + $79 x 8 = $750.5); Round trip flight between Bangkok to Dulles International Airport is estimated at $1,650; local transportation/taxi between hotel and airport per round trip between Chulalongkorn Hospital to airport is estimated at $40, between hotel and the airport in the US is estimated at $200, and $60 for transportation within the region ($300).

F. Participant Support Costs

No participant support is requested.

G. Other Direct Costs (Total $78,803)

Materials and Supplies ($72,830)

PBMCs isolation ($47,000). We request funds to conduct PBMC isolation from collected sample, costs including the reagents and lab consumable and supplies are estimated at $47 per cell for 1,000 cells.

Multiplex microsphere immunoassay detection ($2,275). Funds are requested to perform serological testing of collected human and animal samples. Costs including the reagents and lab consumable and supplies are estimated at $6.5 per sample for 350 samples.

PCR testing and sequencing ($20,475). Funds are requested to perform serological testing of collected human and animal samples. Costs including the reagents and lab consumable and supplies are estimated at average $58.5 per sample 350 samples, given a 10%~15% positive rate.

Field supplies for PBMC collection and processing ($1,225). Funds are requested to perform PBMCs collection and processing in the field. Costs including the reagents and lab consumable and supplies are estimated at average $3.5 per sample 350 samples.

Field supplies for animal sampling ($1,160). Funds are requested to purchase supplies for animal capture, anesthesia, and sample collection. A total cost of $1,160 is estimated

Field work disposable ($668). Funds are requested to purchase disposable materials (tips, tubes, gloves, masks, googles etc.) for field work.

Consultants Service ($3,000)
One (1) zoologist and one (1) veterinarian will provide consultancy service to this project to help 1) select sites for animal sampling and join the sampling trip to 2) identify species; and 3) provide veterinary care in the sample collection of bats, rodents, and shrews. The consultancy fee is estimated at $1,500 per person for the project, in total of $3,000.

Publication Fee ($3,000)
We request $3,000 of publication costs for one peer-review paper generated from the project. The cost is estimated at the reimbursed Open Access fees of $2,900 - $5,000 per manuscript in the past two years for six peer-review papers.

H. Total Direct Costs (Total $138,831)

I. Indirect Costs (Total $11,106)

An indirect cost at 8% is requested.

J. Total Direct and Indirect Costs (Total $149,937)

A total amount of $149,937 is requested.

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: Srimuang, Krongkan

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

POSITION TITLE: Research 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
Chulalongkorn University, Bangkok, Thailand	B.Sc.	03/2012	Medical Technology
Mahidol University, Bangkok, Thailand	Ph.D.	04/2017	Molecular Tropical Medicine and Genetics

A. Personal Statement

I am a medical technologist. My undergraduate training has provided me with a background in multiple biological disciplines including molecular biology, microbiology, and genetics. During my Ph.D., I conducted research with Dr. Mallika Imwong focused on Malaria. My thesis was entitled “Exploring the molecular mechanism of mefloquine resistance in *Plasmodium falciparum*”. I conducted research on drug resistance to malaria in Southeast Asia. Specifically, my focus was on molecular techniques in identifying the genetic mutations involved with drug resistance. Moreover, I got experience in *Plasmodium falciparum* malaria culture. After my Ph.D., I worked at the genetics laboratory at Jetanin Institute of Assisted Conception. My responsibilities included Preimplantation Genetic Testing (PGT) is screen embryos for genetic abnormalities such as chromosome copy number gains or losses (PGT-A, aneuploidy screening), single gene disorders (PGT-M, monogenic or single-gene disease), or structural rearrangement (PGT-SR, structural rearrangement to identify embryos with chromosome imbalanced) by using Next Generation Sequencing. Additionally, I conducted genetic screening in couples for detecting carrier diseases such as glucose-6-phosphate dehydrogenase (G6PD), Alpha thalassemia, Beta thalassemia, and Spinal muscular atrophy (SMA) by using molecular genetics techniques. Currently, I am a Research Scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC EIDCC), King Chulalongkorn Memorial Hospital where I investigate emerging infectious diseases under the guidance of Dr. Supaporn Wacharapluesadee. The research focuses on detecting, identifying, and characterizing pathogens and human genome segments using molecular and serology techniques, including Next Generation Sequencing and Multiplex serology assays as a part of EID-SEARCH project. Additionally, I am the laboratory sequencing lead on the Surveillance System: Emerging Viral Pneumonia and Respiratory tract infections (RTIs) project from patients in Thailand as part of a collaboration with the Department of Disease Control.

1. **Srimuang K**, Miotto O, Lim P, Fairhurst RM, Kwiatkowski DP, Woodrow CJ, Imwong M; Tracking Resistance to Artemisinin Collaboration. “Analysis of anti-malarial resistance markers in *pfmdr1* and *pfcrt* across Southeast Asia in the Tracking Resistance to Artemisinin Collaboration” Malar J. 2016 Nov 8;15(1):541.
2. Reamtong O, **Srimuang K**, Saralamba N, Sangvanich P, Day NP, White NJ, Imwong M. “Protein profiling of mefloquine resistant *Plasmodium falciparum* using mass spectrometry- based proteomics” Int J Mass Spectrom. 2015 Nov 30;391:82-92

3. **Srimuang K**, Udompat P, Thippamom N, Petchrat S, Doung-Ngern P, Kripattanapong S, Supataragul S, Sterling SL, Wacharapluesadee S, Putcharoen O. “Genetic characterization of Norovirus strains from an unusual diarrheal outbreak in the community in Eastern Thailand” (Submitted, January 2023)

B. Positions, Scientific Appointments, and Honors

Positions and Scientific Appointments:

02/2022 – Present	Medical research scientist, Emerging Infectious Diseases Clinical Center (EIDCC), King Chulalongkorn Memorial Hospital
05/2017 - 01/2022	Scientist, Genetics Laboratory, Jetanin Institute for Assisted Reproduction
10/2015 - 03/2016	Fellowship at Laboratory of Molecular and Cellular Parasitology, Department of Microbiology and Immunology, National University of Singapore, Singapore.
11/2011 – 02/2012	Training Medical Technology Programs at Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

Honors:

2012 -2017	Scholarship for Ph.D. from the Royal Golden Jubilee, Ph.D. Grant (14th Batch).

C. Contributions to Science

Undergraduate Research: I participated in research activities under Dr. Attakorn Palasuwan to study in human genetic variants in proteins, such as G6PD, as they relate to the effect of exercise on the reduction of free radicals in the body. We conducted the multiplex PCR to identify the G6PD variants and found 8 variants including Vanua Lava, Mahidol, Mediterranean, Coimbra, Viangchan, Union, Canton, and Kaiping.

Graduate Research: I continued to studies under Dr. Mallika Imwong in the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University. My thesis, “Exploring the molecular mechanism of mefloquine resistance in *Plasmodium falciparum*”, focused on the evolution of resistance to mefloquine, which is commonly used in Southeast Asia in Malaria treatment. The mechanism of mefloquine resistance remains unknown, but I focused on the *Pfmdr1* gene which is involved in the drug resistance mechanism in *Plasmodium falciparum.* I found mutations and polymorphisms in *Pfmdr1* gene.

1. **Srimuang K**, Miotto O, Lim P, Fairhurst RM, Kwiatkowski DP, Woodrow CJ, Imwong M; Tracking Resistance to Artemisinin Collaboration. “Analysis of anti-malarial resistance markers in *pfmdr1* and *pfcrt* across Southeast Asia in the Tracking Resistance to Artemisinin Collaboration” Malar J. 2016 Nov 8;15(1):541.
2. Reamtong O, **Srimuang K**, Saralamba N, Sangvanich P, Day NP, White NJ, Imwong M. “Protein profiling of mefloquine resistant *Plasmodium falciparum* using mass spectrometry- based proteomics” Int J Mass Spectrom. 2015 Nov 30;391:82-92

Post-graduate Research: I was a scientist in genetics lab at Jetanin, a holistic center for infertility, offering counseling and treatment with Assisted Reproductive Technologies such as IVF, ICSI, and Preimplantation Genetic Testing (PGT). My work included the development of standard operating procedures for the lab, including Next Generation Sequencing, for detecting and identifying chromosomes in blastocyst growth to an embryo. Additionally, we assisted couples who had a family history of genetic diseases for screening before pregnancy. The carrier screening focused on highly prevalent diseases in Thailand such as alpha thalassemia, beta thalassemia, G6PD, and SMA. Currently, I am a Research Scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital. I am currently tasked with using molecular and serological techniques in the surveillance of novel, exotic, and known pathogens in wildlife and humans.

1. **Srimuang K**, Udompat P, Thippamom N, Petchrat S, Doung-Ngern P, Kripattanapong S, Supataragul S, Sterling SL, Wacharapluesadee S, Putcharoen O. “Genetic characterization of Norovirus strains from an unusual diarrheal outbreak in the community in Eastern Thailand” (Submitted, January 2023)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contr butors.
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

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: Assistant Professor

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. (hons)	05/2008	Biology
Uniformed Services University, Bethesda, MD	Ph.D.	10/2016	Emerging Infectious Diseases
Uniformed Services University, Bethesda, MD	Postdoc	04/2019	Virology

A. Personal Statement

The spillover of zoonotic viruses into human populations remains an ever-prevalent threat to public and global health. Continual outbreaks of known and unknown zoonotic viruses highlights the paucity of our understanding of the viral diversity, geographic distribution, wildlife hosts, and human populations at-risk for spillover. I am a recently appointed assistant professor dedicated to biosurveillance and virological research of emergent zoonotic viruses. I have completed virological training and spent my time as a post-doctoral fellow applying *in vitro* techniques to characterize the replication of, and mammalian ephrin receptors that mediate cellular entry of emergent henipaviruses. Since the start of the COVID-19 pandemic, I pivoted the focus of my research team to serological analysis of SARS-CoV-2 infection, and then to COVID-19 vaccine-induced antibody durability. In my ongoing research, I develop serological-based approaches to identify the infectome of wildlife and human communities for the assessment of virus spillovers. Since my position as a postdoctoral fellow I have worked collaboratively with national and international researchers including institutional partners at Duke-NUS, Chulalongkorn University, University of Pretoria, and Rocky Mountain Labs towards these goals. Across all of these collaborations, we have aimed to identify known and unknown viruses, the wildlife reservoirs of priority pathogens, the high-risk interfaces for spillover and the biotic/abiotic drivers of virus emergence. Throughout these research projects, we have detected unexpected serological profiles in communities of bats and humans that have not fit our prior understanding of the viral diversity established or detected by genetic techniques. This proposal aims to better understand these serological profiles of wildlife and humans with a targeted focus on henipaviruses and filoviruses. Ongoing projects that I would like to highlight include:

HDTRA12110037. DTRA BRTP, E. Laing (Co-PI). 08/2021-07/2026, “Informing biosurveillance, contribution of pteropodid fruit bats to virus spillover in the Philippines.”
HU00012020067, HU00012120104, HU00012120094.Defense Health Program/CARES Act, NIAID, E. Laing (Associate Investigator). 9/2020-9/2025, “Prospective Assessment of SARS-CoV-2 Seroconversion.”
HDTRA12010025. DTRA BTRP, E. Laing (Co-I). 7/2020 – 6/2025, “Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa.”
U01AI151797. NIH, Centers for Research in Emerging Infectious Diseases, E. Laing (Co-I). 02/2020 – 03/2025, “EID-Southeast Asia Research Collaborative Hub.”
HU00012020067, HU00011920111. Defense Health Program, NIAID, E. Laing (Associate Investigator). 03/2020 – 09/2023, “Epidemiology, Immunology, and Clinical Characteristics of Emerging Infectious Diseases with Pandemic Potential (EpICC-EID).”

B. Positions, Scientific Appointments and Honors

Positions

2021-pres. Assistant Professor, Department of Microbiology and Immunology, School of Medicine, Uniformed Services University, Bethesda, MD.

2021-pres. Joint Appointment, Emerging Infectious Diseases Graduate Program, School of Medicine, Uniformed Services University, Bethesda, MD.

2019-21 Research Assistant Professor, Department of Microbiology and Immunology, Uniformed Services University, Bethesda, MD.

2016-19 Postdoctoral fellow, Henry M. Jackson Foundation, Department of Microbiology, Uniformed Services University, Bethesda, MD.

2010-16 Graduate research student, Department of Microbiology, Uniformed Services University, Bethesda, MD.

Other Experiences and Professional Memberships

2022-pres. Executive committee member, Emerging Infectious Diseases Graduate Program, USUHS

2021-pres. Research center representative, Laboratory Assays Oversight and Quality Working Group, Emerging Infectious Diseases: Southeast Asia Research Collaboration Hub (EID-SEARCH), Centers for Research in Emerging Infectious Diseases (CREID), NIAID, DMID, NIH

2021-pres. Review editor, Frontiers in Virology - Emerging and Reemerging Viruses

2021-pres. Ad hoc reviewer, Frontiers in Immunology, Journal of Clinical Immunology

2021-pres. Steering committee member, JPI/Military Infectious Diseases Research Program, Emerging Infectious Diseases

2019 Ad hoc reviewer, EcoHealthNet 2.0 Program, EcoHealth Alliance

2018 Ad hoc reviewer, Pakistan One-Health Fellowship Program, National Academy of Sciences & Pakistan Academy of Sciences

2014-2019 Member, American Society of Tropical Medicine and Hygiene

2014-2019 Member, American Society of Microbiology

2014-2019 Volunteer, AAAS STEM K-12 Volunteer Program

Mentoring

Postdoctoral fellows Si'Ana A. Coggins, PhD, 2020 - 2022

Graduate students Marana S. Tso, BS, 2021 -
McKenna Roe, BS, 2022 -

Committee member Celeste Huaman, BS, 2021 -
2LT Connor Perry, BS, 2021 -

Honors

2021-2022 Impact Award, USUHS School of Medicine

2021 Outstanding Research Accomplishment/Team/SARS-CoV-2, The EPICC COVID-19 Cohort Team, Military Health System Research Symposium

2020-2021 Impact Award, USUHS School of Medicine

2015-2016 Val G. Hemming Fellowship, Henry M. Jackson Foundation

2015 East Asia and Pacific Summer Institutes Fellowship, National Science Foundation

C. Contributions to Science (†*mentee*, *corresponding)

1. Lyssaviruses and the prototype, rabies virus, remain a public health concern. Beginning with my PhD thesis work, I've researched the virus host-interactions between a rabies-related lyssavirus, Australian bat lyssavirus (ABLV), and its bat host (*Pteropus alecto*). Research has focused on ABLV cellular entry mechanisms, the development of an animal model and ABLV reporters and exploration of novel monoclonal antibodies that neutralize ABLV and other phylogroup I lyssaviruses. Furthermore, comparative bat immunology research was conducted using black flying fox cell lines and ABLV as a model virus/host interaction. Physiological adaptations that accompanied the evolution of flight in bats have been proposed to contribute to the frequent role of bats as asymptomatic hosts of highly pathogenic zoonotic viruses.

Comparatively studying the autophagy pathway in bat cell lines revealed that bat cells had elevated levels of basal autophagy and experienced significantly less cell death when challenged with high virus doses.

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. Virol J. 2013. 11:40. doi: 10.1186/1743-422X-11-40. PMID: 24576301, PMCID: PMC3946599

b. **Laing E.D.***, Sterling S.L., Weir D.L., Beauregard C.R., Smith I.L., Larsen S.E., Wang L-F., Snow A.L., Schaefer B.C., and Broder C.C. Enhanced autophagy contributes to reduced viral infection in black flying fox cells. Viruses. 2019. Mar 14;11(3). pii: E260. doi: 10.3390/v11030260. PMID: 30875748, PMCID: PMC6466025

c. Mastraccio K.E., Huaman C., Warrilow D., Smith G.A., Craig S.B., Weir D.L., **Laing E.D.**, Smith I., Broder C.C. and B.C. Schaefer. Establishment of a longitudinal pre-clinical model of lyssavirus infection. J Virol Methods. 2020 Jul; 281:113882. doi: 10.1016/j.jviromet.2020.113882. Epub 2020 May 12. PMID: 32407866

d. Weir D.L., Coggins S.A., Vu B.K., Coertse J., Yan L., Smith I.L., **Laing E.D.**, Markotter W., Broder C.C., and Schaefer B.C. Isolation and characterization of cross-reactive human monoclonal antibodies that potently neutralize Australian bat lyssavirus variants and other phylogroup 1 lyssaviruses. Viruses. 2021 Mar 1;13(3):391. doi: 10.3390/v13030391. PMID: 33804519; PMCID: PMC8001737.

2. My research experience as a postdoctoral fellow furthered my training in molecular virology techniques. I constructed a recombinant Cedar virus cDNA plasmid and optimized a reverse genetics approach to rescue a recombinant Cedar virus reporter virus, a non-pathogenic *Henipavirus* species. A molecular biology methods chapter detailing recombinant Cedar virus reverse genetics has been submitted and in press. Using this recombinant Cedar virus, we determined that Cedar virus can utilize several non-canonical henipavirus ephrin receptors for cellular entry and explored the structure of the receptor-binding pocket to understand the receptor promiscuity. The non-pathogenic phenotype of CedV creates a potential for CedV to act as a model henipavirus to explore host-pathogen interactions, cellular tropism and factors that determine henipaviral disease pathogenesis. Additionally, I have collaborated on projects detailing henipavirus infection and replication in bat hosts with colleagues at the Rocky Mountain Labs, studying whether specific species of bats are more competent hosts and whether virus-host restriction exists.

a. Amaya M, Broder CC, **Laing ED***. Recombinant Cedar virus: a henipavirus reverse genetics platform. In: Freiberg A.N. and B. Rockx, Nipah Virus: Methods and Protocols, Methods Mol. Biol. (in press)

b. Seifert SN, Letko MC, Bushmaker T, **Laing ED**, Saturday G, Meade-White K, van Doremalen N, Broder CC, Munster VJ. Rousettus aegyptiacus Bats Do Not Support Productive Nipah Virus Replication. J Infect Dis. 2020 May 11;221(Suppl 4):S407-S413. doi: 10.1093/infdis/jiz429. PMID: 31682727; PMCID: PMC7199784.

c. **Laing ED,** Navaratnarajah CK, Cheliout Da Silva S, Petzing SR, Xu Y, Sterling SL, Marsh GA, Wang LF, Amaya M, Nikolov DB, Cattaneo R, Broder CC, Xu K. Structural and functional analyses reveal promiscuous and species specific use of ephrin receptors by Cedar virus. Proc Natl Acad Sci U S A. 2019 Oct 8;116(41):20707-20715. doi: 10.1073/pnas.1911773116. Epub 2019 Sep 23. PMID: 31548390; PMCID: PMC6789926.

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. PMID: 29587789; PMCID: PMC5869790.

3. Bats are increasingly identified as animal reservoirs of emerging zoonotic viruses (e.g. Nipah virus, Ebola virus and SARS-coronavirus). I lead collaborative biosurveillance and research preparedness training including data analysis and interpretations at international partner institutes with lab technicians, field and lab scientists, and masters, doctoral and postdoctoral trainees. Collaborative biosurveillance is presently underway in Thailand (Chulalongkorn University, Bangkok) Malaysia (National Wildlife and Forensic Lab, Universti Purtra Malaysi, National Public Health Lab) via NIH Centers for Research in

Emerging Infectious Diseases, EID-Southeast Asia Research Collaborative Hub. As a collaborator within the DARPA PREEMPT network I supported surveillance for coronaviruses and other priority emerging zoonotic viruses, henipaviruses and filoviruses, in Ghana (Zoological Society of London), Australia (Black Mountain Labs) and Bangladesh (icddr,b). We aim to characterize the geographic distribution of zoonotic filoviruses/henipaviruses/coronaviruses, transmission dynamics in wildlife hosts and generate risk-models for Ebola virus, Nipah virus and SARS-related CoV outbreaks. Results discovered so far suggest a wider geographical footprint of Asiatic filoviruses and have identified several fruit bat species that act as natural reservoirs for these viruses.

a. Paskey AC, Ng JHJ, Rice GK, Chia WN, Philipson CW, Foo RJH, Cer RZ, Long KA, Lueder MR, Lim XF, Frey KG, Hamilton T, Anderson DE, **Laing ED**, Mendenhall IH, Smith GJ, Wang LF, Bishop-Lilly KA. Detection of Recombinant Rousettus Bat Coronavirus GCCDC1 in Lesser Dawn Bats (Eonycteris spelaea) in Singapore. Viruses. 2020 May 14;12(5):539. doi: 10.3390/v12050539. PMID: 32422932; PMCID: PMC7291116.

b. Schulz JE, Seifert SN, Thompson JT, Avanzato V, Sterling SL, Yan L, Letko MC, Matson MJ, Fischer RJ, Tremeau-Bravard A, Seetahal JFR, Ramkissoon V, Foster J, Goldstein T, Anthony SJ, Epstein JH, **Laing ED**, Broder CC, Carrington CVF, Schountz T, Munster VJ. Serological Evidence for Henipa-like and Filo-like Viruses in Trinidad Bats. J Infect Dis. 2020 May 11;221(Suppl 4):S375-S382. doi: 10.1093/infdis/jiz648. PMID: 32034942; PMCID: PMC7213578.

c. Dovih P, **Laing ED**, Chen Y, Low DHW, Ansil BR, Yang X, Shi Z, Broder CC, Smith GJD, Linster M, Ramakrishnan U, Mendenhall IH. Filovirus-reactive antibodies in humans and bats in Northeast India imply zoonotic spillover. PLoS Negl Trop Dis. 2019 Oct 31;13(10):e0007733. doi: 10.1371/journal.pntd.0007733. Erratum in: PLoS Negl Trop Dis. 2021 Nov 16;15(11):e0009836. PMID: 31671094; PMCID: PMC6822707.

d. **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. PMID: 29260678; PMCID: PMC5749470.

4. Responding to the emergence of SARS-CoV-2, our lab developed multiplex serology strategies to identify SARS-CoV-2 antibodies and address research questions related to whether pre-existing antibody memory induced by prior infection with seasonal human coronaviruses effects COVID-19 severity. Our lab's serology efforts supported NIH and DHA collaboratively funded protocols including prospective, longitudinal serological analysis of hospital and community subjects, and health-care workers; and cross-sectional analyses of SARS-CoV-2 infection among deployed military health-care workers.

a. Epsi NJ, Richard SA, Lindholm DA, Mende K, Ganesan A, Huprikar N, Lalani T, Fries AC, Maves RC, Colombo RE, Larson DT, Smith A, Chi SW, Maldonado CJ, Ewers EC, Jones MU, Berjohn CM, Libraty DH, Edwards MS, English C, Rozman JS, Mody RM, Colombo CJ, Samuels EC, Nwachukwu P, Tso MS, Scher AI, Byrne C, Rusiecki J, Simons MP, Tribble D, Broder CC, Agan BK, Burgess TH, **Laing ED**, Pollett SD; EPICC COVID-19 Cohort Study Group. Understanding 'hybrid immunity': comparison and predictors of humoral immune responses to SARS-CoV-2 infection and COVID-19 vaccines. Clin Infect Dis. 2022 May 24:ciac392. doi: 10.1093/cid/ciac392. Epub ahead of print. PMID: 35608504; PMCID: PMC9213853.

b. Lu Z, **Laing ED**, Pena DaMata J, Pohida K, Tso MS, Samuels EC, Epsi NJ, Dorjbal B, Lake C, Richard SA, Maves RC, Lindholm DA, Rozman JS, English C, Huprikar N, Mende K, Colombo RE, Colombo CJ, Broder CC, Ganesan A, Lanteri CA, Agan BK, Tribble D, Simons MP, Dalgard CL, Blair PW, Chenoweth J, Pollett SD, Snow AL, Burgess TH, Malloy AMW; EPICC COVID-19 Cohort Study Group. Durability of SARS-CoV-2-Specific T-Cell Responses at 12 Months Postinfection. J Infect Dis. 2021 Dec 15;224(12):2010-2019. doi: 10.1093/infdis/jiab543. PMID: 34673956; PMCID: PMC8672777.

c. Pollett SD, Richard SA, Fries AC, Simons MP, Mende K, Lalani T, Lee T, Chi S, Mody R, Madar C, Ganesan A, Larson DT, Colombo CJ, Colombo R, Samuels EC, Broder CC, **Laing ED**, Smith DR, Tribble D, Agan BK, Burgess TH. The SARS-CoV-2 mRNA vaccine breakthrough infection phenotype includes significant symptoms, live virus shedding, and viral genetic diversity. Clin Infect Dis. 2021 Jun 12:ciab543. doi: 10.1093/cid/ciab543. Epub ahead of print. PMID: 34117878.

d. Clifton GT, Pati R, Krammer F, **Laing ED**, Broder CC, Mendu DR, Simons MP, Chen HW, Sugiharto VA, Kang AD, Stadlbauer D, Pratt KP, Bandera BC, Fritz DK, Millar EV, Burgess TH, Chung KK. SARS-CoV-2 Infection Risk Among Active Duty Military Members Deployed to a Field Hospital - New York City, April 2020. MMWR Morb Mortal Wkly Rep. 2021 Mar 5;70(9):308-311. doi: 10.15585/mmwr.mm7009a3. PMID: 33661864; PMCID: PMC7948931.

5. In addition to providing serologic assessment of SARS-CoV-2 infection, my research team is actively engaged in examining the durability of COVID-19 vaccine induced humoral immunity. Antibody responses, particularly neutralizing antibodies, are frequently cited as a predictive correlate of protection. With the emergence of variants of concern and waning circulating antibodies, the timing of booster shots remains an important measure for controlling the pandemic. In my lab we evaluate the duration of neutralizing antibodies, durability and breadth of antibody responses against emerging variants of concern, hybrid immune responses, and post-vaccination infections.

a. Wang W, Lusvarghi S, Subramanian R, Epsi NJ, Wang R, Goguet E, Fries AC, Echegaray F, Vassell R, Coggins SA, Richard SA, Lindholm DA, Mende K, Ewers EC, Larson DT, Colombo RE, Colombo CJ, Joseph JO, Rozman JS, Smith A, Lalani T, Berjohn CM, Maves RC, Jones MU, Mody R, Huprikar N, Livezey J, Saunders D, Hollis-Perry M, Wang G, Ganesan A, Simons MP, Broder CC, Tribble DR, **Laing ED**, Agan BK, Burgess TH, Mitre E, Pollett SD, Katzelnick LC, Weiss CD. Antigenic cartography of well-characterized human sera shows SARS-CoV-2 neutralization differences based on infection and vaccination history. Cell Host Microbe. 2022 Dec 14;30(12):1745-1758.e7. doi: 10.1016/j.chom.2022.10.012. Epub 2022 Oct 21. PMID: 36356586; PMCID: PMC9584854.

b. **Laing ED,** Weiss CD, Samuels EC, Coggins SA, Wang W, Wang R, Vassell R, Sterling SL, Tso MS, Conner T, Goguet E, Moser M, Jackson-Thompson BM, Illinik L, Davies J, Ortega O, Parmelee E, Hollis-Perry M, Maiolatesi SE, Wang G, Ramsey KF, Reyes AE, Alcorta Y, Wong MA, Lindrose AR, Duplessis CA, Tribble DR, Malloy AMW, Burgess TH, Pollett SD, Olsen CH, Broder CC, Mitre E. Durability of Antibody Response and Frequency of SARS-CoV-2 Infection 6 Months after COVID-19 Vaccination in Healthcare Workers. Emerg Infect Dis. 2022 Apr;28(4):828-832. doi: 10.3201/eid2804.212037. Epub 2022 Feb 24. PMID: 35203111; PMCID: PMC8962883.

c. Lusvarghi S, Pollett SD, Neerukonda SN, Wang W, Wang R, Vassell R, Epsi NJ, Fries AC, Agan BK, Lindholm DA, Colombo CJ, Mody R, Ewers EC, Lalani T, Ganesan A, Goguet E, Hollis-Perry M, Coggins SA, Simons MP, Katzelnick LC, Wang G, Tribble DR, Bentley L, Eakin AE, Broder CC, Erlandson KJ, **Laing ED**, Burgess TH, Mitre E, Weiss CD. SARS-CoV-2 BA.1 variant is neutralized by vaccine booster-elicited serum but evades most convalescent serum and therapeutic antibodies. Sci Transl Med. 2022 May 18;14(645):eabn8543. doi: 10.1126/scitranslmed.abn8543. Epub 2022 May 18. PMID: 35380448; PMCID: PMC8995032.

d. [†]*Coggins SA*, **Laing ED**, Olsen CH, Goguet E, Moser M, Jackson-Thompson BM, Samuels EC, Pollett SD, Tribble DR, Davies J, Illinik L, Hollis-Perry M, Maiolatesi SE, Duplessis CA, Ramsey KF, Reyes AE, Alcorta Y, Wong MA, Wang G, Ortega O, Parmelee E, Lindrose AR, Snow AL, Malloy AMW, Letizia AG, Ewing D, Powers JH, Schully KL, Burgess TH, Broder CC, Mitre E. Adverse Effects and Antibody Titers in Response to the BNT162b2 mRNA COVID-19 Vaccine in a Prospective Study of Healthcare Workers. Open Forum Infect Dis. 2021 Nov 20;9(1):ofab575. doi: 10.1093/ofid/ofab575. PMID: 35047649; PMCID: PMC8759445.

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: Wacharapluesadee, Supaporn

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

POSITION TITLE: Senior Researcher

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
Chiang Mai University, Thailand	B.S.	02/1991	Medical Technology
Mahidol University, Thailand	M.S.	01/1994	Biochemistry
Chulalongkorn University, Thailand	Ph.D.	03/2006	Biomedical Sciences

A. Personal Statement

I have 29 years in public health research and 20+ years of experience in emerging viral zoonoses. I have managed many internationally funded research projects that involve working with and managing international and local interdisciplinary teams. Most of my research projects are diagnostics development, pathogen discovery, public health surveillance, field surveillance in wild mammals, human behavioral risk surveys, and clinical research. I conduct workshops on the development of novel diagnostic approaches, appropriate sample collection and handling for different pathogens, and viral characterization *in vitro* and *in vivo*. I am a senior researcher at the Thai Red Cross Emerging Infectious Diseases Clinical Center, which conducts research on emerging zoonoses, clinical research, and public health surveillance research. My research background mainly focuses on understanding the process of zoonotic disease emergence, particularly viral zoonoses. This includes identifying the bat origin of Nipah virus, MERS-CoV, and SARS-CoV-2 and pathogenesis and diagnoses of Rabies. My studies on the emergence of novel betacoronaviruses found in Thai bats and Nipah virus have been published. My laboratory has supported the Department of Disease Control (DDC) in diagnosing emerging infectious diseases, communicable diseases, and wastewater surveillance. I am the DDC instructor on the clinical sampling method and consultant for Ebola, MERS, and COVID-19 diagnosis. I am a committee member of the Global Laboratory Leadership Program (GLLP) Thailand, organized by the Department of Medical Sciences. I led the team to diagnose the first human MERS case in 2015 and the first human COVID-19 case outside China in January 2020. I have been the PI on six multidisciplinary research projects that use epidemiology, laboratory, field science, and bioinformatics to diagnose and monitor the emergence of wildlife-origin viral zoonoses, including SARS-CoV, Nipah, and Hendra viruses, Avian influenza, and novel viruses from bats. I am also the Thailand country coordinator for PREDICT project, large contracts from USAID during 2010-2019 involving the successful management of teams of virologists, field biologists, veterinarians, epidemiologists, hospitals, and laboratorians.

1. **Wacharapluesadee S**, Buathong R, Iamsirithawon S, Chaifoo W, Ponpinit T, Ruchisrisarod C, Sonpee C, Katasrila P, Yomrat S, Ghai S, Sirivichayakul S, Okada P, Mekha N, Karnkawinpong O, Uttayamakul S, Vachiraphan A, Plipat T, Hemachudha T. Identification of a Novel Pathogen Using Family-Wide PCR: Initial Confirmation of COVID-19 in Thailand. Front Public Health. 2020 Oct 7;8:555013. doi: 10.3389/fpubh.2020.555013. PMID: 33134237; PMCID: PMC7579402.

2. Buathong R, Chaifoo W, Iamsirithaworn S, **Wacharapluesadee S**, Joyjinda Y, Rodpan A, Ampoot W, Putcharoen O, Paitoonpong L, Suwanpimolkul G, Jantarabenjakul W, Petcharat S, Bunprakob S, Ghai S, Prasithsirikul W, Mungaomklang A, Plipat T, Hemachudha T. Multiple clades of SARS-CoV-2 were introduced to Thailand during the first quarter of 2020. Microbiol Immunol. 2021 Oct;65(10):405-409. doi: 10.1111/1348-0421.12883. Epub 2021 Sep 1. PMID: 33835528; PMCID: PMC8251142.
3. **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. Nat Commun. 2021 Feb 9;12(1):972. doi: 10.1038/s41467-021-21240-1. Erratum in: **Nat Commun**. 2021 Feb 25;12(1):1430.
4. **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 Jul 5;3(1):12. doi: 10.1186/s42522-021-00044-9. PMID: 34218820; PMCID: PMC8255096.

B. Positions, Scientific Appointments, and Honors

Positions and Employment

1994 -97 Biochemical Technician, Department of Entomology, AFRIMS, Thailand
1997 Researcher, Department of Immunology, Chulabhorn Research Institute, Thailand
1997 -00 Medical Technologist, The HIV/AIDS Collaboration Thai-US, Thailand
2000 -16 Laboratory Chief, Neuroscience Centre for Research and Development & WHO Collaborating Centre for Research and Training on Viral Zoonoses, Faculty of Medicine, Chulalongkorn University Hospital, Thai Red Cross Society, Thailand
2016 -21 Deputy Chief of Thai Red Cross Emerging Infectious Diseases Health Science Centre, Faculty of Medicine, Chulalongkorn University Hospital
2021 - Senior Researcher, Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital
Committee member, School of Global Health, Faculty of Medicine, Chulalongkorn University

Other Experience and Professional Membership

2010 -14 PREDICT Thailand Country Coordinator
2014 - Thai Ministry of Public Health (MOPH) Ebola Diagnostic Committee
2015 -19 PREDICT 2 Thailand Country Coordinator
2016 - Steering committee, Bat One Health Research Network, BTRP DTRA
2021 - WHO Scientific Advisory Group for the Origins on Novel Pathogens (SAGO)

C. Contributions to Science

1. **Research on One Health.** One Health is an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals, and ecosystems, as the definition by WHO. Our projects are multidisciplinary studies on human surveillance, wildlife surveillance, wastewater surveillance, and human behavior.
   a. Tangwangvivat R, **Wacharapluesadee S**, Pinyopornpanish P, Petcharat S, Muangnoicharoen H. S, Thippamom N, Phiancharoen C, Hirunpatrawong P, Duangkaewkart P, Chaiden C, Wechsirisan W, Wandee N, Srimuang K, Paitoonpong L, Buathong R, Pawun V, Hinjoy S, Putcharoen O, Iamsirithaworn S. Assessment of SARS-CoV-2 variant wastewater detection strategies in the Bangkok Metropolitan region. **Preprint Research** 2023

b. Keusch GT, Amuasi JH, Anderson DE, Daszak P, Eckerle I, Field H, Koopmans M, Lam SK, Das Neves CG, Peiris M, Perlman S, **Wacharapluesadee S**, Yadana S, Saif L. Pandemic origins and a One Health approach to preparedness and prevention: Solutions based on SARS-CoV-2 and other RNA viruses. **Proc Natl Acad Sci USA**. 2022 Oct 18;119(42):e2202871119. doi: 10.1073/pnas.2202871119. Epub 2022 Oct 10. PMID: 36215506; PMCID: PMC9586299.
c. 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 Infect Dis**. 2022 May 16;22(1):472. doi: 10.1186/s12879-022-07439-7. PMID: 35578171; PMCID: PMC9109443.

2. **Research on Public Health Surveillance in Thailand.** Effective disease control programs rely on effective surveillance and response systems. Our laboratory collaborates with the Department of Diseases Control for active surveillance on syndromic surveillance and outbreak investigation. Both molecular and serology surveillance systems were conducted to identify the cause of the disease.
   a. Plipat T, Buathong R, **Wacharapluesadee S**, Siriarayapon P, Pittayawonganon C, Sangsajja C, Kaewpom T, Petcharat S, Ponpinit T, Jumpasri J, Joyjinda Y, Rodpan A, Ghai S, Jittmittraphap A, Khongwichit S, Smith DR, Corman VM, Drosten C, Hemachudha T (2017). Imported case of Middle East respiratory syndrome coronavirus (MERS-CoV) infection from Oman to Thailand, June 2015. **Euro Surveill** 22(33):pii: 30598.
   b. Okada P, Buathong R, Phuygun S, Thanadachakul T, Parnmen S, Wongboot W, Waicharoen S, **Wacharapluesadee S**, Uttayamakul S, Vachiraphan A, Chittaganpitch M, Mekha N, Janejai N, Iamsirithaworn S, Lee RT, Maurer-Stroh S. Early transmission patterns of coronavirus disease 2019 (COVID-19) in travellers from Wuhan to Thailand, January 2020. **Euro Surveill**. 2020 Feb;25(8):2000097. doi: 10.2807/1560-7917.ES.2020.25.8.2000097. PMID: 32127124; PMCID: PMC7055038.
   c. 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 Feb 12;16(2):e0246864. doi: 10.1371/journal.pone.0246864. PMID: 33577615; PMCID: PMC7880427.
   d. Sangkakam A, Hemachudha P, Saraya AW, Thaweethee-Sukjai B, Cheun-Arom T, Latinne A, Olival KJ, **Wacharapluesadee S**. Detection of influenza virus in rectal swabs of patients admitted in hospital for febrile illnesses in Thailand. **SAGE Open Med**. 2021 Jan 22;9:2050312121989631. doi: 10.1177/2050312121989631. PMID: 33552519; PMCID: PMC7841862.

3. **Research on diagnostic development.** The molecular technique is a gold standard method for pathogen diagnosis and confirmation. The first COVID-19 case outside China in Thailand was primarily detected by family PCR and further confirmed by next-generation sequencing (NGS). The SARS-CoV-2 variants can be identified by the NGS technique or by identifying the mutation markers by MassARRAY technology.
   a. **Wacharapluesadee S**, Kaewpom T, Ampoot W, Ghai S, Khamhang W, Worachotsueptrakun K, Wanthong P, Nopvichai C, Supharatpariyakorn T, Putcharoen O, Paitoonpong L, Suwanpimolkul G, Jantarabenjakul W, Hemachudha P, Krichphiphat A, Buathong R, Plipat T, Hemachudha T. Evaluating the efficiency of specimen pooling for PCR-based detection of COVID-19. **J Med Virol**. 2020 Oct;92(10):2193-2199. doi: 10.1002/jmv.26005. Epub 2020 Jul 21. PMID: 32401343; PMCID: PMC7272832.
   b. **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 R. A, Fernandez S, Putcharoen O. Simultaneous Detection of Omicron and Other SARS-CoV-2 Variants by Multiplex PCR MassARRAY Technology. **Preprint Research** 2023. DOI: https://doi.org/10.21203/rs.3.rs-2482226/v1

c. Joyjinda Y, Rodpan A, Chartpituck P, Suthum K, Yaemsakul S, Cheun-Arom T, Bunprakob S, Olival KJ, Stokes MM, Hemachudha T, **Wacharapluesadee S**. First Complete Genome Sequence of Human Coronavirus HKU1 from a Nonill Bat Guano Miner in Thailand. **Microbiol Resour Announc**. 2019 Feb 7;8(6):e01457-18. doi: 10.1128/MRA.01457-18. PMID: 30746519; PMCID: PMC6368654.

d. Siriyasatien P, **Wacharapluesadee S**, Kraivichian K, Suwanbamrung C, Sutthanont N, Cantos-Barreda A, Phumee A. Development and evaluation of a visible reverse transcription-loop-mediated isothermal amplification (RT-LAMP) for the detection of Asian lineage ZIKV in field-caught mosquitoes. **Acta Trop**. 2022 Dec;236:106691. doi: 10.1016/j.actatropica.2022.106691. Epub 2022 Sep 11. PMID: 36103950.

4. **Research on coronavirus prevalence in Thailand.** Numerous high-impact emerging viruses appear to have bat reservoirs. Our surveillance projects study the diversity of coronavirus (CoV) in bats in Thailand. We have isolated and characterized CoVs from many bat species and detected and sequenced CoV in bat guano collectors. Our surveillance studies continue to analyze the drivers of their emergence and risk factors for spillover.
   a. **Wacharapluesadee S**, Duengkae P, Chaiyes A, Kaewpom T, Rodpan A, Yingsakmongkon S, Petcharat S, Phengsakul P, Maneeorn P, Hemachudha T (2019). Longitudinal study of age-specific pattern of coronavirus infection in Lyle's flying fox (*Pteropus lylei*) in Thailand. **Virol J** 20;15(1):38.
   b. **Wacharapluesadee S**, Duengkae P, Rodparn A, Kaewpom T, Maneeorn P, Kanchanasaka B, Yingsakmongkon S, Sittidetboripat N, Chareesaen C, Khlangsap N, Pidthong A, Leadprathom K, Ghai S, Epstein JH, Daszak P, Olival KJ, Blair PJ, Callahan MV and Hemachudha T (2015). Diversity of Coronavirus in Bats from Eastern Thailand. **Virol J** 12(1):57.
   c. **Wacharapluesadee S**, Sintunawa C, Kaewpom T, Khongnomnan K, Olival KJ, Epstein JH, Rodpan A, Sangsri P, Intarut N, Chindamporn A, Suksawa K, Hemachudha T (2013). Group C betacoronavirus in bat guano fertilizer, Thailand. **Emerg Infect Dis** 19(8).

5. **Research on Nipah virus prevalence in Thai bats.** Nipah virus outbreaks, previously in Thailand's neighboring country, Malaysia, and ongoing in Bangladesh, have high mortality rates. Our surveillance projects study the characterization of Nipah Virus (NiV) in bats in Thailand. In addition, our surveillance studies continue to analyze the drivers of their emergence, understanding their seasonal preferences and risk factors for spillover.
   a. **Wacharapluesadee S**, Samseeneam P, Phermpool M, Kaewpom T, Rodpan A, Maneeorn P, Srongmongkol P, Kanchanasaka B, Hemachudha T (2016). Molecular characterization of Nipah virus from Pteropus hypomelanus in Southern Thailand. **Virol J** 13(1):53
   b. **Wacharapluesadee S**, Jittmittraphap A, Yingsakmongkon S, and Hemachudha T (2016). Molecular Detection of Animal Viral Pathogens. Nipah Virus. **CRC Press**.
   c. **Wacharapluesadee S**, Ngamprasertwong T, Kaewpom T, Kattong P, Rodpana A, Wanghongsa S, Hemachudha T (2013). Genetic characterization of Nipah virus from Thai fruit bats (*Pteropus lylei*). **Asian Biomedicine** 7(6);813-819.
   d. 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 Jun;19(2):175-189. doi: 10.1007/s10393-022-01588-6. Epub 2022 Jun 3. PMID: 35657574.
   e. Breed AC, Meers J, Sendow I, Bossart KN, Barr JA, Smith I, **Wacharapluesadee S**, Wang L, Field HE (2013). The Distribution of Henipaviruses in Southeast Asia and Australasia: Is Wallace's Line a Barrier to Nipah Virus? **PLoS One** 8(4):e61316.

6. **Rabies Neuropathogenesis, diagnosis and management.** The center worked for many years on molecular analyses of rabies, including mutational effects and designing primers to detect the Thai street rabies virus. I regularly organize workshops to teach laboratories in the region how to correctly collect specimens and test for rabies.
    a. Hemachudha T, Ugolini G, Sungkarat W, Laothamatas J, Shuangshoti S, **Wacharapluesadee S** (2013). Human Rabies: neuropathogenesis, diagnosis and management. **Lancet Neurology** 498-513.
    b. Shuangshoti S, Thepa N, Phukpattaranont P, Jittmittraphap A, Intarut N, Tepsumethanon V, **Wacharapluesadee S**, Thorner PS, Hemachudha T (2013). Reduced viral burden in paralytic compared to furious canine rabies is associated with prominent inflammation at the brainstem level. **BMC Vet Res** 14;9(1):31.
    c. Virojanapirom P, Khawplod P, Sawangvaree A, **Wacharapluesadee S**, Hemachudha T, Yamada K, Morimoto K, Nishizono A (2012). Molecular analysis of the mutational effects of Thai street rabies virus with increased virulence in mice after passages in the BHK cell line. **Arch Virol** 157(11):2201-5.
    d. Wilde H, Hemachudha T, **Wacharapluesadee S**, Lumlertdacha B, Tepsumethanon V (2013). Rabies in Asia: The Classical Zoonosis. **Curr Top Microbiol Immunol** 365:185-203.

Co-PI Plan (only needed if applying as Co-PIs):

What each Co-PI will contribute to the proposed research study

As stated in the Mentoring Plan, the co-PIs, Krongkan Srimuang and Spencer Sterling have different educational and training backgrounds. This Co-PI plan is proposed to fill the gaps in knowledge of each scientist and supports a collaborative working relationship for project success. Moreover, this plan was developed to maximize the strengths of each PI. Overall, the project is separated into two objectives, the proposal of this project will be equally managed by Dr. Srimuang and Mr. Sterling. Dr. Srimuang will be responsible for PBMC isolation, sorting, and characterization. Mr. Sterling will be responsible for the technical serological aspects of the project. Both will work closely with the Research Center Mentor, Dr. Eric Laing, to develop this project, protocol development, and network connections. Dr. Supaporn Wacharapluesade, the Co-mentor, will support the wildlife sampling, samples collection, and molecular aspects within Thailand.

How the Co-PIs will jointly work with the affiliated Research Center

Mr. Sterling is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region.

How the Co-PIs will jointly manage the proposed study

This is a great opportunity for both PIs to undergo training in advanced techniques and develop skills in next-generation serology and molecular sequencing. Dr. Srimuang will learn and practice serologic techniques and data analysis from Mr. Sterling. Mr. Sterling will develop skills in cell isolation and molecular sequencing techniques from Dr. Srimuang. Moreover, Mr. Sterling will coordinate planning, sample processing, data analysis, and report preparation for this project with Dr. Srimuang.

References

1. McLinton, E. C., Wagstaff, K. M., Lee, A., Moseley, G. W., Marsh, G. A., Wang, L. F., et al. (2017). Nuclear localization and secretion competence are conserved among henipavirus matrix proteins. J. Gen. Virol. 98, 563–576. doi: 10.1099/jgv.0.000703
2. Zhang, X. A., Li, H., Jiang, F. C., Zhu, F., Zhang, Y. F., Chen, J. J., Tan, C. W., Anderson, D. E., Fan, H., Dong, L. Y., Li, C., Zhang, P. H., Li, Y., Ding, H., Fang, L. Q., Wang, L. F., & Liu, W. (2022). A Zoonotic Henipavirus in Febrile Patients in China. The New England journal of medicine, 387(5), 470–472. https://doi.org/10.1056/NEJMc2202705
3. Lee, S. H., Kim, K., Kim, J., No, J. S., Park, K., Budhathoki, S., Lee, S. H., Lee, J., Cho, S. H., Cho, S., Lee, G. Y., Hwang, J., Kim, H. C., Klein, T. A., Uhm, C. S., Kim, W. K., & Song, J. W. (2021). Discovery and Genetic Characterization of Novel Paramyxoviruses Related to the Genus Henipavirus in Crocidura Species in the Republic of Korea. Viruses, 13(10), 2020. https://doi.org/10.3390/v13102020
4. Madera, S., Kistler, A., Ranaivoson, H. C., Ahyong, V., Andrianiaina, A., Andry, S., Raharinosy, V., Randriambolamanantsoa, T. H., Ravelomanantsoa, N. A. F., Tato, C. M., DeRisi, J. L., Aguilar, H. C., Lacoste, V., Dussart, P., Heraud, J. M., & Brook, C. E. (2022). Discovery and Genomic Characterization of a Novel Henipavirus, Angavokely Virus, from Fruit Bats in Madagascar. Journal of virology, 96(18), e0092122. https://doi.org/10.1128/jvi.00921-22
5. Uwishema, O., Wellington, J., Berjaoui, C., Muoka, K. O., Onyeaka, C. V. P., & Onyeaka, H. (2022). A short communication of Nipah virus outbreak in India: An urgent rising concern. *Annals of medicine and surgery (2012)*, *82*, 104599. https://doi.org/10.1016/j.amsu.2022.104599
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List of abbreviations, acronyms, symbols

AngV: Angavokely virus
BCR: B cell receptor
BSc: Bachelor of Science
BSL2: Biological Safety level 2
BSL3: Biological Safety level 3
cDNA: Complementary DNA
CedV: Cedar virus
Co-PI: Co-principal investigator
CREID: Centers for Research in Emerging Infectious Diseases
DARV: Daeryong virus
DNA: Deoxyribonucleic acid
EHA: EcoHealth Alliance
EID: Emerging Infectious Disease
EID-SEARCH: The Emerging Infectious Disease – South East Asia Research Collaboration Hub
ELISA: Enzyme-linked immunosorbent assay
F: Fusion protein
FACS: Fluorescence-activated cell sorting
FUO: Fever of unknown origin
GAKV: Gamak virus
GhV: Ghana virus
HeV: Hendra virus
HPVs: Henipaviruses
ILI: Influenza-like illness
KCMH: King Chulalongkorn Memorial Hospital
L: large protein
LayV: Langya virus
M: Matrix protein
mAb: Monoclonal antibody
MD: Maryland
MMIA: Multiplex microsphere-based immunoassay
MojV: Mojiang virus
MSc: Master of Science
N: Nucleocapsid
NGS: Next-generation sequencing
NIAID: National Institute of Allergy and Infectious Diseases
NIH: National Institutes of Health
NiV: Nipah virus
P: Phosphoprotein
PBMC: Peripheral blood mononuclear cells
PCR: Polymerase Chain Reaction
PhD: Doctor of Philosophy
PI: Principal investigator
RBP: Receptor-binding protein
RNA: Ribonucleic acid
RT-PCR: Real-time PCR
SARI/ARI: Severe/acute respiratory illness

SARS-CoV-1: Severe acute respiratory syndrome coronavirus 1
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2
SEA: Southeast Asia
Thai-DDC: Department of Disease Control of Thailand
TRC-EIDCC: Thai Red Cross. Emerging Infectious Diseases. Clinical Center
USA: United States
WGS: Whole Genome Sequencing

Facilities and Resources

FACILITIES: Specify the facilities to be used for the conduct of the proposed research.

Laboratory:

The Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC), King Chulalongkorn Memorial Hospital (KCMH) was established by the Committee of the Emerging Infectious Disease Center which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. TRC-EIDCC laboratory facilities include 3 biosafety cabinets (BSL 2, BSL 2+, BSL2+), 3 automated extraction machines, 2 thermocyclers (Proflex), 2 real-time PCR machines (CFX, Bio-rad), 3 Freezers (-80 °C), 2 refrigerators; 4 °C, 2 autoclaves. Our EIDCC uses the Pathogen Asset Control System (PACS) system for sample tracking. Moreover, our Faculty of Medicine, Chulalongkorn University has a central laboratory, the Chula Medical Research Center (Chula MRC), which provide many instruments for medical, masters, and graduate students, Post-doctoral fellows, and researchers at Chulalongkorn University and KCMH. The Chula MRC laboratory facilities are divided into molecular diagnostics (PCR Thermal Cycler, QStudio6 Flex Real-Time PCR, Droplet Digital PCR, GeneXpert, Film array, Illumina MiSeq), immunology facilities (ELISA testing equipment (reader & washer), Luminex, Flow Cytometry), cell sorting machine and a BSL3 laboratory. Moreover, the facility provides general equipment, such as autoclaves, balances, CO2 incubators, Dual Fluorescence Cell Counter, 4 °C refrigerators, -20°C, and -80°C freezers.

Clinical:

KCMH is a 1,000-bed university hospital, in close coordination with the Faculty of Medicine, Chulalongkorn University. The Thai Red Cross Society provides medical treatment, rehabilitation, health promotion and disease prevention services to the general public and the vulnerable. It also provides biopharmaceutical and sterile medicines that are sufficient to meet the needs of patients and safe in accordance with international standards. It serves as a center for providing eye and organ donation services to patients nationwide in line with international standards. It has specialized doctors and public health personnel and also produces specialized nurses. The TRC-EIDCC in the KCMH provides standard medical care to patients with emerging and recurrent diseases and disease spreading prevention.

Animal:

TRC-EIDCC also collaborates with the Department of National Parks, Wildlife and Plant Conservation for detecting the emerging novel pathogens from animals such as bats, rodents, and pangolins in our country. Bats are considered protected wildlife in Thailand by Wildlife Conservation, Department of National Parks, Wildlife and Plant Conservation.

Computer:

TRC-EIDCC used the service from the Office of Information Technology, Chulalongkorn University that provides the application, network, and software such as Office 365, VPN, WIFI, Microsoft Office, Microsoft Windows, SPSS, Adobe, Zoom Meeting, etc. Moreover, our EIDCC use the Pathogen Asset Control System (PACS) for sample tracking.

Office:

The office and laboratory space are located at the EIDCC at 4th Floor, Jongkolnee Watwong Building and 6th Floor, Aor Por Ror Building, respectively. The applicants will have dedicated desks in both buildings.

Other:

Foreign Site Information

If the Pilot Award will have multiple foreign sites, complete this form for EACH research site

Principal Investigator Name:	Krongkan Srimuang
Project Title:	Immune memory bait & capture to identify emerging henipavirus origins
Institution:	King Chulalongkorn Memorial Hospital
Foreign Research Site:	Rama 4 Road, Pathumwan, Bangkok 10330 Thailand
Point of Contact for Research Site:	Krongkan Srimuang Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital Rama 4 Road, Pathumwan, Bangkok 10330 Thailand krongkan.sr@gmail.com

Introduction (3-4 sentences):
Provide a simple description of the overall goals of the project including the work that will be done at the foreign site. Indicate if any of the NIAID grant funds will be sent to the foreign site.

The goals of this project are to know zoonotic reservoirs for pathogen origin by using serologic and cellular immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

To achieve these goals, the investigator will collaborate with NIH PREMISE for Antibody-mediated bait & capture of Thai MojV-like virus.

$149,937 of grant funds will be sent to the site for these studies.

Foreign component (3-4 sentences):
Describe the specific role of the foreign site (i.e. conduct critical experiments?, conduct data analyses?, provide consultations?, provide samples?, study human and/or animal populations?, conduct observational or interventional clinical trials?, collect samples and/or data to be brought to the US?).

The site will 1) conduct all filed work to collect human and animal samples from the study sites; 2) perform laboratory analysis using collected samples; and 3) conduct data analysis as proposed in the project.

Human Subjects (1 word or 1 sentence per bullet):

- **Parent Study IRB approval**
  - **IRB approval number for parent study:** #894ECOH21b (US); #221/64 (Thailand)
  - **IRB approval date:** 12-05-2021 (US); 08-06-2021 (Thailand)
  - **Human Subject Assurance Number: (FWA)#:** #00001102 (external IRB); #00022431 (EcoHealth Alliance); #00000943 (Chulalongkorn University)
- **Does this study require a modification to the IRB approval of a parent study (Yes or No)?**
  - **Yes**
- **Will existing samples from human subjects will be used: (Yes or No)?**
  - **No**
  - **How many subjects provided the existing samples to be used?** N/A
- **Will human subjects be recruited (Yes or No)**
  - **Yes**
  - **Number of human subjects that will be recruited:** 110

- **Population parameters:**
  - **Gender:** 55 males, 55 females
  - **Age Group:** Age >=18 years who provide informed consent.
  -
  - **Race/Ethnicity:** 110 Asian

- **Sample collection will include:**
  - **Blood:** Yes
  - **Urine:** No
  - **Tissues:** No
  - **Other samples (describe):** Nasopharyngeal swab and throat swab

- **Sample collection will be completed in how many visits:** 2 trips

- **Will samples be de-identified (Yes or No)? If No, describe how they will be protected.**
  - Yes

- **Will local IRB approval be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals being sought or state why local IRB approval is not required.**
  - Yes

- **Will informed consent be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals/consents being sought or state why informed consent is not required.**
  - Yes

- **Will samples be brought back to the US (Yes or No)?**
  - No

- **Will data be brought back to the US (Yes or No)?**
  - Yes, some of the data will be shared with EID-SEARCH partners in the US for relevant analysis.

Animal Subjects (1 word or 1 sentence per bullet):

- **Parent study IACUC approval**
  - **IACUC approval number for parent study:** #G2020-42 (US); #019/2563 (Thailand)
  - **IACUC approval date:** 05-05-2020 (US); 03-12-2020 (Thailand)
    Animal Welfare Assurance Number: D16-00572 (A4059-01)
- **Does this study require a modification to the IACUC approval of a parent study (Yes or No)?**
  - **No**

- **Will existing samples from animal subjects will be used: (Yes or No)?**
  - **No**
  - **How many animal subjects provided the existing samples to be used?** N/A
- **Will vertebrate animals be collected (Yes or No)?**
  - **Yes**

- **Species of animals (e.g. rats, mice, rabbits, monkeys):** Bats, rodents, and shrews
- **Animal parameters:**
  - **Total number of animals:** 200 (100 bats, 100 rodents and shrews)
  - **Gender:** 100 males, 100 females
  - **Age range:** 4 - 12 months and elder depending upon species

- **Lab strain (e.g. Sprague-Dawley rats, Balb/C mice):** None
- **Wild animals procured in country (e.g. Rhesus monkeys from a reserve):** No

- **What will be done to them or with them and how often?**
  - Free-ranging bats will be captured using either a mist net or harp trap, and manually restrained during sampling; free-ranging rodents and shrews will be captured through pit traps and box traps.
  - Anesthesia will be conducted for captive rodents and shrews.
  - Once anesthetized blood will be collected.
  - All action will only be performed one time for each animal individual.
  - All animals will be released after sampling.

- **What are the follow-ups?**
  - No follow-up relevant as per protocol, since all animals will be released after sampling ups

- **What will be their fate at the end of the experiments – will they be euthanized?**
  - All animals will not be held longer than 6 hours (typically less than 3 hours) during the sampling process and released after sampling.

- **Will in-country clearances be obtained prior to engaging in any animal research (Yes or No)? If No, describe alternate approvals or state why in-country clearances are not required.**
  - Yes
- **Will samples be brought back to the US (Yes or No)?**
  - No
- **Will data be brought back to the US (Yes or No)?**
  - Yes, some of the data will be shared with EID-SEARCH partners in the US for relevant analysis.

27th January 2022

Krongkan Srimuang Ph.D.
Research Scientist
Thai Red Cross Emerging Infectious Diseases Clinical Center
King Chulalongkorn Memorial Hospital
Rama 4 Road
Pathumwan, Bangkok 10330
Thailand

Subject: Letter of Support for CREID Network Pilot Research Program

Dear Dr. Srimuang,

I am writing to express my support for your CREID Network Pilot Research Program proposal titled, "Immune memory bait and capture to identify emerging henipavirus origins." Your preliminary data demonstrating immunoreactivity against Mojiang henipavirus in individuals sampled as part of the Emerging Infectious Diseases Southeast Asia Research Collaborative Hub (EID-SEARCH) center, provides compelling serologic evidence that novel henipaviruses spill over into human populations. Point-of-care diagnostics and nucleic acid-amplification tests rely on our present understanding of medically relevant pathogens. Thus, emergence of novel zoonotic viruses may go undetected. Serological profiling provides us an ability to measure the human infectome through broad-scale peptide arrays or targeted antigen-based detection.

The research activities conducted at the Thai Red Cross Emerging Infectious Diseases Clinical Center are complementary to the focus of the Vaccine Research Center's PREMISE (Pandemic REsponse REpository through Microbial and Immunological Surveillance and Epidemiology) program. PREMISE works closely with intramural and extramural NIH programs and in partnership with a global network of investigators and collaborators to achieve its objectives through 3 core activities: (1) genetic analysis of zoonotic reservoirs and symptomatic humans for pathogen discovery; (2) development of high throughput, multiplexed serologic and cellular assays; and (3) global blood sampling to identify antigen-specific and cross-reactive immune responses to known and previously unknown viruses of pandemic potential. Biological samples including serum and PBMC are obtained from broad-based population cohorts as well as targeted populations at risk from emerging infections. At the same time, sequence data from known and unknown (through virus discovery) pathogens are analyzed to design and express candidate antigenic proteins of interest. These proteins are then used in high throughput multiplexed assays to screen sera for antibody reactivity. Serum reactivity to candidate antigens is followed by further interrogation of the adaptive immune response at the cellular level using corresponding PBMC samples. Proteins used in the serological arrays are conjugated to fluorophores and used as probes to sort antigen-specific B cells by flow cytometry. In addition, the sequence of such candidate antigens provides the template for the design of overlapping peptide pools to assess T cell responses. Further analysis allows for the identification of

Daniel Douek MD PhD | Chief, Human Immunology Section | Vaccine Research Center
National Institute of Allergy and Infectious Diseases | National Institutes of Health
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neutralizing antibodies specific for the target antigens as well as specific T cell epitopes which may be tested in animal models. Epitope identification informs and guides immunogen design. At the conclusion of this pipeline of assays and analysis, PREMISE delivers the following:

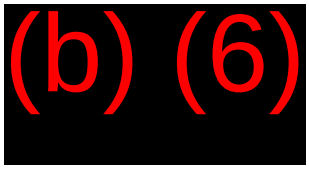
1. reagent and data resources for early detection and diagnosis
2. monoclonal antibodies with therapeutic potential
3. candidate immunogens for further vaccine development

Thus, PREMISE serves as a translational vehicle to integrate serologic and cellular immune discovery, targeting a broad array of pathogens, into product development, and constitutes an anticipatory reagent repository to accelerate the global response to pandemic threats.

As investigators in the Vaccine Research Center at the National Institute of Allergy and Infectious Diseases (NIAID), we are not eligible to be listed as a "co-investigator" on the application but look forward to participating as collaborators. All materials from us will be provided under an approved material transfer or other collaborative agreement. This collaboration is part of our official duties as employees at the NIAID, and no funds from the grant will be used in intramural research, neither will we accept any form of renumeration, whether in the form of salary, honoraria, or travel expenses. We will provide scientific input and mentoring but will not have any duties associated with programmatic stewardship, which will be performed by NIAID extramural program officials. Further, in keeping with the mission of NIAID to promote and facilitate biomedical research and the dissemination of new knowledge, we would supply requested research materials and technical expertise not only to you, but also to other interested and qualified parties for research purposes.

My group has met with Dr. Eric Laing (USU) one of the two mentors for this pilot research program to discuss ways we can collaborate and support your research application. We are enthusiastic about providing the technological and subject-expertise in immunology and translational therapeutic discovery afforded by the PREMISE program that would enhance your knowledge base and broaden your own scientific training.

Sincerely,



Daniel C. Douek

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EID-SEARCH
Emerging Infectious Diseases
South East Asia Research Collaboration Hub

Dr. Krongkan Srimuang, Mr. Spencer Sterling
Thai Red Cross Emerging Infectious Diseases Clinical Center
King Chulalongkorn Memorial Hospital
1873, Rama 4 Road, Pathumwan
Bangkok, Thailand 10330

Dear Krongkan and Spencer,

This letter conveys a strong interest from the Emerging Infectious Disease-Southeast Asia Research Collaboration Hub (EID-SEARCH) at EcoHealth Alliance to collaborate with you at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital on the proposed project titled "*Immune memory bait & capture to identify emerging henipavirus origins"* in Thailand.

The question of the origin of emerging Henipaviruses (HPVs) was identified from the EID-SEARCH human surveillance when a Mojiang-related virus was identified in a local population highly exposed to bats in Thailand. Faced with the challenges of PCR detection and viral isolation, this project will establish and validate a new serological approach to develop MojV-like virus-specific MAbs and define the antigenic spatial relationships between all known rodent- and bat-borne HPVs, revealing the serologic signature of all related undiscovered HPVs. This will significantly contribute to the objectives of EID-SEARCH to conduct targeted and strategic surveillance to identify emerging pathogens with zoonotic potential at the high-risk human-animal interfaces. The research population and site for this project – bat guano collection – represents one of the key scenarios where zoonotic spillover is mostly concerned that requires further in-depth investigation to identify the reservoir hosts. In addition to the gained knowledge, established methods from this project will be shared and used as standards in zoonotic HPVs surveillance and prevention strategies in Thailand and regionally through collaborative and coordinative cooperation among multiple stakeholders.

I am very excited to work with you both on this project, given your critical roles in the previous surveillance work to identify the question and develop the research idea. This project will bring valuable insights to advance disease surveillance and early warning systems for endemic and emerging infectious diseases in Thailand. Members of EID-SEARCH are committed to working closely with you to develop the research project and support the efforts necessary for the success of this project. You will be invited to join all training conducted by EID-SEARCH and EcoHealth Alliance regarding emerging infectious disease surveillance, relevant statistical analysis, and grant writing to expand your skill set. You'll also be supported to present the findings from this research at international conferences, the CREID Network meetings, and external partners and produce high-quality publications from this research.

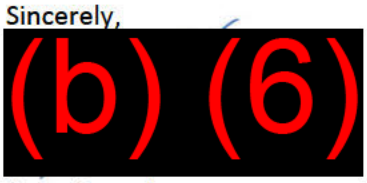
Developing and implementing this project will advance your skills in project management for future research as an independent researcher. Co-Investigators of EID-SEARCH, Dr. Eric Laing from the Uniformed Services University of the Health Sciences and Dr. Supaporn Wacharapluesadee from the Thai Red Cross Emerging Infectious Diseases Clinical Center, have enthusiastically joined the project as your Mentors. Dr. Laing will advise on the study design and provide training on data analysis, and Dr. Wacharapluesadee from your institute will work closely with you to advise on the day-to-day project implementation. Both Mentors will work with you for manuscript writing and provide pertinent and timely career advice to assist in your professional development. They will bring a well-established

EID-SEARCH
Emerging Infectious Diseases
South East Asia Research Collaboration Hub

network of leaders and experts in emerging infectious disease research in Thailand, Southeast Asia, and North America for your network building.

I am confident that this proposed project will bring genuine opportunities for your professional development. And the combined experience in the field and lab and interdisciplinary expertise in serology and public health from the co-PIs will make this a successful and purposeful project to bring public health impacts in a broad region. I look forward to collaborating with you and your team on all phases of this proposed project. I wish you success in the CREID Pilot Research Program application.

Sincerely,



Peter ¯ asza·
Principal Investigator, EID-SEARCH
President, EcoHealth Alliance

30 January 2023

Dr. Krongkan Srimuang & Mr. Spencer Sterling

Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC),

King Chulalongkorn Memorial Hospital

Object: Thai Red Cross Emerging Infectious Diseases Clinical Center support for your application to the CREID Pilot Research Program, entitled "Immune memory bait & capture to identify emerging henipavirus origins."

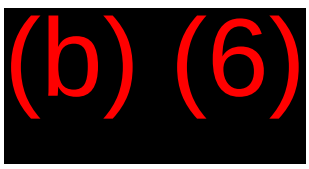
Dear Dr. Srimuang & Mr. Sterling,

Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC) is pleased to support your application to the **CREID Pilot Research Program**, entitled "Immune memory bait & capture to identify emerging henipavirus origins." We are fully committed to executing the statement of work to study zoonotic reservoirs for pathogen origins by using serologic and cellular immune discovery in humans and wildlife and their application as a tool for early detection and identification of novel pathogens in pandemic prevention.

The work proposed in this project is a great opportunity for collaboration with our team, it serves to enhance the use of serology and immunology for the detection of novel virus pathogens in Thailand using new technology and techniques. Moreover, the building of capacity and capability in our laboratory and Faculty will enhance the regional capacity, and the Chulalongkorn network will be used to ensure the success and completion of this project.

This letter expresses my strong interest in you pursuing this application and I fully support the project. I look forward to collaborating with you on this project and I wish you success in the CREID Pilot Research Program application.

Sincerely,



Assistant Professor Opass Putcharoen, MD, Msc

Head of Thai Red Cross Emerging Infectious Diseases Clinical Center

King Chulalongkorn Memorial Hospital

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January 27, 2023

Krongkan Srimuang, Ph.D.
Research Scientist
Thai Red Cross Emerging Infectious Diseases Clinical Center
King Chulalongkorn Memorial Hospital
Rama 4 Road
Pathumwan, Bangkok 10330

Subject: Letter of Support for CREID Network Pilot Research Program

Dear Dr. Srimaung,

I am writing to express my enthusiastic support for your CREID Network Pilot Research Program proposal titled, “Immune memory bait & capture to identify emerging henipavirus origins.” In 2018, I led a training and multiplex serology technology transfer to your mentor Dr. Wacharapluesadee’s research group via DTRA-funded project Chulalongkorn Luminex Training and Research Preparedness (HDTRA1-17-C-0019), and was excited to leverage those activities into ongoing research within the EID-SEARCH. Since 2015 I have been developing serological-approaches for biosurveillance of zoonotic viruses in wildlife and human populations, and I am excited to serve as a co-mentor for this pilot program application.

I hope to provide you with the research and career developmental support for the activities outlined in the proposal. The serological data on henipavirus exposure in Thai guano farmers provides compelling evidence that Mojiang-like henipaviruses can cross the species barrier and may be widely distributed in Southeast Asia. The research aims detailed in the proposal will facilitate critical follow-up sampling of this human cohort to identify whether acute infection causes clinical disease, estimate the sero-attack rate of this novel henipavirus, and will apply a cutting-edge serologic and immunologic approach for monoclonal antibody development and novel emergent virus immunogenic characterization. If awarded, a collaboration with VRC PREMISE program scientists will facilitate an important opportunity to broaden your knowledge base of cellular immunology and B cell biology. You have an extensive background in next-generation sequencing application, and through this proposal would gain a considerable understanding of how next-generation sequencing is used for BCR-sequencing, IgG characterization and monoclonal antibody discovery.

Your proposal is an excellent extension of the EID-SEARCH activities and significant training opportunity.

Sincerely,

(b) (6)

Eric D. Laing
Assistant Professor
Department of Microbiology and Immunology
Uniformed Services University

30th January, 2022

Dr. Krongkan Srimuang & Mr. Spencer Sterling

Thai Red Cross Emerging Infectious

Diseases Clinical Centre (TRC-EIDCC),

King Chulalongkorn Memorial Hospital

Dear Dr. Srimuang & Mr. Sterling,

I am writing in support as Research Center Mentor for your application to the CREID Pilot Research Program, entitled "Immune memory bait & capture to identify emerging henipavirus origins."

With my 20 years of experience in conducting surveillance for novel viruses in wildlife, humans, and bats using molecular technology, I will support the growth of the scientists in the CREID Pilot Research Program. The research aims have the potential to significantly enhance our approach to pathogen surveillance at high-risk human-animal interfaces. Given the understanding of pathogen origins by using serologic and cellular immune discovery in humans, and applying these techniques as a tool for early detection and identification of novel pathogens.

As the head laboratory cheif at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, I believe that this is a great opportunity for our Thailand team to train in the characterization of memory B cells from peripheral blood mononuclear cells (PBMC) and enhanced serological surveillance in novel virus pathogens. Moreover, our laboratory has supported the Department of Disease Control (DDC) in diagnosing emerging infectious diseases and communicable diseases such as Ebola, MERS, and COVID-19. I am also a committee member of the Global Laboratory Leadership Program (GLLP) Thailand, organized by the Department of Medical Sciences. With my experience in diagnosing the first human MERS case in Thailand in 2015 and the first human COVID-19 case outside China in January 2020, I will support multidisciplinary projects involving epidemiology, laboratory, field science, and bioinformatics to diagnose and monitor the emergence of wildlife-origin viral zoonoses, including SARS-CoV, Nipah and Hendra viruses, Avian influenza, and novel viruses from bats. I was also the Thailand country coordinator for the PREDICT project, large contracts from USAID during 2010-2019 involving the successful management of teams of virologists, field biologists, veterinarians, epidemiologists, hospitals, and laboratory staff. I will enthusiastically support the development of a strong knowledge base to build capacity and capability in this project and our team in Thailand.

The CREID Network and the Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH) will support scientists in regions around the world where emerging and reemerging infectious disease outbreaks. Many Southeast Asian partnerships in EID-SEARCH have strong potential to enhance innovations in this area.

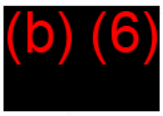
I believe that Dr. Laing, and I will provide scientific skills, expertise, and networks for this project.

Towards that aim, I am committed to supporting your professional development in the following ways:

1. I will maintain regular bi-weekly meetings for the project progresses.
2. I will support the resources in our country and research team in this project.
3. I will create opportunities for scientists network to ensure the success of research project.
4. I will provide and share advice and technical expertise in specific knowledge for this project.

I'm very much looking forward to working closely with you on this 1-year research project and beyond as you continue to develop your surveillance zoonotic disease research program.

Best regards,



Supaporn Wacharapluesadee, Ph.D.

Head Laboratory,

Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC),

King Chulalongkorn Memorial Hospital,

Faculty of Medicine,

Chulalongkorn University.

From: Krongkan Srimuang on behalf of Krongkan Srimuang <krongkan.sr@gmail.com>
To: Sterling, Spencer
Cc: Hongying Li; Laing, Eric; sasiprapa.n@outlook.com; spwa
Subject: Re: Pilot study 2023
Date: Monday, January 30, 2023 2:12:04 AM
Attachments: Pilot study full_V.5.pdf

Dear All,

Please find attached the full application.
I will edit page no. later.

Thank you
Best regards,
KIO

On Mon, Jan 30, 2023 at 11:39 AM Sterling, Spencer <spencer.sterling.ctr@usuhs.edu> wrote:

> I think if we cite the MojV, LayV, and GAKV and DARV papers it should be easily justifiable.
>
> See attached.
>
> On Mon, Jan 30, 2023 at 10:40 AM Hongying Li <li@ecohealthalliance.org> wrote:
>
> > Languages for animal sample size justification, feel free to edit, Eric and Spencer.
> > (I think we are dropping the 100 bats and doing 200 rodents/shrews now?)
> >
> > We will target a total of 200 individuals of rodent/shrew for this project, this sample size is estimated based on a realistic detection rate and required sampling efforts from our previous wildlife surveillance work, given the rodent/shrew population at the study site. Previous work of PMVs in wildlife had an average PCR detection prevalence of ~1.5%, sampling 200 individuals will yield 3 positives. We assume the serology prevalence will be higher, so expect about **5 positives for serology surveillance???**. We acknowledge the challenge of identifying positive results, and very limited serology research has been done to provide guidance. However, we will make the best use of previous data and experience to conduct sampling at the selected site where human positives have been identified and based on the potential seasonality at different time points throughout the project period (Ref. S. Wacharapluesadee *et al.*, A longitudinal study of the prevalence of Nipah virus in Pteropus lylei bats in Thailand: evidence for seasonal preference in disease transmission. *Vector borne and zoonotic diseases (Larchmont, N.Y.)* **10**, 183-190 (2010)). We are confident that this targeted sampling and testing strategy offers the best chance to identify positive results and potentially additional strains henipaviruses.
> >
> > On Mon, Jan 30, 2023 at 10:02 AM Laing, Eric <eric.laing@usuhs.edu> wrote:
> >
> > > Edits - Deleted objective 3, antigenic cartography is not discussed in Aim 2. Make sure you clean up track changes throughout.

Eric D. Laing, Ph.D.
Assistant Professor
Department of Microbiology and Immunology
Uniformed Services University
4301 Jones Bridge Road
Bethesda, MD 20814
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office: (301) 295-3419
lab: (301) 295-9618

eric.laing@usuhs.edu

On Sun, Jan 29, 2023 at 9:12 PM Sterling, Spencer <spencer.sterling.ctr@usuhs.edu> wrote:

> Hi Eric,
>
> We have modified the Specific Aims section, can you please look it over?
>
> -S
>
> On Mon, Jan 30, 2023 at 8:13 AM Hongying Li <li@ecohealthalliance.org> wrote:
>
> > Hi All,
> >
> > 1. Would it be possible to strengthen the sample size justification. I know there might not be any sample size calculation because this focuses on developing the method, but there is a statistical review panel, maybe you can briefly talk about this? E.g. given the estimated positive rate of X%, or total population of the community or animals etc.
> >
> > 2. Are you doing questionnaires when collect samples for humans? If so please describe it in the Method part; if not, please remove these languages from the human subject section.
> >
> > 3. It seems you don't plan to enroll children, then please remove these languages about parents/legal guardians in the human subject protection section.
> >
> > Hongying Li
> >
> > > On Jan 30, 2023, at 7:39 AM, Laing, Eric <eric.laing@usuhs.edu> wrote:
> > >
> > > Hi Kio and Spencer,

My edits are attached. Please revise the specific aims page and look at the previously awarded applications when you make those revisions. Everything else looks good. Spend time on the Aims Page.

Eric D. Laing, Ph.D.
Assistant Professor
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eric.laing@usuhs.edu

On Sun, Jan 29, 2023 at 9:58 AM Krongkan Srimuang <krongkan.sr@gmail.com> wrote:

> Dear All,
>
> Please find attached the word file of the full application for CREID Pilot study Program.
>
> Thank you
> Best regards,
> KIO
>
> On Sun, Jan 29, 2023 at 9:21 PM Krongkan Srimuang <krongkan.sr@gmail.com> wrote:
>
> > Dear Eric, Dr.Supaporn, Spencer, Hongying and Bow
> >
> > Thank you very much for your support in the CREID Pilot study.
> > Please find attached the Full application.
> > If you have any comments please let me know.
> >
> > Thank you very much
> > Best Regards,
> > KIO

<Pilot study full_V.3 (1)-EDL.docx>

--
Spencer Sterling, MPH (he/him)
Scientific Project Coordinator
Broder/Laing Lab
Uniformed Services University of the Health Sciences

> > > WhatsApp- +66 (0) 83-494-5980

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Specific Aims (1-page limit)

This project aims to study zoonotic reservoirs for pathogen origin by using serologic and cellular immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

The hypothesis

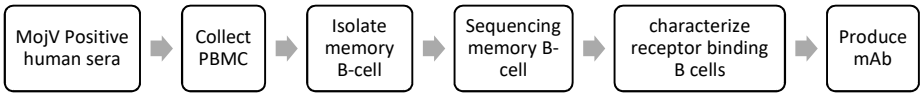
1. Can the memory B-cell immune response be used as a tool for the discovery of novel henipaviruses?
2. Can the spatial relationships between henipaviruses receptor binding proteins be generated that would allow for an understanding of antigenic evolution and functional characterization?

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population.

Human populations near the Khao Chong Phran Non-hunting Area in Ratchaburi province have shown a high seroprevalence to the MojV RBP without confirmed sequencing results. We will follow-up on persons with high MojV RBP IgG titers, sample new people within the population that interact with the local wildlife, and sample shrews and rodents to find the source of the MojV-reactive antibodies.

Aim 2. Antibody-mediated bait & capture of the Thai MojV-like virus

In collaboration with NIH PREMISE, MojV RBP-reactive B cell receptors will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific MAbs will be generated and used for direct antibody-mediated virus capture in people and wildlife.



Objective 1. To use metagenomic and targeted sequencing approaches in human and wildlife populations to detect the Thai MojV-like virus.

Objective 2. To isolate and sequence memory B-cells from humans positive for Mojiang virus in Ratchaburi province, Thailand for antibody production.

Objective 3. To determine epitope binding and antigenic relationships from henipavirus-reactive serum to the known henipaviruses.

Study Rationale (7-page limit)

Nipah virus (NiV) and henipaviral diseases are among the most virulent of zoonotic diseases. The genus *Henipavirus* is currently composed of nine viruses including the prototypes, NiV and Hendra virus (HeV), and Cedar virus (CedV), Ghana virus (GhV), Mojiang virus (MojV) (McLinton et al., 2017), Langya virus (LayV) (Zhang X et al., 2022), Gamak virus (GAKV), Daeryong virus (DARV) (Lee et al., 2021), and Angavokely virus (AngV) (Madera et al., 2022). HeV was first identified in Australia in 1994 as a cause of respiratory illness and fatal encephalitis in humans and horses. Closely following the discovery of HeV, NiV was identified in 1999 in Malaysia after outbreaks of fatal encephalitis in humans, with swine determined to be the intermediate host. Annual outbreaks of NiV have occurred in Bangladesh and are associated with the consumption of raw date palm sap, and since 19 May 2018 annual outbreaks of NiV associated encephalitis of been recorded in Kerala, India (Uwishema et al., 2022). Both HeV and NiV have a broad host range capable of infecting several mammalian orders, and can enter and infect endothelial cells, vasculature, and the central nervous system leading to severe multisystem organ diseases and high mortality. The broad host and tissue tropism of these viruses has been attributed to the use of the ephrinB2 and ephrinB3 ligands, highly conserved proteins with critical functions in evolutionary development, as virus receptors (Bossart et al., 2008). There are currently no vaccines or therapeutics and NiV and henipaviral diseases remain WHO Blueprint list priority pathogens (Middleton and Weingartl, 2012).

A close relative of HeV, CedV was isolated from Australian bats in 2012 and found to be non-pathogenic, failing to cause any clinical symptoms in ferrets and guinea pig animal models (Marsh GA et al., 2012). The natural reservoirs of HeV, NiV, and CedV are flying foxes (*Pteropus sp.*) (Middleton and Weingartl, 2012). GhV was detected in Ghana in straw-colored fruit bats (*Eidolon helvum*) during routine surveillance in 2015 and was the first henipavirus found outside of the Indo-Pacific region. GhV showed a close phylogenetic relationship with HeV and NiV, and like HeV, NiV, and CedV the GhV RBP can mediate binding to ephrinB2 ligand and mediate cellular fusion (Drexler, J. F., 2009). Recent investigations into Madagascan fruit bats (*Eidolon duprenum*) detected a second African henipavirus, AngV. As neither GhV nor AngV have been isolated or rescued, the pathogenic and zoonotic potential of these African fruit bat associated henipaviruses remains unknown.

Multiple genus of small mammals were tested for viral RNA, and MojV was detected in yellow-breasted rats (R. flavipectus) (Wu Z et al., 2014). The discovery of the MojV genome occurred during follow-up investigations into lethal pneumonia from three miners in 2012 in China, however the pathogenic nature of this virus has only been suggested and no isolates have been discovered or tested in an animal model. Additionally, the novel shrew-borne henipaviruses, Gamak virus (GAKV), and Daeryong virus (DARV) were found in the Republic of Korea in 2017-2018. (Lee et al., 2021). Most recently, a close relative of MojV, Langya henipavirus (LayV) was recently isolated from febrile patients in eastern China. Subsequently, LayV was identified in shrews (Zhang et al., 2022), increasing the number of Asiatic rodent-associated henipaviruses to four. These recent discoveries are challenging the paradigms surrounding henipaviruses and their associations with bats and may suggest a novel rodent sub-genus of henipaviruses.

The Henipavirus genome consists of six structural proteins including nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), receptor-binding protein (RBP), and large (L) protein (approximately length of 18,000 nucleotides) (Luby, S. P., & Gurley, E. S., 2012). In HeV and NiV, slippage sites within the P protein lead to the expression of the V, W, and C proteins. These proteins are virulence factors and are absent in CedV.

Table 1 Summary viruses in Henipavirus

Henipavirus	Origin (Year)	Reservoir	Genome (nucleotides)	Symptom for human	Reference
Hendra virus (HeV)	Australia (1994)	Fruit bats	18,234	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Nipah virus (NiV)	Malaysia (1999)	Fruit bat	18,246	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Ghana virus (GhV)	Africa (2009)	Fruit bat (*Eidolon helvum)*	18,530	No data	Drexler, J. F., 2009
Cedar virus (CedV)	Australian (2012)	Fruit bat	18,162	Non-pathogenic	Marsh, G. A et al., 2012
Mojiang virus (MojV)	China (2012)	Rodent	18,404	Febrile	Wu Z et al., 2014
Gamak virus (GAKV)	Korea (2017-2018)	Shrew	18,460	No data	Lee et al., 2021
Daeryong virus (DARV)	Korea (2017-2018)	Shrew	19,471	No data	Lee et al., 2021
Langya virus (LayV)	China (2019)	Shrew	18,402	Acute febrile	Zhang et al., 2022
Angavokely virus	Madagascar (2019)	Fruit bat	16,740	No data	Madera et al., 2022

Viruses in the genus *Henipavirus* have a broad reservoir and may cause high case fatality rates following human infection. The discovery and characterization of the novel Henipavirus is a high public health priority.

The greatest bat diversity around the world especially appears to be found in the tropical rain forests of South East Asia. In Thailand, at least 138 species in 11 families and 45 genera of bats have been recorded, such as members of the genus Pteropus, Rhinolophus, and Hipposideros (Soisook et al., 2011). Khao Chong Phran, a cave in Ratchaburi Province, has possibly the largest colony of bats in Thailand. There are estimated to be around 2 million bats in the cave and 95% are insect-eating bats (Department of National Parks, Wildlife and Plant Conservation, Thailand). Moreover, Khao Chong Phran has many community sites, such as cave entrances near communities, guano collection, and tourism sites, which risk zoonotic spillover. Bats are widely considered the animal hosts of zoonotic pathogens such as rhabdoviruses, coronaviruses, paramyxoviruses, and filoviruses, and many of the host species can be found in Thailand.

MojV was associated with a severe pneumonia-like illness killing three of six miners in 2012 in a mineshaft Yunnan, China. Interestingly, this mineshaft was also site where RaTG13, a beta-coronavirus most closely related with SARS-CoV-2, was detected in *Rhinolophid* bats (Rahalkar and Bahulikar, 2020). Identifying the wildlife reservoir of this novel virus will assist intervention strategies to reduce spillover risk.

Research Gaps

As a part of Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH), serological investigations using a multiplex microsphere-based immunoassay (MMIA) have detected four members of a high-risk cohort with MojV-reactive serum, including one person with a high magnitude and durability of anti-MojV IgG across longitudinal samples. Despite this, genomic surveillance has not detected MojV or any novel MojV-like viruses. For this result, we would to further identify and characterize the origin of the Mojiang virus-reactive serum and to explore the novel MojV-like virus in Thailand.

Serological and cellular immunity is an important resource for virus discovery detection by using the specificities and characteristics of memory B cells from peripheral blood mononuclear cells (PBMC). The immune response will recognize and induce antibodies specific to the infecting pathogen. Characterizing the memory B cell response to an unknown pathogen in humans could in the detection of the exposure pathogen in humans. Molecular techniques such as PCR can detect only the genomic material in the body, but memory B cells are long-lived and thus have a much larger window of detection.

Memory B-cell differentiation is characterized by somatic hypermutation in antibody variable region genes (V) and selection of B cells expressing high-affinity variants of this antigen receptor and high sequence similarity in individuals exposed to the same antigen reflect antigen-driven clonal selection and form shared immunological memory between individuals (Yang et al., 2021). Memory B cells are highly abundant in the human spleen, and can proliferate 45% of the total B cell population in this organ (Hauser et al., 2015). Memory B cells can be detected in the Ig variable (IgV) genes in a fraction of IgM+ B cells. In humans, B cells expressing the memory marker CD27+ can be found in the marginal zone of spleen and circulate in the blood. After re-exposure to antigen, memory B cells can quickly proliferate and differentiate into plasma cells. Many studies the antibody diversity and specificity of the naturally paired immunoglobulin heavy and light chains for affinity maturation of B cell receptor (BCR) after virus infection such as SARS-CoV-2 infection (Yang et al., 2021), influenza (Dougan, S et al., 2013), HIV (Moir, S., & Fauci, A. S.,2017), etc. The characterization of memory B cells can be used to develop monoclonal antibodies and produce an effective theraputic.

Significance and Approach

Southeast Asia is one of the world's highest-risk emerging infectious diseases hotspots. From this region emerged both SARS-CoV-1 and SARS-CoV-2, recurrent outbreaks of novel influenza strains, and the spillover of dangerous viral pathogens such as Nipah virus. The diversity of coronaviruses, henipaviruses, and filoviruses are found in wildlife reservoirs in Southeast Asia is due to the high diversity of wildlife, and the demography of the human population brings humans and wildlife into close contact. The spillover of viruses from animals to humans is often misdiagnosed and the clinical symptoms from patients is underestimated during the pandemic. This study provides a surveillance strategy in human and wildlife populations to better understand immunity to novel pathogens and identify the origin of these pathogens to serve as an early warning system to prevent outbreaks of emerging viruses.

This study uses immunology to identify a novel virus that can aide in effective vaccine discovery and development, monoclonal antibody production for prevention and therapy, and generate data resources for early detection and diagnostic assays. Whereas PCR detection or virus

isolation in wildlife is challenging, this new serological approach may be more successful in identifying reservoir hosts.

The Indo-Pacific region has a high risk of novel viral emergence. This study provides an opportunity for in-country research capacity building by linking local infectious disease scientists with an international collaborative network that has proven capacity to conduct this work and produce significant findings. Moreover, a global sero-surveillance network for surveillance technology extends virologic and immunologic screening to product discovery. This study will benefit and expand the CREID, EID-SEARCH networks, and the global sero-surveillance network to improve our understanding of immunology to identify novel pathogens and their origin to prevent future pandemics.

Research Methods

Aim 1. Longitudinal sampling of humans with MojV RBP-reactive serum, syndromic surveillance and investigations into the shrew and rodent population

Serological profiling provides an indirect measurement of the infecting virus. Despite 19% (54/284) seroprevalence in humans, we have not detected nucleic acid sequences of MojV or MojV-like henipaviruses in this human cohort. In addition to the translational application of mAbs as therapeutics, we intend to utilize these mAb as non-clinical diagnostics surveillance tools for virus/virus antigen capture. Additionally, we intend on expanding our understanding of henipavirus prevalence in this population with longitudinal sampling of the wildlife and human populations in this region.

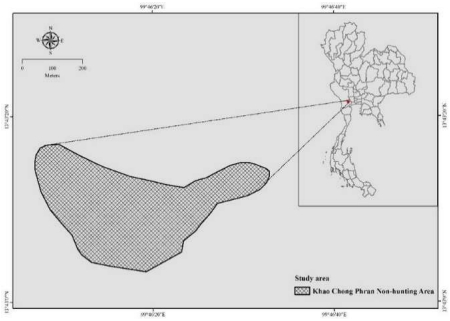


Figure 1**:** Map of Khao Chong Phran Non-hunting Area, Ratchaburi Province, Thailand (Boonpha N et al., 2019)

Site wildlife samples collection

100 bats and 100 shrews or rodents in and around the Khao Chong Phran cave in Ratchaburi Province will be collected for surveillance targeting the Mojiang-like virus. We plan one sampling trip to collect bats, and a second trip to target shrews and rodents. Due to limited knowledge of the local shrew population, we are unsure as to whether we can collect 100 samples, and will supplement the collection with rodents, as needed. Samples will be collected based on the following enrollment criteria: random age, random weight, random sex.

Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and stored. Oral swab and rectal swab will also collect in 100 bats and 100 shrews or rodents. Blood from animals will be collected using a needle to collect veinous blood, and collected blood will be centrifuged and the serum will be removed and stored.

Human surveillance

Four of the samples from prior investigations have shown high MojV RBP-reactive antibody titers and will be selected for follow-up sampling. Additionally, 100 human serum samples, nasopharyngeal swab and throat swab will be collected from persons deemed to be in close contact with wildlife, and people who present with or have recent history of undiagnosed acute febrile illness using the following enrollment criteria;

- Adults (≥ 18 years of age) who sign in the informed consent
- Who present with or have recent history of illness without diagnosis, such as severe/acute respiratory illness (SARI/ARI), Influenza-like illness (ILI), fever of unknown origin (FUO), or respiratory tract infection
- Who is the bat guano collector in the cave
- Who lives or works around wildlife habitats
- Who is involved with livestock or wildlife farming
- Who hunts or works with wildlife
- Who has not been previously sampled as part of the EID-SEARCH and PREICT projects

Sample size justification

Human population

$$n = \frac{N}{1 + Ne^2}$$

n = sample size of the study

N = population size for finite populations: 8,150 (data from 2018)

e = error: 10%

$$n = \frac{8{,}150}{1 + (8{,}510 \text{ x } 0.1^2)}$$

n = 98.79 (round up to 100 samples)

Wildlife population

The sample size of 100 of each species follows the guidelines established during the PREDICT project.

Targeted Viral Sequencing and Serologic testing

Targeted sequencing

For samples from shrews, rodents, and acute individuals, we will use targeted sequencing and metagenomic approaches in an attempt to detect the Thai MojV-like virus. The oral swab and rectal swab will do target sequencing in henipavirus Family PCR and Sanger sequencing. Then we use Next Generation Sequencing (NGS) technology to perform Whole Genome Sequencing (WGS) on the nucleic acid specimens with relatively strong PCR positive signals. WGS libraries will use an enrichment

library preparation (Respiratory Viral Oligos Panel, RVOP) or Viral Surveillance Panel with Illumina RNA Prep with Enrichment kit. Libraries will sequence on the Illumina MiSeq 3000 sequencer (Illumina).

Serological testing

Serum samples will be tested for RBP binding to the known henipavirus species, including the Nipah, Hendra, Cedar, Mojiang, Ghana, Langya, Gamak, and Daerong viruses to further investigate the seroprevalence in this region. Additionally, prior positive samples will be re-tested with the expanded panel to enhance our understanding of the antigenic relationships between the novel shrew-borne henipavirus and the Thai virus.

Data analysis

Sequencing will be analyzed using the SPAdes software (version 3.13.0, http://cab.spbu.ru/software/spades/) in Metagenome mode. Serological data will be analyzed using cluster analysis in RStudio.

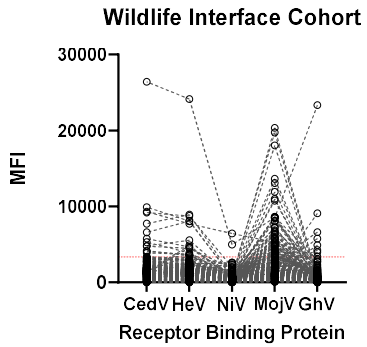
Aim 2. Characterization of monoclonal antibodies from humans with serological profiles consistent with MojV infection.

In collaboration with NIH VRC PREMISE, MojV RBP and F-reactive B cells will be captured, and the B cell receptor will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific mAbs will be generated from the corresponding sequences and will be assessed for binding potential to LayV RBP and F, as well as their neutralization potential.

Importance: The isolation of these mAbs represents a potential therapeutic for MojV, MojV-like henipas, and LayV, depending on how well these cross react.

Human samples collection site

Human positive serum for MojV was found close to Khao Chong Phran in Ratchaburi Province, which has the largest colony of bats in Thailand (Figure 2).



Commented [EL1]: Also propose to pull these out with MojV F.

And that you will test binding of these against LayV G and LayV F. To see cross reaction.

One antibody Broder has against MojV already has demonstrated cross reaction with a LayV protein.

David Veesler can do all the Cyro-EM to look at where these mAbs bind on the proteins.

Figure 2: Seroprevalence of henipavirus proteins from a community with a large bat interface during 2017-2018. MFI is the median fluorescence intensity for IgG reactivity to each protein. Red-dashed line is at 3,357 MFI, the assay cutoff.

Commented [MOU2]: Add in the longitudinal data

PBMC Sorting and B Cell isolation

Whole blood from MojV-positive people will be collected in individual heparin tubes and processed for PBMC isolation using Density Gradient Centrifugation (SepMate™ PBMC Isolation Tubes).

Human PBMCs will be stained with antibodies for positive markers of IgG memory B cells, and antibodies to negatively select and avoid contamination with dead cells, T cells, NK cells or monocytes (live, CD3−,CD14−,CD56−, IgM−, IgA−, CD19+,CD20+,CD27+). Avi-tag for biotinylation and 6xHIS tag for Nickel purification using the HisPur™ Ni-NTA Spin Purification Kit (Thermo Fisher Scientific, Waltham, MA). Single Memory B Cells will be sorted, cells will proliferate, cDNA will synthesized, then Heavy and light chain variable domains will be amplified (Waltari E et al., 2019).

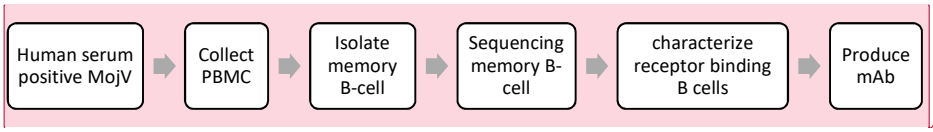
B cell receptor Amplicon Preparation and Recombinant Antibody sorting

Total RNA yields from the PBMC will be determined by absorbance at 260 nm on the NanoDrop™ One (Thermo Fisher Scientific). Total RNA will be used for first strand cDNA synthesis with gene-specific reverse transcription (RT) primers directed to the constant regions. The RT primers for IgG, IgM, IgA, lambda, and kappa will be included. Light chain and heavy chains will be amplified into amplicon. Double-stranded cDNA will be sequenced.

After completion of MiSeq sequencing, antibody repertoires will analyze using methods based on the pipeline. Heavy and light-chain sequences will be assembled into an expression vector and transfected into cells for monoclonal antibody expression. Recombinant antibody will be isolated and purified for downstream applications.

Functional assessment

Neutralization of viruses remains a critical correlate of protection for most vaccine strategies. Next, we will test the cross neutralization potential of these anti-MojV RBP and F mAbs to neutralize LayV (Duke NUS/LinFa should be able to do this, if they have LayV and are propagating it, they can use cell-cell fusion/CPE in a straight PRNT). Decide if you want to incorporate this.



Commented [EL3]: Put this under innovation

Determination of envelope glycoprotein epitope binding

Isolated mAbs will be assessed for binding to MojV RBP and F proteins through multiplex binding serology. Further, the cross reaction potential of these mAbs will be assessed against GamV, DarV, and LayV RBP and F. MojV has not been isolated or rescued, and the virus receptor for MojV has not yet been discovered [2 citations]. Thus, a functional assessment of cross neutralizing potential is not possible. However, through cyro-electron microscopy we can investigate binding epitopes between

these mAbs and MojV and LayV envelope glycoproteins. Epitope identification can then be used to make predictions about neutralizing potential through deductions about the location of henipavirus RBP receptor-binding pockets.

References

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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: Assistant Professor

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. (hons)	05/2008	Biology
Uniformed Services University, Bethesda, MD	Ph.D.	10/2016	Emerging Infectious Diseases
Uniformed Services University, Bethesda, MD	Postdoc	04/2019	Virology

A. Personal Statement

The spillover of zoonotic viruses into human populations remains an ever-prevalent threat to public and global health. Continual outbreaks of known and unknown zoonotic viruses highlights the paucity of our understanding of the viral diversity, geographic distribution, wildlife hosts, and human populations at-risk for spillover. I am a recently appointed assistant professor dedicated to biosurveillance and virological research of emergent zoonotic viruses. I have completed virological training and spent my time as a post-doctoral fellow applying *in vitro* techniques to characterize the replication of, and mammalian ephrin receptors that mediate cellular entry of emergent henipaviruses. Since the start of the COVID-19 pandemic, I pivoted the focus of my research team to serological analysis of SARS-CoV-2 infection, and then to COVID-19 vaccine-induced antibody durability. In my ongoing research, I develop serological-based approaches to identify the infectome of wildlife and human communities for the assessment of virus spillovers. Since my position as a postdoctoral fellow I have worked collaboratively with national and international researchers including institutional partners at Duke-NUS, Chulalongkorn University, University of Pretoria, and Rocky Mountain Labs towards these goals. Across all of these collaborations, we have aimed to identify known and unknown viruses, the wildlife reservoirs of priority pathogens, the high-risk interfaces for spillover and the biotic/abiotic drivers of virus emergence. Throughout these research projects, we have detected unexpected serological profiles in communities of bats and humans that have not fit our prior understanding of the viral diversity established or detected by genetic techniques. This proposal aims to better understand these serological profiles of wildlife and humans with a targeted focus on henipaviruses and filoviruses. Ongoing projects that I would like to highlight include:

- HDTRA12110037. DTRA BRTP, E. Laing (Co-PI). 08/2021-07/2026, “Informing biosurveillance, contribution of pteropodid fruit bats to virus spillover in the Philippines.”
- HU00012020067, HU00012120104, HU00012120094.Defense Health Program/CARES Act, NIAID, E. Laing (Associate Investigator). 9/2020-9/2025, “Prospective Assessment of SARS-CoV-2 Seroconversion.”
- HDTRA12010025. DTRA BTRP, E. Laing (Co-I). 7/2020 – 6/2025, “Biosurveillance for Viral Zoonoses around Bat-Livestock-Human interfaces in Southern Africa.”
- U01AI151797. NIH, Centers for Research in Emerging Infectious Diseases, E. Laing (Co-I). 02/2020 – 03/2025, “EID-Southeast Asia Research Collaborative Hub.”
- HU00012020067, HU00011920111. Defense Health Program, NIAID, E. Laing (Associate Investigator). 03/2020 – 09/2023, “Epidemiology, Immunology, and Clinical Characteristics of Emerging Infectious Diseases with Pandemic Potential (EpICC-EID).”

B. Positions, Scientific Appointments and Honors

Positions

2021-pres. Assistant Professor, Department of Microbiology and Immunology, School of Medicine, Uniformed Services University, Bethesda, MD.
2021-pres. Joint Appointment, Emerging Infectious Diseases Graduate Program, School of Medicine, Uniformed Services University, Bethesda, MD.
2019-21 Research Assistant Professor, Department of Microbiology and Immunology, Uniformed Services University, Bethesda, MD.
2016-19 Postdoctoral fellow, Henry M. Jackson Foundation, Department of Microbiology, Uniformed Services University, Bethesda, MD.
2010-16 Graduate research student, Department of Microbiology, Uniformed Services University, Bethesda, MD.

Other Experiences and Professional Memberships
2022-pres. Executive committee member, Emerging Infectious Diseases Graduate Program, USUHS
2021-pres. Research center representative, Laboratory Assays Oversight and Quality Working Group, Emerging Infectious Diseases: Southeast Asia Research Collaboration Hub (EID-SEARCH), Centers for Research in Emerging Infectious Diseases (CREID), NIAID, DMID, NIH
2021-pres. Review editor, Frontiers in Virology - Emerging and Reemerging Viruses
2021-pres. Ad hoc reviewer, Frontiers in Immunology, Journal of Clinical Immunology
2021-pres. Steering committee member, JPI/Military Infectious Diseases Research Program, Emerging Infectious Diseases
2019 Ad hoc reviewer, EcoHealthNet 2.0 Program, EcoHealth Alliance
2018 Ad hoc reviewer, Pakistan One-Health Fellowship Program, National Academy of Sciences & Pakistan Academy of Sciences
2014-2019 Member, American Society of Tropical Medicine and Hygiene
2014-2019 Member, American Society of Microbiology
2014-2019 Volunteer, AAAS STEM K-12 Volunteer Program

Mentoring
Postdoctoral fellows Si'Ana A. Coggins, PhD, 2020 - 2022
Graduate students Marana S. Tso, BS, 2021 -
McKenna Roe, BS, 2022 -
Committee member Celeste Huaman, BS, 2021 -
2LT Connor Perry, BS, 2021 -

Honors
2021-2022 Impact Award, USUHS School of Medicine
2021 Outstanding Research Accomplishment/Team/SARS-CoV-2, The EPICC COVID-19 Cohort Team, Military Health System Research Symposium
2020-2021 Impact Award, USUHS School of Medicine
2015-2016 Val G. Hemming Fellowship, Henry M. Jackson Foundation
2015 East Asia and Pacific Summer Institutes Fellowship, National Science Foundation

C. Contributions to Science (†*mentee*, *corresponding)
1. Lyssaviruses and the prototype, rabies virus, remain a public health concern. Beginning with my PhD thesis work, I've researched the virus host-interactions between a rabies-related lyssavirus, Australian bat lyssavirus (ABLV), and its bat host (*Pteropus alecto*). Research has focused on ABLV cellular entry mechanisms, the development of an animal model and ABLV reporters and exploration of novel monoclonal antibodies that neutralize ABLV and other phylogroup I lyssaviruses. Furthermore, comparative bat immunology research was conducted using black flying fox cell lines and ABLV as a model virus/host interaction. Physiological adaptations that accompanied the evolution of flight in bats have been proposed to contribute to the frequent role of bats as asymptomatic hosts of highly pathogenic zoonotic viruses. Comparatively studying the autophagy pathway in bat cell lines revealed that bat cells had elevated levels of basal autophagy and experienced significantly less cell death when challenged with high virus doses.

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. Virol J. 2013. 11:40. doi: 10.1186/1743-422X-11-40. PMID: 24576301, PMCID: PMC3946599

b. **Laing E.D.***, Sterling S.L., Weir D.L., Beauregard C.R., Smith I.L., Larsen S.E., Wang L-F., Snow A.L., Schaefer B.C., and Broder C.C. Enhanced autophagy contributes to reduced viral infection in black flying fox cells. Viruses. 2019. Mar 14;11(3). pii: E260. doi: 10.3390/v11030260. PMID: 30875748, PMCID: PMC6466025

c. Mastraccio K.E., Huaman C., Warrilow D., Smith G.A., Craig S.B., Weir D.L., **Laing E.D.**, Smith I., Broder C.C. and B.C. Schaefer. Establishment of a longitudinal pre-clinical model of lyssavirus infection. J Virol Methods. 2020 Jul; 281:113882. doi: 10.1016/j.jviromet.2020.113882. Epub 2020 May 12. PMID: 32407866

d. Weir D.L., Coggins S.A., Vu B.K., Coertse J., Yan L., Smith I.L., **Laing E.D.**, Markotter W., Broder C.C., and Schaefer B.C. Isolation and characterization of cross-reactive human monoclonal antibodies that potently neutralize Australian bat lyssavirus variants and other phylogroup 1 lyssaviruses. Viruses. 2021 Mar 1;13(3):391. doi: 10.3390/v13030391. PMID: 33804519; PMCID: PMC8001737.

2. My research experience as a postdoctoral fellow furthered my training in molecular virology techniques. I constructed a recombinant Cedar virus cDNA plasmid and optimized a reverse genetics approach to rescue a recombinant Cedar virus reporter virus, a non-pathogenic *Henipavirus* species. A molecular biology methods chapter detailing recombinant Cedar virus reverse genetics has been submitted and in press. Using this recombinant Cedar virus, we determined that Cedar virus can utilize several non-canonical henipavirus ephrin receptors for cellular entry and explored the structure of the receptor-binding pocket to understand the receptor promiscuity. The non-pathogenic phenotype of CedV creates a potential for CedV to act as a model henipavirus to explore host-pathogen interactions, cellular tropism and factors that determine henipaviral disease pathogenesis. Additionally, I have collaborated on projects detailing henipavirus infection and replication in bat hosts with colleagues at the Rocky Mountain Labs, studying whether specific species of bats are more competent hosts and whether virus-host restriction exists.

a. Amaya M, Broder CC, **Laing ED***. Recombinant Cedar virus: a henipavirus reverse genetics platform. In: Freiberg A.N. and B. Rockx, Nipah Virus: Methods and Protocols, Methods Mol. Biol. (in press)

b. Seifert SN, Letko MC, Bushmaker T, **Laing ED**, Saturday G, Meade-White K, van Doremalen N, Broder CC, Munster VJ. Rousettus aegyptiacus Bats Do Not Support Productive Nipah Virus Replication. J Infect Dis. 2020 May 11;221(Suppl 4):S407-S413. doi: 10.1093/infdis/jiz429. PMID: 31682727; PMCID: PMC7199784.

c. **Laing ED,** Navaratnarajah CK, Cheliout Da Silva S, Petzing SR, Xu Y, Sterling SL, Marsh GA, Wang LF, Amaya M, Nikolov DB, Cattaneo R, Broder CC, Xu K. Structural and functional analyses reveal promiscuous and species specific use of ephrin receptors by Cedar virus. Proc Natl Acad Sci U S A. 2019 Oct 8;116(41):20707-20715. doi: 10.1073/pnas.1911773116. Epub 2019 Sep 23. PMID: 31548390; PMCID: PMC6789926.

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. PMID: 29587789; PMCID: PMC5869790.

3. Bats are increasingly identified as animal reservoirs of emerging zoonotic viruses (e.g. Nipah virus, Ebola virus and SARS-coronavirus). I lead collaborative biosurveillance and research preparedness training including data analysis and interpretations at international partner institutes with lab technicians, field and lab scientists, and masters, doctoral and postdoctoral trainees. Collaborative biosurveillance is presently underway in Thailand (Chulalongkorn University, Bangkok) Malaysia (National Wildlife and Forensic Lab, Universti Purtra Malaysi, National Public Health Lab) via NIH Centers for Research in Emerging Infectious Diseases, EID-Southeast Asia Research Collaborative Hub. As a collaborator within the DARPA PREEMPT network I supported surveillance for coronaviruses and other priority emerging zoonotic viruses, henipaviruses and filoviruses, in Ghana (Zoological Society of London), Australia (Black

Mountain Labs) and Bangladesh (icddr,b). We aim to characterize the geographic distribution of zoonotic filoviruses/henipaviruses/coronaviruses, transmission dynamics in wildlife hosts and generate risk-models for Ebola virus, Nipah virus and SARS-related CoV outbreaks. Results discovered so far suggest a wider geographical footprint of Asiatic filoviruses and have identified several fruit bat species that act as natural reservoirs for these viruses.

a. Paskey AC, Ng JHJ, Rice GK, Chia WN, Philipson CW, Foo RJH, Cer RZ, Long KA, Lueder MR, Lim XF, Frey KG, Hamilton T, Anderson DE, **Laing ED**, Mendenhall IH, Smith GJ, Wang LF, Bishop-Lilly KA. Detection of Recombinant Rousettus Bat Coronavirus GCCDC1 in Lesser Dawn Bats (Eonycteris spelaea) in Singapore. Viruses. 2020 May 14;12(5):539. doi: 10.3390/v12050539. PMID: 32422932; PMCID: PMC7291116.

b. Schulz JE, Seifert SN, Thompson JT, Avanzato V, Sterling SL, Yan L, Letko MC, Matson MJ, Fischer RJ, Tremeau-Bravard A, Seetahal JFR, Ramkissoon V, Foster J, Goldstein T, Anthony SJ, Epstein JH, **Laing ED**, Broder CC, Carrington CVF, Schountz T, Munster VJ. Serological Evidence for Henipa-like and Filo-like Viruses in Trinidad Bats. J Infect Dis. 2020 May 11;221(Suppl 4):S375-S382. doi: 10.1093/infdis/jiz648. PMID: 32034942; PMCID: PMC7213578.

c. Dovih P, **Laing ED**, Chen Y, Low DHW, Ansil BR, Yang X, Shi Z, Broder CC, Smith GJD, Linster M, Ramakrishnan U, Mendenhall IH. Filovirus-reactive antibodies in humans and bats in Northeast India imply zoonotic spillover. PLoS Negl Trop Dis. 2019 Oct 31;13(10):e0007733. doi: 10.1371/journal.pntd.0007733. Erratum in: PLoS Negl Trop Dis. 2021 Nov 16;15(11):e0009836. PMID: 31671094; PMCID: PMC6822707.

d. **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. PMID: 29260678; PMCID: PMC5749470.

4. Responding to the emergence of SARS-CoV-2, our lab developed multiplex serology strategies to identify SARS-CoV-2 antibodies and address research questions related to whether pre-existing antibody memory induced by prior infection with seasonal human coronaviruses effects COVID-19 severity. Our lab's serology efforts supported NIH and DHA collaboratively funded protocols including prospective, longitudinal serological analysis of hospital and community subjects, and health-care workers; and cross-sectional analyses of SARS-CoV-2 infection among deployed military health-care workers.

a. Epsi NJ, Richard SA, Lindholm DA, Mende K, Ganesan A, Huprikar N, Lalani T, Fries AC, Maves RC, Colombo RE, Larson DT, Smith A, Chi SW, Maldonado CJ, Ewers EC, Jones MU, Berjohn CM, Libraty DH, Edwards MS, English C, Rozman JS, Mody RM, Colombo CJ, Samuels EC, Nwachukwu P, Tso MS, Scher AI, Byrne C, Rusiecki J, Simons MP, Tribble D, Broder CC, Agan BK, Burgess TH, **Laing ED**, Pollett SD; EPICC COVID-19 Cohort Study Group. Understanding 'hybrid immunity': comparison and predictors of humoral immune responses to SARS-CoV-2 infection and COVID-19 vaccines. Clin Infect Dis. 2022 May 24:ciac392. doi: 10.1093/cid/ciac392. Epub ahead of print. PMID: 35608504; PMCID: PMC9213853.

b. Lu Z, **Laing ED**, Pena DaMata J, Pohida K, Tso MS, Samuels EC, Epsi NJ, Dorjbal B, Lake C, Richard SA, Maves RC, Lindholm DA, Rozman JS, English C, Huprikar N, Mende K, Colombo RE, Colombo CJ, Broder CC, Ganesan A, Lanteri CA, Agan BK, Tribble D, Simons MP, Dalgard CL, Blair PW, Chenoweth J, Pollett SD, Snow AL, Burgess TH, Malloy AMW; EPICC COVID-19 Cohort Study Group. Durability of SARS-CoV-2-Specific T-Cell Responses at 12 Months Postinfection. J Infect Dis. 2021 Dec 15;224(12):2010-2019. doi: 10.1093/infdis/jiab543. PMID: 34673956; PMCID: PMC8672777.

c. Pollett SD, Richard SA, Fries AC, Simons MP, Mende K, Lalani T, Lee T, Chi S, Mody R, Madar C, Ganesan A, Larson DT, Colombo CJ, Colombo R, Samuels EC, Broder CC, **Laing ED**, Smith DR, Tribble D, Agan BK, Burgess TH. The SARS-CoV-2 mRNA vaccine breakthrough infection phenotype includes significant symptoms, live virus shedding, and viral genetic diversity. Clin Infect Dis. 2021 Jun 12:ciab543. doi: 10.1093/cid/ciab543. Epub ahead of print. PMID: 34117878.

d. Clifton GT, Pati R, Krammer F, **Laing ED**, Broder CC, Mendu DR, Simons MP, Chen HW, Sugiharto VA, Kang AD, Stadlbauer D, Pratt KP, Bandera BC, Fritz DK, Millar EV, Burgess TH, Chung KK. SARS-CoV-2 Infection Risk Among Active Duty Military Members Deployed to a Field Hospital - New

York City, April 2020. MMWR Morb Mortal Wkly Rep. 2021 Mar 5;70(9):308-311. doi: 10.15585/mmwr.mm7009a3. PMID: 33661864; PMCID: PMC7948931.

5. In addition to providing serologic assessment of SARS-CoV-2 infection, my research team is actively engaged in examining the durability of COVID-19 vaccine induced humoral immunity. Antibody responses, particularly neutralizing antibodies, are frequently cited as a predictive correlate of protection. With the emergence of variants of concern and waning circulating antibodies, the timing of booster shots remains an important measure for controlling the pandemic. In my lab we evaluate the duration of neutralizing antibodies, durability and breadth of antibody responses against emerging variants of concern, hybrid immune responses, and post-vaccination infections.

a. Wang W, Lusvarghi S, Subramanian R, Epsi NJ, Wang R, Goguet E, Fries AC, Echegaray F, Vassell R, Coggins SA, Richard SA, Lindholm DA, Mende K, Ewers EC, Larson DT, Colombo RE, Colombo CJ, Joseph JO, Rozman JS, Smith A, Lalani T, Berjohn CM, Maves RC, Jones MU, Mody R, Huprikar N, Livezey J, Saunders D, Hollis-Perry M, Wang G, Ganesan A, Simons MP, Broder CC, Tribble DR, **Laing ED**, Agan BK, Burgess TH, Mitre E, Pollett SD, Katzelnick LC, Weiss CD. Antigenic cartography of well-characterized human sera shows SARS-CoV-2 neutralization differences based on infection and vaccination history. Cell Host Microbe. 2022 Dec 14;30(12):1745-1758.e7. doi: 10.1016/j.chom.2022.10.012. Epub 2022 Oct 21. PMID: 36356586; PMCID: PMC9584854.

b. **Laing ED,** Weiss CD, Samuels EC, Coggins SA, Wang W, Wang R, Vassell R, Sterling SL, Tso MS, Conner T, Goguet E, Moser M, Jackson-Thompson BM, Illinik L, Davies J, Ortega O, Parmelee E, Hollis-Perry M, Maiolatesi SE, Wang G, Ramsey KF, Reyes AE, Alcorta Y, Wong MA, Lindrose AR, Duplessis CA, Tribble DR, Malloy AMW, Burgess TH, Pollett SD, Olsen CH, Broder CC, Mitre E. Durability of Antibody Response and Frequency of SARS-CoV-2 Infection 6 Months after COVID-19 Vaccination in Healthcare Workers. Emerg Infect Dis. 2022 Apr;28(4):828-832. doi: 10.3201/eid2804.212037. Epub 2022 Feb 24. PMID: 35203111; PMCID: PMC8962883.

c. Lusvarghi S, Pollett SD, Neerukonda SN, Wang W, Wang R, Vassell R, Epsi NJ, Fries AC, Agan BK, Lindholm DA, Colombo CJ, Mody R, Ewers EC, Lalani T, Ganesan A, Goguet E, Hollis-Perry M, Coggins SA, Simons MP, Katzelnick LC, Wang G, Tribble DR, Bentley L, Eakin AE, Broder CC, Erlandson KJ, **Laing ED**, Burgess TH, Mitre E, Weiss CD. SARS-CoV-2 BA.1 variant is neutralized by vaccine booster-elicited serum but evades most convalescent serum and therapeutic antibodies. Sci Transl Med. 2022 May 18;14(645):eabn8543. doi: 10.1126/scitranslmed.abn8543. Epub 2022 May 18. PMID: 35380448; PMCID: PMC8995032.

d. [†]*Coggins SA*, **Laing ED**, Olsen CH, Goguet E, Moser M, Jackson-Thompson BM, Samuels EC, Pollett SD, Tribble DR, Davies J, Illinik L, Hollis-Perry M, Maiolatesi SE, Duplessis CA, Ramsey KF, Reyes AE, Alcorta Y, Wong MA, Wang G, Ortega O, Parmelee E, Lindrose AR, Snow AL, Malloy AMW, Letizia AG, Ewing D, Powers JH, Schully KL, Burgess TH, Broder CC, Mitre E. Adverse Effects and Antibody Titers in Response to the BNT162b2 mRNA COVID-19 Vaccine in a Prospective Study of Healthcare Workers. Open Forum Infect Dis. 2021 Nov 20;9(1):ofab575. doi: 10.1093/ofid/ofab575. PMID: 35047649; PMCID: PMC8759445.

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Proposal Cover Sheet

Project Title	Immune memory bait & capture to identify emerging henipavirus origins
Principal Investigator Name	Krongkan Srimuang, Ph.D.
Position/Title	Research Scientist
Department	Thai Red Cross Emerging Infectious Diseases Clinical Center
Institution Name	King Chulalongkorn Memorial Hospital
Street	Rama 4 Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	Krongkan.sr@gmail.com
Phone	+66 (0) 92-453-0808
Country(ies) where work will be conducted	Thailand
Pathogen(s) focus	Mojiang Virus, Henipavirus, Paramyxovirus
Co-Principal Investigator Name (*If applicable*)	Spencer Sterling, MPH
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Contractual Contact, Title	
Institution Name	
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City, State, Zip Code	
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Phone	

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Name of Mentor	Eric Laing, Ph.D.
Mentor Institution	Uniformed Services University of the Health Sciences
Institution Address	Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814
Permanent location of Mentor	4301 Jones Bridge Road, Bethesda, MD 20814
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Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Name of Mentor	Supaporn Wacharapluesadee, Ph.D.
Mentor Institution	Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital
Institution Address	1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Permanent location of Mentor	4th Floor, Jongkolnee Watwong Building, Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital, 1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
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Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Total Budget	***Repeat this table if there are two Co-PIs***
Direct Costs	
Indirect Costs	
Proposed Start Date	
Proposed End Date	

Project Abstract (250 words)

Prototypical henipaviruses (Hendra and Nipah virus) ~~cause~~ are bat-borne zoonotic ~~diseases~~ viruses, cause ~~with~~ high mortality, and have no~~no~~ effective vaccines or therapeutics. ~~and are hosted by Pteropus flying foxes.~~ The recent detection of closely-related Mojiang and Langya viruses in rats and shrews, and ~~an outbreak~~ isolation of Langya virus from individuals with ~~of~~ acute febrile~~e LayV~~ illness ~~in people~~, challenges the dogma that fruit bats are the key henipavirus reservoirs, and heightens their threat profile. The recently expanded *H~~h~~**enipavirus** genus includes two other shrew-borne viruses (Gamak and Daeryong viruses), and the Madagascan fruit bat-borne Angovkeely virus. ~~In this study~~As part of EID-SEARCH acitivies, we ~~have~~ found serological evidence of infection by a~~ce of a~~ Mojiang-related virus in a community of Thai bat guano collectors who have~~with~~ occupational ~~high~~ exposure to microbats and rodents. Identifying the wildlife reservoir of this likely novel virus will assist intervention strategies to reduce spillover risk. However, PCR detection or virus isolation in wildlife is challenging, and new

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serological approaches may be more successful in identifying reservoir hosts. This project aims to identify novel viruses and ~~study~~ zoonotic reservoirs ~~for pathogen origin~~ through~~by~~ ~~using~~ serologic and cellular immune discovery. We plan to isolate anti-MojV monoclonal antibodies (mAb) from peripheral blood mononuclear cells collected from human study participants we have identified as immunoreactive with MojV antigens, and characterize the cross reactive and cross neutralizing potential of these ~~in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic. We plan to identify seropositive human participants MojV-like virus-specific~~ mAbs to MojV and LayV envelope glycoproteins. ~~MAbs~~Further, we will ~~be generated~~conduct follow-up human and wildlife (bats and rodents) surveillance in the province where the seropositive humans were identified. In addition to our antigen-based approach, we will utilize the mAbs for ~~and used for~~ direct antibody-mediated virus capture~~-~~ of this novel henipavirus from human and wildlife samples, and downstream genomic sequencing~~in people and wildlife~~. ~~We will develop anti-RBP standards to explore the antigenic spatial relationships between all known rodent- and bat-borne HPVs, and the Thai MojV-like virus these will allow the serologic signature of related undiscovered henipaviruses to be identified.~~

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Project Title **Immune memory bait & capture to identify emerging henipavirus origins**

Table of Contents

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Letters of Organizational Support

Letter of Collaboration from CREID Research Center PI

Letter from Research Center Mentor

Letter from Primary Scientific Mentor

Study Personnel ***(1-page limit)***

Krongkan Srimuang, Ph.D., is a researcher at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital and supports the Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia (EID-SEARCH) research. Dr. Srimuang is also a medical technologist working to identify, detect and characterize the spill-over risk of high zoonotic potential viruses from wildlife and humans using serology techniques, Next-generation sequencing, and PCR assays to identify novel viruses. Dr. Srimuang also performs the project Smart Sentinel Surveillance System: Emerging Viral Pneumonia and Respiratory tract infections (RTIs) to identify, detect and characterize the novel/known Respiratory Viruses in our country, Thailand by using multiplex real-time PCR, family PCR and Next-generation sequencing of novel viral pathogens. Dr. Srimuang graduated with Ph.D. in Tropical Medicine from the department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University.

Spencer Sterling, MPH, is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region. Mr. Sterling will planning, sample processing, data analysis, and report preparation for this project.

Dr. Eric Laing will act as Mentor for Dr. Krongkan and Spencer under the Centers for Research in Emerging Infectious Diseases (CREID) Pilot Research Program.

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Dr. Supaporn Wacharapluesadee, Ph.D. is the head laboratory at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, a WHO Collaborating Centre for Research and Training on Viral Zoonoses. Dr. Wacharapluesadee research interests include emerging infectious diseases in bats (including Nipah, Rabies, Coronavirus, and novel pathogens) as well as molecular diagnoses and sequencing of viruses. During the past 10 years, Dr. Wacharapluesadee got funding from USAID PREDICT to conduct the surveillance for novel virus in wildlife and human, and from Biological Threat Reduction Program US DTRA to conduct the EID surveillance in bat using the molecular technology and multiplex serology panel (Luminex technology). TRC-EIDCC is responsible for molecular diagnoses services to the hospital, and is the reference laboratory for rabies virus, MERS-CoV, Ebola, Zika, and other infectious diseases diagnoses for Ministry of Public Health, Thailand and laboratory training center on national and

regional level. Dr. Wacharapluesadee has served and consulted on several WHO and Thai government committees.

Specific Aims (1-page limit)

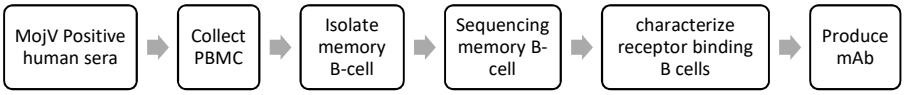
This project aims to study zoonotic reservoirs for pathogen origin by using serologic and cellular immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

The hypothesis

1. Can the memory B-cell immune response be used as a tool for the discovery of novel henipaviruses?
2. Can the spatial relationships between henipaviruses receptor binding proteins be generated that would allow for an understanding of antigenic evolution and functional characterization.

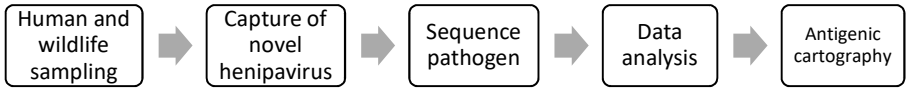
Aim 1. Antibody-mediated bait & capture of Thai MojV-like virus

In collaboration with NIH PREMISE, MojV RBP-reactive B cell receptors will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific MAbs will be generated and used for direct antibody-mediated virus capture in people and wildlife.



Aim 2. Modeling antigenic relationships among known and unknown henipaviruses.

Defining antigenic relationships among these will allow the serologic signature of related undiscovered henipaviruses to be identified. We will develop anti-RBP standards to explore the antigenic spatial relationships between all known rodent- and bat-borne HPVs, and the Thai MojV-like virus.



Objective 1. To isolate and sequence memory B-cells from humans positive for Mojiang virus in Ratchaburi province, Thailand for antibody production.

Objective 2. To capture unknown MojV-like viruses during syndromic surveillance in humans and routine surveillance in wildlife in Ratchaburi province, Thailand.

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Objective 3: To generate antigenic maps using anti-henipavirus RBP standards.

Study Rationale (7-page limit)

Nipah virus (NiV) and henipaviral diseases are among the most virulent of zoonotic diseases. The genus *Henipavirus* is currently composed of nine viruses including the prototypes, NiV and Hendra virus (HeV), and Cedar virus (CedV), Ghana virus (GhV), Mojiang virus (MojV) (McLinton et al., 2017), Langya virus (LayV) (Zhang X et al., 2022), Gamak virus (GAKV), Daeryong virus (DARV) (Lee et al., 2021), and Angavokely virus (AngV) (Madera et al., 2022).

HeV was first identified in Australia in 1994 as thea ~~a~~ cause of respiratory illness and fatal encephalitis in humans and horses. Closely following the discovery of HeV, NiV was identified in 1999 in Malaysia after outbreaks of fatal encephalitis in humans, with swine determined to be the intermediate host. Annual outbreaks of NiV have occurred in Bangladesh and are associated with the consumption of raw date palm sap, and since 19 May 2018 annual outbreaks of NiV associated encephalitis of been recorded in Kerala, India (Uwishema et al., 2022). Both HeV and NiV have a broad host range capable of infecting several mammalian orders, and can enter and infect endothelial cells, vasculature, and the central nervous system leading to severe multisystem organ diseases and high mortality. The broad host and tissue tropism of these viruses has been attributed to the use of the ephrinB2 and ephrinB3 ligands, highly conserved proteins with critical functions in evolutionary development, as virus receptors (Bossart et al., 2008). There are currently no approved vaccines or therapeutics and NiV and henipaviral diseases remain WHO Blueprint list priority pathogens (Middleton and Weingartl, 2012).

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A close relative of HeV, CedV was isolated from Australian bats in 2012 and found to be non-pathogenic, failing to cause any clinical symptoms in ferrets and guinea pig animal models (Marsh GA et al., 2012). The natural reservoirs of HeV, NiV, and CedV are flying foxes (*Pteropus sp.*) (Middleton and Weingartl, 2012). GhV was detected in Ghana in straw-colored fruit bats (*Eidolon helvum*) during routine surveillance in 2015 and was the first henipavirus found outside of the Indo-Pacific region. GhV showed a close phylogenetic relationship with HeV and NiV, and like HeV, NiV, and CedV the GhV RBP can mediate binding to ephrinB2 ligand and mediate cellular fusion (Drexler, J. F., 2009). Recent investigations into Madagascan fruit bats (*Eidolon duprenum*) detected a second African henipavirus, AngV. As neither GhV nor AngV have been isolated or rescued, the pathogenic and zoonotic potential of these African fruit bat associated henipaviruses remains unknown.

During follow-up investigations into lethal pneumonia from three miners in 2012 in China, m~~M~~ultiple genera~~us~~ of small mammals were tested for viral RNA, and MojV was detected in yellow-breasted rats (R. flavipectus) (Wu Z et al., 2014). ~~The discovery of the MojV genome occurred during follow-up investigations into lethal pneumonia from three miners in 2012 in China, however t~~The pathogenic nature of this virus has only been suggested and no isolates have been discovered or tested

in an animal model. Additionally, the novel shrew-borne henipaviruses, Gamak virus (GAKV), and Daeryong virus (DARV) were found in the Republic of Korea in 2017-2018. (Lee et al., 2021). Most recently, a close relative of MojV, Langya henipavirus (LayV) was recently isolated from febrile patients in eastern China. Subsequently, LayV was identified in shrews (Zhang et al., 2022), increasing the number of Asiatic rodent-associated henipaviruses to four. These recent discoveries are challenging the paradigms surrounding henipaviruses and their associations with bats and may suggest a novel rodent sub-genus of henipaviruses.

The Henipavirus genome consists of six structural proteins including nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), receptor-binding protein (RBP), and large (L) protein (approximately length of 18,000 nucleotides) (Luby, S. P., & Gurley, E. S., 2012). In HeV and NiV, slippage sites within the P protein lead to the expression of the V, W, and C proteins. These proteins are virulence factors and are absent in CedV.

Table 1 Summary viruses in Henipavirus

Henipavirus	Origin (Year)	Reservoir	Genome (nucleotides)	Symptom for human	Reference
Hendra virus (HeV)	Australia (1994)	Fruit bats	18,234	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Nipah virus (NiV)	Malaysia (1999)	Fruit bat	18,246	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Ghana virus (GhV)	Africa (2009)	Fruit bat (*Eidolon helvum)*	18,530	No data	Drexler, J. F., 2009
Cedar virus (CedV)	Australian (2012)	Fruit bat	18,162	Non-pathogenic	Marsh, G. A et al., 2012
Mojiang virus (MojV)	China (2012)	Rodent	18,404	Febrile	Wu Z et al., 2014
Gamak virus (GAKV),	Korea (2017-2018)	Shrew	18,460	No data	Lee et al., 2021
Daeryong virus (DARV)	Korea (2017-2018)	Shrew	19,471	No data	Lee et al., 2021
Langya virus (LayV)	China (2019)	Shrew	18,402	Acute febrile	Zhang et al., 2022
Angavokely virus	Madagascar (2019)	Fruit bat	16,740	No data	(Madera et al., 2022)

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Viruses in the genus *Henipavirus* have ~~a broad~~diverse natural reservoirs and several are known to cause disease ~~may cause high case fatality rate~~in ~~s following human infection~~humans. S~~T~~heurveillance for known, and the discovery and characterization novel henipaviruses ~~of the novel Henipavirus is a~~remains a high ~~public health~~ priority for public and global health.

Research Gap

The tropical rain forests of South East Asia host ~~The~~ greatest global diversity of bat ~~diversity~~ species.~~around the world especially appears to be found in the tropical rain forests of South East Asia.~~ In Thailand, at least 138 species in 11 families and 45 genera of bats have been recorded, such as members of the genera~~us~~ *Pteropus, Rhinolophus*, and *Hipposideros* (Soisook et al., 2011). Khao Chong Phran, a cave in Ratchaburi Province, has possibly the largest colony of bats in Thailand. There are estimated to be around 2 million bats in the cave and 95% are insect~~-eating~~ivorous ~~bats~~ (Department of National Parks, Wildlife and Plant Conservation, Thailand). Moreover, Khao Chong Phran has many community sites, such as cave entrances near communities, guano collection, and tourism sites, which represent high-risk interfaces for zoonotic spillover. Bats are widely considered the animal hosts of several zoonotic viral families, including ~~pathogens such as~~ rhabdoviruses, coronaviruses, paramyxoviruses, and filoviruses~~, and many~~ Many of these bat reservoir species can be regionally found~~of the host species can be found~~ in Thailand.

The MojV genome was detected in rodents sampled in a mineshaft in Yunnan, China frequented by miners who died from an undiagnoses ~~was associated with a~~ lethal ~~severe~~ pneumonia-like illness. ~~killing three of six miners in 2012 in a mineshaft Yunnan, China.~~ Interestingly, this mineshaft was also site where RaTG13, a beta-coronavirus most closely related with SARS-CoV-2, was detected in Rhinolophid bats (Rahalkar and Bahulikar, 2020). This was the first time the full genome of a henipavirus had been detected in a non-bat wildlife host. Studies of the MojV virus receptor have yet to identify a cellular receptor, and whether this virus can mediate entry and fusion in human [cite]. It is clear that human ephrinB2 and ephrinB3, canonical receptors for HeV, NiV, CedV, and GhV, do not interact with the MojV receptor-binding glycoprotein (RBP). Thus, the zoonotic potential remains elusive. Subsequently, genomes of GamV and DarV were detected in shrews sampled in South Korea, however, it is not yet clear whether these viruses are zoonotic or pathogenic to humans. The isolation of LayV from humans with febrile illness and isolation from shrews in eastern China was the first evidence that rodent-associated henipavirus are capable of zoonotic transmission and can cause disease in humans. ~~Identifying the wildlife reservoir of this novel virus will assist intervention strategies to reduce spillover risk.~~

As a part of Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH), serological investigations using a antigen-based multiplex microsphere~~-based~~ immunoassay (MMIA) have detected four ~~members~~ individuals of a high-risk human cohort with MojV-reactive serum, including one person with a high magnitude and durability of anti-MojV IgG across longitudinal samples. These results are suprising as ~~Despite this,~~ genomic surveillance has not detected MojV or any novel MojV-like viruses to date in Thailand. These results indicate that the MojV-like henipaviruses may have a regional footprint in Thailand yet remain undetectable by current PCR and sequencing approached. ~~**For this result**~~**Here, we aim to apply serological and immunological techniques to virus discovery that would permis us to~~ ~~**, we would to further**~~ **identify and characterize the** ~~**origin of**~~ **the**~~**the**~~ **MojV**~~**iang-**~~**like virus** ~~**and to explore the novel MojV-like virus**~~ **in Thailand.**

Research Impact

Serological and cellular immunity is an important resource for virus discovery detection by using the specificities and characteristics of memory B cells from peripheral blood mononuclear cells (PBMC). The immune response will recognize and induce antibodies specific to the infecting pathogen. Characterizing the memory B cell response to an unknown pathogen in humans could in the detection of

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the exposure pathogen in humans. Molecular techniques such as PCR can detect only the genomic material in the body, but memory B cells are long-lived and thus have a much larger window of detection.

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Memory B-cell differentiation is characterized by somatic hypermutation in antibody variable region genes (V) and selection of B cells expressing high-affinity variants of this antigen receptor and high sequence similarity in individuals exposed to the same antigen reflect antigen-driven clonal selection and form shared immunological memory between individuals (Yang et al., 2021). Memory B cells are highly abundant in the human spleen, and can proliferate 45% of the total B cell population in this organ (Hauser et al., 2015). Memory B cells can be detected in the Ig variable (IgV) genes in a fraction of IgM+ B cells. In humans, B cells expressing the memory marker CD27+ can be found in the marginal zone of spleen and circulate in the blood. After re-exposure to antigen, memory B cells can quickly proliferate and differentiate into plasma cells. Many studies the antibody diversity and specificity of the naturally paired immunoglobulin heavy and light chains for affinity maturation of B cell receptor (BCR) after virus infection such as SARS-CoV-2 infection (Yang et al., 2021), influenza (Dougan, S et al., 2013), HIV (Moir, S., & Fauci, A. S.,2017), etc. The characterization of memory B cells can be used to develop monoclonal antibodies and produce an effective therapeutic.

Significance and Approach

Southeast Asia is one of the world's highest-risk emerging infectious diseases hotspots. From this region emerged both SARS-CoV-1 and SARS-CoV-2, recurrent outbreaks of novel influenza strains, and the spillover of dangerous viral pathogens such as Nipah virus. The diversity of coronaviruses, henipaviruses, and filoviruses are found in wildlife reservoirs in Southeast Asia is due to the high diversity of wildlife, and the demography of the human population brings humans and wildlife into close contact. The spillover of viruses from animals to humans is often misdiagnosed and the clinical symptoms from patients is underestimated during the pandemic. This study provides a surveillance strategy in human and wildlife populations to better understand immunity to novel pathogens and identify the origin of these pathogens to serve as an early warning system to prevent outbreaks of emerging viruses.

This study uses immunology to identify a novel virus that can aide in effective vaccine discovery and development, monoclonal antibody production for prevention and therapy, and generate data resources for early detection and diagnostic assays. Whereas PCR detection or virus isolation in wildlife is challenging, this new serological approach may be more successful in identifying reservoir hosts.

The Indo-Pacific region has a high risk of novel viral emergence. This study provides an opportunity for in-country research capacity building by linking local infectious disease scientists with an international collaborative network that has proven capacity to conduct this work and produce significant findings. Moreover, a global sero-surveillance network for surveillance technology extends virologic and immunologic screening to product discovery. This study will benefit and expand the CREID, EID-SEARCH networks, and the global sero-surveillance network to improve our understanding of immunology to identify novel pathogens and their origin to prevent future pandemics.

Research Methods

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Aim 1. Characterization of monoclonal antibodies from humans with serological profiles consistent with MojV infection.

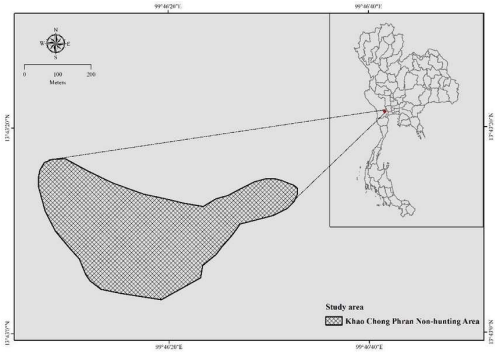
As part of our ongoing virus surveillance conducted through EID-SEARCH, we have detected XX% (XX/XX) MojV seroprevalence in a cohort of guano farmers. In our pan-henipavirus that tested sera against the RBP of presently described henipavirues, sera IgG bound specifically to MojV RBP (Figure 1), demonstrating little cross reaction with the bat-borne ephrinB2 utilizing henipaviruses.

~~In collaboration with NIH VRC PREMISE, MojV RBP-reactive B-cells will be captured, and the B-cell receptor will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific mAbs.~~

Importance: The isolation of these mAbs represents a potential therapeutic for MojV, MojV-like henipas, and LayV, depending on how well these cross react.

~~***Human samples collection site***~~

~~Human positive serum for MojV was found close to Khao Chong Phran in Ratchaburi Province, which has the largest colony of bats in Thailand (Figure 1).~~



~~Figure 1: Map of Khao Chong Phran Non-hunting Area, Ratchaburi Province, Thailand (Boonpha N et al., 2019)~~

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And that you will test binding of these against LayV G and LayV F. To see cross reaction.

One antibody Broder has against MojV already has demonstrated cross reaction with a LayV protein.

David Veesler can do all the Cyro-EM to look at where these mAbs bind on the proteins.

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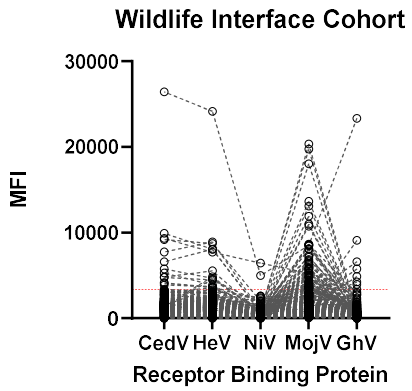

Figure 2: Seroprevalence of henipavirus proteins from a community with a large bat interface. MFI is the median fluorescence intensity for IgG reactivity to each protein. Red-dashed line is at 3,357 MFI, the assay cutoff.

In collaboration with NIH VRC PREMISE, MojV RBP-reactive B cells will be captured, and the B cell receptor will be sequenced from previously-identified seropositive human participants to generate~~.~~ MojV-like virus-specific mAbs.

~~PBMC collection and Single Memory B Cell Isolation~~

Whole blood from MojV-positive people will be collected in individual heparin tubes and processed for PBMC isolation using Density Gradient Centrifugation (SepMate™ PBMC Isolation Tubes).

Human PBMCs will be stained with antibodies for positive markers of IgG memory B cells, and antibodies to negatively select and avoid contamination with dead cells, T cells, NK cells or monocytes (live, CD3−,CD14−,CD56−, IgM−, IgA−, CD19+,CD20+,CD27+). Avi-tag for biotinylation and 6xHIS tag for Nickel purification using the HisPur™ Ni-NTA Spin Purification Kit (Thermo Fisher Scientific, Waltham, MA). Single m~~M~~emory B Cells specific to MojV RBP and F will be probed with these recombinant proteins and ~~will be~~ sorted, cells will proliferate, cDNA will synthesized, then h~~H~~eavy and light chain variable domains will be amplified (Waltari E et al., 2019).

~~B cell receptor Amplicon Preparation~~

Total RNA yields from the PBMC will be determined by absorbance at 260 nm on the NanoDrop™ One (Thermo Fisher Scientific). Total RNA will be used for first strand cDNA synthesis with gene-specific reverse transcription (RT) primers directed to the constant regions. The RT primers for IgG, IgM, IgA, lambda, and kappa will be included. Light chain and heavy chains will be amplified into amplicon. Double-stranded cDNA will be sequenced.

~~B cell receptor Data Analysis~~

After completion of MiSeq sequencing, antibody repertoires will analyze using methods based on the pipeline.

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Commented [EL12]: Also propose to pull these out with MojV F.

And that you will test binding of these against LayV G and LayV F. To see cross reaction.

One antibody Broder has against MojV already has demonstrated cross reaction with a LayV protein.

David Veesler can do all the Cyro-EM to look at where these mAbs bind on the proteins.

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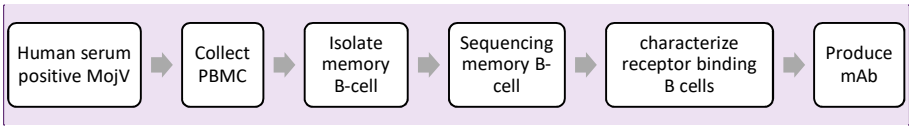
~~***Recombinant Antibody Cloning, Production, and Purification***~~

Heavy and light-chain sequences will be assembled into an expression vector and transfected into cells for monoclonal antibody expression.

Functional assessment

Isolated mAbs will be assessed for binding to MojV RBP and F proteins through multiplex binding serology. Further, the cross reaction potential of these mAbs will be assessed against GamV, DarV, and LayV RBP and F. Neutralization of viruses remains a critical correlate of protection for most vaccine strategies. MojV has not been isolated or rescued, and the virus receptor for MojV has not yet been discovered [2 citations]. Thus, a functional assessment of cross neutralizing potential is not possible.

~~Neutralization of viruses remains a critical correlate of protection for most vaccine strategies.~~ ~~Next~~However, we will test the cross neutralization potential of these anti-MojV RBP and F mAbs to neutralize LayV in collaboration with our EID-SEARCH co-investigators, Dr. Lin-Fa Wang (D~~D~~uke NUS). ~~/LinFa should be able to do this, if they have LayV and are propagating it, they can use cell-cell fusion/CPE in a straight PRNT).~~ Decide if you want to incorporate this.



~~***Antigenic cartography***~~

~~Antisera and monoclonal antibodies to henipavirus RBP, including the novel MojV-like henipavirus, will be generated and tested against all the known RBP. The resulting binding data will be complied, and antigenic relatedness will be determined using RStudio.~~

~~**Determination of envelope glycoprotein epitope binding**~~

~~Isolated mAbs will be assessed for binding to MojV RBP and F proteins through multiplex binding serology. Further, the cross reaction potential of these mAbs will be assessed against GamV, DarV, and LayV RBP and F. MojV has not been isolated or rescued, and the virus receptor for MojV has not yet been discovered [2 citations]. Thus, a functional assessment of cross neutralizing potential is not possible. However, through cyro-electron microscopy we can investigate binding epitopes between these mAbs and MojV and LayV envelope glycoproteins. Epitope identification can then be used to make predictions about neutralizing potential through deductions about the location of henipavirus RBP receptor binding pockets.~~

Aim 2. Utility of Thai MojV-like mAbs for surveillance.

Serological profiling provides an indirect measurement of the infecting virus. Despite XX% (XX/XXX), seroprevalence in humans, we have not detected nucleic acid sequences of MojV or MojV-like heniapviruses in this human cohort. In addition to the translational application of mAbs as therapeutics, we intend to utilize these mAb as non-clinical diagnostics surveillance tools for virus/virus antigen capture.

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Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Site wildlife samples collection

Human positive serum for MojV was found close to Khao Chong Phran in Ratchaburi Province, which has the largest colony of bats in Thailand (Figure 2). Here, we propose to undertake additional field collections, incorporating collections of samples from shrews in the area, which are linked to LayV, GamV, and DarV henipaviruses.

Commented [EL20]: Show serological profile Figure

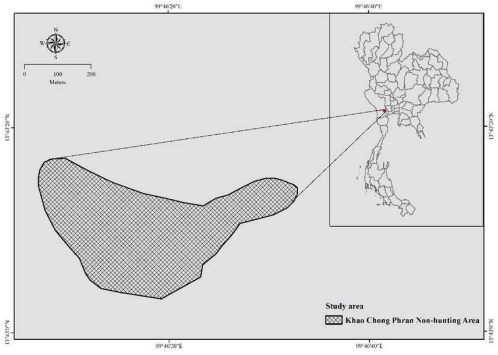

Figure 2: Map of Khao Chong Phran Non-hunting Area, Ratchaburi Province, Thailand (Boonpha N et al., 2019)

100 Rodents 100 bats, and 100 shrews in Khao Chong Phran cave in Ratchaburi Province will be collected for surveillance targeting the Mojiang-like virus. In this study, we can collect only one time for a limited grant and time.

Enrollment criteria: Random age, random weight, random sex.

Blood from animals will collect by using a needle in vein blood using serum to detect the undiscovered henipaviruses.

Human syndromic surveillance

100 human samples will collect serum to detect henipa-like virus near Khao Chong Phran cave in Ratchaburi Province for 1 year.

Enrollment criteria;

- Adults (≥ 18 years of age) who sign in the informed consent
- Who have sick without any reason such as severe/acute respiratory illness (SARI/ARI), Influenza-like illness (ILI), fever of unknown origin (FUO), or respiratory tract infection
- Who is the bat guano collector in the cave
- Who lives or works around wildlife habitats

- Who is involved with livestock or wildlife farming
- Who hunts wildlife or works with wildlife

Data analysis

Virus sequencing….after mAb pulldown.

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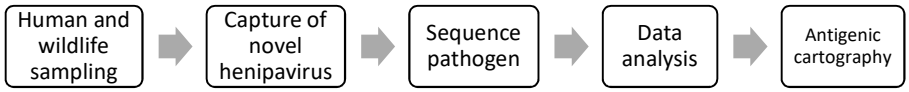

Project Timeline (1-page limit)

Activity	05/23	06/23	07/23	08/23	09/23	10/23	11/23	12/24	01/24	02/24	03/24	04/24
Mentoring and training activities												
1.Training, literature review												
2.Monthly mentoring meeting												
3.Manuscript writing												
4.EID-SEARCH meeting												
Research activities												

1.IRB amendment												
Aim 1												
1. Collect PBMC human sample and isolation memory B-cell for Mojiang virus												
2. B cell receptor Data Analysis												
3. Produce mAb												
Aim 2												
4. Wildlife samples collection												
5.Human samples collection												
5.Identify and characterize novel viral pathogen												
6.Data analysis												

Research Performance Sites *(1-page limit)*

This proposed study will be implemented at the King Chulalongkorn Memorial Hospital (KCMH) collaborating with the Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC). KCMH is a 1,000-bed, university hospital in close coordination with the Faculty of Medicine, Chulalongkorn University. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. It's one of the center hospitals for Emerging Infectious Diseases in Thailand, especially for domestic and international patients.

TRC-EIDCC was established by the Committee of the Emerging Infectious Disease Center, which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. Our clinical center

provides standard medical care to patients with emerging and recurrent diseases and disease transmission. Moreover, the center plans preparedness activities, trains medical and government staff on interventions, hosts conferences for medical and public health professionals, creates networks of medical care for patients with emerging and recurrent diseases, and leads research and development projects for medical care and disease control. Apart from medical innovation in emerging and recurrent diseases, the clinical center strives to be the model of infectious diseases clinical services and pandemic control through research activities surrounding infectious diseases and effective disease control measures.

We also collaborate with the Department of National Parks, Wildlife and Plant Conservation for detecting novel pathogens from animals such as bats, rodents, and shrews in our country.

We also collaborate with the Department of Disease Control of Thailand (Thai-DDC) to support the Thai government and national policy makers through molecular diagnosis, including the identification of the first case of SARS-CoV-2 infection in Thailand. TRC-EIDCC prides itself on its high performance in research laboratory services and is a part of the national reference laboratory for COVID-19 and other emerging infectious diseases confirmation in Thailand.

CREID Research Center Collaboration *(1-page limit)*

The goals and objectives of the proposed research project, "Immune memory bait & capture to identify emerging henipavirus origins", are fully consistent with the specific aims of EID-SEARCH. EID-SEARCH takes a One Health approach to understanding zoonotic disease emergence, with a focus on viral diversity in wildlife at high-risk human animal interfaces. EID-SEARCH aims to understand the frequency and causes of virus spillover in high-risk communities across Southeast Asia and identify strategies and tools to reduce this risk. As the proposed research focuses on the links between farming practices, wildlife diversity, virus diversity, and cross-species transmission in Thailand, we expect the results of the project to make a significant contribution towards the overall goals of EID-SEARCH.

The proposed project is well-designed, leverages cutting-edge technology, and includes a robust ecological component that corresponds to the overall research strategy of EID-SEARCH. The EID-SEARCH team is highly motivated to support the application of metagenomics to ecological questions and assist in

the analysis of the unique dataset that will be generated from this research. With the heightened focus on identifying and limiting risks associated with the complex interactions between humans, wildlife, and domestic mammals that has resulted from the emergence of SARS-CoV-2 from a wet market in China, the proposed research is both timely and likely to yield highly-cited, impactful publications for EID-SEARCH and Thailand partners.

The EID-SEARCH team includes a number of experts in study design, laboratory-based diagnostics, genetic and genomic analyses, and ecological modelling who will contribute to project implementation, data analysis, and manuscript preparation as required. Communication and coordination mechanisms will be set up between the Thailand research team and scientists at the EID-SEARCH research sites for resource sharing, training, knowledge exchange, and mentorship. In particular, EID-SEARCH will facilitate and encourage relationships between our partners in Southeast Asia (based in Malaysia, Singapore, and Thailand) that will facilitate Thailand team professional development and strengthen research capacity in the region. Dr. Eric Laing, the project Mentor, has an established relationship with both Dr. Krongkan and Spencer Sterling, and will act as the liaison between EID-SEARCH and the TRC-EIDCC research team. Further, Dr. Doeuk who is the directior of the VRC PREMISE program is enthuasiatic about the opportunity to collaborate and apply their rapid assembly, transfection and production of immunoglobulin (RATP-Ig) workflow as a serologic/immunologic based approached for therapeutic and virus discovery (see Letter of Support). An expected outcome of this research project will be pilot data to support a joint funding application in the future.

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Mentoring Plan *(2-page limit)*

1. Goals for Pilot study and Career

Krongan Srimuang: My career goal is to be an expert scientist in either an academic research laboratory or a lecturer in medical science. My long-term research interests include the understanding of priority emerging infectious diseases, genetics, and epigenetics using molecular and serologic techniques. Detection of novel pathogens in their wildlife vectors can help characterize the pathogenicity, virulence, and evolution of these and other closely related pathogens. I believe that this pilot study can provide training in new techniques for the detection, identification, and characterization of novel pathogens that are currently unavailable in Thailand. My goal for the Pilot study is to gain more knowledge and skills for a lifelong career in public health-related research. I wish to expand my skills in novel virus detection using

next-generation serologic techniques such as PBMC studies and memory B-cell isolation and sequencing that can contribute to improved surveillance and research capacity at human-animal interfaces. My mentors Dr. Laing and Dr. Wacharapluesadee can provide me with opportunities to develop and apply project skills and improve my scientific writing.

My education has provided me with a strong foundation to expand from for this study. I received my undergraduate degree in Medical Technology from Chulalongkorn University which included training in molecular biology, serology, immunology, chemistry, virology, bacteriology, mycology, parasitology, hematology, clinical microscopy, transfusion medicine, and forensic medicine. My interest in molecular technology led me to pursue a Ph.D. at the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, which provided me with training in several molecular techniques including conventional PCR, real-time PCR, Next Generation Sequencing (NGS), etc. The emergence of COVID-19 motivated me to focus on emerging infectious diseases. I am currently completing my post-doctoral fellowship at the Emerging Infectious Diseases Clinical Center (EIDCC), King Chulalongkorn Memorial Hospital with Dr. Wacharpluesadee. Throughout this fellowship, I have continued learning advanced and innovative techniques to detect, identify, and characterize the novel pathogens. Through this pilot project, I will learn about the Peripheral Blood Mononuclear Cells (PBMC) which are a critical part of many subdivisions of medical research including Infectious diseases, Vaccine development, and Immunology. This project aims to study zoonotic reservoirs for by using serologic and cellular immune discovery in humans, and applying this immune knowledge in wildlife for early detection and identification of novel pathogens. This proposed study will expose me to various techniques and immunology topics that I useful in designing and testing new pipelines as technological advances continue to develop. This study will also allow me to develop, manage, and communicate my research.

Spencer Sterling: My overall career goal is to understand the factors that lead to viral zoonosis and to build epidemiological capacity in regions with the highest risk for zoonotic spillover. Through my training and educational experiences, I have learned laboratory and epidemiological techniques that can assist in the understanding of zoonosis. I wish to continue my education by expanding my repertoire of molecular techniques to include studies of the immune system's response to zoonotic disease in humans and animals, as well as to learn next-generation sequencing techniques. Additionally, I would like to use my experience in epidemiological techniques to train scientists in the Southeast Asian region in order to build local capacity in infectious disease research. Dr. Laing has been a mentor for me throughout my career, and this project would allow for this relationship to expand into new molecular areas, as well as grant writing and project management skills. My professional relationship Dr. Wacharapluesadee with began in 2018 when I participated in a DTRA BTRP funding project that focused on transferring antigen-based multiplex serological technology to her lab group~~Additionally, Dr. Wacharapluesadee~~. Currently, I am a Visiting Scientist at TRCEIDCC and I work closely with Dr. Srimuang, and other members of Dr. Wacharapluesadee's research group on EID-SEARCH activities. Additionally, Dr. Wacharapluesadee has decades of experience in infectious disease research in the region and has mentored scientists that have gone on to careers at the Thai Ministry of Public Health, Department of Disease Control, Department of Parks and Wildlife, academia, and the private sector. Both mentors have encouraged Dr. Srimuang and I to apply for this pilot study as well as pursue other funding mechanisms for our research interests. Both mentors will provide for me the ability to independently pursue my research interests in the zoonotic epidemiology and train me on the best practices of scientific communication.

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Vertebrate Animals Section Requirements

1. Description of Procedures (Vertebrate Animals Section)

Animal samples will be collect in the Khao Chong Phran cave in Ratchaburi Province, including bats, rodents, and shrews as were approved by the IACUC for the project "Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia" (Animal Protocol #G2020-42). Moreover, the project

has been approved by the Thai Department of National Park, Wildlife and Plant Conservation on 0907.4/28372.

1.1 Bats (order Chiroptera): Family Pteropodiae; Family Rhinopomatidae; Family Emballonuridae; Family Craseonycteridae; Family Megadermatidae; Family Rhinolophidae; Family Hipposideridae; Family Vespertilionidae; Family Mollossidae; etc.
Random age, random weight, random sex, and total number 100.

1.2 Rodents (order Rodentia) Maxomys whiteheadi; Rattus exulans; Rattus annandalei; Leopoldamys sabanus; Sundamys muelleri; Chiropodomys gliroides; Niviventer cremoriventer; Maxomys rajah; Hylomys suillus; Niviventer; Maxomys; Maxomys surifer; Rhizomys sumatrensis; Rhinosciurus laticaudatus; Callosciurus notatus; Rattus tiomanicus; Tupaia glis; Petaurillus hosei; Rattus tanezumi; Rattus norvegicus; Rattus rattus; Sundasciurus lowii; Tupaia longipes; Tupaia tana; Callosciurus prevostii; Sundasciurus hippurus; Tupaia minor; Hystrix crassispinis; Maxomys alticola; Rattus argentiventer; Trichys fasciculata; Dremomys everetti; Callosciurus adamsi; Rattus baluensis; Rattus losea; Bandicota indica; Mus cookie; Berylmys bowersi; Rattus nitidus; Mus caroli; Leopoldamys neilli; Leopoldamys edwardsi; Mus cervicolor; Mus fragilicauda; Niviventer confucianus; Niviventer fulvescens; Bandicota Savile; etc.
Random age, random weight, random sex, and total number 100.

1.3 Shrews (order Soricomorpha) Sorex isodon; Crocidura attenuate; Crocidura fuliginosa; Crocidura horsfieldii; Crocidura vorax; etc.
Random age, random weight, random sex, and total number 100.

2. Justifications (Vertebrate Animals Section)

For our Aim 2. Modeling antigenic relationships among known and unknown henipaviruses.
We will develop anti-Henipavirus RBP standards to explore the antigenic spatial relationships between all known rodent- and bat-borne HPVs, and the Thai MojV-like virus. We would like to survey all animals that could be reservoir hosts or intermediate hosts of novel henipaviruses that can be spillover to humans to increase the likelihood of detecting a novel virus. Therefore, the research goals be accomplished using an alternative model (e.g., computational, human, invertebrate, in vitro).

3. Minimization of Pain and Distress (Vertebrate Animals Section)

For animals requiring anesthesia- Blockage of visual stimulus, handling in cloth bags and other methods will be employed to keep handling procedures gentle and to minimize stress.

4. Method of Euthanasia

This study will not use methods of euthanasia in all animals.

Human Subjects Research

This project will enroll human subjects under the approved protocols by the Health Medical Lab Institutional Review Board, US (No. 894ECOH21b) and the Institute Review Board of the Faculty of Medicine, Chulalongkorn University, Thailand (No. 211/64).

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

All applicants proposing human subjects research should address the following elements on the Protection of Human Subjects:

1. Risks to the subjects

Human blood will be collected by a needle from a vein in the arm (approximately 15 ml) into a heparin tube.

Having blood drawn may produce discomfort or minor bleeding and the possibility of bruising at the site of the needle puncture. There is also a slight risk of infection at the site of the

needle puncture. Although rare, some people have experienced nausea, light headedness, and

fainting in association with a blood draw.

2. Adequacy of protection against these risks

Vital signs will be taken at each visit. Vital signs include measurements of your pulse (heart rate), respirations (breaths per minute), blood pressure, height, weight, and body temperature.

3. Potential benefits of the research to the subjects and others

There are no direct benefits to the research subjects. Indirect benefits to the research to subjects is the possible identification of undiscovered henipaviruses infections in this population. Detection can lead to correct diagnostics and proper treatment of the disease

4. Importance of the knowledge gained or to be gained

This study will bring opportunity, experience, and knowledge in immunology and serology to research staff in Thailand. Moreover, the new methods and techniques to identify novel pathogens by using memory B-cell can be used in the study of zoonotic reservoirs and pathogen origins. By using serologic and cellular immune discovery in humans and wildlife, we seek the ability for early detection and identification of novel pathogens to prevent the next pandemic.

5. Country- / institution-specific ethics / IRB regulations addressed

This project will enroll human subjects under the approved protocols by the Health Medical Lab Institutional Review Board, US (No. 894ECOH21b) and the Institute Review Board of the Faculty of Medicine, Chulalongkorn University, Thailand (No. 211/64).

Budget/Budget Justification

No.	Desription	Budget ($)	Unit cost (USD)	month	Unit	Cost
1	Pernonel	63,600.00				19,400.00

	Krongkan Srimuang		1,000.00	12	month (20 hrs/month)	12,000.00
	Spencer		0.00		month (100 hrs/month)	0.00
	Lab Technician (Part-time)		1,000.00	6	month (100 hrs/month)	6,000.00
	Field Technician (Part-time)		600.00	2	month (60 hrs/month)	1,200.00
	Lab admin coordinator (Part-time): coordinate with university (Mrs Chatchawadee)		200.00	1	month (20 hrs/month)	200.00
2	Fleldwork					
	Human sampling	15,348.67				11,388.67
2.1	Human sample collection		70.00	110	case	7,700.00
	Human sample collection field work: 2 days/trip (10 participants) (transportation, accommodation, food, wage, subject gift, etc.)		250.00	1	Trip	
2.2	Human sample collection field work: 2 days/trip (50 participants) (transportation, accommodation, food, wage, subject gift, etc.)		2,500.00	1	Trip	2,500.00
	Office suppiles		1,188.67	1	Time	1,188.67
	Animal sampling	28,000.00				8,000.00
2.3, 2.4	Animal sample collection field work: 7 days /trip (100 bats/trip), (transportation, accomadation, food, wage, etc.)		7,000.00	1	Trip	7,000.00
	Animal sample collection field work: 7 days /trip (100 rodents or shrews /trip), (transportation, accomadation, food, wage, etc.)					
	Field supplies: PPE, gloves, nets, dryice, etc.		1,000.00	1	Time	1,000.00
3	Lab work	84,500.00				43,500.00
3.1, 3.4	PBMC collection and isolation, and sequencing service cost		40.00	1000	cell	40,000.00
3.3	Reagents and supplies for serology testing of human and wildlife samples				test	0.00

3.5, 3.6	MMIA in human and animal samples: reagent and supplies (sheath bufferm 96 wells plate, microcentrifuge tube, pipette tp, pipette, Multi-Channel pipette, etc.)		3.50	1000		3,500.00
6	Indirect costs (8%)	**15,995.89**	15,995.89	1	Time	15,995.89
	Total	**207,444.56**				98,284.56

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Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

List of abbreviations, acronyms, symbols

AngV: Angavokely virus
BCR: B cell receptor
BSc: Bachelor of Science
BSL2: Biological Safety level 2
BSL3: Biological Safety level 3
cDNA: Complementary DNA
CedV: Cedar virus
Co-PI: Co-principal investigator
CREID: Centers for Research in Emerging Infectious Diseases
DARV: Daeryong virus
DNA: Deoxyribonucleic acid
EHA: EcoHealth Alliance
EID: Emerging Infectious Disease
EID-SEARCH: The Emerging Infectious Disease – South East Asia Research Collaboration Hub
ELISA: Enzyme-linked immunosorbent assay
F: Fusion protein
FACS: Fluorescence-activated cell sorting
FUO: Fever of unknown origin
GAKV: Gamak virus
GhV: Ghana virus
HeV: Hendra virus
HPVs: Henipaviruses
ILI: Influenza-like illness
KCMH: King Chulalongkorn Memorial Hospital
L: large protein
LayV: Langya virus
M: Matrix protein
mAb: Monoclonal antibody
MD: Maryland
MMIA: Multiplex microsphere-based immunoassay
MojV: Mojiang virus
MSc: Master of Science
N: Nucleocapsid
NGS: Next-generation sequencing
NIAID: National Institute of Allergy and Infectious Diseases
NIH: National Institutes of Health
NiV: Nipah virus
P: Phosphoprotein
PBMC: Peripheral blood mononuclear cells
PCR: Polymerase Chain Reaction
PhD: Doctor of Philosophy
PI: Principal investigator
RBP: Receptor-binding protein
RNA: Ribonucleic acid
RT-PCR: Real-time PCR
SARI/ARI: Severe/acute respiratory illness

SARS-CoV-1: Severe acute respiratory syndrome coronavirus 1
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2
SEA: Southeast Asia
Thai-DDC: Department of Disease Control of Thailand
TRC-EIDCC: Thai Red Cross. Emerging Infectious Diseases. Clinical Center
USA: United States
WGS: Whole Genome Sequencing

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Facilities and Resources

FACILITIES: Specify the facilities to be used for the conduct of the proposed research.

Laboratory:

The Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC), King Chulalongkorn Memorial Hospital (KCMH) was established by the Committee of the Emerging Infectious Disease Center which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. TRC-EIDCC laboratory facilities include 3 biosafety cabinets (BSL 2, BSL 2+, BSL2+), 3 automated extraction machines, 2 thermocyclers (Proflex), 2 real-time PCR machines (CFX, Bio-rad), 3 Freezers (-80 °C), 2 refrigerators; 4 °C, 2 autoclaves. Our EIDCC uses the Pathogen Asset Control System (PACS) system for sample tracking. Moreover, our Faculty of Medicine, Chulalongkorn University has a central laboratory, the Chula Medical Research Center (Chula MRC), which provide many instruments for medical, masters, and graduate students, Post-doctoral fellows, and researchers at Chulalongkorn University and KCMH. The Chula MRC laboratory facilities are divided into molecular diagnostics (PCR Thermal Cycler, QStudio6 Flex Real-Time PCR, Droplet Digital PCR, GeneXpert, Film array, Illumina MiSeq), immunology facilities (ELISA testing equipment (reader & washer), Luminex, Flow Cytometry), cell sorting machine and a BSL3 laboratory. Moreover, the facility provides general equipment, such as autoclaves, balances, CO2 incubators, Dual Fluorescence Cell Counter, 4 °C refrigerators, -20°C, and -80°C freezers.

Clinical:

KCMH is a 1,000-bed university hospital, in close coordination with the Faculty of Medicine, Chulalongkorn University. The Thai Red Cross Society provides medical treatment, rehabilitation, health promotion and disease prevention services to the general public and the vulnerable. It also provides biopharmaceutical and sterile medicines that are sufficient to meet the needs of patients and safe in accordance with international standards. It serves as a center for providing eye and organ donation services to patients nationwide in line with international standards. It has specialized doctors and public health personnel and also produces specialized nurses. The TRC-EIDCC in the KCMH provides standard medical care to patients with emerging and recurrent diseases and disease spreading prevention.

Animal:

TRC-EIDCC also collaborates with the Department of National Parks, Wildlife and Plant Conservation for detecting the emerging novel pathogens from animals such as bats, rodents, and pangolins in our country. Bats are considered protected wildlife in Thailand by Wildlife Conservation, Department of National Parks, Wildlife and Plant Conservation.

Computer:

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

TRC-EIDCC used the service from the Office of Information Technology, Chulalongkorn University that provides the application, network, and software such as Office 365, VPN, WIFI, Microsoft Office, Microsoft Windows, SPSS, Adobe, Zoom Meeting, etc. Moreover, our EIDCC use the Pathogen Asset Control System (PACS) for sample tracking.

Office:

The office and laboratory space are located at the EIDCC at 4th Floor, Jongkolnee Watwong Building and 6th Floor, Aor Por Ror Building, respectively. The applicants will have dedicated desks in both buildings.

Other:

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

30 January 2023

Dr. Krongkan Srimuang & Mr. Spencer Sterling

Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC),

King Chulalongkorn Memorial Hospital

Object: Thai Red Cross Emerging Infectious Diseases Clinical Center support for your application to the CREID Pilot Research Program, entitled "Immune memory bait & capture to identify emerging henipavirus origins."

Dear Dr. Srimuang & Mr. Sterling,

Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC) is pleased to support your application to the **CREID Pilot Research Program**, entitled "Immune memory bait & capture to identify emerging henipavirus origins." We are fully committed to executing the statement of work to study zoonotic reservoirs for pathogen origin by using serologic and cellular immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

The work proposed in this project will be given a great opportunity and collaboration to our team, it serves very strong knowledge in serology and immunology for the detection of novel virus pathogens in Thailand using new technology and techniques. Moreover, the building capacity and capability of our laboratory and Faculty will access and support to make the success and completion of this project.

This letter expresses my strong interest and drives forward to making this application a success. I lok forward to collaborating with you of this project. I wish you success in the CREID Pilot Research Program application.

Sincerely,

Assistant Professor Opass Putcharoen, MD, Msc

Head of Thai Red Cross Emerging Infectious Diseases Clinical Center

King Chulalongkorn Memorial Hospital

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

30th January, 2022

Dr. Krongkan Srimuang & Mr. Spencer Sterling

Thai Red Cross Emerging Infectious

Diseases Clinical Centre (TRC-EIDCC),

King Chulalongkorn Memorial Hospital

Dear Dr. Srimuang & Mr. Sterling,

I am writing this letter to express my support as Research Center Mentor for your application to the CREID Pilot Research Program, entitled "Immune memory bait & capture to identify emerging henipavirus origins."

My experience in immunology and serology will support them in the CREID Pilot Research Program. The research aim has the potential to have a strong impact on our approach to pathogen surveillance at high-risk human-animal interfaces. Given the understanding of the pathogen origin by using serologic and cellular immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

I believe that this is a great opportunity to use scientific knowledge and expertise to train the characterization of memory B cells from peripheral blood mononuclear cells (PBMC) and serology background to the team at Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital, Thailand. Our stronger partnerships have the potential to really drive forward innovations in this serology and immunology which include the implementation of novel techniques for pathogen surveillance as proposed in your pilot project application.

The CREID Network and the Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH) will support scientists in regions around the world where emerging and reemerging infectious disease outbreaks. Many Southeast Asian partnerships in EID-SEARCH have strong the potential to really drive forward innovations in this area.

I believe that Dr. Wacharapluesadee, Co-PI, and I will provide scientific skills, expertise, and networks for this project.

Towards that aim, I am committed to supporting your professional development in the following ways:

1.

2.

3.

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Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

I'm very much looking forward to working closely with you on this 1-year research project and beyond as you continue to develop your surveillance zoonotic disease research program.

Best regards,

Eric Laing, Ph.D.

Assistance Professor

Uniformed Services University of the Health Sciences

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

30th January, 2022

Dr. Krongkan Srimuang & Mr. Spencer Sterling

Thai Red Cross Emerging Infectious

Diseases Clinical Centre (TRC-EIDCC),

King Chulalongkorn Memorial Hospital

Dear Dr. Srimuang & Mr. Sterling,

I am writing in support as Research Center Mentor for your application to the CREID Pilot Research Program, entitled “Immune memory bait & capture to identify emerging henipavirus origins.”

For my experience in 20 years to conduct the surveillance for novel virus in wildlife, human, and bats using molecular technology and multiplex serology panel (Luminex technology) will support them in the CREID Pilot Research Program. The research aim has the potential to have a strong impact on our approach to pathogen surveillance at high-risk human-animal interfaces. Given the understanding of the pathogen origin by using serologic and virus discovery in bats, humans, and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

As the head laboratory at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, I believe that this is a great opportunity for our Thailand team that can train in the characterization of memory B cells from peripheral blood mononuclear cells (PBMC) and serology surveillance in novel virus pathogens. Moreover, our laboratory has supported the Department of Disease Control (DDC) in diagnosing emerging infectious diseases and communicable diseases such as Ebola, MERS, and COVID-19. I am also a committee member of the Global Laboratory Leadership Program (GLLP) Thailand, organized by the Department of Medical Sciences. With my experience to diagnose the first human MERS case in 2015 and the first human COVID-19 case outside China in January 2020, I will support multidisciplinary projects involving epidemiology, laboratory, field science, and bioinformatics to diagnose and monitor the emergence of wildlife-origin viral zoonoses, including SARS-CoV, Nipah, and Hendra viruses, Avian influenza, and novel viruses from bats. I am also the Thailand country coordinator for PREDICT project, large contracts from USAID during 2010-2019 involving the successful management of teams of virologists, field biologists, veterinarians, epidemiologists, hospitals, and laboratorians. I will be given the strong knowledge to build capacity and capability in this project and our team in Thailand.

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

The CREID Network and the Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH) will support scientists in regions around the world where emerging and reemerging infectious disease outbreaks. Many Southeast Asian partnerships in EID-SEARCH have strong the potential to really drive forward innovations in this area.

I believe that Dr. Laing, and I will provide scientific skills, expertise, and networks for this project.

Towards that aim, I am committed to supporting your professional development in the following ways:

1. I will maintain regular bi-weekly meetings for the project progresses.
2. I will support the resources in our country and research team in this project.
3. I will create opportunities for scientists network to ensure the success of research project.
4. I will provide and share advice and technical expertise in specific knowledge for this project.

I'm very much looking forward to working closely with you on this 1-year research project and beyond as you continue to develop your surveillance zoonotic disease research program.

Best regards,

Supaporn Wacharapluesadee, Ph.D.

Head Laboratory,

Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC),

King Chulalongkorn Memorial Hospital,

Faculty of Medicine,

Chulalongkorn University.

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Pilot Program Application Submission Checklist – Final Application Submission

Title of Application: Immune memory bait & capture to identify emerging henipavirus origins

Submitted by: Krongkan Srimuang, Ph.D.

Submission Date: 1/30/2023

Full Application Submission Requirements

☒ Proposal Cover Sheet

☒ Title and Table of Contents

☒ Study Personnel *(1-page limit)*

- Main Application Body Section Requirements *(7-page limit):*
  - ☒ Research Aims & Objectives
  - ☒ Study Rationale/Research Gap/Impact
  - ☒ Significance and Approach
  - ☒ Research Methods

☒ Project Timeline (*1-page limit*)

☒ Research Performance Sites *(1-page limit)*

☒ CREID Research Center Collaboration *(1-page limit)*

☒ Mentoring Plan *(2-page limit)*

- Vertebrate Animals Section Requirements *(3-page limit):*
  - ☒ **Description of Procedures**
  - ☒ **Justifications**
  - ☒ **Minimization of Pain and Distress**
  - ☒ **Method of Euthanasia (Cover Page Supplement / PHS Fellowship Supplemental Form)**
- Human Subjects Research *(3-page limit):*
  - ☒ Summary of the parent study and IRB approval information for the study
  - ☒ Risks to the subjects
  - ☒ Adequacy of protection against these risks
  - ☒ Potential benefits of the research to the subjects and others
  - ☒ Importance of the knowledge gained or to be gained
  - ☒ Country / institution-specific ethics / IRB regulations addressed
- Research, Related Project Information, and Budget/Budget Justification
  - ☐ R&R Other Project Information Form
  - ☐ Full budget, with total costs of no more than $150,000

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Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

☐ Budget justification which describes the labor and other direct costs

☐ If your institution does not have adequate funds for a cost-reimbursement award and requires pre-payment of funds during the award year, please note this in your budget justification and outline a payment schedule that will function for your project.

- Supporting Documentation
  - Biographical Sketch and Other Support. All applications must include:
    - ☒ Applicant PI Biographical Sketch (*4-page limit*)
    - ☒ Applicant PI Previous/Current/Pending Support (Include funding amounts, *no page limit*)
    - ☐ Mentor Biographical Sketch (*4-page limit*)
    - ☐ Mentor Current/Pending Support (*no page limit*)
    - ☐ Key Personnel Biographical Sketches (*4-page limit each*)
    - ☐ Key Personnel Current/Pending Support (*no page limit*)
  - Co-PI Plan (only needed if applying as Co-PIs) (*1-page limit*)
    - ☐ What each Co-PI will contribute to the proposed research study
    - ☐ How the Co-PIs will jointly work with the affiliated Research Center
    - ☐ How the Co-PIs will jointly manage the proposed study
  - ☒ References Cited (*no page limit*)
  - ☒ List of Abbreviations, Acronyms, and Symbols
  - ☒ Facilities, Existing Equipment, and Other Resources (*template provided*)
  - ☐ NIH Foreign Clearance form (*template provided*)
  - ☐ Letters of Organizational Support *(2-page limit per letter)*
  - ☐ Letter of Collaboration from CREID Research Center PI *(2-page limit per letter)*
  - ☐ Letter from Research Center Mentor (*2-page limit)*
  - ☐ Letter from Primary Scientific Mentor (if different than Research Center mentor) (*2-page limit)*

Commented [el23]: Dear Eric, this is your part.

Commented [el24]: Dear Eric, this is your part.

27th January 2022

Krongkan Srimuang Ph.D.
Research Scientist
Thai Red Cross Emerging Infectious Diseases Clinical Center
King Chulalongkorn Memorial Hospital
Rama 4 Road
Pathumwan, Bangkok 10330
Thailand

Subject: Letter of Support for CREID Network Pilot Research Program

Dear Dr. Srimaung,

I am writing to express my support for your CREID Network Pilot Research Program proposal titled, "Immune memory bait and capture to identify emerging henipavirus origins." Your preliminary data demonstrating immunoreactivity against Mojiang henipavirus in individuals sampled as part of the Emerging Infectious Diseases Southeast Asia Research Collaborative Hub (EID-SEARCH) center, provides compelling serologic evidence that novel henipaviruses spill over into human populations. Point-of-care diagnostics and nucleic acid-amplification tests rely on our present understanding of medically relevant pathogens. Thus, emergence of novel zoonotic viruses may go undetected. Serological profiling provides us an ability to measure the human infectome through broad-scale peptide arrays or targeted antigen-based detection.

The research activities conducted at the Thai Red Cross Emerging Infectious Diseases Clinical Center are complementary to the focus of the Vaccine Research Center's PREMISE (Pandemic REsponse REpository through Microbial and Immunological Surveillance and Epidemiology) program. PREMISE works closely with intramural and extramural NIH programs and in partnership with a global network of investigators and collaborators to achieve its objectives through 3 core activities: (1) genetic analysis of zoonotic reservoirs and symptomatic humans for pathogen discovery; (2) development of high throughput, multiplexed serologic and cellular assays; and (3) global blood sampling to identify antigen-specific and cross-reactive immune responses to known and previously unknown viruses of pandemic potential. Biological samples including serum and PBMC are obtained from broad-based population cohorts as well as targeted populations at risk from emerging infections. At the same time, sequence data from known and unknown (through virus discovery) pathogens are analyzed to design and express candidate antigenic proteins of interest. These proteins are then used in high throughput multiplexed assays to screen sera for antibody reactivity. Serum reactivity to candidate antigens is followed by further interrogation of the adaptive immune response at the cellular level using corresponding PBMC samples. Proteins used in the serological arrays are conjugated to fluorophores and used as probes to sort antigen-specific B cells by flow cytometry. In addition, the sequence of such candidate antigens provides the template for the design of overlapping peptide pools to assess T cell responses. Further analysis allows for the identification of

neutralizing antibodies specific for the target antigens as well as specific T cell epitopes which may be tested in animal models. Epitope identification informs and guides immunogen design. At the conclusion of this pipeline of assays and analysis, PREMISE delivers the following:

1. reagent and data resources for early detection and diagnosis
2. monoclonal antibodies with therapeutic potential
3. candidate immunogens for further vaccine development

Thus, PREMISE serves as a translational vehicle to integrate serologic and cellular immune discovery, targeting a broad array of pathogens, into product development, and constitutes an anticipatory reagent repository to accelerate the global response to pandemic threats.

As investigators in the Vaccine Research Center at the National Institute of Allergy and Infectious Diseases (NIAID), we are not eligible to be listed as a "co-investigator" on the application but look forward to participating as collaborators. All materials from us will be provided under an approved material transfer or other collaborative agreement. This collaboration is part of our official duties as employees at the NIAID, and no funds from the grant will be used in intramural research, neither will we accept any form of renumeration, whether in the form of salary, honoraria, or travel expenses. We will provide scientific input and mentoring but will not have any duties associated with programmatic stewardship, which will be performed by NIAID extramural program officials. Further, in keeping with the mission of NIAID to promote and facilitate biomedical research and the dissemination of new knowledge, we would supply requested research materials and technical expertise not only to you, but also to other interested and qualified parties for research purposes.

My group has met with Dr. Eric Laing (USU) one of the two mentors for this pilot research program to discuss ways we can collaborate and support your research application. We are enthusiastic about providing the technological and subject-expertise in immunology and translational therapeutic discovery afforded by the PREMISE program that would enhance your knowledge base and broaden your own scientific training.

Sincerely,

(b) (6)

Daniel C. Douek

Daniel Douek MD PhD | Chief, Human Immunology Section | Vaccine Research Center
National Institute of Allergy and Infectious Diseases | National Institutes of Health
40 Convent Drive, Bethesda, MD 20892-3022 | Office: +1 301 594 8484

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Proposal Cover Sheet

Project Title	Immune memory bait & capture to identify emerging henipavirus origins
Principal Investigator Name	Krongkan Srimuang, Ph.D.
Position/Title	Research Scientist
Department	Thai Red Cross Emerging Infectious Diseases Clinical Center
Institution Name	King Chulalongkorn Memorial Hospital
Street	Rama 4 Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	Krongkan.sr@gmail.com
Phone	+66 (0) 92-453-0808
Country(ies) where work will be conducted	Thailand
Pathogen(s) focus	Mojiang Virus, Henipavirus, Paramyxovirus
Co-Principal Investigator Name (*If applicable*)	Spencer Sterling, MPH
Position/Title	Scientific Project Coordinator
Department	Microbiology and Immunology
Institution Name	Uniformed Services University of the Health Sciences
Street	Jones Bridge Road
City, State, Zip Code	Bethesda, MD 20814
Country	USA
Email	spencer.sterling.ctr@usuhs.edu
Phone	+66 (0) 83-494-5980

Contact information for institution(s) that would financially manage the award *(please note: changing the managing institution upon award may result in substantial funding delays)*	
Contractual Contact, Title	Chula Unisearch
Institution Name	Chulalongkorn University
Street	254 Phyathai Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	unisearch@chula.ac.th
Phone	0 2218 2880
For Co-Principal Investigator (If applicable):	
Contractual Contact, Title	
Institution Name	
Street	
City, State, Zip Code	
Country	
Email	
Phone	

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Name of Mentor	Eric Laing, Ph.D.
Mentor Institution	Uniformed Services University of the Health Sciences
Institution Address	Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814
Permanent location of Mentor	4301 Jones Bridge Road, Bethesda, MD 20814
Mentor email	eric.laing@usuhs.edu
Mentor phone	+1 (301) 980-8192
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Name of Mentor	Supaporn Wacharapluesadee, Ph.D.
Mentor Institution	Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital
Institution Address	1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Permanent location of Mentor	4th Floor, Jongkolnee Watwong Building, Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital, 1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Mentor email	spwa@hotmail.com
Mentor phone	+66 (0) 89-920-6292
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Total Budget	***Repeat this table if there are two Co-PIs***
Direct Costs	
Indirect Costs	
Proposed Start Date	
Proposed End Date	

Project Abstract (250 words)

Prototypical henipaviruses (Hendra and Nipah virus) cause zoonotic diseases with high mortality and no effective vaccines or therapeutics and are hosted by Pteropus flying foxes. The recent detection of closely-related Mojiang and Langya viruses in rats and shrews, and an outbreak of acute febrile LayV illness in people, challenges the dogma that fruit bats are the key henipavirus reservoirs, and heightens their threat profile. The recently expanded henipavirus includes two other shrew-borne viruses (Gamak and Daeryong viruses), and the Madagascan fruit bat-borne Angovkeely virus. In this study, we have found serological evidence of a Mojiang-related virus in a community of Thai bat guano collectors with high exposure to microbats and rodents. Identifying the wildlife reservoir of this likely novel virus will assist intervention strategies to reduce spillover risk. However, PCR detection or virus isolation in wildlife is challenging, and new serological approaches may be more successful in identifying reservoir hosts. This project aims to study zoonotic reservoirs for pathogen origin by using serologic and cellular

immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic. We plan to identify seropositive human participants MojV-like virus-specific MAbs will be generated and used for direct antibody-mediated virus capture in people and wildlife. We will develop anti-RBP standards to explore the antigenic spatial relationships between all known rodent- and bat-borne HPVs, and the Thai MojV-like virus these will allow the serologic signature of related undiscovered henipaviruses to be identified.

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Project Title **Immune memory bait & capture to identify emerging henipavirus origins**

Table of Contents

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Study Personnel ***(1-page limit)***

Krongkan Srimuang, Ph.D., is a researcher at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital and supports the Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia (EID-SEARCH) research. Dr. Srimuang is also a medical technologist working to identify, detect and characterize the spill-over risk of high zoonotic potential viruses from wildlife and humans using serology techniques, Next-generation sequencing, and PCR assays to identify novel viruses. Dr. Srimuang also performs the project Smart Sentinel Surveillance System: Emerging Viral Pneumonia and Respiratory tract infections (RTIs) to identify, detect and characterize the novel/known Respiratory Viruses in our country, Thailand by using multiplex real-time PCR, family PCR and Next-generation sequencing of novel viral pathogens. Dr. Srimuang graduated with Ph.D. in Tropical Medicine from the department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University.

Spencer Sterling, MPH, is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region. Mr. Sterling will planning, sample processing, data analysis, and report preparation for this project.

Dr. Eric Laing will act as Mentor for Dr. Krongkan and Spencer under the Centers for Research in Emerging Infectious Diseases (CREID) Pilot Research Program.

Commented [el1]: For Eric

Dr. Supaporn Wacharapluesadee, Ph.D. is the head laboratory at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, a WHO Collaborating Centre for Research and Training on Viral Zoonoses. Dr. Wacharapluesadee research interests include emerging infectious diseases in bats (including Nipah, Rabies, Coronavirus, and novel pathogens) as well as molecular diagnoses and sequencing of viruses. During the past 10 years, Dr. Wacharapluesadee got funding from USAID PREDICT to conduct the surveillance for novel virus in wildlife and human, and from Biological Threat Reduction Program US DTRA to conduct the EID surveillance in bat using the molecular technology and multiplex serology panel (Luminex technology). TRC-EIDCC is responsible for molecular diagnoses services to the hospital, and is the reference laboratory for rabies virus, MERS-CoV, Ebola, Zika, and other infectious diseases diagnoses for Ministry of Public Health, Thailand and laboratory training center on national and regional level. Dr. Wacharapluesadee has served and consulted on several WHO and Thai government committees.

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Specific Aims (1-page limit)

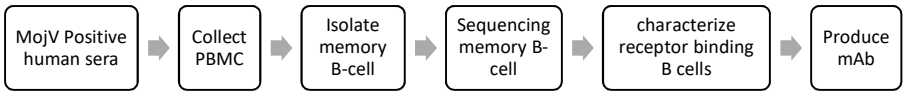
This project aims to study zoonotic reservoirs for pathogen origin by using serologic and cellular immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

The hypothesis

1. Can the memory B-cell immune response be used as a tool for the discovery of novel henipaviruses?
2. Can the spatial relationships between henipaviruses receptor binding proteins be generated that would allow for an understanding of antigenic evolution and functional characterization.

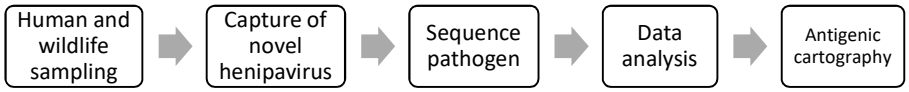
Aim 1. Antibody-mediated bait & capture of Thai MojV-like virus

In collaboration with NIH PREMISE, MojV RBP-reactive B cell receptors will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific MAbs will be generated and used for direct antibody-mediated virus capture in people and wildlife.



Aim 2. Modeling antigenic relationships among known and unknown henipaviruses.

Defining antigenic relationships among these will allow the serologic signature of related undiscovered henipaviruses to be identified. We will develop anti-RBP standards to explore the antigenic spatial relationships between all known rodent- and bat-borne HPVs, and the Thai MojV-like virus.



Objective 1. To isolate and sequence memory B-cells from humans positive for Mojiang virus in Ratchaburi province, Thailand for antibody production.

Objective 2. To capture unknown MojV-like viruses during syndromic surveillance in humans and routine surveillance in wildlife in Ratchaburi province, Thailand.

Objective 3: To generate antigenic maps using anti-henipavirus RBP standards.

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Study Rationale (7-page limit)

Nipah virus (NiV) and henipaviral diseases are among the most virulent of zoonotic diseases. The genus *Henipavirus* is currently composed of nine viruses including the prototypes, NiV and Hendra virus (HeV), and Cedar virus (CedV), Ghana virus (GhV), Mojiang virus (MojV) (McLinton et al., 2017), Langya virus (LayV) (Zhang X et al., 2022), Gamak virus (GAKV), Daeryong virus (DARV) (Lee et al., 2021), and Angavokely virus (AngV) (Madera et al., 2022).

HeV was first identified in Australia in 1994 as a cause of respiratory illness and fatal encephalitis in humans and horses. Closely following the discovery of HeV, NiV was identified in 1999 in Malaysia after outbreaks of fatal encephalitis in humans, with swine determined to be the intermediate host. Annual outbreaks of NiV have occurred in Bangladesh and are associated with the consumption of raw date palm sap, and since 19 May 2018 annual outbreaks of NiV associated encephalitis of been recorded in Kerala, India (Uwishema et al., 2022). Both HeV and NiV have a broad host range capable of infecting several mammalian orders, and can enter and infect endothelial cells, vasculature, and the central nervous system leading to severe multisystem organ diseases and high mortality. The broad host and tissue tropism of these viruses has been attributed to the use of the ephrinB2 and ephrinB3 ligands, highly conserved proteins with critical functions in evolutionary development, as virus receptors (Bossart et al., 2008). There are currently no vaccines or therapeutics and NiV and henipaviral diseases remain WHO Blueprint list priority pathogens (Middleton and Weingartl, 2012).

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A close relative of HeV, CedV was isolated from Australian bats in 2012 and found to be non-pathogenic, failing to cause any clinical symptoms in ferrets and guinea pig animal models (Marsh GA et al., 2012). The natural reservoirs of HeV, NiV, and CedV are flying foxes (*Pteropus sp.*) (Middleton and Weingartl, 2012). GhV was detected in Ghana in straw-colored fruit bats (*Eidolon helvum*) during routine surveillance in 2015 and was the first henipavirus found outside of the Indo-Pacific region. GhV showed a close phylogenetic relationship with HeV and NiV, and like HeV, NiV, and CedV the GhV RBP can mediate binding to ephrinB2 ligand and mediate cellular fusion (Drexler, J. F., 2009). Recent investigations into Madagascan fruit bats (*Eidolon duprenum*) detected a second African henipavirus, AngV. As neither GhV nor AngV have been isolated or rescued, the pathogenic and zoonotic potential of these African fruit bat associated henipaviruses remains unknown.

Multiple genus of small mammals were tested for viral RNA, and MojV was detected in yellow-breasted rats (R. flavipectus) (Wu Z et al., 2014). The discovery of the MojV genome occurred during follow-up investigations into lethal pneumonia from three miners in 2012 in China, however the pathogenic nature of this virus has only been suggested and no isolates have been discovered or tested in an animal model. Additionally, the novel shrew-borne henipaviruses, Gamak virus (GAKV), and Daeryong virus (DARV) were found in the Republic of Korea in 2017-2018. (Lee et al., 2021). Most recently, a close relative of MojV, Langya henipavirus (LayV) was recently isolated from febrile patients in eastern China. Subsequently, LayV was identified in shrews (Zhang et al., 2022), increasing the number of Asiatic rodent-associated henipaviruses to four. These recent discoveries are challenging the paradigms surrounding henipaviruses and their associations with bats and may suggest a novel rodent sub-genus of henipaviruses.

The Henipavirus genome consists of six structural proteins including nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), receptor-binding protein (RBP), and large (L)

protein (approximately length of 18,000 nucleotides) (Luby, S. P., & Gurley, E. S., 2012). In HeV and NiV, slippage sites within the P protein lead to the expression of the V, W, and C proteins. These proteins are virulence factors and are absent in CedV.

Table 1 Summary viruses in Henipavirus

Henipavirus	Origin (Year)	Reservoir	Genome (nucleotides)	Symptom for human	Reference
Hendra virus (HeV)	Australia (1994)	Fruit bats	18,234	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Nipah virus (NiV)	Malaysia (1999)	Fruit bat	18,246	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Ghana virus (GhV)	Africa (2009)	Fruit bat (*Eidolon helvum)*	18,530	No data	Drexler, J. F., 2009
Cedar virus (CedV)	Australian (2012)	Fruit bat	18,162	Non-pathogenic	Marsh, G. A et al., 2012
Mojiang virus (MojV)	China (2012)	Rodent	18,404	Febrile	Wu Z et al., 2014
Gamak virus (GAKV),	Korea (2017-2018)	Shrew	18,460	No data	Lee et al., 2021
Daeryong virus (DARV)	Korea (2017-2018)	Shrew	19,471	No data	Lee et al., 2021
Langya virus (LayV)	China (2019)	Shrew	18,402	Acute febrile	Zhang et al., 2022
Angavokely virus	Madagascar (2019)	Fruit bat	16,740	No data	(Madera et al., 2022)

Viruses in the genus *Henipavirus* have a broad reservoir and may cause high case fatality rates following human infection. The discovery and characterization of the novel Henipavirus is a high public health priority.

Research Gap

The greatest bat diversity around the world especially appears to be found in the tropical rain forests of South East Asia. In Thailand, at least 138 species in 11 families and 45 genera of bats have been recorded, such as members of the genus Pteropus, Rhinolophus, and Hipposideros (Soisook et al., 2011). Khao Chong Phran, a cave in Ratchaburi Province, has possibly the largest colony of bats in Thailand. There are estimated to be around 2 million bats in the cave and 95% are insect-eating bats (Department of National Parks, Wildlife and Plant Conservation, Thailand). Moreover, Khao Chong Phran has many community sites, such as cave entrances near communities, guano collection, and tourism sites, which risk zoonotic spillover. Bats are widely considered the animal hosts of zoonotic pathogens such as rhabdoviruses, coronaviruses, paramyxoviruses, and filoviruses, and many of the host species can be found in Thailand.

MojV was associated with a severe pneumonia-like illness killing three of six miners in 2012 in a mineshaft Yunnan, China. Interestingly, this mineshaft was also site where RaTG13, a beta-coronavirus most closely related with SARS-CoV-2, was detected in Rhinolophid bats (Rahalkar and Bahulikar, 2020). Identifying the wildlife reservoir of this novel virus will assist intervention strategies to reduce spillover risk.

As a part of Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH), serological investigations using a multiplex microsphere-based immunoassay (MMIA) have detected four members of a high-risk cohort with MojV-reactive serum, including one person with a high magnitude and durability of anti-MojV IgG across longitudinal samples. Despite this, genomic surveillance has not detected MojV or any novel MojV-like viruses. For this result, we would to further identify and characterize the origin of the Mojiang virus and to explore the novel MojV-like virus in Thailand.

Research Impact

Serological and cellular immunity is an important resource for virus discovery detection by using the specificities and characteristics of memory B cells from peripheral blood mononuclear cells (PBMC). The immune response will recognize and induce antibodies specific to the infecting pathogen. Characterizing the memory B cell response to an unknown pathogen in humans could in the detection of the exposure pathogen in humans. Molecular techniques such as PCR can detect only the genomic material in the body, but memory B cells are long-lived and thus have a much larger window of detection.

Memory B-cell differentiation is characterized by somatic hypermutation in antibody variable region genes (V) and selection of B cells expressing high-affinity variants of this antigen receptor and high sequence similarity in individuals exposed to the same antigen reflect antigen-driven clonal selection and form shared immunological memory between individuals (Yang et al., 2021). Memory B cells are highly abundant in the human spleen, and can proliferate 45% of the total B cell population in this organ (Hauser et al., 2015). Memory B cells can be detected in the Ig variable (IgV) genes in a fraction of IgM+ B cells. In humans, B cells expressing the memory marker CD27+ can be found in the marginal zone of spleen and circulate in the blood. After re-exposure to antigen, memory B cells can quickly proliferate and differentiate into plasma cells. Many studies the antibody diversity and specificity of the naturally paired immunoglobulin heavy and light chains for affinity maturation of B cell receptor (BCR) after virus infection such as SARS-CoV-2 infection (Yang et al., 2021), influenza (Dougan, S et al., 2013), HIV (Moir, S., & Fauci, A. S.,2017), etc. The characterization of memory B cells can be used to develop monoclonal antibodies and produce an effective therapeutic.

Significance and Approach

Southeast Asia is one of the world's highest-risk emerging infectious diseases hotspots. From this region emerged both SARS-CoV-1 and SARS-CoV-2, recurrent outbreaks of novel influenza strains, and the spillover of dangerous viral pathogens such as Nipah virus. The diversity of coronaviruses, henipaviruses, and filoviruses are found in wildlife reservoirs in Southeast Asia is due to the high diversity of wildlife, and the demography of the human population brings humans and wildlife into close contact. The spillover of viruses from animals to humans is often misdiagnosed and the clinical symptoms from patients is underestimated during the pandemic. This study provides a surveillance

strategy in human and wildlife populations to better understand immunity to novel pathogens and identify the origin of these pathogens to serve as an early warning system to prevent outbreaks of emerging viruses.

This study uses immunology to identify a novel virus that can aide in effective vaccine discovery and development, monoclonal antibody production for prevention and therapy, and generate data resources for early detection and diagnostic assays. Whereas PCR detection or virus isolation in wildlife is challenging, this new serological approach may be more successful in identifying reservoir hosts.

The Indo-Pacific region has a high risk of novel viral emergence. This study provides an opportunity for in-country research capacity building by linking local infectious disease scientists with an international collaborative network that has proven capacity to conduct this work and produce significant findings. Moreover, a global sero-surveillance network for surveillance technology extends virologic and immunologic screening to product discovery. This study will benefit and expand the CREID, EID-SEARCH networks, and the global sero-surveillance network to improve our understanding of immunology to identify novel pathogens and their origin to prevent future pandemics.

Research Methods

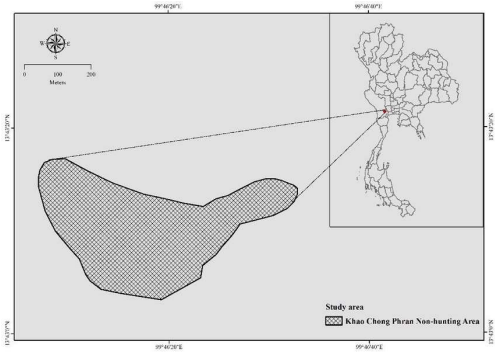
Aim 1. Characterization of monoclonal antibodies from humans with serological profiles consistent with MojV infection.

In collaboration with NIH VRC PREMISE, MojV RBP-reactive B cells will be captured, and the B cell receptor will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific mAbs.

Importance: The isolation of these mAbs represents a potential therapeutic for MojV, MojV-like henipas, and LayV, depending on how well these cross react.

Human samples collection site

Human positive serum for MojV was found close to Khao Chong Phran in Ratchaburi Province, which has the largest colony of bats in Thailand (Figure 1).



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And that you will test binding of these against LayV G and LayV F. To see cross reaction.

One antibody Broder has against MojV already has demonstrated cross reaction with a LayV protein.

David Veesler can do all the Cyro-EM to look at where these mAbs bind on the proteins.

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Figure 1: Map of Khao Chong Phran Non-hunting Area, Ratchaburi Province, Thailand (Boonpha N et al., 2019)

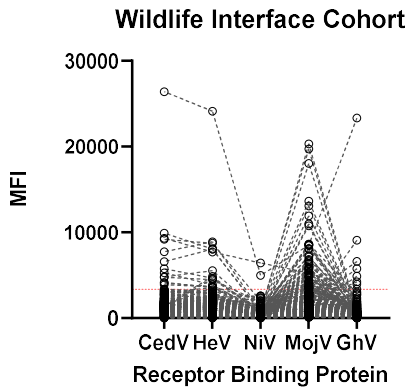

Figure 2: Seroprevalence of henipavirus proteins from a community with a large bat interface. MFI is the median fluorescence intensity for IgG reactivity to each protein. Red-dashed line is at 3,357 MFI, the assay cutoff.

PBMC collection and Single Memory B Cell Isolation

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Whole blood from MojV-positive people will be collected in individual heparin tubes and processed for PBMC isolation using Density Gradient Centrifugation (SepMate™ PBMC Isolation Tubes).

Human PBMCs will be stained with antibodies for positive markers of IgG memory B cells, and antibodies to negatively select and avoid contamination with dead cells, T cells, NK cells or monocytes (live, CD3−,CD14−,CD56−, IgM−, IgA−, CD19+,CD20+,CD27+). Avi-tag for biotinylation and 6xHIS tag for Nickel purification using the HisPur™ Ni-NTA Spin Purification Kit (Thermo Fisher Scientific, Waltham, MA). Single Memory B Cells will be sorted, cells will proliferate, cDNA will synthesized, then Heavy and light chain variable domains will be amplified (Waltari E et al., 2019).

B cell receptor Amplicon Preparation

Total RNA yields from the PBMC will be determined by absorbance at 260 nm on the NanoDrop™ One (Thermo Fisher Scientific). Total RNA will be used for first strand cDNA synthesis with gene-specific reverse transcription (RT) primers directed to the constant regions. The RT primers for IgG, IgM, IgA, lambda, and kappa will be included. Light chain and heavy chains will be amplified into amplicon. Double-stranded cDNA will be sequenced.

B cell receptor Data Analysis

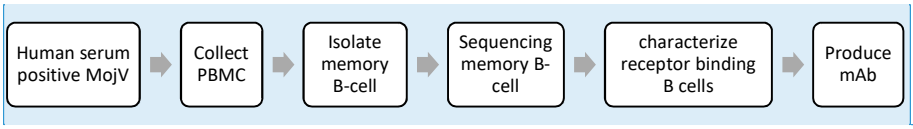
After completion of MiSeq sequencing, antibody repertoires will analyze using methods based on the pipeline.

Recombinant Antibody Cloning, Production, and Purification

Heavy and light-chain sequences will be assembled into an expression vector and transfected into cells for monoclonal antibody expression.

Functional assessment

Neutralization of viruses remains a critical correlate of protection for most vaccine strategies. Next we will test the cross neutralization potential of these anti-MojV RBP and F mAbs to neutralize LayV (Duke NUS/LinFa should be able to do this, if they have LayV and are propagating it, they can use cell-cell fusion/CPE in a straight PRNT). Decide if you want to incorporate this.



Antigenic cartography

Antisera and monoclonal antibodies to henipavirus RBP, including the novel MojV-like henipavirus, will be generated and tested against all the known RBP. The resulting binding data will be complied, and antigenic relatedness will be determined using RStudio.

Determination of envelope glycoprotein epitope binding

Isolated mAbs will be assessed for binding to MojV RBP and F proteins through multiplex binding serology. Further, the cross reaction potential of these mAbs will be assessed against GamV, DarV, and LayV RBP and F. MojV has not been isolated or rescued, and the virus receptor for MojV has not yet been discovered [2 citations]. Thus, a functional assessment of cross neutralizing potential is not possible. However, through cyro-electron microscopy we can investigate binding epitopes between these mAbs and MojV and LayV envelope glycoproteins. Epitope identification can then be used to make predictions about neutralizing potential through deductions about the location of henipavirus RBP receptor-binding pockets.

Aim 2. Utility of Thai MojV-like mAbs for surveillance.

Serological profiling provides an indirect measurement of the infecting virus. Despite XX% (XX/XXX), seroprevalence in humans, we have not detected nucleic acid sequences of MojV or MojV-like heniapviruses in this human cohort. In addition to the translational application of mAbs as therapeutics, we intend to utilize these mAb as non-clinical diagnostics surveillance tools for virus/virus antigen capture.

Site wildlife samples collection

100 Rodents 100 bats, and 100 shrews in Khao Chong Phran cave in Ratchaburi Province will be collected for surveillance targeting the Mojiang-like virus. In this study, we can collect only one time for a limited grant and time.

Enrollment criteria: Random age, random weight, random sex.

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Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Blood from animals will collect by using a needle in vein blood using serum to detect the undiscovered henipaviruses.

Human syndromic surveillance

100 human samples will collect serum to detect henipa-like virus near Khao Chong Phran cave in Ratchaburi Province for 1 year.

Enrollment criteria;

- Adults (≥ 18 years of age) who sign in the informed consent
- Who have sick without any reason such as severe/acute respiratory illness (SARI/ARI), Influenza-like illness (ILI), fever of unknown origin (FUO), or respiratory tract infection
- Who is the bat guano collector in the cave
- Who lives or works around wildlife habitats
- Who is involved with livestock or wildlife farming
- Who hunts wildlife or works with wildlife

Data analysis

Virus sequencing….after mAb pulldown.

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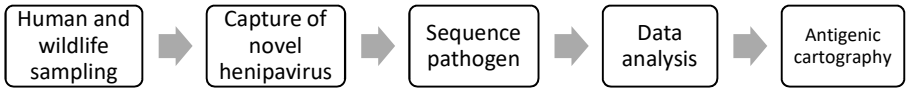

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Project Timeline (1-page limit)

Activity	05/23	06/23	07/23	08/23	09/23	10/23	11/23	12/24	01/24	02/24	03/24	04/24
Mentoring and training activities												
1.Training, literature review												
2.Monthly mentoring meeting												
3.Manuscript writing												
4.EID-SEARCH meeting												
Research activities												
1.IRB amendment												
Aim 1												
1. Collect PBMC human sample and isolation memory B-cell for Mojiang virus												
2. B cell receptor Data Analysis												
3. Produce mAb												
Aim 2												
4. Wildlife samples collection												
5.Human samples collection												
5.Identify and characterize novel viral pathogen												
6.Data analysis												

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Research Performance Sites *(1-page limit)*

This proposed study will be implemented at the King Chulalongkorn Memorial Hospital (KCMH) collaborating with the Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC). KCMH is a 1,000-bed, university hospital in close coordination with the Faculty of Medicine, Chulalongkorn University. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. It's one of the center hospitals for Emerging Infectious Diseases in Thailand, especially for domestic and international patients.

TRC-EIDCC was established by the Committee of the Emerging Infectious Disease Center, which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. Our clinical center provides standard medical care to patients with emerging and recurrent diseases and disease transmission. Moreover, the center plans preparedness activities, trains medical and government staff on interventions, hosts conferences for medical and public health professionals, creates networks of medical care for patients with emerging and recurrent diseases, and leads research and development projects for medical care and disease control. Apart from medical innovation in emerging and recurrent diseases, the clinical center strives to be the model of infectious diseases clinical services and pandemic control through research activities surrounding infectious diseases and effective disease control measures.

We also collaborate with the Department of National Parks, Wildlife and Plant Conservation for detecting novel pathogens from animals such as bats, rodents, and shrews in our country.

We also collaborate with the Department of Disease Control of Thailand (Thai-DDC) to support the Thai government and national policy makers through molecular diagnosis, including the identification of the first case of SARS-CoV-2 infection in Thailand. TRC-EIDCC prides itself on its high performance in research laboratory services and is a part of the national reference laboratory for COVID-19 and other emerging infectious diseases confirmation in Thailand.

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

CREID Research Center Collaboration *(1-page limit)*

The goals and objectives of the proposed research project, "Immune memory bait & capture to identify emerging henipavirus origins", are fully consistent with the specific aims of EID-SEARCH. EID-SEARCH takes a One Health approach to understanding zoonotic disease emergence, with a focus on viral diversity in wildlife at high-risk human animal interfaces. EID-SEARCH aims to understand the frequency and causes of virus spillover in high-risk communities across Southeast Asia and identify strategies and tools to reduce this risk. As the proposed research focuses on the links between farming practices, wildlife diversity, virus diversity, and cross-species transmission in Thailand, we expect the results of the project to make a significant contribution towards the overall goals of EID-SEARCH.

The proposed project is well-designed, leverages cutting-edge technology, and includes a robust ecological component that corresponds to the overall research strategy of EID-SEARCH. The EID-SEARCH team is highly motivated to support the application of metagenomics to ecological questions and assist in the analysis of the unique dataset that will be generated from this research. With the heightened focus on identifying and limiting risks associated with the complex interactions between humans, wildlife, and domestic mammals that has resulted from the emergence of SARS-CoV-2 from a wet market in China, the proposed research is both timely and likely to yield highly-cited, impactful publications for EID-SEARCH and Thailand partners.

The EID-SEARCH team includes a number of experts in study design, laboratory-based diagnostics, genetic and genomic analyses, and ecological modelling who will contribute to project implementation, data analysis, and manuscript preparation as required. Communication and coordination mechanisms will be set up between the Thailand research team and scientists at the EID-SEARCH research sites for resource sharing, training, knowledge exchange, and mentorship. In particular, EID-SEARCH will facilitate and encourage relationships between our partners in Southeast Asia (based in Malaysia, Singapore, and Thailand) that will facilitate Thailand team professional development and strengthen research capacity in the region. Dr. Eric Laing, the project Mentor, has an established relationship with both Dr. Krongkan and Spencer Sterling, and will act as the liaison between EID-SEARCH and the TRC-EIDCC research team. An expected outcome of this research project will be pilot data to support a joint funding application in the future.

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Mentoring Plan *(2-page limit)*

1. Goals for Pilot study and Career

Krongan Srimuang: My career goal is to be an expert scientist in either an academic research laboratory or a lecturer in medical science. My long-term research interests include the understanding of priority emerging infectious diseases, genetics, and epigenetics using molecular and serologic techniques. Detection of novel pathogens in their wildlife vectors can help characterize the pathogenicity, virulence, and evolution of these and other closely related pathogens. I believe that this pilot study can provide training in new techniques for the detection, identification, and characterization of novel pathogens that are currently unavailable in Thailand. My goal for the Pilot study is to gain more knowledge and skills for a lifelong career in public health-related research. I wish to expand my skills in novel virus detection using next-generation serologic techniques such as PBMC studies and memory B-cell isolation and sequencing that can contribute to improved surveillance and research capacity at human-animal interfaces. My mentors Dr. Laing and Dr. Wacharapluesadee can provide me with opportunities to develop and apply project skills and improve my scientific writing.

My education has provided me with a strong foundation to expand from for this study. I received my undergraduate degree in Medical Technology from Chulalongkorn University which included training in molecular biology, serology, immunology, chemistry, virology, bacteriology, mycology, parasitology, hematology, clinical microscopy, transfusion medicine, and forensic medicine. My interest in molecular technology led me to pursue a Ph.D. at the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, which provided me with training in several molecular techniques including conventional PCR, real-time PCR, Next Generation Sequencing (NGS), etc. The emergence of COVID-19 motivated me to focus on emerging infectious diseases. I am currently completing my post-doctoral fellowship at the Emerging Infectious Diseases Clinical Center (EIDCC), King Chulalongkorn Memorial Hospital with Dr. Wacharpluesadee. Throughout this fellowship, I have continued learning advanced and innovative techniques to detect, identify, and characterize the novel pathogens. Through this pilot project, I will learn about the Peripheral Blood Mononuclear Cells (PBMC) which are a critical part of many subdivisions of medical research including Infectious diseases, Vaccine development, and Immunology. This project aims to study zoonotic reservoirs for by using serologic and cellular immune discovery in humans, and applying this immune knowledge in wildlife for early detection and identification of novel pathogens. This proposed study will expose me to various techniques and immunology topics that I useful in designing and testing new pipelines as technological advances continue to develop. This study will also allow me to develop, manage, and communicate my research.

Spencer Sterling: My overall career goal is to understand the factors that lead to viral zoonosis and to build epidemiological capacity in regions with the highest risk for zoonotic spillover. Through my training and educational experiences, I have learned laboratory and epidemiological techniques that can assist in the understanding of zoonosis. I wish to continue my education by expanding my repertoire of molecular techniques to include studies of the immune system's response to zoonotic disease in humans and animals, as well as to learn next-generation sequencing techniques. Additionally, I would like to use my experience in epidemiological techniques to train scientists in the Southeast Asian region in order to build local capacity in infectious disease research. Dr. Laing has been a mentor for me throughout my career, and this project would allow for this relationship to expand into new molecular areas, as well as grant

writing and project management skills. Additionally, Dr. Wacharapluesadee has decades of experience in infectious disease research in the region and has mentored scientists that have gone on to careers at the Thai Ministry of Public Health, Department of Disease Control, Department of Parks and Wildlife, academia, and the private sector. Both mentors have encouraged Dr. Srimuang and I to apply for this pilot study as well as pursue other funding mechanisms for our research interests. Both mentors will provide for me the ability to independently pursue my research interests in the zoonotic epidemiology and train me on the best practices of scientific communication.

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Vertebrate Animals Section Requirements

1. Description of Procedures (Vertebrate Animals Section)

Animal samples will be collect in the Khao Chong Phran cave in Ratchaburi Province, including bats, rodents, and shrews as were approved by the IACUC for the project "Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia" (Animal Protocol #G2020-42). Moreover, the project has been approved by the Thai Department of National Park, Wildlife and Plant Conservation on 0907.4/28372.

1.1 Bats (order Chiroptera): Family Pteropodiae; Family Rhinopomatidae; Family Emballonuridae; Family Craseonycteridae; Family Megadermatidae; Family Rhinolophidae; Family Hipposideridae; Family Vespertilionidae; Family Mollossidae; etc.
Random age, random weight, random sex, and total number 100.

1.2 Rodents (order Rodentia) Maxomys whiteheadi; Rattus exulans; Rattus annandalei; Leopoldamys sabanus; Sundamys muelleri; Chiropodomys gliroides; Niviventer cremoriventer; Maxomys rajah; Hylomys suillus; Niviventer; Maxomys; Maxomys surifer; Rhizomys sumatrensis; Rhinosciurus laticaudatus; Callosciurus notatus; Rattus tiomanicus; Tupaia glis; Petaurillus hosei; Rattus tanezumi; Rattus norvegicus; Rattus rattus; Sundasciurus lowii; Tupaia longipes; Tupaia tana; Callosciurus prevostii; Sundasciurus hippurus; Tupaia minor; Hystrix crassispinis; Maxomys alticola; Rattus argentiventer; Trichys fasciculata; Dremomys everetti; Callosciurus adamsi; Rattus baluensis; Rattus losea; Bandicota indica; Mus cookie; Berylmys bowersi; Rattus nitidus; Mus caroli; Leopoldamys neilli; Leopoldamys edwardsi; Mus cervicolor; Mus fragilicauda; Niviventer confucianus; Niviventer fulvescens; Bandicota Savile; etc.
Random age, random weight, random sex, and total number 100.

1.3 Shrews (order Soricomorpha) Sorex isodon; Crocidura attenuate; Crocidura fuliginosa; Crocidura horsfieldii; Crocidura vorax; etc.
Random age, random weight, random sex, and total number 100.

2. Justifications (Vertebrate Animals Section)

For our Aim 2. Modeling antigenic relationships among known and unknown henipaviruses.
We will develop anti-Henipavirus RBP standards to explore the antigenic spatial relationships between all known rodent- and bat-borne HPVs, and the Thai MojV-like virus. We would like to survey all animals that could be reservoir hosts or intermediate hosts of novel henipaviruses that can be spillover to humans to increase the likelihood of detecting a novel virus. Therefore, the research goals be accomplished using an alternative model (e.g., computational, human, invertebrate, in vitro).

3. Minimization of Pain and Distress (Vertebrate Animals Section)

For animals requiring anesthesia- Blockage of visual stimulus, handling in cloth bags and other methods will be employed to keep handling procedures gentle and to minimize stress.

4. Method of Euthanasia

This study will not use methods of euthanasia in all animals.

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Human Subjects Research
This project will enroll human subjects under the approved protocols by the Health Medical Lab Institutional Review Board, US (No. 894ECOH21b) and the Institute Review Board of the Faculty of Medicine, Chulalongkorn University, Thailand (No. 211/64).

All applicants proposing human subjects research should address the following elements on the Protection of Human Subjects:

1. Risks to the subjects

Human blood will be collected by a needle from a vein in the arm (approximately 15 ml) into a heparin tube.

Having blood drawn may produce discomfort or minor bleeding and the possibility of bruising at the site of the needle puncture. There is also a slight risk of infection at the site of the

needle puncture. Although rare, some people have experienced nausea, light headedness, and

fainting in association with a blood draw.

2. Adequacy of protection against these risks

Vital signs will be taken at each visit. Vital signs include measurements of your pulse (heart rate), respirations (breaths per minute), blood pressure, height, weight, and body temperature.

3. Potential benefits of the research to the subjects and others

There are no direct benefits to the research subjects. Indirect benefits to the research to subjects is the possible identification of undiscovered henipaviruses infections in this population. Detection can lead to correct diagnostics and proper treatment of the disease

4. Importance of the knowledge gained or to be gained

This study will bring opportunity, experience, and knowledge in immunology and serology to research staff in Thailand. Moreover, the new methods and techniques to identify novel pathogens by using memory B-cell can be used in the study of zoonotic reservoirs and pathogen origins. By using serologic and cellular immune discovery in humans and wildlife, we seek the ability for early detection and identification of novel pathogens to prevent the next pandemic.

5. Country- / institution-specific ethics / IRB regulations addressed

This project will enroll human subjects under the approved protocols by the Health Medical Lab Institutional Review Board, US (No. 894ECOH21b) and the Institute Review Board of the Faculty of Medicine, Chulalongkorn University, Thailand (No. 211/64).

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Budget/Budget Justification

No.	Desription	Budget ($)	Unit cost (USD)	month	Unit	Cost
1	Pernonel	63,600.00				19,400.00
	Krongkan Srimuang		1,000.00	12	month (20 hrs/month)	12,000.00
	Spencer		0.00		month (100 hrs/month)	0.00
	Lab Technician (Part-time)		1,000.00	6	month (100 hrs/month)	6,000.00
	Field Technician (Part-time)		600.00	2	month (60 hrs/month)	1,200.00
	Lab admin coordinator (Part-time): coordinate with university (Mrs Chatchawadee)		200.00	1	month (20 hrs/month)	200.00
2	Fleldwork					
	Human sampling	15,348.67				11,388.67
2.1	Human sample collection		70.00	110	case	7,700.00
	Human sample collection field work: 2 days/trip (10 participants) (transportation, accommodation, food, wage, subject gift, etc.)		250.00	1	Trip	
2.2	Human sample collection field work: 2 days/trip (50 participants) (transportation, accommodation, food, wage, subject gift, etc.)		2,500.00	1	Trip	2,500.00
	Office suppiles		1,188.67	1	Time	1,188.67
	Animal sampling	28,000.00				8,000.00
2.3, 2.4	Animal sample collection field work: 7 days /trip (100 bats/trip), (transportation, accomadation, food, wage, etc.)		7,000.00	1	Trip	7,000.00
	Animal sample collection field work: 7 days /trip (100 rodents or shrews /trip), (transportation, accomadation, food, wage, etc.)					
	Field supplies: PPE, gloves, nets, dryice, etc.		1,000.00	1	Time	1,000.00
3	Lab work	84,500.00				43,500.00
3.1, 3.4	PBMC collection and isolation, and sequencing service cost		40.00	1000	cell	40,000.00

3.3	Reagents and supplies for serology testing of human and wildlife samples				test	0.00
3.5, 3.6	MMIA in human and animal samples: reagent and supplies (sheath bufferm 96 wells plate, microcentrifuge tube, pipette tp, pipette, Multi-Channel pipette, etc.)		3.50	1000		3,500.00
6	Indirect costs (8%)	**15,995.89**	15,995.89	1	Time	15,995.89
	Total	**207,444.56**				98,284.56

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Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

List of abbreviations, acronyms, symbols

AngV: Angavokely virus
BCR: B cell receptor
BSc: Bachelor of Science
BSL2: Biological Safety level 2
BSL3: Biological Safety level 3
cDNA: Complementary DNA
CedV: Cedar virus
Co-PI: Co-principal investigator
CREID: Centers for Research in Emerging Infectious Diseases
DARV: Daeryong virus
DNA: Deoxyribonucleic acid
EHA: EcoHealth Alliance
EID: Emerging Infectious Disease
EID-SEARCH: The Emerging Infectious Disease – South East Asia Research Collaboration Hub
ELISA: Enzyme-linked immunosorbent assay
F: Fusion protein
FACS: Fluorescence-activated cell sorting
FUO: Fever of unknown origin
GAKV: Gamak virus
GhV: Ghana virus
HeV: Hendra virus
HPVs: Henipaviruses
ILI: Influenza-like illness
KCMH: King Chulalongkorn Memorial Hospital
L: large protein
LayV: Langya virus
M: Matrix protein
mAb: Monoclonal antibody
MD: Maryland
MMIA: Multiplex microsphere-based immunoassay
MojV: Mojiang virus
MSc: Master of Science
N: Nucleocapsid
NGS: Next-generation sequencing
NIAID: National Institute of Allergy and Infectious Diseases
NIH: National Institutes of Health
NiV: Nipah virus
P: Phosphoprotein
PBMC: Peripheral blood mononuclear cells
PCR: Polymerase Chain Reaction
PhD: Doctor of Philosophy
PI: Principal investigator
RBP: Receptor-binding protein
RNA: Ribonucleic acid
RT-PCR: Real-time PCR
SARI/ARI: Severe/acute respiratory illness

SARS-CoV-1: Severe acute respiratory syndrome coronavirus 1
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2
SEA: Southeast Asia
Thai-DDC: Department of Disease Control of Thailand
TRC-EIDCC: Thai Red Cross. Emerging Infectious Diseases. Clinical Center
USA: United States
WGS: Whole Genome Sequencing

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Facilities and Resources

FACILITIES: Specify the facilities to be used for the conduct of the proposed research.

Laboratory:

The Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC), King Chulalongkorn Memorial Hospital (KCMH) was established by the Committee of the Emerging Infectious Disease Center which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. TRC-EIDCC laboratory facilities include 3 biosafety cabinets (BSL 2, BSL 2+, BSL2+), 3 automated extraction machines, 2 thermocyclers (Proflex), 2 real-time PCR machines (CFX, Bio-rad), 3 Freezers (-80 °C), 2 refrigerators; 4 °C, 2 autoclaves. Our EIDCC uses the Pathogen Asset Control System (PACS) system for sample tracking. Moreover, our Faculty of Medicine, Chulalongkorn University has a central laboratory, the Chula Medical Research Center (Chula MRC), which provide many instruments for medical, masters, and graduate students, Post-doctoral fellows, and researchers at Chulalongkorn University and KCMH. The Chula MRC laboratory facilities are divided into molecular diagnostics (PCR Thermal Cycler, QStudio6 Flex Real-Time PCR, Droplet Digital PCR, GeneXpert, Film array, Illumina MiSeq), immunology facilities (ELISA testing equipment (reader & washer), Luminex, Flow Cytometry), cell sorting machine and a BSL3 laboratory. Moreover, the facility provides general equipment, such as autoclaves, balances, CO2 incubators, Dual Fluorescence Cell Counter, 4 °C refrigerators, -20°C, and -80°C freezers.

Clinical:

KCMH is a 1,000-bed university hospital, in close coordination with the Faculty of Medicine, Chulalongkorn University. The Thai Red Cross Society provides medical treatment, rehabilitation, health promotion and disease prevention services to the general public and the vulnerable. It also provides biopharmaceutical and sterile medicines that are sufficient to meet the needs of patients and safe in accordance with international standards. It serves as a center for providing eye and organ donation services to patients nationwide in line with international standards. It has specialized doctors and public health personnel and also produces specialized nurses. The TRC-EIDCC in the KCMH provides standard medical care to patients with emerging and recurrent diseases and disease spreading prevention.

Animal:

TRC-EIDCC also collaborates with the Department of National Parks, Wildlife and Plant Conservation for detecting the emerging novel pathogens from animals such as bats, rodents, and pangolins in our country. Bats are considered protected wildlife in Thailand by Wildlife Conservation, Department of National Parks, Wildlife and Plant Conservation.

Computer:

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

TRC-EIDCC used the service from the Office of Information Technology, Chulalongkorn University that provides the application, network, and software such as Office 365, VPN, WIFI, Microsoft Office, Microsoft Windows, SPSS, Adobe, Zoom Meeting, etc. Moreover, our EIDCC use the Pathogen Asset Control System (PACS) for sample tracking.

Office:

The office and laboratory space are located at the EIDCC at 4th Floor, Jongkolnee Watwong Building and 6th Floor, Aor Por Ror Building, respectively. The applicants will have dedicated desks in both buildings.

Other:

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

30 January 2023

Dr. Krongkan Srimuang & Mr. Spencer Sterling

Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC),

King Chulalongkorn Memorial Hospital

Object: Thai Red Cross Emerging Infectious Diseases Clinical Center support for your application to the CREID Pilot Research Program, entitled "Immune memory bait & capture to identify emerging henipavirus origins."

Dear Dr. Srimuang & Mr. Sterling,

Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC) is pleased to support your application to the **CREID Pilot Research Program**, entitled "Immune memory bait & capture to identify emerging henipavirus origins." We are fully committed to executing the statement of work to study zoonotic reservoirs for pathogen origin by using serologic and cellular immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

The work proposed in this project will be given a great opportunity and collaboration to our team, it serves very strong knowledge in serology and immunology for the detection of novel virus pathogens in Thailand using new technology and techniques. Moreover, the building capacity and capability of our laboratory and Faculty will access and support to make the success and completion of this project.

This letter expresses my strong interest and drives forward to making this application a success. I lok forward to collaborating with you of this project. I wish you success in the CREID Pilot Research Program application.

Sincerely,

Assistant Professor Opass Putcharoen, MD, Msc

Head of Thai Red Cross Emerging Infectious Diseases Clinical Center

King Chulalongkorn Memorial Hospital

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

30th January, 2022

Dr. Krongkan Srimuang & Mr. Spencer Sterling

Thai Red Cross Emerging Infectious

Diseases Clinical Centre (TRC-EIDCC),

King Chulalongkorn Memorial Hospital

Dear Dr. Srimuang & Mr. Sterling,

I am writing this letter to express my support as Research Center Mentor for your application to the CREID Pilot Research Program, entitled "Immune memory bait & capture to identify emerging henipavirus origins."

My experience in immunology and serology will support them in the CREID Pilot Research Program. The research aim has the potential to have a strong impact on our approach to pathogen surveillance at high-risk human-animal interfaces. Given the understanding of the pathogen origin by using serologic and cellular immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

I believe that this is a great opportunity to use scientific knowledge and expertise to train the characterization of memory B cells from peripheral blood mononuclear cells (PBMC) and serology background to the team at Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital, Thailand. Our stronger partnerships have the potential to really drive forward innovations in this serology and immunology which include the implementation of novel techniques for pathogen surveillance as proposed in your pilot project application.

The CREID Network and the Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH) will support scientists in regions around the world where emerging and reemerging infectious disease outbreaks. Many Southeast Asian partnerships in EID-SEARCH have strong the potential to really drive forward innovations in this area.

I believe that Dr. Wacharapluesadee, Co-PI, and I will provide scientific skills, expertise, and networks for this project.

Towards that aim, I am committed to supporting your professional development in the following ways:

1.

2.

3.

Commented [el11]: Could you please add the supporting?

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

I'm very much looking forward to working closely with you on this 1-year research project and beyond as you continue to develop your surveillance zoonotic disease research program.

Best regards,

Eric Laing, Ph.D.

Assistance Professor

Uniformed Services University of the Health Sciences

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

30th January, 2022

Dr. Krongkan Srimuang & Mr. Spencer Sterling

Thai Red Cross Emerging Infectious

Diseases Clinical Centre (TRC-EIDCC),

King Chulalongkorn Memorial Hospital

Dear Dr. Srimuang & Mr. Sterling,

I am writing in support as Research Center Mentor for your application to the CREID Pilot Research Program, entitled “Immune memory bait & capture to identify emerging henipavirus origins.”

For my experience in 20 years to conduct the surveillance for novel virus in wildlife, human, and bats using molecular technology and multiplex serology panel (Luminex technology) will support them in the CREID Pilot Research Program. The research aim has the potential to have a strong impact on our approach to pathogen surveillance at high-risk human-animal interfaces. Given the understanding of the pathogen origin by using serologic and virus discovery in bats, humans, and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

As the head laboratory at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, I believe that this is a great opportunity for our Thailand team that can train in the characterization of memory B cells from peripheral blood mononuclear cells (PBMC) and serology surveillance in novel virus pathogens. Moreover, our laboratory has supported the Department of Disease Control (DDC) in diagnosing emerging infectious diseases and communicable diseases such as Ebola, MERS, and COVID-19. I am also a committee member of the Global Laboratory Leadership Program (GLLP) Thailand, organized by the Department of Medical Sciences. With my experience to diagnose the first human MERS case in 2015 and the first human COVID-19 case outside China in January 2020, I will support multidisciplinary projects involving epidemiology, laboratory, field science, and bioinformatics to diagnose and monitor the emergence of wildlife-origin viral zoonoses, including SARS-CoV, Nipah, and Hendra viruses, Avian influenza, and novel viruses from bats. I am also the Thailand country coordinator for PREDICT project, large contracts from USAID during 2010-2019 involving the successful management of teams of virologists, field biologists, veterinarians, epidemiologists, hospitals, and laboratorians. I will be given the strong knowledge to build capacity and capability in this project and our team in Thailand.

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

The CREID Network and the Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH) will support scientists in regions around the world where emerging and reemerging infectious disease outbreaks. Many Southeast Asian partnerships in EID-SEARCH have strong the potential to really drive forward innovations in this area.

I believe that Dr. Laing, and I will provide scientific skills, expertise, and networks for this project.

Towards that aim, I am committed to supporting your professional development in the following ways:

1. I will maintain regular bi-weekly meetings for the project progresses.
2. I will support the resources in our country and research team in this project.
3. I will create opportunities for scientists network to ensure the success of research project.
4. I will provide and share advice and technical expertise in specific knowledge for this project.

I'm very much looking forward to working closely with you on this 1-year research project and beyond as you continue to develop your surveillance zoonotic disease research program.

Best regards,

Supaporn Wacharapluesadee, Ph.D.

Head Laboratory,

Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC),

King Chulalongkorn Memorial Hospital,

Faculty of Medicine,

Chulalongkorn University.

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

Pilot Program Application Submission Checklist – Final Application Submission

Title of Application: Immune memory bait & capture to identify emerging henipavirus origins

Submitted by: Krongkan Srimuang, Ph.D.

Submission Date: 1/30/2023

Full Application Submission Requirements

☒ Proposal Cover Sheet

☒ Title and Table of Contents

☒ Study Personnel *(1-page limit)*

- Main Application Body Section Requirements *(7-page limit):*
  - ☒ Research Aims & Objectives
  - ☒ Study Rationale/Research Gap/Impact
  - ☒ Significance and Approach
  - ☒ Research Methods

☒ Project Timeline (*1-page limit*)

☒ Research Performance Sites *(1-page limit)*

☒ CREID Research Center Collaboration *(1-page limit)*

☒ Mentoring Plan *(2-page limit)*

- Vertebrate Animals Section Requirements *(3-page limit):*
  - ☒ **Description of Procedures**
  - ☒ **Justifications**
  - ☒ **Minimization of Pain and Distress**
  - ☒ **Method of Euthanasia (Cover Page Supplement / PHS Fellowship Supplemental Form)**
- Human Subjects Research *(3-page limit):*
  - ☒ Summary of the parent study and IRB approval information for the study
  - ☒ Risks to the subjects
  - ☒ Adequacy of protection against these risks
  - ☒ Potential benefits of the research to the subjects and others
  - ☒ Importance of the knowledge gained or to be gained
  - ☒ Country / institution-specific ethics / IRB regulations addressed
- Research, Related Project Information, and Budget/Budget Justification
  - ☐ R&R Other Project Information Form
  - ☐ Full budget, with total costs of no more than $150,000

Commented [el12]: How should we do about the budget?

Applicant(s) Name (Last, first, middle): (Srimuang, Krongkan)

☐ Budget justification which describes the labor and other direct costs

☐ If your institution does not have adequate funds for a cost-reimbursement award and requires pre-payment of funds during the award year, please note this in your budget justification and outline a payment schedule that will function for your project.

- Supporting Documentation
  - Biographical Sketch and Other Support. All applications must include:
    - ☒ Applicant PI Biographical Sketch (*4-page limit*)
    - ☒ Applicant PI Previous/Current/Pending Support (Include funding amounts, *no page limit*)
    - ☐ Mentor Biographical Sketch (*4-page limit*)
    - ☐ Mentor Current/Pending Support (*no page limit*)
    - ☐ Key Personnel Biographical Sketches (*4-page limit each*)
    - ☐ Key Personnel Current/Pending Support (*no page limit*)
  - Co-PI Plan (only needed if applying as Co-PIs) (*1-page limit*)
    - ☐ What each Co-PI will contribute to the proposed research study
    - ☐ How the Co-PIs will jointly work with the affiliated Research Center
    - ☐ How the Co-PIs will jointly manage the proposed study
  - ☒ References Cited (*no page limit*)
  - ☒ List of Abbreviations, Acronyms, and Symbols
  - ☒ Facilities, Existing Equipment, and Other Resources (*template provided*)
  - ☐ NIH Foreign Clearance form (*template provided*)
  - ☐ Letters of Organizational Support *(2-page limit per letter)*
  - ☐ Letter of Collaboration from CREID Research Center PI *(2-page limit per letter)*
  - ☐ Letter from Research Center Mentor (*2-page limit)*
  - ☐ Letter from Primary Scientific Mentor (if different than Research Center mentor) (*2-page limit)*

Commented [el13]: Dear Eric, this is your part.

Commented [el14]: Dear Eric, this is your part.

From: Krongkan Srimuang on behalf of Krongkan Srimuang <krongkan.sr@gmail.com>
To: Laing, Eric
Cc: Hongying Li; Spencer Sterling; sasiprapa.n@outlook.com; spwa
Subject: Re: Pilot study 2023
Date: Saturday, January 28, 2023 7:54:06 AM
Attachments: Research aim, methods_V.3.docx

Dear Eric,

Please find attached the edit research aim and method.
If you need more information please let me know.

Thank you,
Best regards,
KIO

On Sat, Jan 28, 2023 at 1:52 PM Laing, Eric <eric.laing@usuhs.edu> wrote:

> Hi Kio,
>
> My biosketch and LOS are attached
>
> 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 Fri, Jan 27, 2023 at 4:46 PM Laing, Eric <eric.laing@usuhs.edu> wrote:
>
> > Hi Kio and Spencer,
> >
> > Do you guys have a version in which Aim 2 is more developed? You need to focus on that Aim.
> >
> > LOS from Dr. Douek is attached
> >
> > - Eric
> >
> > Eric D. Laing, Ph.D.
> > Assistant Professor
> > Department of Microbiology and Immunology
> > Uniformed Services University
> > 4301 Jones Bridge Road
> > Bethesda, MD 20814

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eric.laing@usuhs.edu

On Thu, Jan 26, 2023 at 11:02 PM Hongying Li <li@ecohealthalliance.org> wrote:

> Hi Kio, Spencer, Eric,
>
> Attached are
>
> - Vertebrate Animals Section
> - Human Subject Research
> - Foreign Clearance
> - Letter of Collaboration from CREID RC signed by Peter
>
> **You need to read through and edit** based on the project activities, for example, sample types, enrollment of children or not, age group, and other relevant information.
>
> Please also ensure the format, font, size, line spacing, margin, etc. are consistent and follow the guidance.
>
> Let me know if you have any questions! Send me documents to review anytime.
>
> Best,
> Hongying
>
> On Thu, Jan 26, 2023 at 10:23 PM Krongkan Srimuang <krongkan.sr@gmail.com> wrote:
>
> > Dear Eric,
> >
> > Please find attached the Pilot study including all documents.
> > If you need further information please let me know.
> >
> > Thank you very much
> >
> > Best regards,
> > KIO

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: Wacharapluesadee, Supaporn

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

POSITION TITLE: Senior Researcher

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
Chiang Mai University, Thailand	B.S.	02/1991	Medical Technology
Mahidol University, Thailand	M.S.	01/1994	Biochemistry
Chulalongkorn University, Thailand	Ph.D.	03/2006	Biomedical Sciences

A. Personal Statement

I have 29 years in public health research and 20+ years of experience in emerging viral zoonoses. I have managed many internationally funded research projects that involve working with and managing international and local interdisciplinary teams. Most of my research projects are diagnostics development, pathogen discovery, public health surveillance, field surveillance in wild mammals, human behavioral risk surveys, and clinical research. I conduct workshops on the development of novel diagnostic approaches, appropriate sample collection and handling for different pathogens, and viral characterization *in vitro* and *in vivo*. I am a senior researcher at the Thai Red Cross Emerging Infectious Diseases Clinical Center, which conducts research on emerging zoonoses, clinical research, and public health surveillance research. My research background mainly focuses on understanding the process of zoonotic disease emergence, particularly viral zoonoses. This includes identifying the bat origin of Nipah virus, MERS-CoV, and SARS-CoV-2 and pathogenesis and diagnoses of Rabies. My studies on the emergence of novel betacoronaviruses found in Thai bats and Nipah virus have been published. My laboratory has supported the Department of Disease Control (DDC) in diagnosing emerging infectious diseases, communicable diseases, and wastewater surveillance. I am the DDC instructor on the clinical sampling method and consultant for Ebola, MERS, and COVID-19 diagnosis. I am a committee member of the Global Laboratory Leadership Program (GLLP) Thailand, organized by the Department of Medical Sciences. I led the team to diagnose the first human MERS case in 2015 and the first human COVID-19 case outside China in January 2020. I have been the PI on six multidisciplinary research projects that use epidemiology, laboratory, field science, and bioinformatics to diagnose and monitor the emergence of wildlife-origin viral zoonoses, including SARS-CoV, Nipah, and Hendra viruses, Avian influenza, and novel viruses from bats. I am also the Thailand country coordinator for PREDICT project, large contracts from USAID during 2010-2019 involving the successful management of teams of virologists, field biologists, veterinarians, epidemiologists, hospitals, and laboratorians.

1. **Wacharapluesadee S**, Buathong R, Iamsirithawon S, Chaifoo W, Ponpinit T, Ruchisrisarod C, Sonpee C, Katasrila P, Yomrat S, Ghai S, Sirivichayakul S, Okada P, Mekha N, Karnkawinpong O, Uttayamakul S, Vachiraphan A, Plipat T, Hemachudha T. Identification of a Novel Pathogen Using Family-Wide PCR: Initial Confirmation of COVID-19 in Thailand. Front Public Health. 2020 Oct 7;8:555013. doi: 10.3389/fpubh.2020.555013. PMID: 33134237; PMCID: PMC7579402.

2. Buathong R, Chaifoo W, Iamsirithaworn S, **Wacharapluesadee S**, Joyjinda Y, Rodpan A, Ampoot W, Putcharoen O, Paitoonpong L, Suwanpimolkul G, Jantarabenjakul W, Petcharat S, Bunprakob S, Ghai S, Prasithsirikul W, Mungaomklang A, Plipat T, Hemachudha T. Multiple clades of SARS-CoV-2 were introduced to Thailand during the first quarter of 2020. Microbiol Immunol. 2021 Oct;65(10):405-409. doi: 10.1111/1348-0421.12883. Epub 2021 Sep 1. PMID: 33835528; PMCID: PMC8251142.
3. **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. Nat Commun. 2021 Feb 9;12(1):972. doi: 10.1038/s41467-021-21240-1. Erratum in: **Nat Commun**. 2021 Feb 25;12(1):1430.
4. **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 Jul 5;3(1):12. doi: 10.1186/s42522-021-00044-9. PMID: 34218820; PMCID: PMC8255096.

B. Positions, Scientific Appointments, and Honors

Positions and Employment

1994 -97 Biochemical Technician, Department of Entomology, AFRIMS, Thailand
1997 Researcher, Department of Immunology, Chulabhorn Research Institute, Thailand
1997 -00 Medical Technologist, The HIV/AIDS Collaboration Thai-US, Thailand
2000 -16 Laboratory Chief, Neuroscience Centre for Research and Development & WHO Collaborating Centre for Research and Training on Viral Zoonoses, Faculty of Medicine, Chulalongkorn University Hospital, Thai Red Cross Society, Thailand
2016 -21 Deputy Chief of Thai Red Cross Emerging Infectious Diseases Health Science Centre, Faculty of Medicine, Chulalongkorn University Hospital
2021 - Senior Researcher, Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital
Committee member, School of Global Health, Faculty of Medicine, Chulalongkorn University

Other Experience and Professional Membership

2010 -14 PREDICT Thailand Country Coordinator
2014 - Thai Ministry of Public Health (MOPH) Ebola Diagnostic Committee
2015 -19 PREDICT 2 Thailand Country Coordinator
2016 - Steering committee, Bat One Health Research Network, BTRP DTRA
2021 - WHO Scientific Advisory Group for the Origins on Novel Pathogens (SAGO)

C. Contributions to Science

1. **Research on One Health.** One Health is an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals, and ecosystems, as the definition by WHO. Our projects are multidisciplinary studies on human surveillance, wildlife surveillance, wastewater surveillance, and human behavior.
   a. Tangwangvivat R, **Wacharapluesadee S**, Pinyopornpanish P, Petcharat S, Muangnoicharoen H. S, Thippamom N, Phiancharoen C, Hirunpatrawong P, Duangkaewkart P, Chaiden C, Wechsirisan W, Wandee N, Srimuang K, Paitoonpong L, Buathong R, Pawun V, Hinjoy S, Putcharoen O, Iamsirithaworn S. Assessment of SARS-CoV-2 variant wastewater detection strategies in the Bangkok Metropolitan region. **Preprint Research** 2023

b. Keusch GT, Amuasi JH, Anderson DE, Daszak P, Eckerle I, Field H, Koopmans M, Lam SK, Das Neves CG, Peiris M, Perlman S, **Wacharapluesadee S**, Yadana S, Saif L. Pandemic origins and a One Health approach to preparedness and prevention: Solutions based on SARS-CoV-2 and other RNA viruses. **Proc Natl Acad Sci USA**. 2022 Oct 18;119(42):e2202871119. doi: 10.1073/pnas.2202871119. Epub 2022 Oct 10. PMID: 36215506; PMCID: PMC9586299.
c. 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 Infect Dis**. 2022 May 16;22(1):472. doi: 10.1186/s12879-022-07439-7. PMID: 35578171; PMCID: PMC9109443.

2. **Research on Public Health Surveillance in Thailand.** Effective disease control programs rely on effective surveillance and response systems. Our laboratory collaborates with the Department of Diseases Control for active surveillance on syndromic surveillance and outbreak investigation. Both molecular and serology surveillance systems were conducted to identify the cause of the disease.
   a. Plipat T, Buathong R, **Wacharapluesadee S**, Siriarayapon P, Pittayawonganon C, Sangsajja C, Kaewpom T, Petcharat S, Ponpinit T, Jumpasri J, Joyjinda Y, Rodpan A, Ghai S, Jittmittraphap A, Khongwichit S, Smith DR, Corman VM, Drosten C, Hemachudha T (2017). Imported case of Middle East respiratory syndrome coronavirus (MERS-CoV) infection from Oman to Thailand, June 2015. **Euro Surveill** 22(33):pii: 30598.
   b. Okada P, Buathong R, Phuygun S, Thanadachakul T, Parnmen S, Wongboot W, Waicharoen S, **Wacharapluesadee S**, Uttayamakul S, Vachiraphan A, Chittaganpitch M, Mekha N, Janejai N, Iamsirithaworn S, Lee RT, Maurer-Stroh S. Early transmission patterns of coronavirus disease 2019 (COVID-19) in travellers from Wuhan to Thailand, January 2020. **Euro Surveill**. 2020 Feb;25(8):2000097. doi: 10.2807/1560-7917.ES.2020.25.8.2000097. PMID: 32127124; PMCID: PMC7055038.
   c. 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 Feb 12;16(2):e0246864. doi: 10.1371/journal.pone.0246864. PMID: 33577615; PMCID: PMC7880427.
   d. Sangkakam A, Hemachudha P, Saraya AW, Thaweethee-Sukjai B, Cheun-Arom T, Latinne A, Olival KJ, **Wacharapluesadee S**. Detection of influenza virus in rectal swabs of patients admitted in hospital for febrile illnesses in Thailand. **SAGE Open Med**. 2021 Jan 22;9:2050312121989631. doi: 10.1177/2050312121989631. PMID: 33552519; PMCID: PMC7841862.

3. **Research on diagnostic development.** The molecular technique is a gold standard method for pathogen diagnosis and confirmation. The first COVID-19 case outside China in Thailand was primarily detected by family PCR and further confirmed by next-generation sequencing (NGS). The SARS-CoV-2 variants can be identified by the NGS technique or by identifying the mutation markers by MassARRAY technology.
   a. **Wacharapluesadee S**, Kaewpom T, Ampoot W, Ghai S, Khamhang W, Worachotsueptrakun K, Wanthong P, Nopvichai C, Supharatpariyakorn T, Putcharoen O, Paitoonpong L, Suwanpimolkul G, Jantarabenjakul W, Hemachudha P, Krichphiphat A, Buathong R, Plipat T, Hemachudha T. Evaluating the efficiency of specimen pooling for PCR-based detection of COVID-19. **J Med Virol**. 2020 Oct;92(10):2193-2199. doi: 10.1002/jmv.26005. Epub 2020 Jul 21. PMID: 32401343; PMCID: PMC7272832.
   b. **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 R. A, Fernandez S, Putcharoen O. Simultaneous Detection of Omicron and Other SARS-CoV-2 Variants by Multiplex PCR MassARRAY Technology. **Preprint Research** 2023. DOI: https://doi.org/10.21203/rs.3.rs-2482226/v1

c. Joyjinda Y, Rodpan A, Chartpituck P, Suthum K, Yaemsakul S, Cheun-Arom T, Bunprakob S, Olival KJ, Stokes MM, Hemachudha T, **Wacharapluesadee S**. First Complete Genome Sequence of Human Coronavirus HKU1 from a Nonill Bat Guano Miner in Thailand. **Microbiol Resour Announc**. 2019 Feb 7;8(6):e01457-18. doi: 10.1128/MRA.01457-18. PMID: 30746519; PMCID: PMC6368654.

d. Siriyasatien P, **Wacharapluesadee S**, Kraivichian K, Suwanbamrung C, Sutthanont N, Cantos-Barreda A, Phumee A. Development and evaluation of a visible reverse transcription-loop-mediated isothermal amplification (RT-LAMP) for the detection of Asian lineage ZIKV in field-caught mosquitoes. **Acta Trop**. 2022 Dec;236:106691. doi: 10.1016/j.actatropica.2022.106691. Epub 2022 Sep 11. PMID: 36103950.

4. **Research on coronavirus prevalence in Thailand.** Numerous high-impact emerging viruses appear to have bat reservoirs. Our surveillance projects study the diversity of coronavirus (CoV) in bats in Thailand. We have isolated and characterized CoVs from many bat species and detected and sequenced CoV in bat guano collectors. Our surveillance studies continue to analyze the drivers of their emergence and risk factors for spillover.
   a. **Wacharapluesadee S**, Duengkae P, Chaiyes A, Kaewpom T, Rodpan A, Yingsakmongkon S, Petcharat S, Phengsakul P, Maneeorn P, Hemachudha T (2019). Longitudinal study of age-specific pattern of coronavirus infection in Lyle's flying fox (*Pteropus lylei*) in Thailand. **Virol J** 20;15(1):38.
   b. **Wacharapluesadee S**, Duengkae P, Rodparn A, Kaewpom T, Maneeorn P, Kanchanasaka B, Yingsakmongkon S, Sittidetboripat N, Chareesaen C, Khlangsap N, Pidthong A, Leadprathom K, Ghai S, Epstein JH, Daszak P, Olival KJ, Blair PJ, Callahan MV and Hemachudha T (2015). Diversity of Coronavirus in Bats from Eastern Thailand. **Virol J** 12(1):57.
   c. **Wacharapluesadee S**, Sintunawa C, Kaewpom T, Khongnomnan K, Olival KJ, Epstein JH, Rodpan A, Sangsri P, Intarut N, Chindamporn A, Suksawa K, Hemachudha T (2013). Group C betacoronavirus in bat guano fertilizer, Thailand. **Emerg Infect Dis** 19(8).

5. **Research on Nipah virus prevalence in Thai bats.** Nipah virus outbreaks, previously in Thailand's neighboring country, Malaysia, and ongoing in Bangladesh, have high mortality rates. Our surveillance projects study the characterization of Nipah Virus (NiV) in bats in Thailand. In addition, our surveillance studies continue to analyze the drivers of their emergence, understanding their seasonal preferences and risk factors for spillover.
   a. **Wacharapluesadee S**, Samseeneam P, Phermpool M, Kaewpom T, Rodpan A, Maneeorn P, Srongmongkol P, Kanchanasaka B, Hemachudha T (2016). Molecular characterization of Nipah virus from Pteropus hypomelanus in Southern Thailand. **Virol J** 13(1):53
   b. **Wacharapluesadee S**, Jittmittraphap A, Yingsakmongkon S, and Hemachudha T (2016). Molecular Detection of Animal Viral Pathogens. Nipah Virus. **CRC Press**.
   c. **Wacharapluesadee S**, Ngamprasertwong T, Kaewpom T, Kattong P, Rodpana A, Wanghongsa S, Hemachudha T (2013). Genetic characterization of Nipah virus from Thai fruit bats (*Pteropus lylei*). **Asian Biomedicine** 7(6);813-819.
   d. 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 Jun;19(2):175-189. doi: 10.1007/s10393-022-01588-6. Epub 2022 Jun 3. PMID: 35657574.
   e. Breed AC, Meers J, Sendow I, Bossart KN, Barr JA, Smith I, **Wacharapluesadee S**, Wang L, Field HE (2013). The Distribution of Henipaviruses in Southeast Asia and Australasia: Is Wallace's Line a Barrier to Nipah Virus? **PLoS One** 8(4):e61316.

6. **Rabies Neuropathogenesis, diagnosis and management.** The center worked for many years on molecular analyses of rabies, including mutational effects and designing primers to detect the Thai street rabies virus. I regularly organize workshops to teach laboratories in the region how to correctly collect specimens and test for rabies.
    a. Hemachudha T, Ugolini G, Sungkarat W, Laothamatas J, Shuangshoti S, **Wacharapluesadee S** (2013). Human Rabies: neuropathogenesis, diagnosis and management. **Lancet Neurology** 498-513.
    b. Shuangshoti S, Thepa N, Phukpattaranont P, Jittmittraphap A, Intarut N, Tepsumethanon V, **Wacharapluesadee S**, Thorner PS, Hemachudha T (2013). Reduced viral burden in paralytic compared to furious canine rabies is associated with prominent inflammation at the brainstem level. **BMC Vet Res** 14;9(1):31.
    c. Virojanapirom P, Khawplod P, Sawangvaree A, **Wacharapluesadee S**, Hemachudha T, Yamada K, Morimoto K, Nishizono A (2012). Molecular analysis of the mutational effects of Thai street rabies virus with increased virulence in mice after passages in the BHK cell line. **Arch Virol** 157(11):2201-5.
    d. Wilde H, Hemachudha T, **Wacharapluesadee S**, Lumlertdacha B, Tepsumethanon V (2013). Rabies in Asia: The Classical Zoonosis. **Curr Top Microbiol Immunol** 365:185-203.

Event	No./Specimen type				
Bat	**BPN**	**BPS**	**BLV**	**BLP**	**BSN**
NIH_Bats_Ratchaburi-Wat Khao Chong Pran-B-2020SEP11_B20738-837	100	-	-	-	-
NIH_Bats_Ratchaburi-Wat Khao Chong Pran-B-2021Mar26_B21001-101	-	90	-	-	-
NIH_NRCT_Bats_Ratchaburi-Wat Khao Chong Pran-B-2021Oct15_B21102-301	192	-	103	-	-
NIH_Bats_Chachoengsao-Khao Ang Rue Nai-B-2022Jan28-B22001-53-B22103-153	104	-	29	-	-
NIH_Bats_Chachoengsao-Wang Nam Yen Botanical Gardens-B-2022Jan29-B22054-22102	49	-	31	-	-
Rodent					
NIH_Rodents_Ratchaburi-Wat Khao Chong Pran Non-hunting Area-2021Sep5-R21001-106	92	-	33	-	-
Macaques					
NIH_Macaques_Ratchaburi-Wat Tham Nam-P-2021Mar31_P21001-100	100	-	100	-	-
NIH_Macaques_Phetchaburi-Bang Tabun-P-2022Apr21_P22001-100	-	-	-	-	99
Total	637	90	296		99

BPN	Blood plasma no medium
BPS	Blood plasma in normal saline
BLV	Whole blood in VTM
BLP	Whole blood in PBS
BSN	Blood serum no medium

Event	No./Specimen type				
DB-Bat	**BPN**	**BPS**	**BLV**	**BLP**	**BSN**
DNP-Bats-Phetchabun-Wang Pong-Khao Ta No-2020Jun22_B20301-439 (B20301-400)	-	-	-	100	-
DNP-Bats-Phetchabun-Wang Pong-Pha Cong Cave-2020Jun24_B20440-542 (B20440-539)	-	-	-	100	-
DNP-Bats-Phetchaburi-Khao-Nang-Phanthurat-2020Oct02-B20938-1077	134	-	-	-	-
DNP-Bats-Pathum Thani-Slumberland-2020Nov06_B201078-1177	92	-	-	-	-
DNP_Bats_Ratchaburi-Wat Khao Chong Pran-B-2022Aug19_B22232-331	91	-	50	-	-
DC-Natural wildlife					
DNP-Natural wildlife-DC20001-20307	4	-	-	-	148
DWL					
DWL-DWL22001-22161	-	-	-	-	41
Total	321		50	200	189

Event	
Bat	**BPN**
TH-Loei-Phu Luang-B-2018Jan19_B18001-116	99
TH-Chonburi-Wat Luang Temple-B-2018Feb16_B18117-246	119
TH-Chonburi-Wat Luang Temple-B-2018May18_B18322-421	100
TH-Ratchaburi-Wat Khao Chong Pran-B-2018Jun08_B18541-590	45
TH-Loei-Pha Ya Cave-B-2018Jul06_B18591-690	99
TH-Chiangmai-B-2019Jan13_B19001-042	11
TH-Ratchaburi-Wat Khao Chong Pran-B-2019Jun14_B19043-142	97
TH-Chonburi-Wat Khao Cha-ang-B-2019Jul24_B19143-242	98
TH-RatChaburi-Wat Khao Chong Pran-B-2019Aug16_B19257-336	79
TH-Chonburi-Wat Khao Cha-ang-B-2019Oct17_B19402-501	100
TH-Chantaburi-Khao Soi Dao-B-2019Nov08_B19514-713	181
TH-Chantaburi-Khao Soi Dao-B-2020Jun11_B20001-20200	198
TH-Chachoengsao-Khao Ang Rue Nai -B-2020Jun19_B20201-300	98
TH-Chachoengsao-Khao Ang Rue Nai -B-2020Jul22_B20543-640	96
TH-Ratchaburi-Wat Khao Chong Pran-B-2020Sep11_B20838-937	100
TH-Phetchaburi-Khao Nang Phanthurat-B-2020Nov27_B201178-1277	94
TH-Ratchaburi-Wat Khao Chong Pran-B-2020Dec11_B201278-1377	100
Total	1714

No./Specimen type			
BPS	**BLV**	**BLP**	**BSN**
-	-	-	-
-	-	-	-
-	-	-	-
-	-	-	-
-	-	-	-
-	35	-	-
-	-	-	-
-	-	-	-
-	-	-	-
-	-	-	-
-	-	-	-
-	-	-	-
-	-	-	-
-	-	-	-
-	-	-	-
-	-	-	-
-	-	-	-
	35		

	EID-SEARCH		
	Archived specimens		Future plan
	Y2	**Y3**	**Y4**
Community	56	50	50
Hospital/Clinic	3	100	100
Total	59	150	150

	PREDICT_Ratchaburi		PREDICT_Chonburi		
	Archived specimens				
Y5	**2017**	**2018**	**2017**	**2018**	
50	113	115	115	128	
100					
150	113	115	115	128	980

The King Chulalongkorn Memorial Hospital and Faculty of Medicine, Thailand

Facilities and Equipment

The Thai Red Cross Emerging Infectious Diseases Clinical Center (EIDCC) has 100 square meters of office space, 200 square meters of molecular and serology lab spaces, and > 3,000 square meters of shared lab space with the faculty of Medicine (Chula Medical Research Center, MRC). There is twenty staff including 6 clinicians, 8 scientists, 1 bioinformatician 1 research nurse, and 4 admin staff. EIDCC lab supports molecular and serology diagnostics to the hospital and government partners, including Department of Diseases Control (DDC) and Department of National Parks, Wildlife, and Plant Conservation (DNP). EIDCC conducts research projects granted by the Thai government and international partners. The lab lead, Dr. Wacharapluesadee, has experience in emerging infectious zoonotic surveillance, especially from bats, for over 20 years. The research network includes the DDC, Faculty of the Veterinary Science, Chulalongkorn University and Faculty of Forestry, Kasetsart University.

The Chula MRC laboratory is equipped for virology, cell culture, serology, and molecular biology. There is one BSL-3 lab facility and 3 cell culture lab spaces. In addition, MRC has 8 PCR machines, 5 real-time PCR machines, 2 gel document imaging, 3 horizontal electrophoresis, 3 UV-visible spectrophotometers, 3 ultra-centrifuge, 2 high-speed centrifuge machines, 3 refrigerated centrifuge, 2 refrigerated incubator shakers, 5 autoclaves, 2 hot air oven, 6 upright microscopes, 3 inverted microscopes, 2 inverted microscopes with fluorescence, 9 CO_2 incubators, 5 Biosafety cabinets, freeze dryer, speed vacuum concentrator, extraction machine, ImmunoSpot ultimate analyzer, flow cytometer, Bio-plex multiplex suspension array system, microplate reader and washer, droplet digital PCR, automated electrophoresis system, dual fluorescence cell counter, NanoSight, florescence and phosphor imager, Chemiluminescence western blot scanner, CO_2 incubator with shaker, Automate Electrophoresis System, Automated Nucleic Acid and Protein Extraction System, Fluorescent Table and DNA analyzer.

The EIDCC lab is equipped for serology, molecular biology, and next-generation sequencing. There are 3 PCR machines, 4 deep Freezers, 3 freezers, 3 refrigerators, 4 Biosafety cabinets, 2 micro-centrifuge, 2 refrigerated centrifuges, 3 real-time PCR machines, automated gel electrophoresis, Next-generation sequencer (MiSeq system), NGS library preparation system, and MinION (Oxford Nanopore Technologies). The specimen repository system uses the “Pathogen Asset Control System, PACS” software supported by US DTRA. There are 4 PACS computers. The computer server of the faculty is available to access and use for genomic analysis.

From:	Laing, Eric on behalf of Laing, Eric <eric.laing@usuhs.edu>
To:	Noam Ross
Subject:	Fwd: New NIH proposal on serology methods
Date:	Wednesday, January 25, 2023 10:12:31 PM
Attachments:	Biosketch_Dr. Supaporn Wacharapluesadee_NIH 2023_23-Jan-23.docx Blood specimens_animal-human_25-Jan-23.xlsx EIDCC facilities and equipment_26-Jan-23.docx

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see attachments in the forwarded email

Eric D. Laing, Ph.D.
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---------- Forwarded message ---------
From: **Sasiprapa Ninwattana** <sasiprapa.n@outlook.com>
Date: Wed, Jan 25, 2023 at 10:08 PM
Subject: Re: New NIH proposal on serology methods
To: Laing, Eric <eric.laing@usuhs.edu>
Cc: Spencer Sterling <spencer.sterling.ctr@usuhs.edu>, Supaporn Wacharapluesadee <spwa@hotmail.com>, Hongying Li <li@ecohealthalliance.org>, Khwankamon Rattanatumhi <khwankamon.r@gmail.com>, Krongkan Srimuang <krongkan5678@gmail.com>

Dear Eric,

Attached please find the NIH-biosketch of Dr. Supaporn, the facilities and equipment, and the excel file containing the number of animal and human sera samples we have at our EIDCC lab.

We would be grateful if you could send us a draft of the letter of support for Dr. Supaporn to sign.

Let me know if you require any additional information or if there is anything I can help with.

Best regards,
Bow

Sasiprapa Ninwattana (she/her)
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 11 Jan 2023, at 21:44, Hongying Li <li@ecohealthalliance.org> wrote:

Hi Bow and Spencer,

I have sent the following info and attachments to Noam before, but just resending for your reference.

Attached please find the latest versions of relevant materials I have:

- Supapron's NIH-biosketch - this needs to be updated with the new format (no section D) https://grants.nih.gov/grants/forms/biosketch.htm
- Facilities and equipment
- Other support - this need to be updated with the new format and other grants https://grants.nih.gov/grants/forms/othersupport.htm - *but you will only need this for the Just-In-Time procedure,* ***not*** *now for proposal submission*

Other things you need from Chula are:

- Budget
- Budget justification

And if you need to fill in IRB approval information to use human samples under EID-SEARCH (on the cover page):

- US: Health Medical Lab Institutional Review Board (No. 894ECOH21b) on May 12, 2021
- Thailand: Institute Review Board of the Faculty of Medicine, Chulalongkorn University (No. 211/64) on June 8, 2021

Let me know if anything else I can help with.

Best,
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

---------- Forwarded message ---------
From: **Laing, Eric** <eric.laing@usuhs.edu>
Date: Tue, Jan 10, 2023 at 4:54 PM
Subject: Re: New NIH proposal on serology methods
To: Supaporn Wacharapluesadee <spwa@hotmail.com>
Cc: Noam Ross <ross@ecohealthalliance.org>, Hongying Li <li@ecohealthalliance.org>, Peter Daszak <daszak@ecohealthalliance.org>, Laing, Eric <eric.laing.ctr@usuhs.edu>, Kevin Olival <olival@ecohealthalliance.org>, Sterling, Spencer <spencer.sterling.ctr@usuhs.edu>, Opass ID <opassid@gmail.com>

Hi Chu,

My lab is working on expanding our filo/henipa panel to a 28-plex, most of which should be available for further testing under EID-SEARCH. If this proposal is funded Noam and I would like to make sure that there are additional funds in this R01 proposal to support retesting in your lab.

Which groups of the samples (host species and project name) do you plan to test? Now I have moved to work at the new lab, and it isn't easy to access the old samples (before 2020). But it is possible. For humans and wildlife, how many samples/species have you collected now and what is your projected target for the remainder of the EID-SEARCH POP? What would be really helpful would be a cost estimate for retesting all those samples under this R01 with the optimized in vitro and in silico serologic approaches.

We'd like to list you as a Co-Investigator, which means we'd need a biosketch, LOS, equipment/facilities pages, and that additional budget that we would include in YRS4-5 of this proposal to supplement additional testing. Spencer can be involved, just let me know whether salary support is coming from my end as HJF employee or whether he'd be listed as key personnel in your lab directly.

Best regards,
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 Thu, Jan 5, 2023 at 5:51 AM Supaporn Wacharapluesadee <spwa@hotmail.com> wrote:

> Dear Noam and Eric,
> Thank you for your email and invitation to collaborate on the new proposal. I would love to join and have Spencer be responsible for lab work if it is funded. However, I would like to clarify objective 3 on retesting the archived samples. **Which groups of the samples (host species and project name) do you plan to test?** Now I have moved to work at the new lab, and it isn't easy to access the old samples (before 2020). But it is possible.
>
> Best,
> Supaporn
>
> *Supaporn Wacharapluesadee, PhD*
> Thai Red Cross Emerging Infectious Diseases Clinical Center
> King Chulalongkorn Memorial Hospital
> Rama4 road, Patumwan, Bangkok, Thailand 10330
> Skype ID: supapornwa
>
> ---
>
> **From:** Noam Ross <ross@ecohealthalliance.org>
> **Sent:** Thursday, January 5, 2023 4:48 AM
> **To:** S Wacharapluesadee <spwa@hotmail.com>
> **Cc:** Hongying Li <li@ecohealthalliance.org>; Peter Daszak <daszak@ecohealthalliance.org>; Laing, Eric <eric.laing.ctr@usuhs.edu>; Kevin Olival <olival@ecohealthalliance.org>
> **Subject:** New NIH proposal on serology methods
>
> Dear Supaporn,
>
> Happy New Yea! I hope this finds you well. I'm writing because I am putting together a new NIH grant proposal at EcoHealth together with Eric Laing at USU that we wanted to include you in. The aim of the grant is to improve both lab and statistical methods for luminex serology. The main elements of the project would be to:
>
> 1) Develop statistical methods to simultaneously analyze multiplex serology to

quantify the probability that signals from multiple beads represent antibodies from exposures to different viruses, cross-reactions of viruses in the multiplex panel, or signals of new, previously unknown viruses.
2) Develop discovery-optimized panels for filoviruses and henipaviruses that mix high-specificity and high-sensitivity beads so as to maximize the ability to identify signals of novel viruses.
3) Re-test and re-anlayze results from samples from previous serology surveillance studies that have ambiguous results to identify signals of exposure to previously unknown viruses, and conduct antigenic mapping to estimate where they fall phenotypically in relation to known viruses.

We anticipate sharing all our work across partners over the course of the project (if/when funded). However, we specifically hope to collaborate with you on #3 above. We'd like to use this to look deeper at EID-SEARCH results and ideally retest samples (such as the Mojiang-seropositive samples) to see what the new panels yield. For our proposed project, some of the new panels will be developed while EID-SEARCH is still running. If this new project is funded, we will work with you to determine what should be done concurrently with EID-SEARCH or after EID-SEARCH is completed and the best way to budget this (mind you, EcoHealth also hopes to get a continuation on our EID-SEARCH project so it is likely both will be concurrent). Assuming the best case, we submit this in February (Due date Feb 3, 2023) and start work in Fall 2023. Depending on how we stage our tasks, re-testing work could start as early as late 2024 or 2025.

One additional note – this project would be subject to new data sharing requirements NIH has enacted for projects starting 2023 or later. In short, all data we generate out of the project would be published at the end of the project period, whether or not publications are complete. We don't anticipate this would be an issue but want to make sure everyone is aware of this from the start of the grant so we can plan data and manuscript publication accordingly.

We'd like to have you as Key Personnel on this proposal. If you are interested, we'd love to have you aboard and get your thoughts and questions. Eric and I will also follow up next week with a proposal budget. We would also need an NIH Biosketch, Other Support form, and Facilities information from you by January 20. We have recent versions of all of these for EID-SEARCH, so we can send templates for you to modify and approve early next week.

Please let us know what you think, and we'd be happy to have a call to discuss.

Best,

Noam
--

Dr. Noam Ross
Principal Scientist, Computational Research

EcoHealth Alliance

520 Eighth Avenue, Suite 1200
New York, NY 10018
www.ecohealthalliance.org

+1.212.380.4471 (direct)
+1.212.380.4465 (fax)
@noamross@ecoevo.social (fediverse)
noamross.net

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

<2022 EIDCC facilities and equipment_TRC EIDCC.docx><Biosketch_(Wacharapluesadee,Supaporn)_EIDRC_RFA-AI_19-028_(PI-Daszak)_v02.docx>

Pilot Program Application Submission Checklist – Final Application Submission
Title of Application: Click or tap here to enter text.
Submitted by: Click or tap here to enter text.
Submission Date: Click or tap to enter a date.

Full Application Submission Requirements

- ☒ Proposal Cover Sheet
- ☒ Title and Table of Contents
- ☒ Study Personnel *(1-page limit)*
- Main Application Body Section Requirements *(7-page limit):*
  - ☒ Research Aims & Objectives
  - ☒ Study Rationale/Research Gap/Impact
  - ☒ Significance and Approach
  - ☐ Research Methods
- ☐ Project Timeline (*1-page limit*)
- ☒ Research Performance Sites *(1-page limit)*
- ☒ CREID Research Center Collaboration *(1-page limit)*
- ☒ Mentoring Plan *(2-page limit)*
- Vertebrate Animals Section Requirements *(3-page limit):*
  - ☐ Description of Procedures
  - ☐ Justifications
  - ☐ Minimization of Pain and Distress
  - ☐ Method of Euthanasia (Cover Page Supplement / PHS Fellowship Supplemental Form)
- Human Subjects Research *(3-page limit):*
  - ☐ Summary of the parent study and IRB approval information for the study
  - ☐ Risks to the subjects
  - ☐ Adequacy of protection against these risks
  - ☐ Potential benefits of the research to the subjects and others
  - ☐ Importance of the knowledge gained or to be gained
  - ☐ Country / institution-specific ethics / IRB regulations addressed
- Research, Related Project Information, and Budget/Budget Justification
  - ☐ R&R Other Project Information Form
  - ☐ Full budget, with total costs of no more than $150,000
  - ☐ Budget justification which describes the labor and other direct costs
  - ☐ If your institution does not have adequate funds for a cost-reimbursement award and requires pre-payment of funds during the award year, please note this in your budget justification and outline a payment schedule that will function for your project.
- Supporting Documentation
  - Biographical Sketch and Other Support. All applications must include:
    - ☒ Applicant PI Biographical Sketch (*4-page limit*)
    - ☒ Applicant PI Previous/Current/Pending Support (Include funding amounts, *no page limit*)
    - ☐ Mentor Biographical Sketch (*4-page limit*)
    - ☐ Mentor Current/Pending Support (*no page limit*)
    - ☐ Key Personnel Biographical Sketches (*4-page limit each*)
    - ☐ Key Personnel Current/Pending Support (*no page limit*)
  - Co-PI Plan (only needed if applying as Co-PIs) (*1-page limit*)

Commented [el1]: How should we do about the budget?

Commented [el2]: Dear Eric, this is your part.

Commented [el3]: Dear Spencer, this is your part.

Pilot Program Application Submission Checklist – Final Application Submission
Title of Application: Click or tap here to enter text.
Submitted by: Click or tap here to enter text.
Submission Date: Click or tap to enter a date.

- ☐ What each Co-PI will contribute to the proposed research study
- ☐ How the Co-PIs will jointly work with the affiliated Research Center
- ☐ How the Co-PIs will jointly manage the proposed study

☒ References Cited (*no page limit*)
☒ List of Abbreviations, Acronyms, and Symbols
☒ Facilities, Existing Equipment, and Other Resources (*template provided*)
☐ NIH Foreign Clearance form (*template provided*)
☐ Letters of Organizational Support *(2-page limit per letter)*
☐ Letter of Collaboration from CREID Research Center PI *(2-page limit per letter)*
☐ Letter from Research Center Mentor (*2-page limit)*
☐ Letter from Primary Scientific Mentor (if different than Research Center mentor) (*2-page limit)*

Commented [el4]: Dear Eric, this is your part.

Proposal Cover Sheet

Project Title	Immune memory bait & capture to identify emerging henipavirus origins
Principal Investigator Name	Krongkan Srimuang, Ph.D.
Position/Title	Research Scientist
Department	Thai Red Cross Emerging Infectious Diseases Clinical Center
Institution Name	King Chulalongkorn Memorial Hospital
Street	Rama 4 Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	Krongkan.sr@gmail.com
Phone	+66 (0) 92-453-0808
Country(ies) where work will be conducted	Thailand
Pathogen(s) focus	Mojiang Virus, Henipavirus, Paramyxovirus
Co-Principal Investigator Name (*If applicable*)	Spencer Sterling, MPH
Position/Title	Scientific Project Coordinator
Department	Microbiology and Immunology
Institution Name	Uniformed Services University of the Health Sciences
Street	Jones Bridge Road
City, State, Zip Code	Bethesda, MD 20814
Country	USA
Email	spencer.sterling.ctr@usuhs.edu
Phone	+66 (0) 83-494-5980

Contact information for institution(s) that would financially manage the award *(please note: changing the managing institution upon award may result in substantial funding delays)*	
Contractual Contact, Title	Chula Unisearch
Institution Name	Chulalongkorn University
Street	254 Phyathai Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	unisearch@chula.ac.th
Phone	0 2218 2880
For Co-Principal Investigator (If applicable):	
Contractual Contact, Title	
Institution Name	
Street	
City, State, Zip Code	
Country	
Email	
Phone	

Name of Mentor	Eric Laing, Ph.D.
Mentor Institution	Uniformed Services University of the Health Sciences
Institution Address	Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814
Permanent location of Mentor	4301 Jones Bridge Road, Bethesda, MD 20814
Mentor email	eric.laing@usuhs.edu
Mentor phone	+1 (301) 980-8192
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Name of Mentor	Supaporn Wacharapluesadee, Ph.D.
Mentor Institution	Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital
Institution Address	1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Permanent location of Mentor	4th Floor, Jongkolnee Watwong Building, Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital, 1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Mentor email	spwa@hotmail.com
Mentor phone	+66 (0) 89-920-6292
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Total Budget	***Repeat this table if there are two Co-PIs***
Direct Costs	
Indirect Costs	
Proposed Start Date	
Proposed End Date	

Project Abstract (250 words)

Prototypical henipaviruses (Hendra and Nipah virus) cause zoonotic diseases with high mortality and no effective vaccines or therapeutics and are hosted by Pteropus flying foxes. The recent detection of closely-related Mojiang and Langya viruses in rats and shrews, and an outbreak of acute febrile LayV illness in people, challenges the dogma that fruit bats are the key henipavirus reservoirs, and heightens their threat profile. The recently expanded henipavirus includes two other shrew-borne viruses (Gamak and Daeryong viruses), and the Madagascan fruit bat-borne Angovkeely virus. In this study, we have found serological evidence of a Mojiang-related virus in a community of Thai bat guano collectors with high exposure to microbats and rodents. Identifying the wildlife reservoir of this likely novel virus will assist intervention strategies to reduce spillover risk. However, PCR detection or virus isolation in wildlife is challenging, and new serological approaches may be more successful in identifying reservoir hosts. This project aims to study zoonotic reservoirs for pathogen origin by using serologic and cellular

immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic. We plan to identify seropositive human participants MojV-like virus-specific MAbs will be generated and used for direct antibody-mediated virus capture in people and wildlife. We will develop anti-RBP standards to explore the antigenic spatial relationships between all known rodent- and bat-borne HPVs, and the Thai MojV-like virus these will allow the serologic signature of related undiscovered henipaviruses to be identified.

Study Personnel ***(1-page limit)***

Krongkan Srimuang, Ph.D., is a researcher at the Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital and supports the Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of Southeast Asia (EID-SEARCH) research. Dr. Srimuang is also a medical technologist working to identify, detect and characterize the spill-over risk of high zoonotic potential viruses from wildlife and humans using serology techniques, Next-generation sequencing, and PCR assays to identify novel viruses. Dr. Srimuang also performs the project Smart Sentinel Surveillance System: Emerging Viral Pneumonia and Respiratory tract infections (RTIs) to identify, detect and characterize the novel/known Respiratory Viruses in our country, Thailand by using multiplex real-time PCR, family PCR and Next-generation sequencing of novel viral pathogens. Dr. Srimuang graduated with Ph.D. in Tropical Medicine from the department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University.

Spencer Sterling, MPH, is currently employed by the Henry M. Jackson Foundation for the Advancement of Medical Research (MD, USA), supports research activities for the Laing Lab at the Uniformed Services University, and is a visiting scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University. Mr. Sterling is supporting the serological investigations of the EID-SEARCH project, as well as other projects in the Indo-Pacific region. Mr. Sterling will planning, sample processing, data analysis, and report preparation for this project.

Commented [el1]: For Spencer

Dr. Eric Laing will act as Mentor for Dr. Krongkan and Spencer under the Centers for Research in Emerging Infectious Diseases (CREID) Pilot Research Program.

Commented [el2]: For Eric

Dr. Supaporn Wacharapluesadee, Ph.D. is the head laboratory at the Thai Red Cross Emerging Infectious Diseases Clinical Centre (TRC-EIDCC), King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, a WHO Collaborating Centre for Research and Training on Viral Zoonoses. Dr. Wacharapluesadee research interests include emerging infectious diseases in bats (including Nipah, Rabies, Coronavirus, and novel pathogens) as well as molecular diagnoses and sequencing of viruses. During the past 10 years, Dr. Wacharapluesadee got funding from USAID PREDICT to conduct the surveillance for novel virus in wildlife and human, and from Biological Threat Reduction Program US DTRA to conduct the EID surveillance in bat using the molecular technology and multiplex serology panel (Luminex technology). TRC-EIDCC is responsible for molecular diagnoses services to the hospital, and is the reference laboratory for rabies virus, MERS-CoV, Ebola, Zika, and other infectious diseases diagnoses for Ministry of Public Health, Thailand and laboratory training center on national and regional level. Dr. Wacharapluesadee has served and consulted on several WHO and Thai government committees.

Research Performance Sites *(1-page limit)*

This proposed study will be implemented at the King Chulalongkorn Memorial Hospital (KCMH) collaborating with the Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC). KCMH is a 1,000-bed, university hospital in close coordination with the Faculty of Medicine, Chulalongkorn University. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. It's one of the center hospitals for Emerging Infectious Diseases in Thailand, especially for domestic and international patients.

TRC-EIDCC was established by the Committee of the Emerging Infectious Disease Center, which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. Our clinical center provides standard medical care to patients with emerging and recurrent diseases and disease transmission. Moreover, the center plans preparedness activities, trains medical and government staff on interventions, hosts conferences for medical and public health professionals, creates networks of medical care for patients with emerging and recurrent diseases, and leads research and development projects for medical care and disease control. Apart from medical innovation in emerging and recurrent diseases, the clinical center strives to be the model of infectious diseases clinical services and pandemic control through research activities surrounding infectious diseases and effective disease control measures.

We also collaborate with the Department of National Parks, Wildlife and Plant Conservation for detecting novel pathogens from animals such as bats, rodents, and shrews in our country.

We also collaborate with the Department of Disease Control of Thailand (Thai-DDC) to support the Thai government and national policy makers through molecular diagnosis, including the identification of the first case of SARS-CoV-2 infection in Thailand. TRC-EIDCC prides itself on its high performance in research laboratory services and is a part of the national reference laboratory for COVID-19 and other emerging infectious diseases confirmation in Thailand.

CREID Research Center Collaboration *(1-page limit)*

The goals and objectives of the proposed research project, "Immune memory bait & capture to identify emerging henipavirus origins", are fully consistent with the specific aims of EID-SEARCH. EID-SEARCH takes a One Health approach to understanding zoonotic disease emergence, with a focus on viral diversity in wildlife at high-risk human animal interfaces. EID-SEARCH aims to understand the frequency and causes of virus spillover in high-risk communities across Southeast Asia and identify strategies and tools to reduce this risk. As the proposed research focuses on the links between farming practices, wildlife diversity, virus diversity, and cross-species transmission in Thailand, we expect the results of the project to make a significant contribution towards the overall goals of EID-SEARCH.

The proposed project is well-designed, leverages cutting-edge technology, and includes a robust ecological component that corresponds to the overall research strategy of EID-SEARCH. The EID-SEARCH team is highly motivated to support the application of metagenomics to ecological questions and assist in the analysis of the unique dataset that will be generated from this research. With the heightened focus on identifying and limiting risks associated with the complex interactions between humans, wildlife, and domestic mammals that has resulted from the emergence of SARS-CoV-2 from a wet market in China, the proposed research is both timely and likely to yield highly-cited, impactful publications for EID-SEARCH and Thailand partners.

The EID-SEARCH team includes a number of experts in study design, laboratory-based diagnostics, genetic and genomic analyses, and ecological modelling who will contribute to project implementation, data analysis, and manuscript preparation as required. Communication and coordination mechanisms will be set up between the Thailand research team and scientists at the EID-SEARCH research sites for resource sharing, training, knowledge exchange, and mentorship. In particular, EID-SEARCH will facilitate and encourage relationships between our partners in Southeast Asia (based in Malaysia, Singapore, and Thailand) that will facilitate Thailand team professional development and strengthen research capacity in the region. Dr. Eric Laing, the project Mentor, has an established relationship with both Dr. Krongkan and Spencer Sterling, and will act as the liaison between EID-SEARCH and the TRC-EIDCC research team. An expected outcome of this research project will be pilot data to support a joint funding application in the future.

Mentoring Plan *(2-page limit)*

1. Goals for Pilot study and Career

Krongan Srimuang: My career goal is to be an expert scientist in either an academic research laboratory or a lecturer in medical science. My long-term research interests include the understanding of priority emerging infectious diseases, genetics, and epigenetics using molecular and serologic techniques. Detection of novel pathogens in their wildlife vectors can help characterize the pathogenicity, virulence, and evolution of these and other closely related pathogens. I believe that this pilot study can provide training in new techniques for the detection, identification, and characterization of novel pathogens that are currently unavailable in Thailand. My goal for the Pilot study is to gain more knowledge and skills for a lifelong career in public health-related research. I wish to expand my skills in novel virus detection using next-generation serologic techniques such as PBMC studies and memory B-cell isolation and sequencing that can contribute to improved surveillance and research capacity at human-animal interfaces. My mentors Dr. Laing and Dr. Wacharapluesadee can provide me with opportunities to develop and apply project skills and improve my scientific writing.

My education has provided me with a strong foundation to expand from for this study. I received my undergraduate degree in Medical Technology from Chulalongkorn University which included training in molecular biology, serology, immunology, chemistry, virology, bacteriology, mycology, parasitology, hematology, clinical microscopy, transfusion medicine, and forensic medicine. My interest in molecular technology led me to pursue a Ph.D. at the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, which provided me with training in several molecular techniques including conventional PCR, real-time PCR, Next Generation Sequencing (NGS), etc. The emergence of COVID-19 motivated me to focus on emerging infectious diseases. I am currently completing my post-doctoral fellowship at the Emerging Infectious Diseases Clinical Center (EIDCC), King Chulalongkorn Memorial Hospital with Dr. Wacharpluesadee. Throughout this fellowship, I have continued learning advanced and innovative techniques to detect, identify, and characterize the novel pathogens. Through this pilot project, I will learn about the Peripheral Blood Mononuclear Cells (PBMC) which are a critical part of many subdivisions of medical research including Infectious diseases, Vaccine development, and Immunology. This project aims to study zoonotic reservoirs for by using serologic and cellular immune discovery in humans, and applying this immune knowledge in wildlife for early detection and identification of novel pathogens. This proposed study will expose me to various techniques and immunology topics that I useful in designing and testing new pipelines as technological advances continue to develop. This study will also allow me to develop, manage, and communicate my research.

Spencer Sterling: My overall career goal is to understand the factors that lead to viral zoonosis and to build epidemiological capacity in regions with the highest risk for zoonotic spillover. Through my training and educational experiences, I have learned laboratory and epidemiological techniques that can assist in the understanding of zoonosis. I wish to continue my education by expanding my repertoire of molecular techniques to include studies of the immune system's response to zoonotic disease in humans and animals, as well as to learn next-generation sequencing techniques. Additionally, I would like to use my experience in epidemiological techniques to train scientists in the Southeast Asian region in order to build local capacity in infectious disease research. Dr. Laing has been a mentor for me throughout my career, and this project would allow for this relationship to expand into new molecular areas, as well as grant writing and project management skills. Additionally, Dr. Wacharapluesadee has decades of experience in infectious disease research in the region and has

mentored scientists that have gone on to careers at the Thai Ministry of Public Health, Department of Disease Control, Department of Parks and Wildlife, academia, and the private sector. Both mentors have encouraged Dr. Srimuang and I to apply for this pilot study as well as pursue other funding mechanisms for our research interests. Both mentors will provide for me the ability to independently pursue my research interests in the zoonotic epidemiology and train me on the best practices of scientific communication.

Applicant Name (Last, first, middle):

Facilities and Resources

FACILITIES: Specify the facilities to be used for the conduct of the proposed research.

Laboratory:

The Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC-EIDCC), King Chulalongkorn Memorial Hospital (KCMH) was established by the Committee of the Emerging Infectious Disease Center which recognizes the importance of emerging and recurrent diseases, and emphasizes the importance of pandemic preparedness. The Center was approved to be a part of KCMH by the Thai Red Cross Society, being responsible for medical care in patients with emerging and recurrent diseases. KCMH is a public general and tertiary referral hospital in Bangkok, Thailand. It is one of the largest hospitals in Thailand, and as one of Thailand's leading medical school affiliates is widely considered one of the best public hospitals in the country. TRC-EIDCC laboratory facilities include 3 biosafety cabinets (BSL 2, BSL 2+, BSL2+), 3 automated extraction machines, 2 thermocyclers (Proflex), 2 real-time PCR machines (CFX, Bio-rad), 3 Freezers (-80 °C), 2 refrigerators; 4 °C, 2 autoclaves. Our EIDCC uses the Pathogen Asset Control System (PACS) system for sample tracking. Moreover, our Faculty of Medicine, Chulalongkorn University has a central laboratory, the Chula Medical Research Center (Chula MRC), which provide many instruments for medical, masters, and graduate students, Post-doctoral fellows, and researchers at Chulalongkorn University and KCMH. The Chula MRC laboratory facilities are divided into molecular diagnostics (PCR Thermal Cycler, QStudio6 Flex Real-Time PCR, Droplet Digital PCR, GeneXpert, Film array, Illumina MiSeq), immunology facilities (ELISA testing equipment (reader & washer), Luminex, Flow Cytometry), and a BSL3 laboratory. Moreover, the facility provides general equipment, such as autoclaves, balances, CO2 incubators, Dual Fluorescence Cell Counter, 4 °C refrigerators, -20°C, and -80°C freezers.

Clinical:

KCMH is a 1,000-bed university hospital, in close coordination with the Faculty of Medicine, Chulalongkorn University. The Thai Red Cross Society provides medical treatment, rehabilitation, health promotion and disease prevention services to the general public and the vulnerable. It also provides biopharmaceutical and sterile medicines that are sufficient to meet the needs of patients and safe in accordance with international standards. It serves as a center for providing eye and organ donation services to patients nationwide in line with international standards. It has specialized doctors and public health personnel and also produces specialized nurses. The TRC-EIDCC in the KCMH provides standard medical care to patients with emerging and recurrent diseases and disease spreading prevention.

Animal:

TRC-EIDCC also collaborates with the Department of National Parks, Wildlife and Plant Conservation for detecting the emerging novel pathogens from animals such as bats, rodents, and pangolins in our country. Bats are considered protected wildlife in Thailand by Wildlife Conservation, Department of National Parks, Wildlife and Plant Conservation.

Applicant Name (Last, first, middle):

Computer:

TRC-EIDCC used the service from the Office of Information Technology, Chulalongkorn University that provides the application, network, and software such as Office 365, VPN, WIFI, Microsoft Office, Microsoft Windows, SPSS, Adobe, Zoom Meeting, etc. Moreover, our EIDCC use the Pathogen Asset Control System (PACS) for sample tracking.

Office:

The office and laboratory space are located at the EIDCC at 4th Floor, Jongkolnee Watwong Building and 6th Floor, Aor Por Ror Building, respectively. The applicants will have dedicated desks in both buildings.

Other:

Specific Aims (1-page limit)

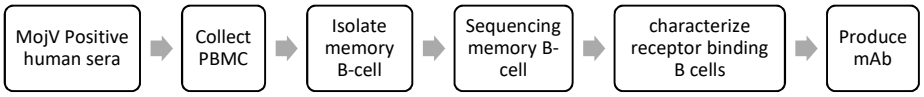
This project aims to study zoonotic reservoirs for pathogen origin by using serologic and cellular immune discovery in humans and wildlife applying to be used as a tool for early detection and identification of novel pathogens to prevent the next pandemic.

The hypothesis

1. Can the memory B-cell immune response be used as a tool for the discovery of novel henipaviruses?
2. Can the spatial relationships between henipaviruses receptor binding proteins be generated that would allow for an understanding of antigenic evolution and functional characterization.

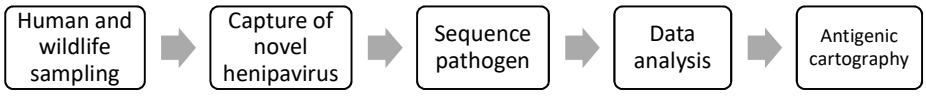
Aim 1. Antibody-mediated bait & capture of Thai MojV-like virus

In collaboration with NIH PREMISE, MojV RBP-reactive B cell receptors will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific MAbs will be generated and used for direct antibody-mediated virus capture in people and wildlife.



Aim 2. Modeling antigenic relationships among known and unknown henipaviruses.

Defining antigenic relationships among these will allow the serologic signature of related undiscovered henipaviruses to be identified. We will develop anti-RBP standards to explore the antigenic spatial relationships between all known rodent- and bat-borne HPVs, and the Thai MojV-like virus.



Objective 1. To isolate and sequence memory B-cells from humans positive for Mojiang virus in Ratchaburi province, Thailand for antibody production.

Objective 2. To capture unknown MojV-like viruses during syndromic surveillance in humans and routine surveillance in wildlife in Ratchaburi province, Thailand.

Objective 3: To generate antigenic maps using anti-henipavirus RBP standards.

Study Rationale/Research Gap/Impact (7-page limit)

The genus Henipavirus is currently composed of nine viruses including Hendra virus (HeV), Nipah virus (NiV), Cedar virus (CedV), Ghana virus (GhV), Mojiang virus (MojV) (McLinton et al., 2017), Langya virus (LayV) (Zhang X et al., 2022), Gamak virus (GAKV), Daeryong virus (DARV) (Lee et al., 2021), and Angavokely virus (AngV) (Madera et al., 2022).

HeV was first identified in Australia in 1994 as a cause of encephalitis in humans and horses. NiV was identified in 1999 in Malaysia as a cause of encephalitis in humans, with swine determined to be the intermediate host. Additionally, annual outbreaks of NiV have occurred in Bangladesh and are associated with the consumption of raw date palm sap. Both viruses cause severe diseases with high mortality and there are currently no vaccines or therapeutics (Middleton and Weingartl, 2012). CedV was identified in Australian bats in 2012 and found to be non-pathogenic, failing to cause any clinical symptoms in ferrets and guinea pig animal models (Marsh GA et al., 2012). GhV was detected in Ghana in straw-colored fruit bats (*Eidolon helvum*) during routine surveillance in 2015 and was the first henipavirus found outside of the Indo-Pacific region. GhV showed a close phylogenetic relationship with HeV and NiV (Drexler, J. F., 2009). The natural reservoirs of HeV, NiV, and CedV are flying foxes (*Pteropus sp.*) (Middleton and Weingartl, 2012). Recent investigations into Madagascan fruit bats detected a second African paramoxyvirus, AngV. MojV, was identified during follow-up investigations into lethal pneumonia from three miners in 2012 in China. Multiple genus of small mammals were tested for viral RNA, and MojV was detected in yellow-breasted rats (R. flavipectus) (Wu Z et al., 2014). Langya henipavirus (LayV), a close relative of MojV, was recently identified in febrile patients in eastern China and is closely linked to shrews (Zhang et al., 2022). The novel shrew-borne henipaviruses, Gamak virus (GAKV), and Daeryong virus (DARV) were found in the Republic of Korea in 2017-2018. (Lee et al., 2021). These recent discoveries are challenging the paradigms surrounding henipaviruses and their associations with bats and may suggest a novel rodent sub-genus of henipaviruses.

The Henipavirus genome consists of six structural proteins including nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), receptor-binding protein (RBP), and large (L) protein (approximately length of 18,000 nucleotides) (Luby, S. P., & Gurley, E. S., 2012). In HeV and NiV, slippage sites within the P protein lead to the expression of the V, W, and C proteins. These proteins are virulence factors and are absent in CedV.

Table 1 Summary viruses in Henipavirus

Henipavirus	Origin (Year)	Reservoir	Genome (nucleotides)	Symptom for human	Reference
Hendra virus (HeV)	Australia (1994)	Fruit bats	18,234	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Nipah virus (NiV)	Malaysia (1999)	Fruit bat	18,246	Encephalitis	Luby, S. P., & Gurley, E. S., 2012
Ghana virus (GhV)	Africa (2009)	Fruit bat (*Eidolon helvum)*	18,530	No data	Drexler, J. F., 2009
Cedar virus (CedV)	Australian (2012)	Fruit bat	18,162	Non-pathogenic	Marsh, G. A et al., 2012

Mojiang virus (MojV)	China (2012)	Rodent	18,404	Febrile	Wu Z et al., 2014
Gamak virus (GAKV),	Korea (2017-2018)	Shrew	18,460	No data	Lee et al., 2021
Daeryong virus (DARV)	Korea (2017-2018)	Shrew	19,471	No data	Lee et al., 2021
Langya virus (LayV)	China (2019)	Shrew	18,402	Acute febrile	Zhang et al., 2022
Angavokely virus	Madagascar (2019)	Fruit bat	16,740	No data	(Madera et al., 2022)

Viruses in the genus Henipavirus have a broad reservoir and may cause high case fatality rates following human infection. The discovery and characterization of the novel Henipavirus is a high public health priority.

The greatest bat diversity around the world especially appears to be found in the tropical rain forests of South East Asia. In Thailand, at least 138 species in 11 families and 45 genera of bats have been recorded, such as members of the genus Pteropus, Rhinolophus, and Hipposideros (Soisook et al., 2011). Khao Chong Phran, a cave in Ratchaburi Province, has possibly the largest colony of bats in Thailand. There are estimated to be around 2 million bats in the cave and 95% are Insect-eating bats (Department of National Parks, Wildlife and Plant Conservation, Thailand). Moreover, Khao Chong Phran has many community sites, such as cave entrances near communities, guano collection, and tourism sites, which risk zoonotic spillover. Bats are widely considered the animal hosts of zoonotic pathogens such as rhabdoviruses, coronaviruses, paramyxoviruses, and filoviruses, and many of the host species can be found in Thailand.

MojV was associated with a severe pneumonia-like illness killing three of six miners in 2012 in a mineshaft Yunnan, China. Interestingly, this mineshaft was also site where RaTG13, a beta-coronavirus most closely related with SARS-CoV-2, was detected in Rhinolophid bats (Rahalkar and Bahulikar, 2020). Identifying the wildlife reservoir of this novel virus will assist intervention strategies to reduce spillover risk.

As a part of Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH), serological investigations using a Multiplex microsphere-based immunoassay (MMIA) have detected four members of a high-risk cohort with MojV-reactive serum, including one person with saturating levels of antibody from multiple years. Despite this, genomic surveillance has not detected MojV or any novel MojV-like viruses. For this result, we would to further identify and characterize the origin of the Mojiang virus and to explore the novel MojV-like virus in Thailand.

Serological and cellular immunity is an important resource for virus discovery detection by using the specificities and characteristics of memory B cells from peripheral blood mononuclear cells (PBMC). The immune response will recognize and induce antibodies specific to the infecting pathogen. Characterizing the memory B cell response to an unknown pathogen in humans could in the detection of the exposure pathogen in humans. Molecular techniques such as PCR can detect only the genomic material in the body, but memory B cells are long-lived and thus have a much larger window of detection.

Memory B-cell differentiation is characterized by somatic hypermutation in antibody variable region genes (V) and selection of B cells expressing high-affinity variants of this antigen receptor and high sequence similarity in individuals exposed to the same antigen reflect antigen-driven clonal selection and form shared immunological memory between individuals (Yang et al., 2021). Memory B

cells are highly abundant in the human spleen, and can proliferate 45% of the total B cell population in this organ (Hauser et al., 2015). Memory B cells can be detected in the Ig variable (IgV) genes in a fraction of IgM+ B cells. In humans, B cells expressing the memory marker CD27+ can be found in the marginal zone of spleen and circulate in the blood. After re-exposure to antigen, memory B cells can quickly proliferate and differentiate into plasma cells. Many studies the antibody diversity and specificity of the naturally paired immunoglobulin heavy and light chains for affinity maturation of B cell receptor (BCR) after virus infection such as SARS-CoV-2 infection (Yang et al., 2021), influenza (Dougan, S et al., 2013), HIV (Moir, S., & Fauci, A. S.,2017), etc. The characterization of memory B cells can be used to develop monoclonal antibodies and produce an effective theraputic.

Significance and Approach

Southeast Asia is one of the world's highest-risk emerging infectious diseases hotspots. From this region emerged both SARS-CoV-1 and SARS-CoV-2, recurrent outbreaks of novel influenza strains, and the spillover of dangerous viral pathogens such as Nipah virus. The diversity of coronaviruses, henipaviruses, and filoviruses are found in wildlife reservoirs in Southeast Asia is due to the high diversity of wildlife, and the demography of the human population brings humans and wildlife into close contact. The spillover of viruses from animals to humans is often misdiagnosed and the clinical symptoms from patients is underestimated during the pandemic. This study provides a surveillance strategy in human and wildlife populations to better understand immunity to novel pathogens and identify the origin of these pathogens to serve as an early warning system to prevent outbreaks of emerging viruses.

This study uses immunology to identify a novel virus that can aide in effective vaccine discovery and development, monoclonal antibody production for prevention and therapy, and generate data resources for early detection and diagnostic assays. Whereas PCR detection or virus isolation in wildlife is challenging, this new serological approach may be more successful in identifying reservoir hosts.

The Indo-Pacific region has a high risk of novel viral emergence. This study provides an opportunity for in-country research capacity building by linking local infectious disease scientists with an international collaborative network that has proven capacity to conduct this work and produce significant findings. Moreover, a global sero-surveillance network for surveillance technology extends virologic and immunologic screening to product discovery. This study will benefit and expand the CREID, EID-SEARCH networks, and the global sero-surveillance network to improve our understanding of immunology to identify novel pathogens and their origin to prevent future pandemics.

Research Methods

For aim 1. Antibody-mediated bait & capture of Thai MojV-like virus

In collaboration with NIH PREMISE, MojV RBP-reactive B cells will be captured, and the B cell receptor will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific MAbs will be generated and used for direct antibody-mediated virus capture in people and wildlife.

Human samples collection site

Human positive serum for MojV was found close to Khao Chong Phran in Ratchaburi Province, which has the largest colony of bats in Thailand (Figure 1).

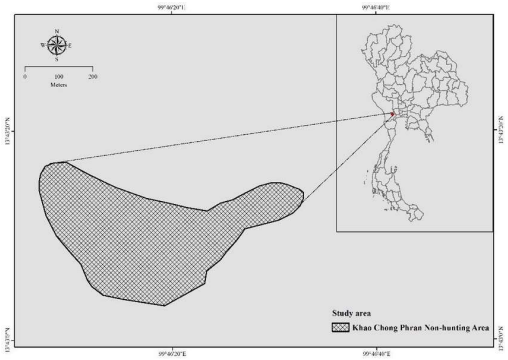


Figure 1**:** Map of Khao Chong Phran Non-hunting Area, Ratchaburi Province, Thailand (Boonpha N et al., 2019)

Isolation PBMC

Whole blood from MojV-positive people will be collected in individual heparin tubes and processed for PBMC isolation using Density Gradient Centrifugation (SepMate™ PBMC Isolation Tubes).

Single Memory B Cell Isolation

Human PBMCs will be stained with antibodies for positive markers of IgG memory B cells, and antibodies to negatively select and avoid contamination with dead cells, T cells, NK cells or monocytes (live, CD3−,CD14−,CD56−, IgM−, IgA−, CD19+,CD20+,CD27+). Avi-tag for biotinylation and 6xHIS tag for Nickel purification using the HisPur™ Ni-NTA Spin Purification Kit (Thermo Fisher Scientific, Waltham, MA). Single Memory B Cells will be sorted, cells will proliferate, cDNA will synthesized, then Heavy and light chain variable domains will be amplified (Waltari E et al., 2019).

B cell receptor Amplicon Preparation

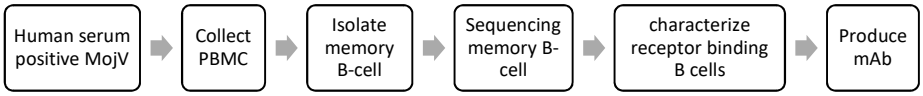
Total RNA yields from the PBMC will be determined by absorbance at 260 nm on the NanoDrop™ One (Thermo Fisher Scientific). Total RNA will be used for first strand cDNA synthesis with gene-specific reverse transcription (RT) primers directed to the constant regions. The RT primers for IgG, IgM, IgA, lambda, and kappa will be included. Light chain and heavy chains will be amplified into amplicon. Double-stranded cDNA will be sequenced.

B cell receptor Data Analysis

After completion of MiSeq sequencing, antibody repertoires will analyze using methods based on the pipeline.

Recombinant Antibody Cloning, Production, and Purification

Heavy and light-chain sequences will be assembled into an expression vector and transfected into XXXXX cells for monoclonal antibody expression.



Aim 2. Modeling antigenic relationships among known and unknown henipaviruses.

Defining antigenic relationships among these viral species will allow the serologic signature of related undiscovered henipaviruses to be identified. We will develop anti-RBP standards to explore the antigenic spatial relationships between all known rodent- and bat-borne HPVs, and the Thai MojV-like virus.

Site wildlife samples collection

50 Rodents 50 bats, and 50 shrews in Khao Chong Phran cave in Ratchaburi Province will be collected for surveillance targeting the Mojiang-like virus.

Commented [s1]: Will we take multiple sampling trips or just one?

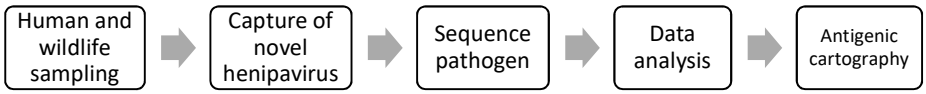
Human syndromic surveillance

50 patients who have Influenza-Like-Illness or Severe acute respiratory infection or respiratory tract infection will collect serum to detect henipa-like virus.

Antigenic cartography

Antisera and monoclonal antibodies to henipavirus RBP, including the novel MojV-like henipavirus, will be generated and tested against all the known RBP. The resulting binding data will be complied, and antigenic relatedness will be determined using RStudio.

Data analysis



Project Timeline (1-page limit)

Activity	05/23	06/23	07/23	08/23	09/23	10/23	11/23	12/24	01/24	02/24	03/24	04/24
Mentoring and training activities												
1.Training, literature review												
2.Bi-weekly mentoring meeting												
Research activities												
1. Collect PBMC human sample and isolation memory B-cell for Mojiang virus	←	→										
2. B cell receptor Data Analysis		←	→									
3. Produce mAb			←	→								
4. Wildlife samples collection					←	→						
5.Identify and characterize novel viral pathogen						←					→	
6.Data analysis											←	→

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Applicant Name (Last, first, middle):

Research and Related Other Project Information

1. Are Human Subjects Involved?	Yes	X	No													
1.a. If YES to Human Subjects																
Is the Project Exempt from Federal regulations?	Yes		No													
If yes, check appropriate exemption number	1		2		3		4		5		6		7		8	
If no, is the IRB review Pending?	Yes	X	No													
IRB Approval Date:																
Human Subject Assurance Number																

Commented [SN1]: We are still waiting for the global IRB approval to use for our IRB amendment.

2. Are Vertebrate Animals Used?	Yes	X	No	
2.a. If YES to Vertebrate Animals				
Is the IACUC review Pending?	Yes	X	No	
IACUC Approval Date				
Animal Welfare Assurance Number				

Commented [SN2]: In the approved IACUC version, we stated that 3cc **non-heparinized** syringe is used for blood sample collection for bats weigh > 200 grams. Do we need to amend this if different types of tube will be used for PBMC collection?

3. Is proprietary/privileged information included in the application?	Yes		No	

4.a. Does this project have an Actual or Potential impact -- positive or negative -- on the environment?	Yes		No	
4.b. If yes, please explain				
4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed?	Yes		No	
4.d. If yes, please explain				

Applicant Name (Last, first, middle):

5. If the research performance site designated, or eligible to be designated, as a historic place?	Yes		No	
5.a. If yes, please explain				

6. Does this project involve activities outside of the United States or partnership with international collaborators?	Yes		No	
6.a. If yes, identify countries				
6.b. Optional explanation				

From: Krongkan Srimuang on behalf of Krongkan Srimuang <krongkan5678@gmail.com>
To: Hongying Li; Sterling, Spencer
Cc: Supaporn Wacharapluesadee; Sasiprapa Ninwattana; Eric Laing
Subject: Pilot study 2023-Thailand
Date: Sunday, January 15, 2023 10:16:02 PM
Attachments: Submission Checklist_Application.docx
1 Application Cover Sheet.docx
Study Personnel_V.2.docx
Research Performance Sites_V.2.docx
CREID Research Center Collaboration.docx
Mentoring Plan_V.2.docx
Facilities and Resources_V.2.docx
Research aim, methods_V.2.docx
2 Research and Related Other Project Information_SN.docx

Dear Hongying,

I and Spencer are writing the Pilot application.
We have some parts not finished which are labeled on the Submission Checklist.

I have attached files to this email for your consideration following;
1. Submission Checklist_Application
2. Proposal Cover Sheet
3. Study Personnel
4. Research Performance Sites
5. CREID Research Center Collaboration
6. Mentoring Plan
7. Facilities, Existing Equipment, and Other Resources
8. Research aim, methods
9. Research and Related Other Project Information_from BOW

I don't know what to do with the IRB part.
Can you give us some advice?
Thank you very much
Best regards,
KIO

The King Chulalongkorn Memorial Hospital and Faculty of Medicine, Thailand

Facilities

The Thai Red Cross Emerging Infectious Diseases Clinical Center (EID-CC) has 100 square meters of office space, 400 square meters of lab space, and more than 3,000 square meters of shared lab space with the faculty of Medicine (Chula Medical Research Center, MRC). There is twenty-one staff including 6 clinicians, 7 scientists, 1 bioinformatician, 2 research nurses, and 4 admin staff. EID-CC lab supports molecular and serology diagnostics to the hospital and government partners, including the Department of Diseases Control (DDC) and the Department of National Parks, Wildlife, and Plant Conservation (DNP). EID-CC conducts research projects granted by the Thai government and international partners. The lab leader, Dr. Wacharapluesadee, has experience in emerging infectious zoonotic surveillance, especially in bats, for over 20 years. The research network includes the Faculty of Forestry, Kasetsart University, DNP, DDC, and the faculty of the Veterinary Science Rajamangala University of Technology Srivijaya.

Equipment

The Chula MRC laboratory is equipped for virology, cell culture, serology, and molecular biology. There is one BSL-3 lab facility and 3 cell culture lab spaces. In addition, the Chula MRC has 8 PCR machines, 5 real-time PCR machines, 2 gel document imaging, 3 horizontal electrophoresis, 3 UV-visible spectrophotometers, 3 ultra-centrifuge, 2 high-speed centrifuge machines, 3 refrigerated centrifuge, 2 refrigerated incubator shakers, 5 autoclaves, 2 hot air oven, 6 upright microscopes, 3 inverted microscopes, 2 inverted microscopes with fluorescence, 9 CO_2 incubators, 5 Biosafety cabinets, freeze dryer, speed vacuum concentrator, extraction machine, immunoSpot ultimate analyzer, flow cytometer, Bio-plex multiplex suspension array system, microplate reader and washer, droplet digital PCR, automated electrophoresis system, dual fluorescence cell counter, NanoSight, florescence and phosphor imager, Chemiluminescence western blot scanner, CO_2 incubator with shaker, Automate Electrophoresis System, Automated Nucleic Acid and Protein Extraction System, Fluorescent Table and DNA analyzer.

The EIDCC lab is equipped for serology, molecular biology, and next-generation sequencing. There are 3 PCR machines, 4 deep Freezers, 3 freezers, 3 refrigerators, 4 Biosafety cabinets, 2 micro-centrifuge, 3 real-time PCR machines, automated gel electrophoresis, Nex-generation sequencer (MiSeq system), and NGS library preparation system. The specimen repository system uses the “Pathogen Asset Control System, PACS” software supported by US DTRA. There are 4 PACS computers. The computer server of the faculty of Medicine is available to access and use for genomic analysis.

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: Wacharapluesadee, Supaporn

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6)

POSITION TITLE: Laboratory Chief

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
Chiang Mai University, Thailand	B.S.	02/1991	Medical Technology
Mahidol University, Thailand	M.S.	01/1994	Biochemistry
Chulalongkorn University, Thailand	Ph.D.	03/2006	Biomedical Sciences

A. Personal Statement

I have 20+ years in research and 15+ years of experience in emerging viral zoonoses. I have managed many internationally funded research projects, that involves working with and managing international and local interdisciplinary teams. Majority of my research projects are field surveillance in wild mammals, human behavioral risk surveys, and clinical sampling. I conduct workshops on development of novel diagnostic approaches, appropriate sample collection and handling for different pathogens, and viral characterization *in vitro* and *in vivo*. I am the Deputy Chief of Thai Red Cross Emerging Infectious Diseases Health Science Centre which conducts research on emerging zoonoses. My research background is focused on understanding the process of zoonotic disease emergence, particularly viral zoonoses. This includes identifying the bat origin of Nipah virus and MERS-CoV, and pathogenesis and diagnoses of Rabies. My study on the emergence of novel betacoronaviruses found in Thai bats, as well as Nipah virus have been published. Our centre was the first laboratory to correctly diagnose the first human MERS case in Thailand, which led to swift execution of containment measures preventing a MERS outbreak in Thailand. We are now the government's reference laboratory for emerging infectious diseases. I have been the PI on 5 multidisciplinary research projects that use epidemiology, laboratory, field science and bioinformatics to diagnose and monitor the emergence of wildlife-origin viral zoonoses, including SARS-CoV, Nipah and Hendra virus, Avian influenza and novel viruses from bats. I am also the Thailand country manager for large contracts from USAID involving successful management of teams of virologists, field biologists, veterinarians, epidemiologists, hospitals and laboratorians.

1. Phumee A, Buathong R, Boonserm R, Intayot P, Aungsananta N, Jittmittraphap A, Joyjinda Y, **Wacharapluesadee S**, Siriyasatien P (2019). Molecular Epidemiology and Genetic Diversity of Zika Virus from Field-Caught Mosquitoes in Various Regions of Thailand. **Pathogens** 8(1)pii:E30.
2. Joyjinda Y, Rodpan A, Chartpituck P, Suthum K, Yaemsakul S, Cheun-Arom T, Bunprakob S, Olival KJ, Stokes MM, Hemachudha T, **Wacharapluesadee S** (2019). First Complete Genome Sequence of Human Coronavirus HKU1 from a Nonill Bat Guano Miner in Thailand. **Microbiol Resour Announc** 8(6)pii:e01457-18.

3. **Wacharapluesadee S**, Duengkae P, Chaiyes A, Kaewpom T, Rodpan A, Yingsakmongkon S, Petcharat S, Phengsakul P, Maneeorn P, Hemachudha T (2019). Longitudinal study of age-specific pattern of coronavirus infection in Lyle's flying fox (Pteropus lylei) in Thailand. **Virol J** 20;15(1):38.
4. **Wacharapluesadee S**, Sintunawa C, Kaewpom T, Khongnomnan K, Olival KJ, Epstein JH, Rodpan A, Sangsri P, Intarut N, Chindamporn A, Suksawa K, Hemachudha T (2013). Group C betacoronavirus in bat guano fertilizer, Thailand. **Emerg Infect Dis** 19(8).

B. Positions and Honors

Positions and Employment

1994 -97 Biochemical Technician, Department of Entomology, AFRIMS, Thailand
1997 Researcher, Department of Immunology, Chulabhorn Research Institute, Thailand
1997 -00 Medical Technologist, The HIV/AIDS Collaboration Thai-US, Thailand
2000 -16 Laboratory Chief, Neuroscience Centre for Research and Development & WHO Collaborating Centre for Research and Training on Viral Zoonoses, Faculty of Medicine, Chulalongkorn University Hospital, Thai Red Cross Society, Thailand
2016 - Deputy Chief of Thai Red Cross Emerging Infectious Diseases Health Science Centre, Faculty of Medicine, Chulalongkorn University Hospital

Other Experience and Professional Membership

2010 -14 PREDICT Thailand Country Coordinator
2014 - Thai Ministry of Public Health (MOPH) Ebola Diagnostic Committee
2015 - PREDICT 2 Thailand Country Coordinator
2016 - Steering committee, Bat One Health Research Network, BTRP DTRA

C. Contribution to Science

1. Research on coronavirus prevalence in Thailand. Numerous high impact emerging viruses appear to have bat reservoirs. Our surveillance projects study the diversity of coronavirus (CoV) in bats in Thailand. We have isolated and characterized CoVs from many bat species, and detected and sequenced CoV in bat guano miner. Our surveillance studies continue to analyze the drivers of their emergence, and risk factors for spillover.

a. Joyjinda Y, Rodpan A, Chartpituck P, Suthum K, Yaemsakul S, Cheun-Arom T, Bunprakob S, Olival KJ, Stokes MM, Hemachudha T, **Wacharapluesadee S** (2019). First Complete Genome Sequence of Human Coronavirus HKU1 from a Nonill Bat Guano Miner in Thailand. **Microbiol Resour Announc** 8(6).pii:e01457-18.
b. **Wacharapluesadee S**, Duengkae P, Chaiyes A, Kaewpom T, Rodpan A, Yingsakmongkon S, Petcharat S, Phengsakul P, Maneeorn P, Hemachudha T (2019). Longitudinal study of age-specific pattern of coronavirus infection in Lyle's flying fox (Pteropus lylei) in Thailand. **Virol J** 20;15(1):38.
c. Plipat T, Buathong R, **Wacharapluesadee S**, Siriarayapon P, Pittayawonganon C, Sangsajja C, Kaewpom T, Petcharat S, Ponpinit T, Jumpasri J, Joyjinda Y, Rodpan A, Ghai S, Jittmittraphap A, Khongwichit S, Smith DR, Corman VM, Drosten C, Hemachudha T (2017). Imported case of Middle East respiratory syndrome coronavirus (MERS-CoV) infection from Oman to Thailand, June 2015. **Euro Surveill** 22(33):pii: 30598.
d. **Wacharapluesadee S**, Duengkae P, Rodparn A, Kaewpom T, Maneeorn P, Kanchanasaka B, Yingsakmongkon S, Sittidetboripat N, Chareesaen C, Khlangsap N, Pidthong A, Leadprathom K, Ghai S, Epstein JH, Daszak P, Olival KJ, Blair PJ, Callahan MV and Hemachudha T (2015). Diversity of Coronavirus in Bats from Eastern Thailand. **Virol J** 12(1):57.

2. **Research on Nipah virus prevalence in Thai bats.** Nipah virus outbreaks, previously in Thailand's neighbouring country, Malaysia, and ongoing in Bangladesh have high mortality rate. Our surveillance projects study the characterization of Nipah Virus (NiV) in bats in Thailand. Our surveillance studies continue to analyze the drivers of their emergence, understanding their seasonal preference, and risk factors for spillover.
    a. **Wacharapluesadee S**, Samseeneam P, Phermpool M, Kaewpom T, Rodpan A, Maneeorn P, Srongmongkol P, Kanchanasaka B, Hemachudha T (2016). Molecular characterization of Nipah virus from Pteropus hypomelanus in Southern Thailand. **Virol J** 13(1):53
    b. **Wacharapluesadee S**, Jittmittraphap A, Yingsakmongkon S, and Hemachudha T (2016). Molecular Detection of Animal Viral Pathogens. Nipah Virus. CRC Press.
    c. **Wacharapluesadee S**, Ngamprasertwong T, Kaewpom T, Kattong P, Rodpana A, Wanghongsa S, Hemachudha T (2013). Genetic characterization of Nipah virus from Thai fruit bats (*Pteropus lylei*). **Asian Biomedicine** 7(6);813-819.
    d. Breed AC, Meers J, Sendow I, Bossart KN, Barr JA, Smith I, **Wacharapluesadee S**, Wang L, Field HE (2013). The Distribution of Henipaviruses in Southeast Asia and Australasia: Is Wallace's Line a Barrier to Nipah Virus? **PLoS One** 8(4):e61316.
3. **Rabies Neuropathogenesis, diagnosis and management.** The centre worked many years on molecular analyses of rabies, including mutational effects, and designing primers to detect Thai street rabies virus. I regularly organize workshops to teach laboratories in the region on how to correctly collect specimen and test for rabies.
    a. Hemachudha T, Ugolini G, Sungkarat W, Laothamatas J, Shuangshoti S, **Wacharapluesadee S** (2013). Human Rabies: neuropathogenesis, diagnosis and management. **Lancet Neurology** 498-513.
    b. Shuangshoti S, Thepa N, Phukpattaranont P, Jittmittraphap A, Intarut N, Tepsumethanon V, **Wacharapluesadee S**, Thorner PS, Hemachudha T (2013). Reduced viral burden in paralytic compared to furious canine rabies is associated with prominent inflammation at the brainstem level. **BMC Vet Res** 14;9(1):31.
    c. Virojanapirom P, Khawplod P, Sawangvaree A, **Wacharapluesadee S**, Hemachudha T, Yamada K, Morimoto K, Nishizono A (2012). Molecular analysis of the mutational effects of Thai street rabies virus with increased virulence in mice after passages in the BHK cell line. **Arch Virol** 157(11):2201-5.
    d. Wilde H, Hemachudha T, **Wacharapluesadee S**, Lumlertdacha B, Tepsumethanon V (2013). Rabies in Asia: The Classical Zoonosis. **Curr Top Microbiol Immunol** 365:185-203.
4. **Investigating causes of encephalitis**. More than 50% of patients presenting with fever remain undiagnosed. Our centre has focused a lot of research into diagnosing fever of unknown origins (FUO). We study epidemiology, pathology and conduct surveillance studies into viral pathogens, and autoimmune diseases.
    a. Hemachudha P, **Wacharapluesadee S**, Buathong R, Petcharat S, Bunprakob S, Ruchiseesarod C, Roeksomtawin P, Hemachudha T (2019). Lack of Transmission of Zika Virus Infection to Breastfed Infant. **Clin Med Insights Case Rep** 12:1179547619835179.
    b. Phumee A, Buathong R, Boonserm R, Intayot P, Aungsananta N, Jittmittraphap A, Joyjinda Y, **Wacharapluesadee S,** Siriyasatien P (2019). Molecular Epidemiology and Genetic Diversity of Zika Virus from Field-Caught Mosquitoes in Various Regions of Thailand. **Pathogens** 8(1).pii: E30.
    c. Phumee A, Chompoosri J, Intayot P, Boonserm R, Boonyasuppayakorn S, Buathong R, Thavara U, Tawatsin A, Joyjinda Y, **Wacharapluesadee S**, Siriyasatien P (2019). Vertical transmission of Zika virus in Culex quinquefasciatus Say and Aedes aegypti (L.) mosquitoes. **Scientific reports** 9(1):5257.

d. Thanprasertsuk S, Pleumkanitkul S, **Wacharapluesadee S**, Ponpinit T, Hemachudha T, Suankratay C (2017). HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis: the First Case Report in Southeast Asia. **AIDS Res Hum Retroviruses**.

D. Additional Information: Research Support and/or Scholastic Performance

Ongoing Research Support

USAID Emerging Pandemic Threats Mazet (PI) 10/01/14 – 09/30/19
PREDICT-2
The goal is to conduct surveillance for novel pathogens in wildlife, livestock and people; characterize human risk behavior; analyze EID risk; and design interventions in >20 countries
Role: Thailand country coordinator

Multi-Echelon Diagnostics Wacharapluesadee (PI) 07/01/18 – 08/31/19
The goal is to support the Defense Threat Reduction Agency's (DTRA) Multi-Echelon Diagnostics evaluation program. Responsibilities include using the specified point-of-care diagnostic(s) through the procurement of supplies, enrollment of subjects according to inclusion and exclusion criteria, testing the samples, and reporting the data to the Naval Health Research Center (NHRC).

Pathogen Surveillance for Viral Zoonoses Wacharapluesadee (PI) 12/15/16 – 06/30/20
Disease surveillance analysis of wildlife-domestic animal-human interfaces, in coordination with PREDICT USAID project.

Surveillance for Emerging Infectious Disease Pathogens at the Animal-Human Interfaces in Thailand, in Coordination with PREDICT USAID Project and the Bat Serology Study
Wacharapluesadee (PI) 06/01/18 – 04/30/20
The goal of the study is to understand and mitigate zoonotic disease using multiplex serology developed by Utah State University (USU). This is also a disease surveillance analysis of wildlife-domestic animal-human interfaces, in coordination with PREDICT USAID project.

Completed Research Support (last 3 years only) out of 14 prior awards

NSTDA Viral Laboratory Network Wacharapluesadee (PI) 04/01/16 – 03/31/19
The goal of this study was to establish a viral laboratory network in Thailand for Emerging Infectious Disease preparedness among the university laboratories and government public health laboratory.

From:	Hongying Li on behalf of Hongying Li <li@ecohealthalliance.org>
To:	Spencer Sterling; Sasiprapa Ninwattana
Cc:	Supaporn Wacharapluesadee; eric.laing_usuhs
Subject:	Fwd: New NIH proposal on serology methods
Date:	Wednesday, January 11, 2023 9:46:05 AM
Attachments:	2022 EIDCC facilities and equipment_TRC EIDCC.docx Biosketch_(Wacharapluesadee,Supaporn)_EIDRC_RFA-AI_19-028_(PI-Daszak)_v02.docx

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Hi Bow and Spencer,

I have sent the following info and attachments to Noam before, but just resending for your reference.

Attached please find the latest versions of relevant materials I have:

- Supapron's NIH-biosketch - this needs to be updated with the new format (no section D) https://grants.nih.gov/grants/forms/biosketch.htm
- Facilities and equipment
- Other support - this need to be updated with the new format and other grants https://grants.nih.gov/grants/forms/othersupport.htm - *but you will only need this for the Just-In-Time procedure,* ***not*** *now for proposal submission*

Other things you need from Chula are:

- Budget
- Budget justification

And if you need to fill in IRB approval information to use human samples under EID-SEARCH (on the cover page):

- US: Health Medical Lab Institutional Review Board (No. 894ECOH21b) on May 12, 2021
- Thailand: Institute Review Board of the Faculty of Medicine, Chulalongkorn University (No. 211/64) on June 8, 2021

Let me know if anything else I can help with.

Best,
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

---------- Forwarded message ---------
From: **Laing, Eric** <eric.laing@usuhs.edu>
Date: Tue, Jan 10, 2023 at 4:54 PM
Subject: Re: New NIH proposal on serology methods
To: Supaporn Wacharapluesadee <spwa@hotmail.com>
Cc: Noam Ross <ross@ecohealthalliance.org>, Hongying Li <li@ecohealthalliance.org>, Peter Daszak <daszak@ecohealthalliance.org>, Laing, Eric <eric.laing.ctr@usuhs.edu>, Kevin Olival <olival@ecohealthalliance.org>, Sterling, Spencer <spencer.sterling.ctr@usuhs.edu>, Opass ID <opassid@gmail.com>

Hi Chu,

My lab is working on expanding our filo/henipa panel to a 28-plex, most of which should be available for further testing under EID-SEARCH. If this proposal is funded Noam and I would like to make sure that there are additional funds in this R01 proposal to support retesting in your lab.

Which groups of the samples (host species and project name) do you plan to test? Now I have moved to work at the new lab, and it isn't easy to access the old samples (before 2020). But it is possible.
For humans and wildlife, how many samples/species have you collected now and what is your projected target for the remainder of the EID-SEARCH POP? What would be really helpful would be a cost estimate for retesting all those samples under this R01 with the optimized in vitro and in silico serologic approaches.

We'd like to list you as a Co-Investigator, which means we'd need a biosketch, LOS, equipment/facilities pages, and that additional budget that we would include in YRS4-5 of this proposal to supplement additional testing. Spencer can be involved, just let me know whether salary support is coming from my end as HJF employee or whether he'd be listed as key personnel in your lab directly.

Best regards,
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 Thu, Jan 5, 2023 at 5:51 AM Supaporn Wacharapluesadee <spwa@hotmail.com> wrote:

> Dear Noam and Eric,
> Thank you for your email and invitation to collaborate on the new proposal. I would love to join and have Spencer be responsible for lab work if it is funded.
> However, I would like to clarify objective 3 on retesting the archived samples. **Which groups of the samples (host species and project name) do you plan to test?** Now I have moved to work at the new lab, and it isn't easy to access the old samples (before 2020). But it is possible.
>
> Best,
> Supaporn
>
> *Supaporn Wacharapluesadee, PhD*
> Thai Red Cross Emerging Infectious Diseases Clinical Center
> King Chulalongkorn Memorial Hospital
> Rama4 road, Patumwan, Bangkok, Thailand 10330
> Skype ID: supapornwa
>
> ---
>
> **From:** Noam Ross <ross@ecohealthalliance.org>
> **Sent:** Thursday, January 5, 2023 4:48 AM
> **To:** S Wacharapluesadee <spwa@hotmail.com>
> **Cc:** Hongying Li <li@ecohealthalliance.org>; Peter Daszak <daszak@ecohealthalliance.org>; Laing, Eric <eric.laing.ctr@usuhs.edu>; Kevin Olival <olival@ecohealthalliance.org>
> **Subject:** New NIH proposal on serology methods
>
> Dear Supaporn,
>
> Happy New Yea! I hope this finds you well. I'm writing because I am putting together a new NIH grant proposal at EcoHealth together with Eric Laing at USU that we wanted to include you in. The aim of the grant is to improve both lab and statistical methods for luminex serology. The main elements of the project would be to:
>
> 1) Develop statistical methods to simultaneously analyze multiplex serology to quantify the probability that signals from multiple beads represent antibodies from exposures to different viruses, cross-reactions of viruses in the multiplex panel, or signals of new, previously unknown viruses.
> 2) Develop discovery-optimized panels for filoviruses and henipaviruses that mix high-specificity and high-sensitivity beads so as to maximize the ability to identify signals of novel viruses.
> 3) Re-test and re-anlayze results from samples from previous serology surveillance studies that have ambiguous results to identify signals of exposure to previously unknown viruses,

and conduct antigenic mapping to estimate where they fall phenotypically in relation to known viruses.

We anticipate sharing all our work across partners over the course of the project (if/when funded). However, we specifically hope to collaborate with you on #3 above. We'd like to use this to look deeper at EID-SEARCH results and ideally retest samples (such as the Mojiang-seropositive samples) to see what the new panels yield. For our proposed project, some of the new panels will be developed while EID-SEARCH is still running. If this new project is funded, we will work with you to determine what should be done concurrently with EID-SEARCH or after EID-SEARCH is completed and the best way to budget this (mind you, EcoHealth also hopes to get a continuation on our EID-SEARCH project so it is likely both will be concurrent). Assuming the best case, we submit this in February (Due date Feb 3, 2023) and start work in Fall 2023. Depending on how we stage our tasks, re-testing work could start as early as late 2024 or 2025.

One additional note – this project would be subject to new data sharing requirements NIH has enacted for projects starting 2023 or later. In short, all data we generate out of the project would be published at the end of the project period, whether or not publications are complete. We don't anticipate this would be an issue but want to make sure everyone is aware of this from the start of the grant so we can plan data and manuscript publication accordingly.

We'd like to have you as Key Personnel on this proposal. If you are interested, we'd love to have you aboard and get your thoughts and questions. Eric and I will also follow up next week with a proposal budget. We would also need an NIH Biosketch, Other Support form, and Facilities information from you by January 20. We have recent versions of all of these for EID-SEARCH, so we can send templates for you to modify and approve early next week.

Please let us know what you think, and we'd be happy to have a call to discuss.

Best,

Noam
--

Dr. Noam Ross
Principal Scientist, Computational Research

EcoHealth Alliance
520 Eighth Avenue, Suite 1200
New York, NY 10018
www.ecohealthalliance.org

+1.212.380.4471 (direct)
+1.212.380.4465 (fax)
@noamross@ecoevo.social (fediverse)
noamross.net

> *conservation.*

Updated: 10/28/22

CREID Pilot Research Program
2023 Letter of Intent

Please use this template to submit your Letter of Intent (LOI) for the 2023 Pilot Research Program. Be sure to also submit a Biosketch for the applicant PI and the co-PI if one is included.

LOIs are due December 5, 2022, 5pm ET.

Project Title	Immune memory bait & capture to identify emerging henipavirus origins
Principal Investigator Name	Krongkan Srimuang, Ph.D.
Position/Title	Research Scientist
Department	Thai Red Cross Emerging Infectious Diseases Clinical Center
Institution Name	King Chulalongkorn Memorial Hospital
Street	1873, Rama 4 Road, Pathumwan
City, State, Zip Code	Bangkok 10330
Country	Thailand
Email	Krongkan.sr@gmail.com
Phone	+66 (0) 92-453-0808
CREID Research Center affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)
The category of eligibility of the applicant(s) (see Section 1.3 of Call for Applications)	LMIC Applicant
Co-Principal Investigator Name (*If applicable*)	Spencer Sterling, MPH
Position/Title	Scientific Project Coordinator
Department	Microbiology and Immunology
Institution Name	Uniformed Services University of the Health Sciences
Street	Jones Bridge Road
City, State, Zip Code	Bethesda, MD 20814
Country	USA
Email	spencer.sterling.ctr@usuhs.edu
Phone	+66 (0) 83-494-5980
CREID Research Center affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)
The category of eligibility of the applicant(s) (see Call for Applications, Section 1.3)	The applicant is a visiting scientist based in Thailand, who is recommended by the RC PI given his critical role in lab capacity building in the region. The applicant or his affiliated institution will not receive funding from this project.

Name of Mentor	Eric Laing, Ph.D.
Mentor Institution	Uniformed Services University of the Health Sciences
Institution Address	Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814
Permanent location of Mentor	4301 Jones Bridge Road, Bethesda, MD 20814

Updated: 10/28/22

Mentor email	eric.laing@usuhs.edu
Mentor phone	+1 (301) 980-8192
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Name of Mentor	Supaporn Wacharapluesadee, Ph.D.
Mentor Institution	Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital
Institution Address	1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Permanent location of Mentor	4th Floor, Jongkolnee Watwong Building, Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital, 1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Mentor email	spwa@hotmail.com
Mentor phone	+66 (0) 89-920-6292
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Contact information for institution(s) that would financially manage the award *(please note: changing the managing institution upon award may result in substantial funding delays)*	
Contractual Contact, Title	Chula Unisearch
Institution Name	Chulalongkorn University
Street	254 Phyathai Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	unisearch@chula.ac.th
Phone	0 2218 2880
For Co-Principal Investigator (If applicable):	
Contractual Contact, Title	
Institution Name	
Street	
City, State, Zip Code	
Country	
Email	
Phone	

Overview of proposed research project	
Pathogen(s) focus	Mojiang Virus, Henipavirus, Paramyxovirus
Country(ies) where work will be conducted	Thailand
Research Aims and Objectives (250 words)	

Prototypical henipaviruses (Hendra and Nipah virus) cause zoonotic diseases with high mortality and no effective vaccines or therapeutics and are hosted by Pteropus flying foxes. The recent detection of closely-related Mojiang and Langya viruses in rats and shrews, and an outbreak of acute febrile LayV illness in people, challenges the dogma that fruit bats are the key henipavirus reservoirs, and heightens their threat profile. We have found serological evidence of a Mojiang-related virus in a community of Thai bat guano collectors with

high exposure to microbats and rodents. Identifying the wildlife reservoir of this likely novel virus will assist intervention strategies to reduce spillover risk. However, PCR detection or virus isolation in wildlife is challenging, and new serological approaches may be more successful in identifying reservoir hosts.

Aim 1. Antibody-mediated bait & capture of Thai MojV-like virus

In collaboration with NIH PREMISE, MojV RBP-reactive B cell receptors will be sequenced from previously-identified seropositive human participants. MojV-like virus-specific MAbs will be generated and used for direct antibody-mediated virus capture in people and wildlife.

Aim 2. Modeling antigenic relationships among known and unknown henipaviruses.

The henipavirus genus has recently expanded to include LayV, MojV, two other shrew-borne viruses (Gamak and Daeryong viruses), and the Madagascan fruit bat-borne Angovkeely virus. Defining antigenic relationships among these will allow the serologic signature of related undiscovered henipaviruses to be identified. We will develop anti-RBP standards to explore the antigenic spatial relationships between all known rodent- and bat-borne HPVs, and the Thai MojV-like virus.

If you are proposing a human subjects' or animal research study, please describe the already approved study it is nested within and your plan to obtain an IRB or IACUC modification for the proposed study within 2 months of award.

This project will enroll human subjects under the approved protocols by the Health Medical Lab Institutional Review Board, US (No. 894ECOH21b) and the Institute Review Board of the Faculty of Medicine, Chulalongkorn University, Thailand (No. 211/64). We will obtain an IRB amendment approval for peripheral blood mononuclear cells (PBMCs) collection within two months of award.

If you are proposing a study involving a select agent, indicate the select agent and address the issue identified in the Call for Applications, Section 1.2 about prior approval.

Not applicable.

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: Krongkan Srimuang

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

POSITION TITLE: Research 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
Chulalongkorn University, Bangkok, Thailand	B.Sc.	03/2012	Medical Technology
Mahidol University, Bangkok, Thailand	Ph.D.	04/2017	Molecular Tropical Medicine and Genetics

A. Personal Statement

I am a medical technologist. My undergraduate training has provided me with a background in multiple biological disciplines including molecular biology, microbiology, and genetics. During my Ph.D., I conducted research with Dr. Mallika Imwong focused on Malaria. My thesis was entitled “Exploring the molecular mechanism of mefloquine resistance in *Plasmodium falciparum*”. I conducted research on drug resistance to malaria in Southeast Asia. Specifically, my focus was on molecular techniques in identifying the genetic mutations involved with drug resistance. Moreover, I got experience in *Plasmodium falciparum* malaria culture. After my Ph.D., I worked at the genetics laboratory at Jetanin Institute of Assisted Conception. My responsibilities included Preimplantation Genetic Testing (PGT) is screen embryos for genetic abnormalities such as chromosome copy number gains or losses (PGT-A, aneuploidy screening), single gene disorders (PGT-M, monogenic or single-gene disease), or structural rearrangement (PGT-SR, structural rearrangement to identify embryos with chromosome imbalanced) by using Next Generation Sequencing. Additionally, I conducted genetic screening in couples for detecting carrier diseases such as glucose-6-phosphate dehydrogenase (G6PD), Alpha thalassemia, Beta thalassemia, and Spinal muscular atrophy (SMA) by using molecular genetics techniques. Currently, I am a Research Scientist at the Thai Red Cross Emerging Infectious Diseases Clinical Center (TRC EIDCC), King Chulalongkorn Memorial Hospital where I investigate emerging infectious diseases under the guidance of Dr. Supaporn Wacharapluesadee. The research focuses on detecting, identifying, and characterizing pathogens and human genome segments using molecular and serology techniques, including Next Generation Sequencing and Multiplex serology assays as a part of EID-SEARCH project. Additionally, I am the laboratory sequencing lead on the Surveillance System: Emerging Viral Pneumonia and Respiratory tract infections (RTIs) project from patients in Thailand as part of a collaboration with the Department of Disease Control.

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B. Positions, Scientific Appointments, and Honors

Positions and Scientific Appointments:

02/2022 – Present	Medical research scientist, Emerging Infectious Diseases Clinical Center (EIDCC), King Chulalongkorn Memorial Hospital
05/2017 - 01/2022	Scientist, Genetics Laboratory, Jetanin Institute for Assisted Reproduction
10/2015 - 03/2016	Fellowship at Laboratory of Molecular and Cellular Parasitology, Department of Microbiology and Immunology, National University of Singapore, Singapore.
11/2011 – 02/2012	Training Medical Technology Programs at Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

Honors:

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NAME: Sterling, Spencer Lee

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

POSITION TITLE: Research Project Coordinator

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University of Nebraska Medical Center	MPH	05/2020	Epidemiology

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From: Krongkan Srimuang on behalf of Krongkan Srimuang <krongkan.sr@gmail.com>
To: linfa.wang@duke-nus.edu.sg
Cc: Eric Laing; Hongying Li; Peter Daszak; Spencer Sterling; opassid@gmail.com; spwa
Subject: Re: Letter of Intent submission for Pilot Research Program 2023
Date: Sunday, December 11, 2022 8:41:53 PM
Attachments: LOI - EID-SEARCH (Thailand).pdf
Biosketch-Krongkan Srimuang.pdf
Biosketch-SterlingSL.pdf

Dear Prof.Linfa Wang,

My apologies as I did not send the attachment so here it is attached.

Best regards,
Krongkan Srimuang, Ph.D.
Thai Red Cross Emerging Infectious Diseases Clinical Centre,
King Chulalongkorn Memorial Hospital,
Rama4 road, Patumwan, Bangkok, Thailand 10330

On Sun, Dec 11, 2022 at 8:16 AM Krongkan Srimuang <krongkan.sr@gmail.com> wrote:

> Dear Prof.Linfa Wang
>
> I applied the NIH pilot study in title “Immune memory bait & capture to identify emerging henipavirus origins” last week, please find the attached file.
> This includes the PBMC study in Mojiang antibody positive from human in Ratchaburi. Dr. Eric is the mentor in this study.
> Please advice how three labs can collaborate on this study.
>
> Thank you very much
>
> Best regards,
> Krongkan Srimuang
>
> On Tue, 6 Dec 2565 BE at 00:32 CREID Info <info@creid-network.org> wrote:
>
> > Dear Krongkan,
> >
> > Thank you for submitting a Letter of Intent for the CREID Network Pilot Research Program. We will follow up with you this week if we have questions. As a reminder, full applications are due by **January 30, 2023 at 5pm ET**.
> >
> > Best wishes,
> > CREID Coordinating Center
> > info@creid-network.org
> > https://creid-network.org/pilot-program

From: Krongkan Srimuang <krongkan.sr@gmail.com>
Sent: Monday, December 5, 2022 3:05 AM
To: CREID Info <info@creid-network.org>
Cc: Peter Daszak <daszak@ecohealthalliance.org>; Hongying Li <li@ecohealthalliance.org>; Eric Laing <eric.laing@usuhs.edu>; spwa <spwa@hotmail.com>; Spencer Sterling <spencer.sterling.ctr@usuhs.edu>
Subject: Letter of Intent submission for Pilot Research Program 2023

To whom it may concern,

We are part of EID-SEARCH research group and we would like to submit for the Pilot Research Program 2023.

Please find attached the three documents including;

1. Letter of Intent (LOI)

2. Biosketch of the applicant PI

3. Biosketch of the applicant Co-PI

If you require any further information, please do not hesitate to contact us.

Best regards,

Krongkan Srimuang

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King Chulalongkorn Memorial Hospital,
Rama4 road, Patumwan, Bangkok, Thailand 10330

BIOGRAPHICAL SKETCH

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Updated: 10/28/22

CREID Pilot Research Program
2023 Letter of Intent

Please use this template to submit your Letter of Intent (LOI) for the 2023 Pilot Research Program. Be sure to also submit a Biosketch for the applicant PI and the co-PI if one is included.

LOIs are due December 5, 2022, 5pm ET.

Project Title	Seizing immune memory to identify emerging henipaviruses
Principal Investigator Name	Krongkan Srimuang, Ph.D.
Position/Title	Research scientist
Department	Thai Red Cross Emerging Infectious Diseases Clinical Center
Institution Name	King Chulalongkorn Memorial Hospital
Street	1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District
City, State, Zip Code	Bangkok 10330
Country	Thailand
Email	Krongkan.sr@gmail.com
Phone	+66 (0) 92-453-0808
CREID Research Center affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)
The category of eligibility of the applicant(s) (see Section 1.3 of Call for Applications)	LMIC Applicants
Co-Principal Investigator Name (*If applicable*)	Spencer Sterling, MPH
Position/Title	Scientific Project Coordinator
Department	Microbiology and Immunology
Institution Name	Uniformed Services University of the Health Sciences
Street	Jones Bridge Road
City, State, Zip Code	Bethesda, MD 20814
Country	USA
Email	spencer.sterling.ctr@usuhs.edu
Phone	+66 (0) 83-494-5980
CREID Research Center affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)
The category of eligibility of the applicant(s) (see Call for Applications, Section 1.3)	

Name of Mentor	Eric Laing, Ph.D.
Mentor Institution	Uniformed Services University of the Health Sciences
Institution Address	Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814
Permanent location of Mentor	4301 Jones Bridge Road, Bethesda, MD 20814
Mentor email	eric.laing@usuhs.edu
Mentor phone	(301) 980-8192

Updated: 10/28/22

Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Name of Mentor	Supaporn Wacharapluesadee, Ph.D.
Mentor Institution	Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital
Institution Address	1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Permanent location of Mentor	4th Floor, Jongkolnee Watwong Building, Thai Red Cross Emerging Infectious Diseases Clinical Center, King Chulalongkorn Memorial Hospital, 1873, Rama 4 Road, Pathumwan Sub-district, Pathumwan District, Bangkok 10330.
Mentor email	spwa@hotmail.com
Mentor phone	+66 (0) 89-920-6292
Research Center Affiliation	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)

Contact information for institution(s) that would financially manage the award *(please note: changing the managing institution upon award may result in substantial funding delays)*	
Contractual Contact, Title	Chula Unisearch
Institution Name	Chulalongkorn University
Street	254 Phyathai Road
City, State, Zip Code	Pathumwan, Bangkok 10330
Country	Thailand
Email	unisearch@chula.ac.th
Phone	0 2218 2880
For Co-Principal Investigator (If applicable):	
Contractual Contact, Title	
Institution Name	
Street	
City, State, Zip Code	
Country	
Email	
Phone	

Overview of proposed research project	
Pathogen(s) focus	Mojiang Virus, Henipavirus, Paramyxovirus
Country(ies) where work will be conducted	Thailand
Research Aims and Objectives (250 words)	

The recent isolation of Langya virus, a novel henipavirus, from patients with acute febrile illness has provided further evidence that henipaviruses remain an emerging zoonotic virus threat to public health. The prototypical henipaviruses, Hendra virus and Nipah virus, are zoonotic viruses hosted by flying foxes (Pteropus genus) across mainland south/southeast Asia and Australia. The detection of henipaviruses, Mojiang virus and Langya virus, infections in rats and shrews changed the standing dogma that fruit bats were the primary wildlife reservoirs of henipaviruses. Continued vigilant zoonotic virus surveillance of sentinel populations remains critical to detect and identify novel henipaviruses before outbreaks of clinical disease that facilitate virus isolation. However, the

detection of viremia and virus isolation from wildlife populations remains elusive for these emerging rodent-borne henipaviruses.

Aim 1. Antibody-mediated capture of the Thai MojV-like virus and discovery

Utilize the MojV RBP antigen to bait and capture MojV-like virus specific memory B cells from seropositive human participants. With support from NIH PREMISE, MojV RBP-reactive B cell receptors will be sequenced to generate monoclonal antibodies specific to the MojV-like virus. These monoclonal antibodies will be used for direct antibody-mediated virus capture in ongoing human and wildlife surveillance.

Aim 2. Multidimensional spatial modeling to characterize antigenic relationships between known and unknown heniapviruses.
In addition to LayV and MojV the henipavirus genus has expanded to include two other shrew-borne viruses, Gamak virus and Daeryong virus, and a Madagascan fruit bat-borne Angovkeely virus. Defining the antigenic relationships of these henipaviruses remains a valuable tool for characterizing the serologic signature of related undiscovered henipaviruses. Anti-RBP standards will be developed and utilized to explore serogroups and the antigenic spatial relationships between known rodent-borne, known bat-borne, and the Thai MojV-like virus.

If you are proposing a human subjects' or animal research study, please describe the already approved study it is nested within and your plan to obtain an IRB or IACUC modification for the proposed study within 2 months of award.

This project will collect samples under approved protocols (EcoHealth Alliance) and (King Chulalongkorn Medical Hospital): (Protocol Numbers). We will seek an IRB amendment for human sampling so that we can collect peripheral blood mononuclear cells (PBMC) during the serological sampling for use in Aim 1.

If you are proposing a study involving a select agent, indicate the select agent and address the issue identified in the Call for Applications, Section 1.2 about prior approval.

From: Krongkan Srimuang on behalf of Krongkan Srimuang <krongkan5678@gmail.com>
To: Hongying Li
Cc: Spencer Sterling; Sasiprapa Ninwattana; Supaporn Wacharapluesadee; eric.laing_usuhs; Peter Daszak
Subject: Re: CREID Network pilot research proposal 2023
Date: Friday, December 2, 2022 6:26:22 AM
Attachments: Biosketch-Krongkan Srimuang.pdf
Biosketch-SterlingSL.pdf
1 LOI Thailand.docx

Dear Hongying,

Please find attached the draft of the LOI from Thailand. The abstract was modified by Eric.
The attached includes my and Spencer's biosketch.

Thank you very much

Best regards,
Krongkan Srimuang

On Tue, Nov 29, 2022 at 8:47 AM Hongying Li <li@ecohealthalliance.org> wrote:

> Dear All,
>
> Thank you for confirming your availability, let's have a quick meeting on:
>
> Wednesday, Nov 30 at 8:00 AM (BKK time)
> Tuesday, Nov 29 at 8:00 PM (Eastern time)
>
> We will discuss the general idea to confirm the information for the LOI (template attached)
>
> I have sent you a calendar invite, also including the Zoom link below, look forward to speaking with you!
>
> Eric - it will be helpful if you're free to join the discussion.
>
> Best regards,
> Hongying
>
> Join Zoom Meeting
> https://ecohealthalliance-org.zoom.us/j/94509492583?pwd=eUwyL05vUWZLUDV3RFljbDFaZVJhdz09
>
> Meeting ID: 945 0949 2583
> Passcode: thailand
>
> On Wed, Nov 23, 2022 at 11:09 PM Krongkan Srimuang <krongkan5678@gmail.com> wrote:
>
> > Dear Hongying,
> >
> > Thank you for the great news!
> > I am available on Monday. What time do you prefer?
> > Please let us know.
> >
> > Thank you very much
> > Best regards,
> > Krongkan
> >
> > On Thu, Nov 24, 2022 at 7:43 AM Spencer Sterling <spencer.sterling.ctr@usuhs.edu> wrote:
> >
> > > Hi Hongying,
> > >
> > > Great news! I will be free either day.
> > >
> > > V/R,
> > > Spencer

Sent from my iPhone

> On Nov 23, 2022, at 11:44 PM, Hongying Li <li@ecohealthalliance.org> wrote:
>
> Dear Krongkan and Spencer,
>
> Thanks for sharing the proposal abstract!
>
> Peter and Kevin reviewed all proposed ideas and think this one perfectly aligns with EID-SEARCH's research objectives and will help answer some important questions.
>
> However, can we meet to further discuss the title and writing regarding the significance of this work? The current text sounds a bit like adding additional lab procedures that have already been covered by current EID-SEARCH work, so we need to make it sound more significant...And please read through the instruction here for the proposal preparation https://creid-network.org/documents/pilot-program/2023/CREIDPilotProgram_CallforApplications_2023.pdf to see the research and objective priorities (e.g., well-defined hypothesis, and human subject research in LOI, etc.)
>
> Please let me know when you will be available for a quick *call anytime next Monday or Tuesday.*
>
> I'm re-attaching two successful applications, as well as some LOIs examples for your reference.
>
> Best,
> Hongying
>
> On Sat, Nov 19, 2022 at 8:42 AM Krongkan Srimuang <krongkan5678@gmail.com> wrote:
>> Dear Hongying,
>>
>> Please find attached the abstract for applying to the CREID Pilot Research Program 2023 from Emerging Infectious Diseases Clinical Center (EIDCC), Thailand.
>> If you require any further information, please feel free to contact us.
>>
>> Thank you very much
>>
>> Best Regards,
>> Krongkan Srimuang
>>
>> On Wed, Nov 16, 2022 at 5:08 PM Hongying Li <li@ecohealthalliance.org> wrote:
>>> Thank you, Bow!
>>>
>>> Kio and Spencer,
>>>
>>> Attached are the two successful applications for 2021 and 2022 for your reference.
>>>
>>> And can you please quickly write up one paragraph of your proposed idea (like the abstract) **before Monday next week**? We have received eight requests to collaborate this year, so Kevin, Peter, and I need to review all of them together on Monday to decide which three to support, and it would be great if you can send something written-down.
>>>
>>> Best,
>>> 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

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

On Wed, Nov 16, 2022 at 4:01 PM Sasiprapa Ninwattana <sasiprapa.n@outlook.com> wrote:

> Dear All,
>
> Attached please find the proposal that we submitted for last year's pilot research program for CREID network for your reference.
> Please note that the letter of intent needs to be submitted by December 5, 2022.
>
> Please feel free to ask me or Hongying any questions you may have.
>
> 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

<Final - CREIDApplication.NguyenVanCuong.Proposal.pdf>
<CREID_Pilot_Program_Application_SIEGERS_HEANG_FINAL_PDF_FOR_SUBMISSION.pdf>
<LOI.Cuong.EID-SEARCH.pdf>
<LOI final signed Vireak Heang Jurre Siegers.pdf>

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: Vincent J. Munster

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6)

POSITION TITLE: Chief, Virus Ecology Unit

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
Utrecht University	MSc	1999 - 2001	Molecular Microbiology
Erasmus University	PhD	2002 - 2006	Molecular Virology
Erasmus Medical Center	Postdoctoral	2006 - 2009	Influenza transmission
Rocky Mountain Laboratories, NIH/NIAID	Postdoctoral	2009 - 2012	Virus Ecology

A. Personal statement

Past outbreaks of bat-borne zoonotic viruses such as coronaviruses, henipaviruses and filoviruses, have had an enormous impact on human and wildlife health. The unpredictability of the zoonotic introductions of these bat-borne limits the potential for effective intervention strategies. One of the main reasons for the absence of successful pre-emptive strategies is our lack of understanding of the ecology, evolution and interaction with the immune system of these pathogens in their respective natural reservoirs. I have gained extensive laboratory and field experience. I have expanded my expertise over the last year towards the field of bat-borne zoonotic diseases (Ebola, Marburg, Nipah and MERS-CoV), conducting field studies in the Republic of Congo and Mali and experimental studies at the Rocky Mountain Laboratories high and maximum containment facility (BSL3 and BSL4). Within the NIAID Virus Ecology unit (http://www.niaid.nih.gov/labsandresources/labs/aboutlabs/lv/virusecology/Pages/default.aspx#niaid_inlineNav_Anchor) my work focuses on natural reservoirs of emerging viruses and elucidation of the underlying biotic and abiotic drivers of zoonotic and cross-species transmission events. Over two decades, I have a demonstrated record of accomplished and productive research resulting in over 190 peer-reviewed publications.

B. Positions and Honors

Positions

2013 – Present Chief, Virus Ecology Unit, Laboratory of Virology, Rocky Mountain Laboratories, NIAID/NIH
2009 – 2012 Post-doctoral fellow, Disease Modelling and Transmission Section, NIAID/NIH.
2006 – 2009 Postdoctoral research fellow at the Department of Virology, Erasmus Medical Center.

Other Experiences and Professional Memberships

2021 Member WHO virus evolution working group
2020 Member WHO animal working group
2020 Member Operation Warp Speed / CAG
2020 NIAID coronavirus response
2018 DRC Ebola Outbreak: NIAID Internal Coordination Working Group
2018 NIAID Viral Pathogen Preparedness Working Group
2018 Scientific Advisory Board, DTRA Western Asia bat research project
2017 FAO-OIE-WHO global technical meeting on MERS-CoV, Geneva
2017 WHO Global Outbreak and Alert Network workshop meeting, Hong Kong
2017 WHO Environmental Contamination of MERS-CoV meeting, Hong Kong
2016 Graduate faculty appointment, Marshall University, Joan C. Edwards School of Medicine

2016	PhD thesis examiner, University of Melbourne, Australia
2015-2016	Member the Scientific Advisory Group of the NIAID workshop on MERS animal models
2015	Member the Scientific Advisory Group organizing the NIAID workshop on MERS animal models.
2015	Member of the ASPR SPIRiT Ebola environmental working group.
2015	Member of the ASPR Science Disaster Preparedness working group.
2014-2015	Team lead of the combined WHO - CDC/NIH diagnostic laboratory during the Ebola virus outbreak, Monrovia, Liberia.
2014	American Society for Virology, Program Planning Committee.
2014	Moderator, IOM/NRC Workshop on Research Priorities to Inform Public Health and Medical Practice for Domestic Ebola Virus Disease, Institute of Medicine of the National academy of Sciences.
2014	Organizer of the fifth ESWI Influenza Conference in Riga, Latvia.
2014	Scientific organizing committee of the Endemic and Emerging Viral Diseases of Priority in the Middle East and North Africa.
2013	WHO-ISARIC joint MERS-CoV Outbreak Readiness Workshop.
2013	Member Coronavirus Therapeutics Interagency Working Group (NIH, CDC, BARDA and DoD).
2013	Moderator, NIAID MERS-CoV Research: Current Status and Future Priorities Meeting.
2013	Editor for PLoS One, One Health and Frontiers in Cellular and Infection Microbiology
2011-2014	Board member of the European Scientific Working group on Influenza.
2008	Member of the OIE ad hoc Group on Wildlife Disease Surveillance.
2005-present	Reviewer for journals including: Lancet Infectious Diseases, Nature, Nature Medicine, PNAS and Science.

Grant application reviewer

2019	AAAS for the Saudi Arabia's Ministry of Education Research
2018	BBSRC, UK
2018	Italian Ministry of Health
2017	BBSRC (Biotechnology and Biological Sciences Research Council), UK
2016	USAID Combating Zika and Future Threats Grand Challenge
2016	FINOVI Foundation, France
2015	Italian Ministry of Health
2015	Referee Panel for Health and Medical research Fund, HKSAR
2015	Human Frontier Science Program
2012	Referee Panel for Health and Medical research Fund, HKSAR
2008-2009	National Medical Research Council, Singapore
2008	Minnesota Center of Excellence for Influenza Research and Surveillance, USA
2007	Department for Environment, Food and Rural Affairs, UK

Miscellaneous

2014-2015	Team lead of the combined CDC/NIH diagnostic laboratory under GOARN/WHO during the Ebola virus outbreak, Monrovia, Liberia, providing diagnostic services to several Ebola treatment units in the Monrovia area
2015-2018	Thompson Reuters / Clarivate highly cited researcher 2015, 2016, 2017 and 2018

Honors

2020	AAAS Golden Goose award, COVID-19 response
2016	European Society for Virology young investigator award
2015	NIH Director's award, in recognition of the establishment and running of diagnostic field laboratory during the Ebola virus outbreak in West Africa.
2014	NIAID merit award for the development of a nonhuman primate disease model and a treatment strategy for MERS-CoV
2014	ASM IAAC young investigator award
2014	NIH Director's award, in recognition of the exceptional and rapid response to the emergence of MERS-coronavirus.
2011	European Scientific Working group on Influenza, Best Body of Work award for Young Scientists.

C. Contributions to Science

1 During my PhD working on avian influenza at Erasmus Medical Center, it became clear to me that there was a lack of integration between bench virology and fieldwork. Being trained as a "classical" molecular virologist I could very well answer questions on what mutations would make a virus more pathogenic, but the translation of this knowledge to real world situations remained elusive. Integrating various disciplines helped me to understand the ecology and drivers of avian influenza and pandemic influenza outbreaks.

- **Munster VJ**, Wallensten A, Baas C, Rimmelzwaan GF, Schutten M, Olsen B, Osterhaus AD, Fouchier RA. Mallards and highly pathogenic avian influenza ancestral viruses, northern Europe. Emerg Infect Dis. 2005;11(10):1545-51
- Olsen B, **Munster VJ**, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA. Global patterns of influenza a virus in wild birds. Science. 2006;312(5772):384-8
- **Munster VJ**, Baas C, Lexmond P, Waldenstrom J, Wallensten A, Fransson T, Rimmelzwaan GF, Beyer WE, Schutten M, Olsen B, Osterhaus AD, Fouchier RA. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog. 2007;3(5):e61
- **Munster VJ**, de Wit E, van den Brand JM, Herfst S, Schrauwen EJ, Bestebroer TM, van de Vijver D, Boucher CA, Koopmans M, Rimmelzwaan GF, Kuiken T, Osterhaus AD, Fouchier RA. Pathogenesis and transmission of swine-origin 2009 A(H1N1) influenza virus in ferrets. Science. 2009;325(5939):481-3
- Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, de Wit E, **Munster VJ**, Sorrell EM, Bestebroer TM, Burke DF, Smith DJ, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. Airborne transmission of influenza A/H5N1 virus between ferrets. Science. 2012;336(6088):1534-41.

2 My research group at the Rocky Mountain Laboratories of the NAID/NIH is built around the concept of complete integration of fieldwork, experimental work and computational modeling to allow study of emerging viruses in their natural, intermediate and human hosts. It is clear that past outbreaks of zoonotic viruses have had an enormous impact on human and wildlife health and that the unpredictability of cross-species transmission events limits the potential for effective intervention strategies. My lab aims to identify the underlying changes in virus-host ecology that allow these viral pathogens to cross the species barrier. Recognizing the strengths and weaknesses of a unilateral focus on field research on one hand and experimental research on the other, we have combined both approaches in one research program. This integrated approach has contributed greatly to the rapid advancements in our knowledge on the emergence of MERS-CoV. Using a combination of fundamental concepts of host species restriction of MERS-CoV and protein-protein binding interaction modeling we predicted potential MERS-CoV host species, which were tested in experimental infections. Our field studies in the Middle East confirmed camels as the primary reservoir for MERS-CoV.

- **Munster VJ**, de Wit E, Feldmann H. Pneumonia from human coronavirus in a macaque model. N Engl J Med. 2013;368(16):1560-2.
- de Wit E, Rasmussen AL, Falzarano D, Bushmaker T, Feldmann F, Brining DL, Fischer ER, Martellaro C, Okumura A, Chang J, Scott D, Benecke AG, Katze MG, Feldmann H, **Munster VJ**. Middle East respiratory syndrome coronavirus (MERS-CoV) causes transient lower respiratory tract infection in rhesus macaques. Proc Natl Acad Sci U S A. 2013;110(41):16598-603
- Alagaili AN, Briese T, Mishra N, Kapoor V, Sameroff SC, Burbelo PD, de Wit E, **Munster VJ**, Hensley LE, Zalmout IS, Kapoor A, Epstein JH, Karesh WB, Daszak P, Mohammed OB, Lipkin WI. Middle East respiratory syndrome coronavirus infection in dromedary camels in Saudi Arabia. MBio. 2014;5(2):e00884-14
- van Doremalen N, Miazgowicz KL, Milne-Price S, Bushmaker T, Robertson S, Scott D, Kinne J, McLellan JS, Zhu J, **Munster VJ**. Host species restriction of Middle East respiratory syndrome coronavirus through its receptor, dipeptidyl peptidase 4. J Virol. 2014;88(16):9220-32.
- Adney DR, van Doremalen N, Brown VR, Bushmaker T, Scott D, de Wit E, Bowen RA, **Munster VJ**. Replication and shedding of MERS-CoV in upper respiratory tract of inoculated dromedary camels. Emerg Infect Dis. 2014;20(12):1999-2005.
- **Munster VJ**, Adney DR, van Doremalen N, Brown VR, Miazgowicz KL, Milne-Price S, Bushmaker T, Rosenke R, Scott D, Hawkinson A, de Wit E, Schountz T, Bowen RA. Replication and shedding of MERS-CoV in Jamaican fruit bats (*Artibeus jamaicensis*). Sci Rep. 2016;6:21878.
- de Wit E, van Doremalen N, Falzarano D, **Munster VJ**. SARS and MERS: recent insights into emerging coronaviruses. Nat Rev Microbiol. 2016. doi: 10.1038/nrmicro.2016.81.
- van Doremalen N, Falzarano D, Ying T, de Wit E, Bushmaker T, Feldmann F, Okumura A, Wang Y, Scott DP, Hanley PW, Feldmann H, Dimitrov DS, **Munster VJ**. Efficacy of antibody-based therapies against Middle East respiratory syndrome coronavirus (MERS-CoV) in common marmosets. Antiviral Res. 2017. doi: 10.1016/j.antiviral.2017.03.025.
- van Doremalen N, Hijazeen ZS, Holloway P, Omari BA, McDowell C, Adney D, Talafha HA, Guitian J, Steel J, Amarin N, Tibbo M, Abu-Basha E, Al-Majali AM, **Munster VJ**, Richt JA. High Prevalence of Middle East Respiratory Coronavirus in Young Dromedary Camels in Jordan. Vector Borne Zoonotic Dis. 2016. doi: 10.1089/vbz.2016.2062.
- Schountz T, Baker ML, Butler J, **Munster VJ**. Immunological Control of Viral Infections in Bats and the Emergence of Viruses Highly Pathogenic to Humans. Front Immunol. 2017;8:1098. doi: 10.3389/fimmu.2017.01098.

3 My research group was directly involved in the response to several outbreaks including the Ebola virus outbreak in West Africa by providing diagnostic support at the request of the WHO for several Ebola treatment units in Monrovia, Liberia and SARS-CoV-2. The emergence of Ebola virus in West Africa highlighted significant gaps in our knowledge, including fundamental ecological questions surrounding zoonotic and human-to-human transmission. In order to understand the drivers of transmission, we examined the stability of the virus within tissues and on body surfaces and determined the potential for transmission. The results from this study directly aided the interpretation of epidemiologic data collected from human corpses and are also applicable to interpreting samples collected from remains of wildlife infected with Ebola virus, especially nonhuman primates, and helped to assess the risk of zoonotic transmission. With the current COVID19 pandemic we are actively involved in the development of medical countermeasures and providing critical experimental data supporting direct public health decisions and interventions.

- Mate SE, Kugelman JR, Nyenswah TG, Ladner JT, Wiley MR, Cordier-Lassalle T, Christie A, Schroth GP, Gross SM, Davies-Wayne GJ, Shinde SA, Murugan R, Sieh SB, Badio M, Fakoli L, Taweh F, de Wit E, van Doremalen N, **Munster VJ**, Pettitt J, Prieto K, Humrighouse BW, Stroher U, DiClaro JW, Hensley LE, Schoepp RJ, Safronetz D, Fair J, Kuhn JH, Blackley DJ, Laney AS, Williams DE, Lo T, Gasasira A, Nichol ST, Formenty P, Kateh FN, De Cock KM, Bolay F, Sanchez-Lockhart M, Palacios G. Molecular Evidence of Sexual Transmission of Ebola Virus. N Engl J Med. 2015;373(25):2448-54
- de Wit E, Falzarano D, Onyango C, Rosenke K, Marzi A, Ochieng M, Juma B, Fischer RJ, Prescott JB, Safronetz D, Omballa V, Owuor C, Hoenen T, Groseth A, van Doremalen N, Zemtsova G, Self J, Bushmaker T, McNally K, Rowe T, Emery SL, Feldmann F, Williamson B, Nyenswah TG, Grolla A, Strong JE, Kobinger G, Stroeher U, Rayfield M, Bolay FK, Zoon KC, Stassijns J, Tampellini L, de Smet M, Nichol ST, Fields B, Sprecher A, Feldmann H, Massaquoi M, **Munster VJ**. The Merits of Malaria Diagnostics during an Ebola Virus Disease Outbreak. Emerg Infect Dis. 2016;22(2)
- Miller MR, McMinn RJ, Misra V, Schountz T, Muller MA, Kurth A, **Munster VJ**. Broad and Temperature Independent Replication Potential of Filoviruses on Cells Derived From Old and New World Bat Species. J Infect Dis. 2016
- **Munster V,** Munoz-Fontela C, Olson SH, Seifert SN, Sprecher A, Ntoumi F, Massaquoi M, Mombouli JV. Outbreaks in a Rapidly Changing Central Africa - Lessons from Ebola. N Engl J Med. 2018.
- van Doremalen N, Bushmaker T, Morris DH, Holbrook MG, Gamble A, Williamson BN, Tamin A, Harcourt JL, Thornburg NJ, Gerber SI, Lloyd-Smith JO, de Wit E, **Munster VJ**. Aerosol and Surface Stability of SARS-CoV-2 as Compared with SARS-CoV-1. N Engl J Med. 2020.
- Letko M, Marzi A, **Munster V**. Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. Nat Microbiol. 2020.
- **Munster VJ**, Koopmans M, van Doremalen N, van Riel D, de Wit E. A Novel Coronavirus Emerging in China - Key Questions for Impact Assessment. N Engl J Med. 2020;382(8):692-4.
- **Munster VJ**, Feldmann F, Williamson BN, van Doremalen N, Perez-Perez L, Schulz J, Meade-White K, Okumura A, Callison J, Brumbaugh B, Avanzato VA, Rosenke R, Hanley PW, Saturday G, Scott D, Fischer ER, de Wit E. Respiratory disease in rhesus macaques inoculated with SARS-CoV-2. Nature. 2020
- van Doremalen N, Lambe T, Spencer A, Belij-Rammerstorfer S, Purushotham JN, Port JR, Avanzato VA, Bushmaker T, Flaxman A, Ulaszewska M, Feldmann F, Allen ER, Sharpe H, Schulz J, Holbrook M, Okumura A, Meade-White K, Perez-Perez L, Edwards NJ, Wright D, Bissett C, Gilbride C, Williamson BN, Rosenke R, Long D, Ishwarbhai A, Kailath R, Rose L, Morris S, Powers C, Lovaglio J, Hanley PW, Scott D, Saturday G, de Wit E, Gilbert SC, **Munster VJ**. ChAdOx1 nCoV-19 vaccine prevents SARS-CoV-2 pneumonia in rhesus macaques. Nature. 2020
- Victoria A. Avanzato, M. Jeremiah Matson, Stephanie N. Seifert, Rhys Pryce, Brandi N. Williamson, Sarah L. Anzick, Kent Barbian, Seth D. Judson, Elizabeth R. Fischer, Craig Martens, Thomas A. Bowden, Emmie de Wit, Francis X. Riedo, **Vincent J. Munster**. Prolonged infectious SARS-CoV-2 shedding from an immunocompromised patient with chronic lymphocytic leukemia and acquired hypogammaglobulinemia. Cell 2021.

Publications in peer-reviewed journals: 190
Citations: >28000
H-factor: 61

ORCID: 0000-0002-2288-3196
ResearcherID: I-7607-2018

Research Support

2019 CEPI ChAd-Ox vaccine development for MERS-CoV, Nipah and Lassa virus, $1,281,810
2018 CEPI Lassa mRNA vaccine, CureVac, $998,000

2018	EDCTP (European & Developing Countries Clinical Trial Partnership). EDCTP2 Call for Proposals - Mobilization of research funds in case of Public Health Emergencies - RIA2018 Emergency Funding Mechanism Proposal: RIA2018EF-2082 — EPIRISK-EBOV. €500,000.
2018	Department of Defense, DARPA grant 'PREEMPT', $1,358,133
2018	CEPI MERS-DNA vaccine "Translational portfolio program encompassing cGMP manufacturing and clinical development of DNA vaccine candidates against both Lassa virus and MERS coronavirus." $143,980.
2016	Department of Defense, DARPA grant 'Thunder', $414,000
2015-2016	ARCUS foundation, Improving Laboratory Capacity in the Republic of Congo as a Foundation for Understanding and Mitigating the Threat of Ebola to Great Apes and People, Co-PI.
2013-2020	NIH, NIAID Division of Intramural Research support for the Virus Ecology Unit, PI.
2013-2020	International Centers of Excellence in Research Center, Brazzaville, Republic of Congo, PI.
2014-2015	Biomedical Advanced Research and Development Authority, MERS-CoV countermeasures, PI.
2013-2014	US Fish and Wildlife, wildlife without borders –Africa program. "Satellite telemetry and the landscape ecology of Hammer-headed fruit bats (*Hypsignathus monstrosus*) in Odzala National Park, Republic of Congo", PI.

From: Munster, Vincent (NIH/NIAID) [E] on behalf of Munster, Vincent (NIH/NIAID) [E] <vincent.munster@nih.gov>
To: Madeline Salino; Laing, Eric; Sarah Munro; Jon Epstein
Subject: RE: [EXTERNAL] NIH Biosketch for Liberia R01 proposal
Date: Tuesday, November 29, 2022 12:16:26 PM
Attachments: biosketchMunsternew.docx

Vincent Munster, PhD
Chief Virus Ecology Section
Rocky Mountain Laboratories
NIAID/NIH

From: Madeline Salino <salino@ecohealthalliance.org>
Sent: Tuesday, November 29, 2022 10:01 AM
To: Laing, Eric <eric.laing@usuhs.edu>; Munster, Vincent (NIH/NIAID) [E] <vincent.munster@nih.gov>; Sarah Munro <munro@ecohealthalliance.org>; Jon Epstein <epstein@ecohealthalliance.org>
Subject: [EXTERNAL] NIH Biosketch for Liberia R01 proposal

Hello, all!

Can you please send me your current NIH-formatted biosketches for inclusion in the Liberia R01 proposal? I would like to collect them before the holidays, so if you could send them by December 21st, that would be excellent.

I've attached the current format (and here is the NIH biosketch webpage). Please let me know if I can answer any questions.

Thank you,
Madeline

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Madeline Salino
Science and Outreach Administrative Assistant
EcoHealth Alliance
520 Eighth Avenue, Ste. 1200
New York, NY 10018
Office: (212) 380-4460 x4513

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

Application Cover Sheet

Project Title	Mixed-species farming, viral diversity, and risks of cross-species transmission in the Central Highlands of Viet Nam
Principal Investigator Name	Nguyen Van Cuong
Position/Title	Project Coordinator and PhD candidate (viva data: 03/15/21)
Department	Centre for Tropical Medicine
Institution Name	Oxford University Clinical Research Unit
Street	764 Vo Van Kiet, Ward 1, District 5
City, State, Zip Code	Ho Chi Minh City, TP Ho Chi Minh, 700000
Country	Viet Nam
Email	cuongnv@oucru.org
Phone	+84 8 39237954
Country(ies) where work will be conducted	Viet Nam
Pathogen(s) focus	Zoonoses, Coronaviruses, Paramyxoviruses

Collaborating CREID Research Center	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)
Contact PI	Peter Daszak
CREID RC Primary Point of Contact	Hongying Li
CREID RC Primary Point of Contact Location or Institution	EcoHealth Alliance (EHA)

Name of Mentor	Cadhla Firth
Mentor Institution	EcoHealth Alliance
Institution Address	520 Eighth Avenue, Ste. 1200 New York, NY 10018
Permanent location of Mentor	New York, NY
Mentor email	firth@ecohealthalliance.org
Mentor phone	+61 447 361 669

Total Budget	**$149,970.58**
Direct Costs	$138,861.64
Indirect Costs	$11,041.17
Proposed Start Date	06/01/2021
Proposed End Date	05/31/2021

Project Abstract

Viet Nam sits within a critical Southeast Asian emerging infectious disease hotspot with diverse wildlife and a rapidly increasing human population. In this region, wildlife farming is a growing but inadequately regulated industry that exhibits many features thought to contribute to increased risks of cross-species transmission and zoonotic disease emergence. We will focus on a key zoonotic disease pathway that was involved in the emergence of Nipah virus, SARS-CoV, and potentially SARS-CoV-2 – the spillover of viruses from wildlife to multi-species farms, and subsequently to people. We will test a key hypothesis: viral diversity and cross-species transmission will increase with species

diversity on mixed-species farms, and this pattern will be further amplified by the presence of wildlife species. We will access a previously untouched sample set from 64 mixed-species farms in a biodiverse region of central Viet Nam where wildlife and domestic species are farmed both together and separately. We will characterize viral diversity in single- and mixed-species farms using metagenomics and PCR, identify their zoonotic potential using ecological and phylogenetic methods, and estimate risk of emergence by analyzing distribution and prevalence. To inform the local community of our results, we will develop a two-way dialogue with farmers and government stakeholders in Viet Nam regarding potential disease risks associated with wildlife and mixed-species farming. Importantly, this project builds capacity for emerging infectious disease research in Southeast Asia by supporting the in-country development of analytically robust, hypothesis-driven research into the ecology and evolution of emerging infectious diseases.

Applicant Name (Last, first, middle): Nguyen, Cuong, Van

Title

Mixed-species farming, viral diversity, and risks of cross-species transmission in the Central Highlands of Viet Nam.

Study Personnel

The PI on this project, Dr. Cuong Nguyen, is ideally suited to conduct and manage this project. Dr. Nguyen is trained as a veterinarian and will be defending his PhD thesis (Open University, United Kingdom (UK)) focused on veterinary epidemiology and antimicrobial usage on farms in Viet Nam on 15 March 2021. Dr. Nguyen possesses experience in performing both laboratory- and field-based research, as well as translating research outcomes into locally-relevant interventions and policy recommendations. These efforts have resulted in 20 peer-reviewed publications since 2018. Dr. Nguyen has worked with the Oxford University Clinical Research Unit (OUCRU) since 2011, during which time he acted as a coordinator of veterinary fieldwork for the Wellcome Trust-funded Vietnamese Initiative for Zoonotic Infections (VIZIONS) project, which ended in 2016, and under which the samples for the proposed project were collected. Dr. Nguyen will be responsible for project coordination, data analysis, public engagement, and presentation/publication of the research conducted under the proposed project. He will be allocated 100% protected time to work on this project and his professional development over the one-year period. To ensure that the proposed research can be accomplished on time and within budget, all laboratory work will be completed by a Senior Research Assistant who will be supervised directly by Dr. Nguyen within the Molecular Epidemiology group at OUCRU. Several members of this research group were part of the VIZIONS team and are familiar with the sampling and laboratory protocols required to ensure the completion of this study in a timely manner.

As co-lead of OUCRU's Molecular Epidemiology research group, Dr. Maia Rabaa will be a collaborator on this project and will supervise Dr. Nguyen's research throughout the proposed project. Dr. Rabaa is a molecular epidemiologist with eight years of experience working at OUCRU, and contributed to the design, implementation, and analysis of data related to the VIZIONS project. Dr. Rabaa and her team have access to the relevant samples, were involved in the design and conduct of the VIZIONS project, have significant expertise in sequencing and phylogenetic analysis, and will provide additional support and advice during Dr. Nguyen's laboratory work, analysis, and writing. As the group lead, Dr. Rabaa will also supervise Dr. Nguyen's progress and administrative tasks and assist in the further development of his budget and human resources management skills as he progresses toward scientific independence.

Dr. Cadhla Firth will act as Mentor for Dr. Nguyen under the Centers for Research in Emerging Infectious Diseases (CREID) Pilot Research Program. Dr. Firth is Senior Research Scientist and Program Coordinator at EcoHealth Alliance (EHA), which houses the Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH) program. Dr Firth has more than 15 years' experience conducting research on emerging infectious diseases, with a focus on the ecology and evolution of viruses in animal populations. Dr. Firth's expertise in study design, molecular biology, metagenomic sequencing, and the analysis of metagenomic and ecological data match well with Dr. Nguyen's interests and the proposed project. Dr. Firth will provide scientific guidance, analytical training, mentoring, and career advice, as well as networking opportunities that support Dr. Nguyen's progression toward independence.

As colleagues on the VIZIONS project, Dr. Nguyen and Dr. Rabaa have built a strong collaborative foundation that complements the peer mentorship that Dr. Rabaa and Dr. Firth established in 2008 during their respective PhD studies under Prof Edward Holmes, and which continues to this day. With Dr. Firth's recent move to EHA, Dr. Rabaa's promotion to Group Head and subsequent collaboration with EHA, and the completion of Dr. Nguyen's PhD work, this team is now in an advantageous position to deliver a strong research project with tangible outcomes under the CREID Pilot Research Program.

Research Aims and Objectives

This project aims to answer the question: "How does species diversity influence viral diversity and the risk of cross-species transmission on mixed-species farms?".

We hypothesize that: (i) viral diversity and cross-species transmission will increase with species diversity on mixed-species farms, (ii) the presence of both wildlife and livestock species on a farm will be associated with increased viral diversity, and (iii) the diversity of viruses with zoonotic potential will increase with species diversity on mixed-species farms. To test these hypotheses, we will access a unique sample set from farms in the Central Highlands of Viet Nam, where a variety of wildlife (e.g., deer, bamboo rats, civets, wild boars, rabbits, porcupines) and domestic species (e.g., chickens, ducks, cats, pigs, goats) are farmed both together and separately. Mixed-species farms create new interfaces between wildlife, domestic animals, and people that may provide opportunities for cross-species transmission of viruses to occur (**Figure 1**).

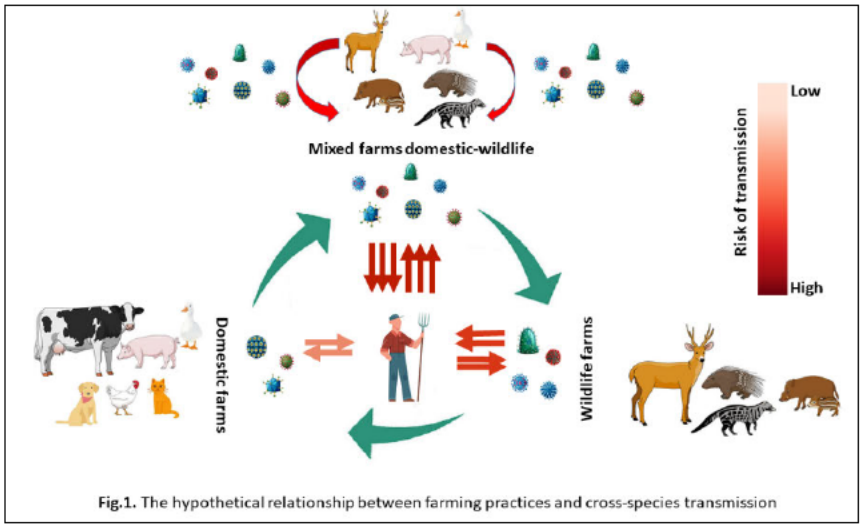


Fig.1. The hypothetical relationship between farming practices and cross-species transmission

The proposed project has three aims:

AIM 1: Estimate the frequency of viral sharing between animals of different species under mixed- and single-species farming conditions and identify 'risky' viruses that commonly cross the species barrier under each of these conditions.

AIM 2: Identify environmental and ecological factors associated with variations in viral prevalence, diversity, or rates of cross-species transmission of 'risky' viruses.

AIM 3: Identify farm management policies and practices to increase awareness and reduce the risk of cross-species transmission of viruses associated with mixed-species farms.

The proposed project has three objectives:

OBJ 1: Characterize the viral diversity present in samples from animals housed on single and mixed-species farms using a combination of metagenomics and polymerase chain reaction (PCR)-based screening.

OBJ 2: Identify viruses with known or predicted zoonotic potential using ecological and phylogenetic methods. Estimate the distribution, prevalence, and relatedness of these viruses across animal species and farms.

OBJ 3: In collaboration with the policy and public engagement departments at OUCRU, engage in two-way dialogues with government stakeholders and the farming community in the Central Highlands about potential disease risks associated with wildlife and mixed-species farming.

Study Rationale/Research Gap/Impact

On July 23, 2020, the Prime Minister of Viet Nam issued a new directive (Directive 29/CT-TTg) [1] calling for increased enforcement of legislation regarding the control of wildlife hunting and trade, which has been widely interpreted as a move toward a complete ban on wildlife trading [2]. This follows similar legislation passed in China in February 2020, which introduced new regulations banning almost all consumption and trade of terrestrial wild animals in the country, and which is expected to cause knock-on policy effects across Asia [3]. Commercial wildlife farming has been proposed as a potential solution to problems caused by a ban on wildlife hunting and trade by creating a regulated and monitored source for wildlife products, while supporting conservation efforts and addressing cultural, economic, and food security issues that may otherwise drive these practices into unregulated marketplaces [4–6]. However, wildlife have also been associated with the emergence of many zoonotic diseases, and several features of wildlife or mixed-species farms suggest that they may pose a greater risk to people than domestic farms alone [7–10].

More than 175 wildlife species are farmed in Viet Nam, including those previously associated with zoonotic transmission risks (e.g., civets, primates, rodents) [11]. However, a recent survey of more than 4000 wildlife farms in southern Viet Nam revealed that 70% of farms also raised domestic species (including livestock) [11]. Critically, pathogens with broad host ranges are significantly more likely to emerge in human populations, which suggests that the presence of multiple species (both wild and domestic) on the same farms may be cause for concern due to the potential for cross-species transmission and viral amplification [12–15]. The risks posed by wildlife farming are further complicated by the high frequency of wildlife laundering, which was the dominant form of wildlife production on 26 Vietnamese farms surveyed between 2014 and 2015 [4]. The frequent movement of wildlife from the natural environment into a domestic setting creates a conduit for the emergence of novel pathogens, including those with zoonotic potential. In addition, the majority of farmed wildlife are raised for food, yet there is a lack of veterinary oversight and infection control measures on Vietnamese wildlife farms that is not mirrored in the farming of domestic species in the region. In a census of more than 4000 wildlife farms in southern Viet Nam, an absence of veterinary care was noted in nearly all cases, along with high numbers of sick and dead animals from unknown causes [11].

Despite the clear risks associated with wildlife farming in Viet Nam, there is very limited understanding of the impacts of wildlife and mixed-species farming practices on viral diversity, virus sharing, and zoonotic transmission [15]. However, the renewed interest in wildlife hunting, farming, and trading that has resulted from the hunt for the origin of SARS-CoV-2 has led to a resurgence in effort to understand the impacts of the policies and practices around wildlife farm management. Although a stated aim of this work is to reduce human and animal disease risk, and zoonotic transmission in particular, there remains an extreme paucity of data related to the risks posed by wildlife farming, or specific farming practices that contribute to this risk. For the first time in the proposed project, we will explicitly test the relationships between species diversity and composition, viral diversity, and cross-species transmission on wildlife and mixed-species farms.

Thus, in line with the goals of the CREID network and the EID-SEARCH team, we aim to use previously collected samples from Vietnamese farms to systematically characterize the diversity and transmission of viruses within animal populations on mixed-species farms. We will assess the risk these viruses may pose to human and animal populations, and use these data to assist in the development of farm management practices and policies to mitigate such risks.

Significance and approach

Wildlife farming, hunting, and trade have repeatedly been associated with the emergence of new zoonotic diseases, including SARS-CoV, SARS-CoV-2, monkeypox virus, and Ebola and Marburg filoviruses [16]. However, strikingly little is known about the drivers of disease emergence from wildlife, particularly with respect to the food chain, including wildlife farms. Previous studies on virus diversity and viral sharing along wildlife supply chains have relied exclusively on consensus PCR-based virus detection or have focused on only a single animal species, even on mixed-species farms [15,17,18]. In contrast, this project will use cutting-edge genomic approaches in ecology and evolution within a hypothesis-driven framework to reveal the determinants of viral diversity, abundance, and transmission within mixed-species farms. To our knowledge, this will be the first data-driven project with the goal of providing a foundation for understanding the roles of mixed-species farming, wildlife farming, and species-species interactions as drivers of disease emergence.

Data collected as part of the VIZIONS project represents a unique opportunity to build a holistic picture of the links between wildlife farming practices, viral diversity, and cross-species transmission. VIZIONS, which ended in 2016, involved international research institutions collaborating with Vietnamese governmental organizations and hospitals to collect samples at three sites across Viet Nam to study clinical illness, epidemiology, pathogen diversity, and social and behavioral factors within a One Health framework. The VIZIONS project had two fundamental components: 1) a hospital disease surveillance program to characterize endemic infections, novel infections, and diseases of unknown origin in humans, and 2) a high-risk human sentinel cohort (HRSC) to assess zoonotic disease incidence and cross-species transmission through sampling of both HRSC members and the animals with which they had contact [19]. Most HRSC cohort members (72.8%) were individuals living on farms, with 53.7% of members in Dak Lak Province reporting exposure to farmed wildlife, including deer, bamboo rats, civets, wild boars, rabbits, and porcupines [20]. We have access to untouched animal samples from this unique dataset from Dak Lak Province, which are linked to publicly available metagenomic data [21,22] from co-located samples from farm workers, and to questionnaire data designed to assess zoonotic disease risk. This provides an unprecedented opportunity to examine multiple dimensions of risk in a complex and highly topical system.

This study also provides an opportunity to link zoonotic disease research back to the communities most at risk of novel disease emergence, including the farmers and government health officials who make daily decisions that directly influence disease risk. Community and government engagement activities were key components of the VIZIONS project and these relationships have been maintained by the OUCRU public engagement team. This will enable a dialogue between the project team and a network of key stakeholders (i.e., farmers, provincial and regional animal health officers, public health officials, and representatives of the Departments of Agriculture and Forestry), through which we can identify potential risks associated with mixed-species farming and wildlife trade in these communities. The result of this engagement activity will be an understanding of how policy is implemented at the local level, and the identification of avenues for future collaborative research and engagement that will benefit the community and expand the CREID and EID-SEARCH networks to improve our understanding and response to the risk of zoonotic virus emergence in Viet Nam and across the region.

Research Methods

Site and sample selection.

Our metagenomic analysis will use animal samples previously collected as part of a three-year high-risk cohort study conducted across Dak Lak Province in Viet Nam's Central Highlands (**Figure 2**). From March 2012 to September 2015, human and animal samples were collected from 64 farms across Dak Lak at multiple time points (median of five visits per farm), including during reported episodes of human illness. Human samples included nasopharyngeal swabs, rectal swabs, and sera, and were associated with questionnaire data to assess human-animal contact and potential risk factors for zoonotic transmission. Human samples collected during illness have been previously analyzed by metagenomic analysis [21,22], and the data are available for comparison with the results of this study. Animal samples primarily included feces and/or rectal swabs (N = 3675), nasal and/or throat swabs (N = 2499), and sera (N = 1245), which were collected from 2251 live animals, comprised of 19 species (12 domestic species, seven wildlife species). These uninterrogated animal samples have been stored at OUCRU in -80°C freezers since the time of collection and are available for metagenomic sequencing in this study.

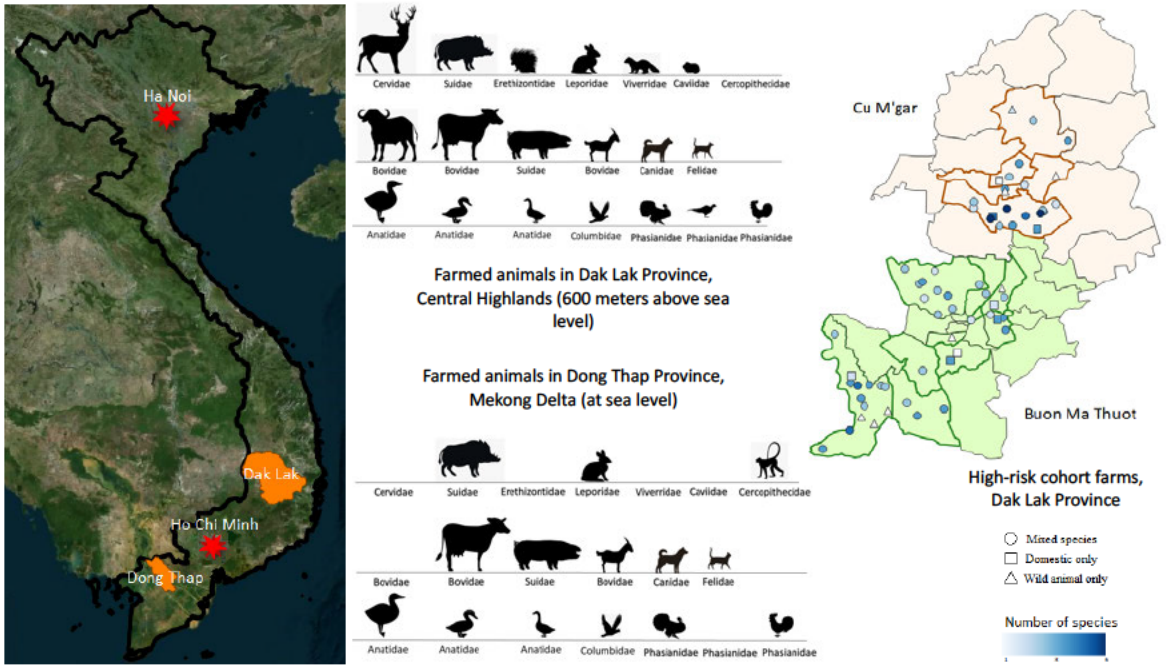


Figure 2. Summary of farmed animals sampled within the VIZIONS project (Dak Lak & Dong Thap Provinces), and distribution of domestic, wildlife, and mixed farms in Dak Lak Province, Viet Nam.

Of the 64 farms sampled as part of the cohort study, 67% contained both domestic and wildlife species, 27% contained domestic species only, and 6% contained wildlife only. The number of species per farm ranged from one to nine, with a median value of five species/farm. To explore the relationships between mixed-species farming, viral diversity, and cross-species transmission, we will select a subset of sampled farms for metagenomics that best capture a gradient of farming intensity in this region. These will include farms with low, medium and high species diversity, as well as farms with and without wildlife (**Figure 3**). Farms will also be selected to achieve maximum overlap in species composition, whereby the species sampled on low diversity farms are also represented on high diversity farms. As such, we will initially target the most frequently sampled domestic (e.g., chickens, ducks, pigs, cats) and wildlife species (e.g., porcupines, rabbits, wild boar, deer) across farms.

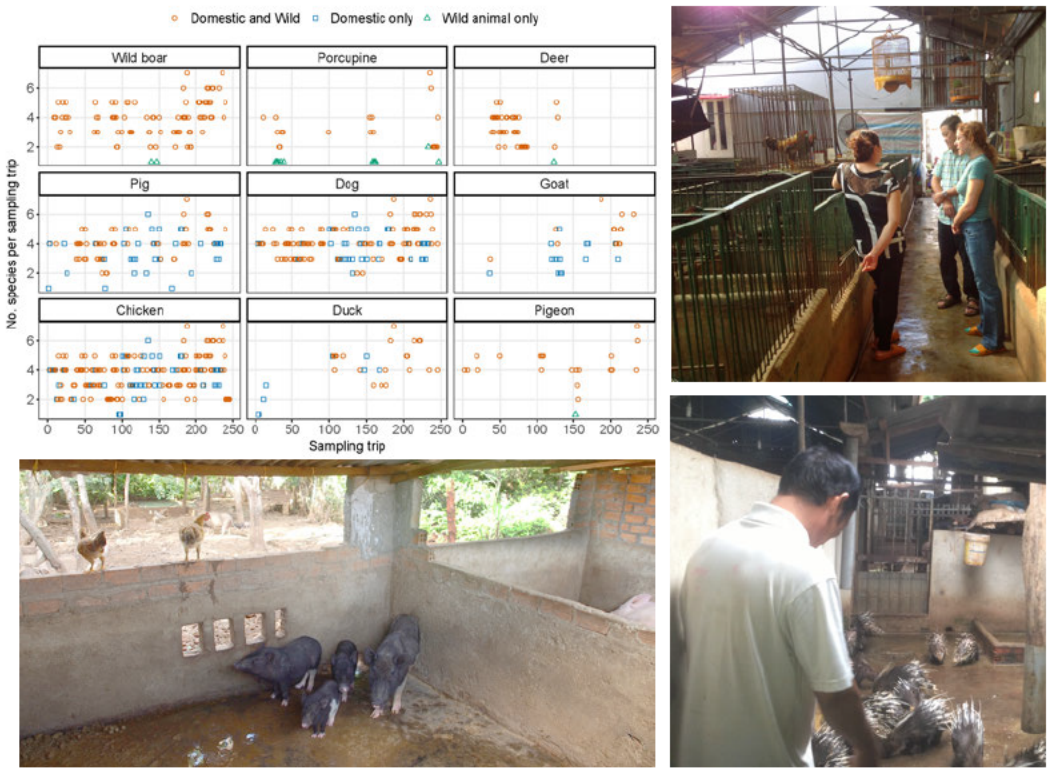


Figure 3. The number of animal species sampled per sampling trip, according to species (select data shown). For example, in the top left corner, wild boar were the only species present on a farm at two sampling events, while one or more other species was sampled on all other sampling trips involving wild boar. Different shapes and colors differentiate farm types (domestic, wild, and mixed wild/domestic). Additional images illustrate farming practices and species diversity on Dak Lak farms.

RNA isolation and metagenomic sequencing.

It has been estimated that only a very small fraction of viral diversity on earth has been described, such that our ability to contextualize disease emergence and cross-species transmission is strongly skewed towards viral families that have already been well-characterized [23]. This bias is further amplified by the widespread use of 'consensus PCR' assays as a mechanism of virus discovery, as this approach attempts to detect new viruses by using sequences from conserved regions of the genome (i.e., primers) of known viruses. As such, this method is likely to be unable to detect viruses that are significantly divergent from those which are already known. In contrast, many recent studies have demonstrated that a metagenomic approach has the ability to reveal the entire virus composition of a sample (i.e., the virome), as well as the relative abundances of each virus [23,24]. We will use this approach to provide a comprehensive view of all viruses present in our dataset.

To comprehensively characterize viromes at both the farm and species level, high quality total ribonucleic acid (RNA) will be extracted from 1000 fecal samples and/or rectal swabs, and 1000 nasal and/or throat swabs (henceforth respiratory swabs) from animals sampled from the subset of farms described above. Fecal samples were selected due to their association with high viral diversity (e.g., coronaviruses, picornaviruses) and the potential importance of fecal-oral transmission in viral emergence and spread [25–27]. Respiratory swabs were selected due to the potential to detect respiratory viruses, which have been associated with multiple significant emerging viral diseases (e.g., SARS, COVID-19, influenza) in the past [28,29]. Extracted RNA will be quantified and samples will be pooled by sample type, species, and farm to achieve a total of 120 pools with a maximum of 10

samples/pool. When animal numbers on a farm are low, pooling will be by taxonomic order (e.g., Rodentia, Carnivora, Artiodactyla), rather than by species. Sequencing libraries will be generated from each pool using the Stranded Total RNA Prep with Ribo-Zero Plus kit (Illumina), which includes a ribosomal RNA depletion step to reduce the amount of animal and bacterial RNA sequenced. Libraries will be assessed for quality on the Agilent 2100 Bioanalyzer and sequenced using the Illumina HiSeq-2500 platform at Macrogen (South Korea).

Sequence analysis.
Raw sequencing reads from each pool will be filtered for quality and assembled *de novo* using Trinity RNA-Seq [30]. Assembled sequences will be identified and annotated by comparing them to the complete nonredundant nucleotide and protein databases available through GenBank using E-value cutoffs of 1E-10 and 1E-4, respectively, and the program Diamond [31]. Sequences representing viral genomes will be further characterized using phylogenetic analysis targeting conserved domains (e.g., the RNA polymerase) that will include related viruses from GenBank to estimate evolutionary relationships. Phylogenies will be inferred using the maximum likelihood method implemented in IQ-Tree, employing the best-fit amino acid substitution models for each virus family [32]. The resultant phylogenies will be used along with the Virus-Host DB [33] to identify putative host species for viruses found in feces or rectal swabs, which commonly also contain plant and invertebrate viruses ingested as part of an animal's diet. Only viruses predicted or known to infect vertebrate animals will be investigated in downstream analyses.

PCR-based screening.
A subset of viruses identified from the metagenomic data will be selected for additional analyses based on phylogenetic relationships and host associations, as described above. As a priority, we will select: (i) viruses with known or predicted zoonotic potential (e.g., coronaviruses, paramyxoviruses), and (ii) viruses found at high prevalence and in multiple species that will facilitate the analysis needed to achieve AIM 2. Primers will be designed from the metagenomic data and used to screen relevant individual samples (i.e., not pools). An effort will be made to target regions of the genome likely to contain enough genetic diversity to support phylogenetic resolution at the farm and species level (e.g., the capsid gene, glycoprotein genes). Positive PCRs will be sequenced in both forward and reverse directions using Sanger sequencing technology (Macrogen) and assembled *de novo* using Geneious [34].

Assessing viral sharing.
To address AIM 1, the prevalence of each known or putative vertebrate virus will be estimated for all pools using the reference mapping software BWA, with RNA polymerase sequences from the metagenomic data as a reference [35]. The abundance of each vertebrate virus within a pool will be estimated from the sequencing data using RNA-Seq by Expectation-Maximization (RSEM) [36], implemented in Trinity. These data will be used to compare viral community structure across farms and host species using standard measures from community ecology, including species richness, alpha diversity, and beta diversity, which will be calculated using a range of R packages [37]. Permutational multivariate analysis of variance tests (permanova) will be used to assess differences in virome composition between farms and species.

For a more detailed analysis of the processes underlying viral sharing, the sequence data generated from our PCR-based screening on individual samples will be used to infer virus-specific phylogenetic trees, using maximum likelihood methods (as above) as well as Bayesian inference where phylogenetic resolution is high. Unlike our previous analysis, these phylogenies will contain only samples from this study and will reflect viral genetic variation across individuals, host species, and farms. To assess phylogenetic clustering and cross-species transmission at the farm and species level, we will utilize statistical frameworks developed for the phylogenetic analysis of discrete character states. These will

include association indices [38], parsimony scores [39], and maximum exclusive single-state clade sizes [40]. Further, we will impose models of discrete trait evolution [41], often utilized in phylogeographical analyses, to investigate viral sharing and the directionality of cross-species transmission between host species and between farms.

For AIM 2, we will use non-metric multidimensional scaling (NMDS) to ordinate each farm based on a set of ecological and environmental variables using R [37]. This analysis will be performed at two scales – local and regional. The local analysis will include data about each farm that was acquired during sampling or from questionnaires, such as the abundance and diversity of animal species, the presence of sick animals (and type of illness), and human activities previously associated with increased disease risk (e.g., butchering/slaughtering, selling wild animals, eating raw meat, etc.) [42]. In addition, the regional analysis will include variables representing the environmental and ecological context of each farm, which will be estimated from publicly available databases. Examples of these variables include average rainfall, average temperature, the mean normalized difference vegetation index (using LANDSAT data [43]), distance to an urban area, distance to water, and an index of wild mammal diversity (using International Union for Conservation of Nature [IUCN] data). Critically, the regional analysis will also include virus data previously generated and published from farms in Dong Thap Province (**Figure 2**) in the Mekong Delta, which were sampled under the same protocols (ENA accession numbers PRJEB6505, PRJEB26687, PRJEB27881) [18,44,45]. The addition of this second sampling location will allow us to separate the effects of farming practices on viral sharing (local scale) from the impacts of the surrounding environment (regional scale). The ordination axes resulting from the NMDS performed at each scale will be used as predictor variables in independent generalized linear models (GLMs) to examine their influence on virus presence and abundance for the subset of prioritized viruses using R. Model fit will be assessed using the Hosmer-Lemeshow goodness of fit test. The resulting models will be used to identify behavioral, environmental, and ecological factors that best predict the presence of 'risky' viruses on mixed-species farms.

Community and policy engagement.
To address AIM 3, we will utilize the results from the above analyses to identify potentially 'risky' farming practices, such as high farm-level species diversity, specific host-virus interactions, or interactions between farming practices and the surrounding environment, to engage stakeholders in discussions about the perception and management of risk in mixed-species farming. We will first organize meetings to engage stakeholders responsible for animal health, human health, and farm management within Dak Lak Province. These include provincial and regional animal and human health officials, and provincial representatives of the Departments of Agriculture (overseeing domestic farm management) and Forestry (overseeing wildlife farm management). We will use these meetings to create a two-way dialogue around these issues and better understand how recommendations on farm health and management are disseminated through public policy and farm management structures. Where recommendations on farm management can be made from our findings, we (with the OUCRU public engagement and policy teams) will work within the identified structures to determine the best practices with which to disseminate these findings to animal health workers and farming communities.

Working with these government stakeholders, we will identify opportunities to engage former members of the Dak Lak HRSC from the VIZIONS project in order to share our findings and better understand current perceptions of the risks associated with wildlife farming, particularly in light of potential links to the COVID-19 pandemic. With the OUCRU public engagement team, we will design surveys to initiate future discussions (extending beyond the scope of this proposal) that consider questions related to bans on wildlife farming and consumption at the policy level and their potential economic impacts, as well as perceived risks and approaches to protecting animal and human health at the farm level.

Research Performance Sites

OUCRU in Ho Chi Minh City (HCMC) was the primary site of the VIZIONS project, which ran from 2012-2016. This project involved two components: 1) hospital-based surveillance of pathogens causing selected human illnesses and 2) three cohorts (~300 humans/cohort) of individuals considered at high occupational risk of contracting zoonotic infections. These cohorts were sampled over three years, along with the animals with which they were in contact. The proposed project will focus on animal samples collected under component 2 of the VIZIONS project in Dak Lak Province. All samples needed for this work have been maintained in OUCRU HCMC laboratory freezers at -80°C from the time of collection and can be easily linked to human questionnaire, serological, and pathogen data collected from the human cohorts within OUCRU's in-house data management system (CliRes).

These unique samples and data are available only at OUCRU, which is committed to facilitating the proposed research. Leadership and several staff members within OUCRU's Molecular Epidemiology group (including Dr. Nguyen) were involved in the design and conduct of the VIZIONS project, and will facilitate access to samples and data, as well as assist in sample processing and analysis. Expertise in molecular biology, viral surveillance, phylogenetics, and molecular epidemiology within the research group will ensure that Dr. Nguyen has the support necessary to complete the proposed project.

OUCRU have established state-of-the-art clinical diagnostics, as well as basic and applied research laboratories that will facilitate successful completion of all aspects of the proposed project. Laboratory facilities include: clinical laboratories (ISO 15189:2012 certified), Biosafety level (BSL) 2 and BSL3 laboratories, and a bio-archiving facility. The laboratories are equipped with safety cabinets (fume hoods, biosafety category II and III), shaking & stationary incubators, refrigerated centrifuges, thermocyclers, real-time quantitative PCR machines, a gel imaging platform (chemiluminescence, ultraviolet, and visible light), gel boxes, water baths, mixing devices, capillary DNA sequencers, Illumina MiSeq sequencers, Oxford Nanopore MinION sequencers, an ultra-centrifuge, a Luminex Flexmap 3D, -80°C freezers, -20°C freezers, refrigerators, liquid nitrogen tanks, ice machines, and autoclaves.

Molecular epidemiology research at OUCRU is conducted in facilities across the laboratories, including a specimen processing facility, molecular diagnostic facility, genomics facility, and an immune-biology facility with serology and cell culture capabilities. High-risk laboratory processes are conducted in the BSL3 laboratory. OUCRU maintains a bio-archiving facility (-20°C, -86°C, liquid nitrogen storage facility) containing 2.5 million samples with continuous temperature monitoring and sample tracking (Lab guard system, Freezerworks system, and biometric access control). Dr. Nguyen will have access to all of the infrastructure and support associated with these facilities to complete the proposed project.

All OUCRU employees have around-the-clock access to computer servers, a virtual private network (VPN), encryption software, information technology (IT) support, and all necessary software including: Git and Github (hosted software revision/audit service), Oracle Virtualbox virtual machines, Google Apps (hosted email and collaboration web-based software), Python, NodeJS, and R programming languages, Meteor (Javascript framework), Jenkins (Continuous Integration server), Microsoft Office, and Adobe CS6. OUCRU also has a dedicated 150+ core Linux server with 8 terabyte (TB) hard drives, and the Molecular Epidemiology group has two dedicated 16-core Mac Pro Servers with 4TB hard drives. Either server individually or in combination may be used for intensive sequence analysis, computational modeling and/or database processing by Dr. Nguyen to ensure successful completion of the project.

CREID Research Center Collaboration

The goals and objectives of the proposed research project, "*Mixed-species farming, viral diversity, and risks of cross-species transmission in the Central Highlands of Viet Nam"*, are fully consistent with the specific aims of EID-SEARCH. EID-SEARCH takes a One Health approach to understanding zoonotic disease emergence, with a focus on viral diversity in wildlife at high-risk human animal interfaces. EID-SEARCH aims to understand the frequency and causes of virus spillover in high-risk communities across Southeast Asia and identify strategies and tools to reduce this risk. As the proposed research focuses on the links between farming practices, wildlife diversity, virus diversity, and cross-species transmission in Viet Nam, we expect the results of the project to make a significant contribution towards the overall goals of EID-SEARCH.

The proposed project is well-designed, leverages cutting-edge technology, and includes a robust ecological component that corresponds to the overall research strategy of EID-SEARCH. The EID-SEARCH team is highly motivated to support the application of metagenomics to ecological questions and assist in the analysis of the unique dataset that will be generated from this research. With the heightened focus on identifying and limiting risks associated with the complex interactions between humans, wildlife, and domestic mammals that has resulted from the emergence of SARS-CoV-2 from a wet market in China, the proposed research is both timely and likely to yield highly-cited, impactful publications for EID-SEARCH and its OUCRU partners.

The EID-SEARCH team includes a number of experts in study design, laboratory-based diagnostics, genetic and genomic analyses, and ecological modelling who will contribute to project implementation, data analysis, and manuscript preparation as required. Communication and coordination mechanisms will be set up between the OUCRU research team and scientists at the EID-SEARCH research sites for resource sharing, training, knowledge exchange, and mentorship. In particular, EID-SEARCH will facilitate and encourage relationships between Dr. Nguyen and our partners in Southeast Asia (based in Malaysia, Singapore, and Thailand) that will facilitate Dr. Nguyen's professional development and strengthen research capacity in the region. Dr. Cadhla Firth, Senior Scientist at EID-SEARCH and the project Mentor, has an established relationship with both Dr. Nguyen and his supervisor, Dr. Maia Rabaa, and will act as the liaison between EID-SEARCH and the OUCRU research team. An expected outcome of this research project will be pilot data to support a joint funding application in the future, led by Dr. Nguyen.

Furthermore, we expect this research to not only improve scientific understanding of zoonotic disease emergence, but also to raise risk awareness among government and local stakeholders and help inform local policies. EID-SEARCH has a team with a strong policy and social science background and extensive experience working with communities and engaging local governments. The EID-SEARCH team will support this project by helping the OUCRU team to develop locally-relevant evidence-based messages, and will strengthen multi-sectoral collaboration in developing practical risk-mitigation strategies. Dr. Nguyen has nearly ten years of experience engaging with local officials and farming communities to develop locally-acceptable research and One Health interventions on the ground in Viet Nam. These relationships Dr. Nguyen has cultivated during his time at OUCRU will facilitate the establishment of a sustainable scientific platform for ongoing research in emerging infectious diseases in Southeast Asia, as well as provide a direct pathway for policy impacts under the EID-SEARCH program.

Mentoring Plan

Although I am scheduled to complete my PhD on March 15, 2021 (viva date), I have been actively involved in research as a veterinarian and Program Coordinator at OUCRU since 2011. As a result, I have a broad understanding of the challenges involved in developing and implementing a research project, and a clear idea of how I would like to progress my career. My overall research goal is to improve the health of people in Viet Nam using a One Health approach that will allow me to maximize the impacts of both my veterinary and academic training. However, my aspirations are not just limited to the creation of scientific knowledge; instead, I wish to actively use this knowledge to address social issues in Viet Nam, as demonstrated by the focus of my PhD research on antimicrobial stewardship on poultry farms.

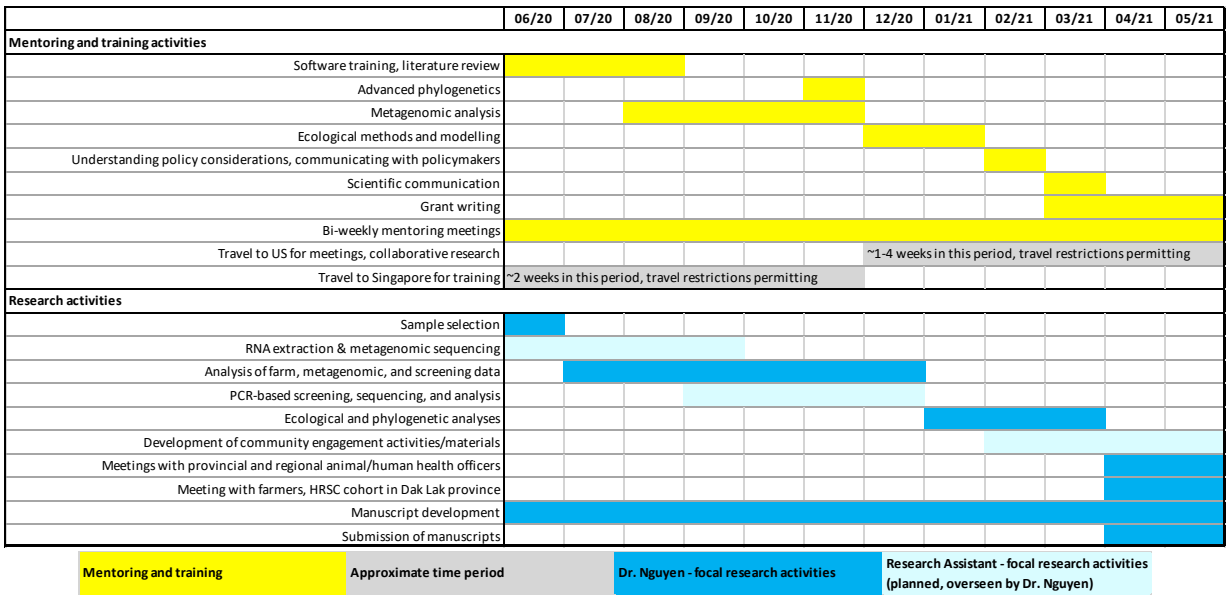
My previous community engagement experiences have focused primarily on highlighting the need for data-driven policy, and I have had only limited experience working with political agencies to translate scientific evidence into laws and regulations. Through the public engagement aim of the proposed project, I will be able to strengthen my relationships with government and local stakeholders, grow my network across Viet Nam, and enable my long-term success using data-driven, community-led research to generate real-world impacts. By working closely with members of EHA, who have a strong background in community engagement and policy development, and the recently formed Policy Engagement group at OUCRU, I feel that this project will provide the perfect opportunity for me to develop my career vision as an independent scientist.

Throughout my career, I have had experience and training in veterinary medicine, laboratory-based science, program coordination, and epidemiology. However, it is clear that genomics (and metagenomics) is now an increasingly common aspect of emerging infectious disease research. There is a pressing need for increased capacity in this field in Viet Nam, as well as in many other lower- and middle-income countries, to ensure that we have access to the best methods and analytical tools available to support a thriving science industry. I believe that, given the opportunity to further strengthen my analytical skills to include metagenomic data analysis, I will be in possession of a comprehensive skillset that will allow me to transition from a junior scientist to an independent science leader in Viet Nam, capable of initiating and delivering hypothesis-driven research to solve locally relevant problems.

With more than 15 years' experience working on emerging infectious diseases, Dr. Firth (my Mentor) has built a research program with a strong multi-disciplinary focus that is only enhanced by her recent appointment as Senior Scientist with EHA. Her expertise includes nearly 10 years' experience in the generation and analysis of metagenomic sequence data, which she has used in both clinically relevant and veterinary contexts, as well as to address a range of ecological and evolutionary questions [25,46–50]. Dr. Firth also has significant experience with field and laboratory science and has led or participated in projects in multiple countries, including Malaysia, Viet Nam, Australia, Brazil, The United States, and Canada. As a result, she has a broad scientific network that involves global leaders in infectious disease research, including those within Southeast Asia (e.g., Prof. Linfa Wang, Director, Emerging Infectious Diseases Program at Duke-NUS Medical School, Singapore; Prof. David Perera, Director, Institute of Health & Community Medicine, UNIMAS). Dr. Firth will actively create opportunities for me to interact with Prof. Wang and other leading scientists through the proposed research project, which will greatly increase my research network. A two-week visit to Prof. Wang's laboratory has been budgeted as part of the proposed project and will provide me with the opportunity to learn cutting-edge laboratory techniques, as well as interact directly with members of his lab. Dr. Firth has also committed to creating opportunities for me to discuss my metagenomic data directly with Prof. Edward Holmes (a world leader in virus discovery and metagenomics), who is highly supportive of the proposed project.

In addition to her scientific skills and network, Dr. Firth has previous experiences training and mentoring scientists in lower- and middle-income countries. This should increase the effectiveness of her mentorship since I am also a junior scientist from one of these countries in Southeast Asia. Dr. Firth also has a close personal relationship with my in-country project supervisor and long-term collaborator, Dr. Maia Rabaa, who is co-lead of OUCRU's Molecular Epidemiology research group. Drs. Firth and Rabaa completed their PhD degrees together and have maintained a strong relationship focused on peer mentorship since that time. As a result of their joint commitment to both this research project and my professional development, I will have access to an extremely high level of support and mentorship both locally and internationally. The combined mentoring, experience, and expertise I will gain through the CREID Pilot Research Program will assist me in my progression toward scientific independence by providing opportunities to develop my grant writing, budgeting, study management, and networking skills, in addition to the new scientific skills I will gain. The data generated as a result of the proposed project will also provide critical pilot data for future grant applications that will form the foundation of a long-term collaborative relationship with EHA.

Through the process of writing the application for the CREID Pilot Research Program, Dr. Firth and I have already established the foundation for an effective communication plan that will continue throughout the project. We have created a WhatsApp group that also includes Dr. Rabaa, which we use to communicate in an informal setting and in real-time. This has been invaluable for sharing ideas, asking questions, and facilitating the development of a good personal relationship that is a pre-requisite for effective mentorship. We have also agreed to a regularly scheduled Zoom meeting that will occur every two weeks, be agenda-driven, and focused on a topic or issue of direct relevance to the research project. Both Dr. Firth and I will be responsible for setting the agenda and following up on action items. I will also attend weekly academic meetings at OUCRU and EHA (over Zoom), which will expose me to the breadth of research at these institutions, and which I have found to be extremely useful for me in the past. Dr. Firth and I have also agreed to extend my visit to the United States to attend the annual CREID meeting to include a visit to EHA in New York City. The timing of this visit will allow me to bring some metagenomic data from the project with me and work through data analysis with Dr. Firth and other EHA scientists. Finally, Dr. Firth, Dr. Rabaa, and I will travel to Dak Lak province in Viet Nam at the end of the project to present the study outputs to the community members that participated in the VIZIONS study, as part of the public engagement aim.



Vertebrate Animals Section Requirements

No new animal sampling will be conducted in the proposed research study. All samples and data to be used in the proposed research project were previously obtained as part of the VIZIONS project, which concluded in 2016. Samples were collected under a protocol approved by the Oxford Tropical Research Ethics Committee (OxTREC code 157-12), and were locally reviewed and approved by the ethical review board of the Hospital for Tropical Diseases in HCMC, with local review and acceptance at all sites. Viet Nam did not have an animal ethics review board at the time that the VIZIONS project was conducted.

Details on the VIZIONS project are available in a 2015 publication [19].

The principal aims of the VIZIONS project were:

1. To establish a model international collaborative consortium with an integrated approach to human and animal health research.
2. To estimate the burden of viral and zoonotic diseases in Viet Nam.
3. To investigate the disease epidemiology of specified clinical syndromes and infections in a cohort of high-risk individuals occupationally exposed to animals, with targeted sampling from domestic animals and wildlife associated with these individuals.
4. To elucidate the etiology of infectious diseases of unknown origin in the human population and provide a repository of putative pathogens for further study.
5. To characterize genetic diversity within virus populations on either side of the species barrier to understand cross-species transmission and disease emergence.
6. To identify socio-demographic, environmental, and behavioral drivers for disease emergence.
7. To create a platform and resource for further research on zoonotic disease agents.

Key in-country partners on the VIZIONS project included:

- Hospitals (The Hospital for Tropical Diseases, HCMC; Dong Thap General Hospital, Cao Lanh City, Dong Thap Province; Dak Lak General Hospital, Buon Ma Thuot City, Dak Lak Province; Khanh Hoa General Hospital, Nha Trang City, Khanh Hoa Province; Hue Central Hospital, Hue City, Thua Thien Hue Province; National Hospital for Tropical Diseases, Ha Noi; Ba Vi District Hospital, Ha Noi)
- Academic institutions (OUCRU, HCMC; OUCRU, Ha Noi; Hanoi Medical University)
- Regional Animal Health Offices (RAHO5, Buon Ma Thuot City, Dak Lak Province)
- Sub-departments of Animal Health (sDAH) (Dak Lak sDAH, Buon Ma Thuot City, Dak Lak Province; Dong Thap sDAH, Cao Lanh City, Dong Thap Province)
- Preventive Medicine Centers (PMCs) (Dak Lak PMC, Buon Ma Thuot City, Dak Lak Province; Dong Thap PMC, Cao Lanh City, Dong Thap Province; Ba Vi District PMC, Ha Noi)
- Ba Vi District Veterinary Station

Applicant Name (Last, first, middle): Nguyen, Cuong, Van

Human Subjects Research

No human subjects, samples, or data will be involved in this research.

1. Are Human Subjects Involved?			No													
1.a. If YES to Human Subjects																
Is the Project Exempt from Federal regulations?	Yes		No													
If yes, check appropriate exemption number	1		2		3		4		5		6		7		8	
If no, is the IRB review Pending?	Yes		No													
IRB Approval Date:																
Human Subject Assurance Number																
2. Are Vertebrate Animals Used?			No													
2.a. If YES to Vertebrate Animals																
Is the IACUC review Pending?	Yes		No													
IACUC Approval Date																
Animal Welfare Assurance Number																
3. Is proprietary/privileged information included in the application?			No													
4.a. Does this project have an Actual or Potential impact -- positive or negative -- on the environment?			No													
4.b. If yes, please explain																
4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed?	Yes		No													
4.d. If yes, please explain																
5. If the research performance site designated, or eligible to be designated, as a historic place?			No													
5.a. If yes, please explain																
6. Does this project involve activities outside of the United States or partnership with international collaborators?	Yes															
6.a. If yes, identify countries	Viet Nam															
6.b. Optional explanation																

Budget Justification

In total, we request $149,970.57 for Dr. Nguyen for this one-year project, starting 1 June 2021, including: $50,878.00 for personnel, $1,200.00 for regulatory fees, $1,000 for equipment, $76,473.76 for consumables, $9,309.88 for travel, and $11,108.93 for indirect costs. Detailed expenses are calculated as follows:

A. Personnel ($50,878.00)

Dr. Nguyen, PI, will commit 12 months (1 FTE) per year to this project for study design, supervising the laboratory work, training in new analytical methods, and conducting the metagenomic and epidemiological analyses. Dr. Nguyen is a veterinary epidemiologist specializing in pathogen surveillance at the human-animal interface and will have just completed his PhD studies (15 March 2021). We request $28,563.00 total salary for Dr. Nguyen for the period of 12 months.

We also request funding for a senior research assistant who will commit 12 months (1 FTE) per year to this project to perform laboratory work and assist in the development of community engagement activities and materials. The research assistant will have significant experience working with and performing virus surveillance/sequencing on samples collected under the VIZIONS project, and will have experience participating in public engagement projects in Viet Nam. We request $22,315.00 total salary for this research assistant for the period of 12 months.

B. Regulatory fees ($1,200.00)

An import license from the Ministry of Health will be required for importation of any consumables, equipment, reagents, and biological materials related to this project, at the cost of $1,200.00.

C. Equipment ($1,000.00)

No equipment will be purchased with the exception of one laptop computer for Dr. Nguyen. We request budget ($1,000.00) for one laptop computer for use by Dr. Nguyen.

D. Consumables ($76,437.76)

RNA Extractions ($29,170.00). We request ($29,170.00, $14.59 per sample) for RNA extractions. This total covers the cost of all kits, reagents, and consumables (MagNA Pure 96 DNA and Viral NA SV Kits, MagNA Pure 96 Processing Cartridges, MagNA Pure 96 Filter Tips, MagNA Pure 96 System Fluid, MagNA Pure 96 Sealing Foil, MagNA Pure 96 Output Plates, Qubit™ RNA HS Assay Kits, Qubit™ Assay Tubes, and TURBO™ DNase) for 2000 extractions.

Metagenomics ($32,500.00). We request ($32,500.00, $270.83 per 10-sample pool) for metagenomic sequencing. This total covers the cost of reagents and consumables for library prep (Illumina Stranded Total RNA Prep with Ribo-Zero Plus kit, Illumina Sequencing Adapters, Agilent AMPure Beads, RNA Clean XP) and sequencing on a HiSeq-4000 at Macrogen (South Korea), for 120 pools.

Follow-up PCRs ($14,173.56). We request ($14,173.56, $11.81 per sample) for targeted RT-PCR following results of the metagenomic analysis. This total covers the cost of all kits, reagents, and consumables (LightCycler 480 Multiwell Plates, 96–Well Half Skirt PCR Plates, Bubble Strip Caps for PCR Strips, SuperScript III One-Step RT-PCR, Primers, Agarose, SYBR™ Safe DNA Gel Stain, DNA Clean & Concentrator-5, Amplicon Sequencing) to cover 1200 reactions.

Other reagents & consumables ($630.21). We request ($630.21) to cover miscellaneous consumables. This total covers the cost of tubes, tips, personal protective equipment, and other consumables (varied

Axygen™ Aerosol Filter Tips, Water - Molecular Biology Reagent, Absolute Ethanol, LoBind Eppendorf tubes, nitrile gloves) required to process samples in the laboratory.

E. Travel ($9,309.88)

International Travel ($8,229.00). We request: 1) $4,335.00 for one trip to the United States for Dr. Nguyen to the CREID meeting in Rockville, Maryland. This total covers one round-trip flight ($1,600.00), lodging and per diem for 4 days/nights ($250 per day; total $1,000), ground transportation costs ($150.00), insurance and visas ($185.00), and quarantine costs for 14 days on return to Viet Nam ($100.00 per day; total $1,400.00). Additional costs for travel to New York City, lodging, and subsistence during this trip will be covered by EHA. 2) We also request an additional $3,894.00 for one trip to meet and train with EID-SEARCH partners in Singapore. This total covers one round-trip flight ($400.00), lodging and per diem for 14 days/nights ($140.00 per day; total $1,960.00), ground transportation costs ($100.00), insurance ($34.00), and quarantine costs for 14 days on return to Viet Nam ($100.00 per day; total $1,400.00).

Inter-province Travel ($1080.88). We request $1080.88 for Dr. Nguyen and two OUCRU staff members (one from the Molecular Epidemiology group and one from the Public Engagement team) to travel to Buon Ma Thuot city, Dak Lak Province for discussions with stakeholders and engagement planning activities. This total covers three round-trip flights ($200.00 per person; total $600.00), lodging and per diem for 3 people for 3 days ($43.43 per person per day; total $390.88), and ground transportation costs ($30.00). Additional costs for engagement activities will be covered by internal OUCRU funding for Public Engagement activities.

Additional travel

EID-SEARCH (EcoHealth Alliance) will provide additional funding for international travel costs related to additional face-to-face meetings and collaborative work periods for Dr. Nguyen and Dr. Firth.

F. Indirect costs ($11,108.93)

We are requesting 8% indirect costs on all direct costs (total $11,108.93).

BIOGRAPHICAL SKETCH

NAME: Nguyen, Cuong, Van

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

POSITION TITLE: Project coordinator

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	Completion Date	FIELD OF STUDY
Agriculture and Forestry University (VN)	DVM	09/2008	Veterinary Medicine
University of Paris XI (France)	MSc	09/2011	Biotechnology (Genome, Cells)
The Open University (UK) (thesis submitted, viva March 2021)	PhD	03/2021	Life, Health and Chemical Sciences

A. Personal Statement

I have strong motivation to lead a research project characterizing viral diversity across multiple animal species, particularly in wildlife/domestic mixed species farms. I was involved in the implementation within the VIZIONS project from 2012-2016, acting as a research assistant and field coordinator. My research interests are focused on community-based studies investigating high potential zoonotic diseases and antimicrobial resistance at the animal-human interface. My primary research work during the VIZIONS study was coordinating the collection of high-quality data and samples, as well as establishing critical community partnerships in both the human and animal health sectors. Beyond my field coordinator role, I also processed and tested for the presence of known viruses in animal samples from the VIZIONS project. These studies collected and have reported on varied sources of data (i.e. epidemiological, clinical, laboratory, genomic, and behavioral data) to better understand the epidemiology and emergence of viral zoonoses. I currently coordinate an intervention project (ViParc: Vietnamese Platform for Antimicrobial Reduction in Chicken production) that aims to reduce antimicrobial usage in chicken production in southern Viet Nam. I now have nearly completed my PhD work characterizing antimicrobial usage in chicken production. I have delivered a number of high impact publications focused on key issues in veterinary epidemiology. I am also committed to providing training for local veterinary students on statistics and epidemiology, veterinary public health, and animal welfare (visiting university lecturer).

a. **Cuong, NV**, Carrique-Mas J, Thu HTV, Hien ND, Hoa NT, Nguyet LA, Anh PH, Bryant JE (2014). *Serological and virological surveillance for porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, and influenza A viruses among smallholder swine farms of the Mekong Delta, Vietnam*. **Journal Swine Health and Production**; 22(5):224–231.

b. **Van Cuong N**, Carrique-Mas J, Vo Be H, An NN, Tue NT, Anh NL, Anh PH, Phuc NT, Baker S, Voutilainen L, Jääskeläinen A, Huhtamo E, Utriainen M, Sironen T, Vaheri A, Henttonen H, Vapalahti O, Chaval Y, Morand S, Bryant JE (2015). *Rodents and risk in the Mekong Delta of Vietnam: seroprevalence of selected zoonotic viruses in rodents and humans*. **Vector Borne Zoonotic Dis**; 15(1):65-72.

c. **Cuong NV,** Truc VN, Nhung NT, Thanh TT, Chieu TT, Hieu TQ, Men NT, Mai HH, Chi HT, Boni MF, van Doorn HR, Thwaites GE, Carrique-Mas JJ, Hoa NT. (2016). *Highly pathogenic avian influenza virus A/H5N1 infection in vaccinated meat duck flocks in the Mekong Delta of Vietnam*. **Transboundary and Emerging Diseases**; 63(2):127–135.

d. **Van Cuong N**, Nhung NT, Nghia NH, Mai Hoa NT, Trung NV, Thwaites G, Carrique-Mas J (2016). *Antimicrobial consumption in medicated feeds in Vietnamese pig and poultry production*. **Eco Health**; 13(3):490-498.

B. Positions and Honors

YEAR(S)	POSITION
2011-2013	Research Assistant, Oxford University Clinical Research Unit (OUCRU), Viet Nam
2014-2016	Senior Research Assistant, VIZIONs Field Coordinator, OUCRU, Viet Nam
2017-2021	PhD candidate, ViPARC project coordinator, OUCRU, Viet Nam
2020-present	Visiting University Lecturer at the Applied Science Institute, Ho Chi Minh University of Technology (HUTECH), Viet Nam

C. Contributions to Science

1. Zoonotic transmission at the human-animal interface

I played a crucial role in implementing a community-based study of zoonotic infections in Viet Nam under the VIZIONS project. By coordinating high quality data and sample collection, I contributed to the success and unique study design of many VIZIONS-related studies, driving a number of sub-studies investigating pathogen evolution and epidemiology. These studies provided insight into viral, bacterial, and parasitological zoonotic infections presenting in communities in Viet Nam. I played multiple roles on the project, including field work and sampling, laboratory-based screening, and complex epidemiological investigations of pathogen diversity in domestic and wild animal farms, as well as in wild animal populations (capturing bats in the jungle and rats in rice fields).

a. Loan HK, **Van Cuong N,** Takhampunya R, Kiet BT, Campbell J, Them LN, Bryant JE, Tippayachai B, Van Hoang N, Morand S, Hien VB, Carrique-Mas JJ (2015). *How important are rats as vectors of leptospirosis in the Mekong Delta of Vietnam?* **Vector Borne Zoonotic Dis**; 15(1):56-64.

b. Van Dung N, Anh PH, **Van Cuong N**, Hoa NT, Carrique-Mas J, Hien VB, Sharp C, Rabaa M, Berto A, Campbell J, Baker S, Farrar J, Woolhouse ME, Bryant JE, Simmonds P (2016). *Large-scale screening and characterization of enteroviruses and kobuviruses infecting pigs in Vietnam*. **J Gen Virol**; 97(2):378-388.

c. Van Nguyen VC, Le Buu C, Desquenes M, Herder S, Nguyen PHL, Campbell JI, **Nguyen VC**, Yimming B, Chalermwond P, Jittapalapong S, Franco JR, Ngo TT, Rabaa MA, Carrique-Mas JJ, Thanh TPT, Nga TVT, Berto A, Hoa NT, Hoang NVM, Tu NC, Chuyen NK, Wills B, Hien TT, Thwaites GE, Yacoub S, Baker S (2016). *A clinical and epidemiological investigation of the first reported human case of the zoonotic parasite* Trypanosoma evansi *in Vietnam*. **Clin Inf Dis**; 62(8):1002-8.

d. Van Nguyen D, **Van Nguyen C**, Bonsall D, Ngo TT, Carrique-Mas J, Pham AH, Bryant JE, Thwaites G, Baker S, Woolhouse M, Simmonds P (2018). *Detection and Characterization of Homologues of Human Hepatitis Viruses and Pegiviruses in Rodents and Bats in Vietnam.* **Viruses**; 10(3):102.

2. Usage and drivers of antimicrobial resistance in animal production

Antimicrobial resistance (AMR) is a global threat to the health and wealth of nations. The AMR crisis has been attributed to the overuse and misuse of antimicrobials. Excessive use of antimicrobials in animal production is one of the contributing factors to this global threat. In my PhD work, I characterized antimicrobial usage (AMU) in small-scale chicken farms in the Mekong Delta region of Viet Nam. This includes consumption of antimicrobials mixed with water by the farmer as well those included in commercial feeds as antimicrobial growth promoters (AGPs). The epidemiological data that I gathered in

this project was used to investigate the relationship between AMU and disease. Results from this thesis suggest that efforts to promote responsible use of antimicrobials and limit excessive AMU are effective in reducing AMU in animal production, and thus should focus on: 1) educating farmers on good farming practices and simple veterinary diagnostics, and 2) strengthening veterinary systems, including farm audits and provision of effective advice from animal health experts. These studies have also shown that that the probability of effective treatment is often very low. My research concluded that the message 'prophylactic AMU does not reduce the probability of disease in flocks' should be further disseminated to poultry farming communities to reduce unnecessary AMU on farms.

a. **Cuong N**, Padungtod P, Thwaites G, Carrique-Mas JJ (2018). *Antimicrobial Usage in Animal Production: A Review of the Literature with a Focus on Low- and Middle-Income Countries*. **Antibiotics** (Basel); 7(3):75.

b. **Cuong NV**, Phu DH, Van NTB, Dinh Truong B, Kiet BT, Hien BV, Thu HTV, Choisy M, Padungtod P, Thwaites G, Carrique-Mas J (2019). *High-Resolution Monitoring of Antimicrobial Consumption in Vietnamese Small-Scale Chicken Farms Highlights Discrepancies Between Study Metrics*. **Frontiers in Vet Sci**; 6:174.

c. Choisy M, **Van Cuong N**, Bao TD, Kiet BT, Hien BV, Thu HV, Chansiripornchai N, Setyawan E, Thwaites G, Rushton J, Carrique-Mas J (2019) *Assessing antimicrobial misuse in small-scale chicken farms in Vietnam from an observational study*. **BMC Vet Res**; 15(1):206.

d. Carrique-Mas JJ, Choisy M, **Cuong NV**, Thwaites GE, Baker S (2020). *An estimation of total antimicrobial usage in humans and animals in Vietnam*. **Antimicrobial resistance and infection control;** 9:16.

3. Understanding AMR in commensal and pathogenic microorganisms in animal production

A better understanding of AMR in both commensal and pathogenic microorganisms in animal production is vital to improve treatment and control, particularly in the context of preventing those bacterial infections in animals as well as the transfer of their genes into human pathogenic bacteria. Epidemiological data from my studies have shown that high levels of AMU resulted in high levels of AMR in both commensal and pathogenic bacteria. These data have resulted in improved treatment and diagnostic policies for animal production across Viet Nam.

a. Yen NTP, Nhung NT, Van NTB, **Cuong NV**, Kiet BT, Phu DH, Hien VB, Campbell J, Chansiripornchai N, E Thwaites G, Carrique-Mas JJ (2020). *Characterizing Antimicrobial Resistance in Chicken Pathogens: A Step towards Improved Antimicrobial Stewardship in Poultry Production in Vietnam*. **Antibiotics (Basel);** 9(8):499.

b. Nguyen NT, Nguyen HM, **Nguyen CV**, Nguyen TV, Nguyen MT, Thai HQ, Ho MH, Thwaites G, Ngo HT, Baker S, Carrique-Mas J (2016). *Use of Colistin and Other Critical Antimicrobials on Pig and Chicken Farms in Southern Vietnam and Its Association with Resistance in Commensal* Escherichia coli *Bacteria*. **Appl Environ Microbiol**; 82(13):3727-3735.

c. Tu LT, Hoang NV, **Cuong NV**, Campbell J, Bryant JE, Hoa NT, Kiet BT, Thompson C, Duy DT, Phat VV, Hien VB, Thwaites G, Baker S, Carrique-Mas JJ (2015). *High levels of contamination and antimicrobial-resistant non-typhoidal* Salmonella *serovars on pig and poultry farms in the Mekong Delta of Vietnam*. **Epidemiol Infect**; 143(14):3074-86.

d. Nhung NT, **Cuong NV**, Campbell J, Hoa NT, Bryant JE, Truc VN, Kiet BT, Jombart T, Trung NV, Hien VB, Thwaites G, Baker S, Carrique-Mas J (2015). *High levels of antimicrobial resistance among* Escherichia coli *isolates from livestock farms and synanthropic rats and shrews in the Mekong Delta of Vietnam*. **Appl Environ Microbiol**; 81(3):812-20.

Complete List of Published Work in MyBibliography:

https://www.ncbi.nlm.nih.gov/myncbi/1NKsW-nQefwcQc/bibliography/public/

BIOGRAPHICAL SKETCH

NAME: Nguyen, Cuong, Van

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

D. Additional Information: Research Support and/or Scholastic Performance

Completed Research Support

My PhD fellowship was funded by the ViPARC project (12/2016-01/2021). This project was funded by the Wellcome Trust through an Intermediate Clinical Fellowship awarded to Dr. Juan J. Carrique-Mas (Grant Reference Number 110085/Z/15/Z).

BIOGRAPHICAL SKETCH

NAME: Firth, Cadhla

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6)

POSITION TITLE: Senior Research Scientist and Program Coordinator, EcoHealth Alliance

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	Completion Date	FIELD OF STUDY
University of Guelph, Guelph, CAN	BS	06/2003	Zoology
University of Guelph, Guelph, CAN	MS	06/2005	Evolutionary Ecology
The Pennsylvania State University, State College, PA	PhD	05/2010	Biology
Columbia University, New York, NY	Postdoctoral	12/2012	Infectious Diseases & Public Health

A. Personal Statement

I am a molecular and evolutionary biologist who has been working on emerging infectious diseases for almost 15 years. In my current research, I explore the ecological and evolutionary dynamics of emerging zoonoses at the human-animal interface, with a focus on rapidly changing environments. I have more than seven years' experience leading independent research projects in this field, including collaborations with industry and local government. Over time, I have been responsible for the successful completion of every aspect of these studies, including project design, funding acquisition, implementation, and reporting/publishing. In 2015 I initiated a research program to investigate the response of zoonotic pathogens to urbanization in Malaysian Borneo using metagenomics, landscape ecology, and population genomics. This research has a strong multi-disciplinary component that includes evolutionary biology, molecular ecology, virology, pathogen genetics and genomics, field biology, and public health, and has required me to become adept at combining and analyzing multiple types of data, including environmental, ecological, and genomic data. As such, my experience, skills, and training are ideally suited to the proposed research project, which builds naturally on my previous work. I also have a strong track record in the generation and use of genetic and genomic data to explore the transmission dynamics and evolution of emerging viruses, with more than 20 publications in this field, seven of which have been cited more than 100 times since 2010. The mastery of phylogenetic and phylodynamic methods I have developed throughout my career are directly applicable to the proposed research. Further, I am strongly committed to supporting diversity and equity both within the scientific community and in the communities we serve, and have led several projects and initiatives aimed at improving science education and health service delivery for underserved communities in tropical northern Australia. I am also committed to providing training and mentorship for low- and middle-income country scientists and scholars, and in my previous role worked to develop protocols and bioinformatics pipelines to enable portable metagenomic sequencing in rural and remote regions in Australia and the Pacific. I am currently supervising three PhD students and three postdoctoral researchers (James Cook University, AUS).

B. Positions and Honors

Positions and Employment

2001-2006 Research Assistant, Department of Zoology, The University of Guelph, Guelph, CAN
2006-2010 PhD Scholar, The Pennsylvania State University, State College, PA
2010-2012 Postdoctoral Fellow, Center for Infection and Immunity, Columbia University, New York, NY
2013 Associate Research Scientist, Center for Infection and Immunity, Columbia University, New York, NY

2013-2017	Research Scientist/ Australian Research Council (ARC) Discovery Early Career Research Fellow, Health and Biosecurity, The Commonwealth Scientific and Industrial Research Organization, Geelong, AUS
2017-2018	Australian Research Council (ARC) Discovery Early Career Research Fellow, School of BioSciences, The University of Melbourne, Parkville, AUS
2018-2020	HOT North Career Development Fellow, Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, AUS
2021-	Senior Research Scientist and Program Coordinator, EcoHealth Alliance, New York, NY

Honors

2003-2005	Ontario Graduate Scholarship, The Province of Ontario (Canada)
2006-2009	Postgraduate Scholarship, Natural Science and Engineering Research Council of Canada
2015-2018	Discovery Early Career Researcher Award, The Australian Research Council
2019-2021	HOT North Career Development Fellowship, Northern Australia Tropical Disease Collaborative Research Programme, National Health and Medical Research Council (AUS)

C. Contributions to Science

Throughout my career, my research has focused on many aspects of infectious disease emergence and has encompassed evolutionary biology, molecular ecology, virology, pathogen genetics and genomics, field biology, and public/veterinary health. My most significant contributions include:

1. The use of genomic and metagenomic sequencing techniques to discover new pathogens, study their evolution, and explore microbial diversity.

I have successfully developed and used cutting-edge metagenomic techniques and bespoke bioinformatics pipelines for: (i) pathogen discovery, including a case of acute encephalitis in an immune-suppressed patient, (ii) studies of viral diversity, including coronaviruses and flaviviruses in bats; and (iii) genomics, including the epizoology of bluetongue virus, the evolution of rhabdoviruses, and the characterization of Australian bunyaviruses. I have also co-authored two perspectives on the utility of next-generation sequencing for pathogen discovery in people and animals. These included an invited submission that described an updated framework for proof of causation in the genomics era and discussed the emerging integration of on-the-ground metagenomics-based diagnostics with molecular epidemiology.

In 2013, I initiated a study that used metagenomics to characterize the viruses and bacteria carried by New York City rats across a range of habitats, with a focus on the built environment. At the time, little was known about the range of potential pathogens carried by urban rodents or the risk factors associated with zoonotic transmission in a city environment. To begin to address this, I designed the first comprehensive molecular survey of rodent-borne microbes in a U.S. city, and demonstrated that urban rats frequently carry a range of zoonotic pathogens. We also found evidence of substantial heterogeneity in pathogen distribution within and between cities, suggesting that the associated risks of human disease may be similarly heterogeneous.

a. **Firth C**, Bhat M, Firth MA, Williams SH, Frye MJ, Simmonds P, Conte JM, et al. 2014. Detection of zoonotic pathogens and characterization of novel viruses carried by commensal Rattus norvegicus in New York City. mBio 5: e01933-14. PMCID: PMC4102142

b. Quan PL, **Firth C**, Conte JM, Williams SH, Zambrana-Torrelio CM, Anthony SJ, Ellison JA, et al. 2013. Bats are a major natural reservoir for hepaciviruses and pegiviruses. Proceedings of the National Academy of Sciences 110: 8194-9. PMCID: PMC3657805

c. **Firth C**, Lipkin WI. 2013. The genomics of emerging pathogens. Annual Review of Genomics and Human Genetics 14: 281-300. PMID: 24003855

d. Quan PL, **Firth C**, Street C, Henriquez JA, Petrosov A, Tashmukhamedova A, Hutchison SK, et al. 2010. Identification of a severe acute respiratory syndrome coronavirus-like virus in a leaf-nosed bat in Nigeria. mBio 1: e00208-10. PMCID: PMC2975989

e. Quan P-L, Wagner T, Briese T, Kapur RP, Torgerson TR, Hornig M, **Firth C**, et al. 2010. Astrovirus encephalitis in X-linked agammaglobulinemia. Emerging Infectious Diseases 16: 918-25. PMCID: PMC4102142

2. I have helped develop the emerging field of urban zoonotic disease ecology.

In 2015, I pioneered an ongoing research program to investigate the response of zoonotic pathogens to urbanization using metagenomic sequencing, landscape ecology, and population genomics. Using a multi-disciplinary approach, this research explores how changes in the environment (e.g., land-use changes, microclimates, etc.), and host community and population structure (e.g., biodiversity, connectivity, density), influence pathogen community composition and zoonotic potential. Initial results of this work indicate that while mammalian diversity decreases with increasing urbanization, connectivity between populations increases, as does microbial species richness and the prevalence of known zoonotic pathogens. This suggests that some features of the built environment may inadvertently support pathogen persistence and spread, and as a result, some zoonotic pathogens may be more likely to emerge with intensifying urbanization. My reputation as an emerging researcher in this field has led to multiple invitations to present as a plenary or symposium speaker at national and international meetings, including the Ecological Society of Australia's Annual Conference (2019) and the Annual Meeting of the American Society of Mammalogists (2019), and the Joint Conference of the Asian Society of Conservation Medicine and the Wildlife Disease Association Australasia (2018).

a. Blasdell KR, Morand S, Perera D, **Firth C.** 2019. Association of rodent-borne *Leptospira* spp. with urbanizing environments in Sarawak, Malaysian Borneo. PloS Neglected Tropical Diseases 13: e0007141. PMCID: PMC6411199

b. Blasdell KR, Perera D, **Firth C.** 2018. High prevalence of rodent-borne *Bartonella* spp. in urbanizing environments in Sarawak, Malaysian Borneo. American Journal of Tropical Medicine and Hygiene 100: 506-9. PMCID: PMC6402934

c. Peterson AC, Ghersi BM, Alda F, **Firth C**, Frye MJ, Bai Y, Osikowicz LM, et al. 2017. Rodent-borne Bartonella infection varies according to host species within and among cities. EcoHealth 14:771-82. PMID: 29164472

d. Frye MJ, **Firth C**, Bhat M, Firth MA, Che X, Lee D, Williams SH, Lipkin WI. 2015. Preliminary survey of ectoparasites and associated pathogens from Norway rats in New York City. Journal of Medical Entomology 52: 253-9. PMCID: PMC4481720

e. **Firth C**, Bhat M, Firth MA, Williams SH, Frye MJ, Simmonds P, Conte JM, et al. 2014. Detection of zoonotic pathogens and characterization of novel viruses carried by commensal Rattus norvegicus in New York City. mBio 5: e01933-14. PMCID: PMC4102142

3. I challenged established beliefs about the ecological and evolutionary processes linked to viral disease emergence, and the timescale over which they occur.

During my PhD, I was able to make significant contributions to this field by questioning the idea that viruses evolve either by cross-species transmission (spillover) or by co-evolution with their hosts, but rarely both. My work demonstrated that viruses with many genomic structures are capable of jumping species barriers, and that evolution through both co-divergence and cross-species transmission may be a

general trend of many virus systems. This has since been supported by numerous subsequent studies that have highlighted the complex evolutionary dynamics of both RNA and DNA viruses.

a. Pagan I, **Firth C**, Holmes EC. 2010. Phylogenetic analysis reveals rapid evolutionary dynamics in the plant RNA virus genus tobamovirus. Journal of Molecular Evolution 71: 298-307. PMID: 20838783

b. **Firth C**, Kitchen A, Shapiro B, Suchard MA, Holmes EC, Rambaut A. 2010. Using time-structured data to estimate evolutionary rates of double-stranded DNA viruses. Molecular Biology and Evolution 27: 2038-51. PMCID: PMC3107591

c. Sali AA, Faye O, Diallo M, **Firth C**, Kitchen A, Holmes EC. 2010. Yellow fever virus exhibits slower evolutionary dynamics than dengue virus. Journal of Virology 84: 765-72. PMCID: PMC2798388

d. **Ramsden C***, Holmes EC, Charleston MA. Hantavirus evolution in relation to its rodent and insectivore hosts: no evidence for co-divergence. 2009. Molecular Biology and Evolution 26: 143-53. PMID: 18922760

* Name changed from Ramsden to Firth in 2009

4. I was among the first to apply novel phylodynamic approaches to reconstruct the spatiotemporal processes behind disease emergence events.

Phylodynamic methods enable the synthesis of epidemiological, geographic, and phylogenetic data to reconstruct the processes behind infectious disease emergence and spread through time and space. These methods are of particular use for investigating the emergence and spread of viruses, as they have a rapid evolutionary rate that closely matches the timescale of virus transmission. I began to explore these approaches during my PhD and have now successfully used them to: (i) explore the genetics underpinning the rapid emergence and spread of human enterovirus 68; (ii) examine the patterns and processes that influence the diversity and geographic distribution of New World hantaviruses; (iii) explore the epizoology of porcine circovirus 2; iv) understand the evolutionary and ecological drivers behind the distribution of bluetongue virus in Australia; and (v) assess the likely origins of hepatitis C virus.

a. **Firth C**, Blasdell KR, Amos-Ritchie R, Sendow I, Agnihotri K, Boyle DB, Daniels P, Kirkland PD, Walker PJ. 2017. Genomic analysis of bluetongue virus episystems in Australia and Indonesia. Veterinary Research 48: 82. PMCID: PMC5701493

b. **Firth C,** Tokarz R, Simith DB, Nunes MR, Bhat M, Rosa ES, Medeiros DB, Palacios G, Vasconcelos PF, Lipkin WI. 2012. Diversity and distribution of hantaviruses in South America. Journal of Virology 86: 13756-66. PMCID: PMC3503106

c. Tokarz R, **Firth C**, Madhi SA, Howie SR, Wu W, Sall AA, Haq S, Briese T, Lipkin WI. 2012. Worldwide emergence of multiple clades of enterovirus 68. Journal of General Virology 93: 1952-8. PMCID: PMC3542132

d. Kapoor A, Simmonds P, Gerold G, Qaisar N, Jain K, Henriquez JA, **Firth C**, et al. 2011. Characterization of a canine homolog of hepatitis C virus. Proceedings of the National Academy of Sciences 108: 11608-13. PMCID: PMC3136326

e. **Firth C**, Charleston MA, Duffy S, Shapiro B, Holmes EC. 2009. Insights into the evolutionary history of an emerging livestock pathogen: porcine circovirus 2. Journal of Virology 83: 12813-21. PMCID: PMC2786836

Complete List of Published Work in MyBibliography:
https://www.ncbi.nlm.nih.gov/myncbi/1pcDCsap4HMk2/bibliography/public/

BIOGRAPHICAL SKETCH

NAME: Firth, Cadhla

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6)

POSITION TITLE: Senior Research Scientist and Program Coordinator, EcoHealth Alliance

D. Additional Information: Research Support and/or Scholastic Performance

Pending Research Support

National Institute Health (Grant 13220428) Daszak P (PI) 2021-2026

Title: Analyzing the potential for future bat coronavirus emergence in Southeast Asia.

Role: Co-Investigator. My role will include project co-ordination across all country partners, data collection, management and analysis, the preparation of manuscripts, and the communication of results to partners and the public.

Goal: The goal of this project is to build a platform that pinpoints the risk of future coronavirus emergence in the region where SARS-CoV-2 likely originated, informs strategies to intervene and prevent future pandemics, and provides critical reagents, therapeutic interventions and recombinant viruses for public health preparedness against future SARS- and COVID-like events.

Current Research Support

National Institutes of Health (1U01AI151797) Daszak P (PI) 2020-2025

Title: Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of South East Asia.

Role: Key Personnel. My role is to help drive the research-focused agenda of the project, including supporting in-country partners in Southeast Asia and developing new collaborations to support project goals. I am also responsible for assisting in data collection, management and analysis, and the preparation of manuscripts for publication.

Goal: The goals of this project are to identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife; identify evidence of novel virus spillover and key risk pathways for transmission; and identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts.

Tropical Australian Academic Health Centre Research Seed Grant McBride J (PI) 2019-2021

Title: Can portable genome sequencing provide a rapid, comprehensive, point-of-care diagnostic test for Far North Queensland hospitals and healthcare centers?

Role: Co-Principal Investigator. My role is project management and testing of the resultant platform *in situ* at Cairns Hospital. I am also leading data analysis and interpretation, and liaising with the clinical co-investigators throughout the project.

Goal: To trial the clinical metagenomics platform we developed as a point-of-care diagnostic test for fever, sepsis, and pneumonia at Cairns Hospital, and test the hypothesis that this approach will increase the proportion of infections that are diagnosed while also reducing time-to-diagnosis.

Previous Research Support

QIMR Berghofer Coronavirus Research Grant Reid D (PI) 2020

Title: Prognostics, Diagnostics and Therapeutic targets in response to infectious threats to the Australian population; (Phase 1) COVID-19.

Role: Co-Investigator. My role was to sequence the genomes of SARS-CoV-2 from patients and perform relevant analyses.

Goal: To predict clinical outcomes from SARS-CoV-2 infection using a combination of immunological and pathogen data collected from nasopharyngeal swabs, blood, and urine samples.

Tropical Australian Academic Health Centre Research Assistance Brown J (PI) 2020

Title: Building Research Support for Cairns Hospital.

Role: Co-Investigator. My role was to collaborate with and support clinicians engaging in infectious disease research, and help build a culture that fosters scientific research.

Goal: Cairns Hospital is a regional facility serving as a referral hospital for remote communities across Far North Queensland, yet does not have a strong culture of scientific research. This grant supported collaborations between academics and clinicians working in emerging infectious diseases in this region to improve research output for rural/remote Australia.

HOT North Pilot Project Grant Firth C (PI) 2019-2020

Title: Portable genome sequencing as a point-of-care diagnostic test in remote tropical Australia

Role: Principal Investigator. My role was to design the patient and sampling strategies, design a laboratory workflow suitable for uptake in a clinical diagnostic lab, assist in the development of novel bioinformatics pipelines for clinical metagenomics, and validate the approach.

Goal: To develop a clinical metagenomics ID diagnostic platform suitable for use in rural and remote hospitals and health services in tropical Northern Australia.

Australian Research Council Discovery Early Career Researcher Firth C (PI) 2015-2018

Title: Characterizing the impact of urbanization on viral diversity, ecology and disease emergence

Role: Principal Investigator. My role was project design and management, fieldwork and sample collection, metagenomic sequencing, data analysis and interpretation.

Goal: This project examined the viral response to changes in reservoir host and vector population structure and dynamics that occur as a result of urbanization, and identified viral characteristics associated with survival in an urban environment in Southeast Asia.

NIEHS Center for Environmental Health Pilot Project Grant Firth C (PI) 2013

Title: Identification of rodent-borne human pathogens in an urban environment and the features that influence their presence and prevalence

Role: Principal Investigator. My role was project design and management, fieldwork and sample collection, metagenomic sequencing, data analysis, and interpretation.

Goal: To characterize the zoonotic pathogens present in rats and their ectoparasites in New York City.

BIOGRAPHICAL SKETCH

NAME: Rabaa, Maia Anita

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6)

POSITION TITLE: University Research Lecturer in Medicine, Molecular Epidemiology Group Head

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	Completion Date	FIELD OF STUDY
Walsh University	BS	05/2001	Biology
Johns Hopkins Bloomberg School of Public Health	MHS	05/2006	International Health, Disease Epidemiology and Control
The Pennsylvania State University	PhD	12/2012	Biology

A. Personal Statement

I have the experience, expertise, training, and motivation needed to successfully support the proposed research project to analyze viral diversity and cross-species transmission on mixed-species farms in Viet Nam. I am a molecular epidemiologist at OUCRU Viet Nam. I have 8 years of experience in the design and implementation of research in human and animal health in Viet Nam, with a focus on emerging infections, viral zoonoses and antimicrobial resistance. I currently lead the Molecular Epidemiology group at OUCRU: a group of 28 staff (including one postdoctoral scientist, PhD students, clinicians, research assistants and technicians, nurses, and experienced project coordinators).

My primary research interests are laboratory, community, and hospital-based studies investigating the epidemiology, ecology, and evolution of pathogens in human populations and at the animal-human interface. My postdoctoral work focused on the development and implementation of a countrywide study of virus epidemiology and viral zoonoses, called VIZIONS, in which I designed and implemented scientific research and assisted in the management of collaborations across 7 Vietnamese hospitals and 3 local and regional animal health offices. I am now the PI on laboratory work and analyses of samples collected within the VIZIONS study, including identification of pathogens in farmed and wild animal populations. These studies use genomics, epidemiology, clinical, laboratory, and field science to test hypotheses on the emergence of viral zoonoses. Project coordinators currently on my team also worked on the VIZIONS study and maintain valuable links to collaborating institutions focused on human and animal health across the country. My team's previous experience and our nationwide network of collaborators will facilitate a scientifically rigorous study with important buy-in from local communities and health authorities. I have managed various institutional grants and delivered a number of significant and highly visible publications focused on a portfolio of varied pathogens and public health issues.

Further, I am committed to providing training and mentorship for low- and middle-income country researchers. In addition to junior research staff, I have supervised 3 PhD students to completion (University of Oxford [UK], Open University [UK]), and am currently supervising 2 PhD students (University of Oxford [UK]) and 1 postdoctoral scientist.

a. **Rabaa MA**, Ngo TT, Tran MP, Carrique-Mas J, Saylors K, Cotten M, Bryant JE, Ho DTN, Nguyen VC, Ngo TH, Wertheim H, Nadjm B, Monagin C, van Doorn HR, Rahman M, Campbell, Boni MF, Pham TTT, Simmonds P, Rambaut A, Nguyen VVC, Wolfe ND, Kellam P, Farrar J, Tran TH, Thwaites GE, Woolhouse MEJ, Baker S. (2015) The Vietnamese initiative on zoonotic infections (VIZIONS): a strategic approach to studying emerging zoonotic infectious diseases across Vietnam. **EcoHealth**. 12(4):726-35.

b. Pham HA, Nguyen VC, Nguyen ST, Ngo TT, Kosoy M, Woolhouse MEJ, Baker S, Bryant JE, Thwaites G, Carrique-Mas JJ, **Rabaa MA**. (2015) Diversity of *Bartonella* identified in bats in southern Vietnam. **Emerging Infectious Diseases**. 21(7):1266-67.

c. Phan M, Anh PH, Cuong NV, Munnink BO, van der Hoek L, Phuc TM, Tue NT, Bryant JE, Baker S, Thwaites G, Woolhouse ME, Kellam P, **Rabaa MA**, and Cotten M. (2016) Unbiased whole-genome deep sequencing of human and porcine stool samples reveals circulation of multiple rotavirus genogroups and putative zoonotic infection. **Virus Evolution**. 2(2):vew027.

d. Berto A, Pham HA, Carrique-Mas JJ, Simmonds P, Van Nguyen C, Tri TN, Nguyen DV, Woolhouse ME, Smith I, Marsh GA, Bryant JE, Thwaites GE, Baker S, **Rabaa MA**. (2017) The detection of potentially novel paramyxoviruses and coronaviruses in bats and rats in the Mekong Delta region of southern Viet Nam. **Zoonoses and Public Health**. 65(1):30-42.

B. Positions and Honors

YEAR(S)	POSITION
2007-2008	Research Associate at the Fogarty International Center, U.S. National Institutes of Health, Maryland, USA
2008-2009	Campbell Distinguished Graduate Fellowship, The Pennsylvania State University, USA
2009-2012	Graduate Research Fellowship, National Science Foundation, USA
2012-2015	Postdoctoral Research Fellow at the University of Edinburgh, Edinburgh, UK
2012-2020	Lead Molecular Epidemiologist at OUCRU, Ho Chi Minh City, VN
2015-2020	Research Fellow at the Nuffield Department of Medicine, University of Oxford, UK
2020-present	Molecular Epidemiology Research Group Head at OUCRU, Ho Chi Minh City, VN
2020-present	University Research Lecturer at the Nuffield Department of Medicine, University of Oxford, UK

C. Contributions to Science

1. Pathogen diversity and exchange at the human-animal interface

With Southeast Asia considered a hub of zoonotic transfer, I, with colleagues at OUCRU, the University of Edinburgh, and the Sanger Institute, sought to track the frequency and risks for pathogen transfer at the human-animal interface. I played a pivotal role in designing and implementing a national hospital- and community-based study of zoonotic infections in Viet Nam, driving a number of substudies and conducting analyses investigating pathogen evolution and epidemiology. These studies have uncovered frequent zoonotic infections presenting in clinical settings, and I am now the PI for several continuing analyses using archived samples and data to perform virological and serological investigations of zoonotic infections, as well as investigations of pathogen diversity in farmed and wild animal populations, including bats.

a. Van Nguyen VC, Le Buu C, Desquenes M, Herder S, Nguyen PHL, Campbell JI, Nguyen VC, Yimming B, Chalermwond P, Jittapalapong S, Franco JR, Ngo TT, **Rabaa MA**, Carrique-Mas JJ, Thanh TPT, Nga TVT, Berto A, Hoa NT, Hoang NVM, Tu NC, Chuyen NK, Wills B, Hien TT, Thwaites GE, Yacoub S, Baker S. (2016) A clinical and epidemiological investigation of the first reported human case of the zoonotic parasite *Trypanosoma evansi* in Vietnam. **Clinical Infectious Disease**. 62(8):1002-8.

b. Lu L, Dung NV, Ivens A, Bogaardt C, O'Toole A, Bryant JE, Carrique-Mas JC, Cuong NV, Anh PH, **Rabaa MA**, Tue NT, Thwaites GE, Baker S, Simmonds P, Woolhouse ME. (2018) Genetic diversity and cross-species transmission of kobuvirus in Vietnam. **Virus Evolution**. 4(1):vey002.

c. Campbell JI, Lan NPH, Phuong PM, Buu Chau L, Duc TP, Guzmán-Verri C, Ruiz-Villalobos N, Tam PTM, Muñoz Álvaro PM, Moreno E, Thwaites GE, **Rabaa MA**, Vinh Chau NV, Baker S. (2017)

Human *Brucella melitensis* infections in southern Vietnam. **Clinical Microbiology and Infection**. 23(11):788-790.

d. Tra My PV, **Rabaa MA**, Donato C, Cowley D, Vinh PV, Ngoc DT, Pham HA, Bryant JE, Woolhouse ME, Kirkwood CD, Baker S. (2014) Novel porcine-related G26P[19] rotavirus identified in pediatric diarrhea patients in Ho Chi Minh City. **Journal of General Virology**. 95(12):2727-33.

2. Evolution and epidemiology of flaviviruses

In my PhD and postdoctoral work, I performed detailed phylogenetic and epidemiological analyses of flavivirus populations to identify the determinants of novel flavivirus emergence in endemic and epidemic settings. This research showed the utility of connecting epidemiological and genomic data in understanding the spread of vector-borne disease, identifying risk factors (population connectivity, immunity, and climate) for flavivirus emergence at various spatial scales. Genomic studies of viruses sampled during phase III dengue vaccine trials showed that dengue virus genetics play a limited role in determining vaccine efficacy and identified genomic regions of the virus that may be under selection for vaccine escape. In these studies, I designed sampling strategies and conducted analyses of sequence data, clinical and epidemiological surveillance data, and geographical, climatological, and census data.

a. **Rabaa MA**, Girerd-Chambaz Y, Hue KDT, Tuan TV, Wills B, Bonaparte M, van der Vliet D, Langevin E, Cortes M, Zambrano B, Dunod C, Wartel-Tram A, Jackson N, Simmons CP. (2017) Genetic epidemiology of dengue virus populations in phase III trials of the CYD tetravalent dengue vaccine and implications for efficacy. **eLife**. 6:e24196.

b. Ho ZJM, Hapuarachchi HC, Barkham T, Chow A, Ng LC, Lee JMV, Leo YS, Prem K, Lim YHG, de Sessions PF, **Rabaa MA**, Chong CS, Tan CH, Rajarethinam J, Tan J, Anderson DE, Ong X, Cook AR, Chong CY, Hsu LY, Yap G, Lai YL, Chawla T, Pan L, Sim S, Chen IM, Thoon KC, Yung CF, Li JH, Ng HLD, Nandar K, Ooi PL, Lin RTP, Aw P, Uehara A, Pratim De P, Soon W, Hibberd ML, Ng HH, Maurer-Stroh S, Sessions OM. (2017) Outbreak of Zika in Singapore – an epidemiological, entomological, virological and clinical account. **Lancet Infectious Diseases**. 17(8):813-821.

c. **Rabaa MA**, Simmons CP, Fox A, Le MQ, Nguyen TTT, Le HY, Gibbons RV, Nguyen XT, Holmes EC, Aaskov JG. (2013) Dengue virus in sub-tropical northern and central Viet Nam: population immunity and climate shape patterns of viral invasion and maintenance. **PLoS Neglected Tropical Disease**. 7(12):e2581

d. **Rabaa MA**, Hang VTT, Wills B, Farrar J, Simmons CP, Holmes EC. (2010) Phylogeography of recently emerged DENV-2 in southern Viet Nam. **PLoS Neglected Tropical Disease**. 4(7):e766.

3. Mechanisms and determinants of antimicrobial resistance (AMR) emergence

Continual emergence of antimicrobial resistance is among the greatest threats to public health. As lead molecular epidemiologist at OUCRU, I have led various investigations of the genetics of AMR and its global spread. Working with collaborators across Asia, I drive both computational and lab-based experimental evolution studies to determine the drivers of the emergence and maintenance of AMR in human and animal populations. These studies have shown that plasmid transfer from healthy human gut microbiota can drive the emergence of resistance in pathogenic *Shigella*, and that inappropriate antimicrobial usage may enhance the frequency of plasmid transfer. Our studies also identify South and Southeast Asia, where inappropriate antimicrobial usage is rampant, as important hotspots of AMR emergence.

a. Duy PT, Nguyen TNT, Duong VT, Chung The H, Boinett C, Thanh HND, Tuyen HT, Thwaites GE, **Rabaa MA***, Baker S*. (2020) Commensal Escherichia coli are a reservoir for the transfer of XDR plasmids into epidemic fluoroquinolone-resistant *Shigella sonnei*. **Nature Microbiology**. doi:10.1038/s41564-019-0645-9.

b. Chung The H, Boinett C, Duy PT, Jenkins C, Weill F-X, Howden BP, Valcanis M, De Lappe N, Cormican M, Wangchuk S, Bodhidatta L, Mason CJ, Nguyen TNT, Tuyen HT, Phat VV, Duong VT, Lan NPH, Turner P, Wick R, Ceyssens P-J, Thwaites GE, Holt KE, Thomson NR, **Rabaa MA***, Baker S*. (2019) Dissecting the molecular evolution of fluoroquinolone-resistant *Shigella sonnei*. **Nature Communications**. 10:4828.

c. The HC, **Rabaa MA**, Thanh DP, Delappe N, Cormican M, Valcanis M, Howden BP, Wangchuk S, Bodhidatta L, Mason CJ, Nguyen TNT, Thuy DV, Thompson CN, Nguyen PHL, Phat VV, Thanh TH, Turner P, Sar P, Thwaites G, Thomson NR, Holt KE, Baker S. (2016) South Asia as a reservoir for the global spread of ciprofloxacin resistant *Shigella sonnei*. **PLoS Medicine**. 13(8):e1002055.

d. The HC, Karkey A, Thanh DP, Boinett CJ, Cain AK, Ellington M, Baker K, Dongol S, Thompson C, Harris SR, Jombart T, Phuong TLT, Hoang NTD, Joshi S, Basnyat B, Thwaites GE, Thomson NR*, **Rabaa MA***, Baker S*. (2015) A high-resolution genomic cross-sectional analysis of dual multi-drug resistant hospital outbreaks of *Klebsiella pneumoniae* in a low-income setting. **EMBO Molecular Medicine**. 7(3):227-39.

4. Typhoid epidemiology, genomics, and the role of carriage in transmission

A better understanding of the role of genetics in typhoid epidemiology is needed to improve diagnostics, treatment, and control, particularly in the context of introduction of typhoid conjugate vaccine in endemic regions. I have recently led and collaborated on studies that have utilized bacterial genomics to investigate the relationships between treatment failure, AMR, and vaccination, as well as to identify the selective pressures induced by gallbladder carriage and to better characterize the potential roles of typhoid carriers in typhoid transmission following large-scale vaccination. Our studies have shown that novel emerging clades of AMR *Salmonella* Typhi can rapidly undermine accepted treatment regimens; these data have resulted in improved treatment and diagnostic policies across Nepal. Novel studies of gallbladder carriage are expected to inform typhoid surveillance, including the identification and treatment of typhoid carriers, thus aiding elimination efforts.

a. Duy PT, Nga TVT, To NTN, Dan Thanh HN, Dongol S, Karkey A, Carey M, Basnyat B, Thwaites GE, **Rabaa MA***, Baker S*. Gallbladder carriage generates genetic variation and genome degradation in *Salmonella* Typhi. **PLoS Pathogens**. 16(10):e1008998.

b. Duy PT, Dongol S, Giri A, To NTN, Dan Thanh HN, Quynh NPN, Trung ND, Thwaites GE, Basnyat B, Baker S, **Rabaa MA***, Karkey A*. The emergence of azithromycin-resistant *Salmonella* Typhi in Nepal. **JAC-Antimicrobial Resistance**. 2(4):dlaa109.

c. Dyson ZA, Thanh DP, Bodhidatta L, Mason CJ, **Rabaa MA**, Phat VV, Tuyen HT, Thwaites GE, Baker S, Holt KE. (2017) Whole genome sequence analysis of *Salmonella* Typhi isolated in Thailand before and after the introduction of a national immunization program. **PLoS Neglected Tropical Diseases**. 11(1):e0005274.

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* equal contribution

Complete List of Published Work in MyBibliography:
https://www.ncbi.nlm.nih.gov/myncbi/1292rmtPpBsAK/bibliography/public/

BIOGRAPHICAL SKETCH

NAME: Rabaa, Maia Anita

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6)

POSITION TITLE: University Research Lecturer in Medicine, Molecular Epidemiology Group Head

D. Additional Information: Research Support and/or Scholastic Performance

Pending Research Support

NIAID/NIH (Grant 13220428) Daszak (PI) 07/2021 – 06/2026

Title: Analyzing the potential for future bat coronavirus emergence in Southeast Asia

Goal: The overall goal of this study is to build a platform to pinpoint the risk of future coronavirus emergence in a region where COVID-19 likely originated, inform strategies to intervene and prevent future pandemics, and provide critical reagents, therapeutic interventions, and recombinant viruses for public health preparedness against future SARS- and COVID-like events.

Role: Co-Investigator (country PI)

Pfizer (Investigator Sponsored Research: Pre-clinical/Clinical) Pham (PI) 03/2021 – 03/2023

Title: The bacterial etiology and antimicrobial susceptibility patterns of lower respiratory and intra-abdominal infections in Viet Nam

Goal: The goal of this study is to characterize and compare the microbiology, antimicrobial resistance patterns, and clinical outcomes of lower respiratory tract and intra-abdominal infections in 6 metropolitan hospitals in Viet Nam between 2019 and 2020.

Role: Co-Investigator

Current Research Support

COVID-19 Research Response Fund, University of Oxford Baird (PI) 05/2020 – 03/2021

Title: Validating GeneXpert platform for SARS-CoV-2 diagnosis for remote sites in Eastern Indonesia

Goal: The goal is to validate the GeneXpert platform using various sample types and to estimate the prevalence of SARS-CoV-2 infection among patients seeking treatment for febrile illness in Sumba, Indonesia.

Role: Co-Investigator

Research grant, Oxford University Clinical Research Unit Rabaa (PI) 02/2020 – 12/2021

Title: Molecular epidemiology of K. pneumoniae in bloodstream infections

Goal: The goal is to investigate the epidemiology and evolution of *K. pneumoniae* causing bloodstream infections in southern Viet Nam from 2010-2019.

Role: PI

Research grant, Oxford University Clinical Research Unit Rabaa (PI) 02/2020 – 12/2021

Title: A novel molecular assay for simultaneous detection of pathogen and AMR genes

Goal: The goal is to develop a rapid, inexpensive assay that will allow for rapid screening and pathogen/AMR diagnostics for bloodstream infections in resource-poor hospitals.

Role: PI

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List of abbreviations, acronyms, symbols

AGP: antimicrobial growth promoter
AMR: antimicrobial resistance
AMU: antimicrobial usage
AUS: Australia
BS: Bachelor of Science
BSL: biosafety level
CAN: Canada
CREID: Centers for Research in Emerging Infectious Diseases
DNA: deoxyribonucleic acid
Duke-NUS: Duke-National University of Singapore
DVM: Doctor of Veterinary Medicine
EHA: EcoHealth Alliance
EID: emerging infectious disease
EID-SEARCH: The Emerging Infectious Disease – South East Asia Research Collaboration Hub
ELISA: enzyme-linked immunosorbent assay
FACS: fluorescence-activated cell sorting
GCP: good clinical practices
GLM: generalized linear model
GLP: good laboratory practices
HCMC: Ho Chi Minh City
HPLC: high-performance liquid chromatography
HRSC: high-risk human sentinel cohort
HUTECH: Ho Chi Minh University of Technology
IT: information technology
IUCN: International Union for Conservation of Nature
MHS: Master of Health Science
MoH: Ministry of Health, Viet Nam
MS: Master of Science
MSc: Master of Science
NGS: next-generation sequencing
NIAID: National Institute of Allergy and Infectious Diseases
NIH: National Institutes of Health
NMDS: non-metric multidimensional scaling
NY: New York State
OUCRU: Oxford University Clinical Research Unit
OxTREC: Oxford Tropical Research Ethics Committee
PA: Pennsylvania State
PCR: polymerase chain reaction
PhD: Doctor of Philosophy
PI: principal investigator
PMC: Preventative Medicine Center
RAHO: Regional Animal Health Office
RNA: ribonucleic acid
RSEM: RNA-Seq by expectation-maximization
RT-PCR: real-time PCR
sDAH: sub-departments of Animal Health

SEA: Southeast Asia
TB: terabyte
UK: United Kingdom
UNIMAS: Universiti Malaysia Sarawak
USA: United States of America
ViPARC: Vietnamese Platform for Antimicrobial Reduction in Chicken production
VIZIONS: Vietnamese Initiative for Zoonotic Infections
VN: Viet Nam
VPN: virtual private network
WGS: whole genome sequencing

Facilities, Existing Equipment, and Other Resources

Laboratory:

OUCRU, in partnership with the Hospital for Tropical Diseases, have established state-of-the-art clinical diagnostic, basic, and applied research laboratories. The laboratory facilities include: clinical laboratories (ISO 15189:2012 certified), BSL2 research laboratories (GLP and GCLP compliance), BSL3 laboratories (MoH, Viet Nam certified), and a bio-archiving facility (Biosecurity compliance).

OUCRU laboratories in HCMC and Hanoi are collectively equipped with 29 safety cabinets (fume hoods, biosafety category II and III), 21 shaking and stationary incubators, 36 centrifuges including refrigerated centrifuges that accommodate swinging bucket and fixed angle rotors, six refrigerated microfuges, 12 thermocyclers, five real-time PCR machines, four UV/Visible microplate readers, a gel imaging platform (chemiluminescence, UV, and visible light), gel boxes, water baths, and mixing devices, 11 water-jacketed CO_2 incubators, five inverted microscopes, three fluorescence-activated flow cytometers (one with cell sorting capability), three capillary DNA sequencers, two Illumina MiSeq sequencers, Oxford Nanopore MinION sequencers, two HPLC systems, one GenXpert machine, one ultra-centrifuge, two Chef Mapper XA Systems, one Luminex Flexmap 3D, one Bio-Plex Suspension Array System, 26 -80°C freezers, 77 -20°C freezers, 33 refrigerators, eight liquid nitrogen tanks, a liquid nitrogen shipper, ice machines, autoclaves, and film developers.

Molecular Epidemiology research is conducted in several facilities across the laboratories including a specimen processing facility, a molecular diagnostic facility (GeneXpert and RT-PCR based diagnosis), a genomics facility (NGS facility using Illumina MiSeq and Oxford Nanopore MinION systems), and an immune-biology facility (ELISA, Luminex, FACS Lyrics system, Fluoresce microscopy, and cell culture facility). High-risk laboratory processes are conducted in the BSL3 laboratory. OUCRU maintains a bio-archiving facility (-20°C, -80°C, and liquid nitrogen storage facility) containing 2.5 million samples with continuous temperature monitoring and sample tracking system (Lab guard system, Freezerworks system and biometric access control system).

Clinical:

NA

Animal:

NA

Computer:

OUCRU employees have around-the-clock access to servers, VPN, encryption software, IT support, and all necessary software including: Git and Github (Hosted software revision/audit service), BBEdit text editors, Oracle Virtualbox virtual machines, Google Apps (Hosted email and collaboration web based software), Python, NodeJS, and R programming languages, Meteor (Javascript framework), Bash shell scripts, Jenkins (Continuous Integration server), Microsoft Office and Adobe CS6 running on Apple Mac OS X, Ubuntu Linux, and Windows Operating Systems. OUCRU has a dedicated 150+ core Linux server with 8TB hard drives, and the Molecular Epidemiology group has two dedicated 16-core Mac Pro Servers with 4TB hard drives. Either server individually or in combination may be used for intensive sequence analysis, computational modeling and/or database processing by Dr. Nguyen. Access to supercomputing services is maintained by a dedicated staff member from the IT team.

Office:

The applicant will have a dedicated desk in the office of the Molecular Epidemiology Group under the supervision of Drs. Maia Rabaa and Duy Pham Thanh, with expertise in both wet and dry laboratory research.

Other:

NA

Applicant Name (Last, first, middle): Nguyen, Cuong, Van

Letter of Organizational Support

28th February 2021

Dear CREID pilot research program funding committee,

Proposal from Nguyen Van Cuong

As Director of the Oxford University Clinical Research Unit (OUCRU) in Vietnam I would like to confirm my strongest possible support for the application from Nguyen Van Cuong.

Cuong has worked at OUCRU in Ho Chi Minh City since 2011, first as a Research Assistant and Project Coordinator for the Wellcome Trust funded Vietnam Initiative on Zoonotic infections (VIZIONS) project, and since 2017 as a PhD student and Project Coordinator for the ViPARC project (a Veterinary Intervention to Reduce Antimicrobial Usage in Animal Production). He has submitted an excellent PhD thesis (Open University, UK) and is scheduled to defend it on 15 March, 2021. In Cuong's tenure at OUCRU, he has developed strong skills in veterinary epidemiology, project coordination, and biostatistical analysis, along with varied wet laboratory skills. He will make an excellent post-doctoral researcher.

As Cuong moves into his postdoctoral career, the CREID pilot research program presents an excellent opportunity for him to grow in his independence as a scientist while gaining new scientific and program management skills as principal investigator of the proposed project. The proposed project will allow Cuong to develop additional expertise in study design, metagenomic sequencing, and bioinformatics. Additionally, the built-in mentorship program and regional and global networks that will be established through this collaboration with EcoHealth Alliance and the EID-SEARCH team will aid Cuong in designing and developing future impactful research on emerging infectious diseases at the human-animal interface, a topic that is in line with both Cuong's personal scientific agenda and the mission of OUCRU.

If funding is obtained for Cuong's proposal from the CREID pilot research program, OUCRU is committed to providing sufficient support to complete the project. Under this scheme, Cuong will have 100% protected time to complete the laboratory and analytical work to achieve the aims of this 1-year study, while working as a postdoctoral researcher within the OUCRU Molecular Epidemiology group, jointly led by Drs. Maia Rabaa and Duy Pham Thanh.

Cuong will have access to a remarkable collection of archived samples and data from the VIZIONS project for screening and downstream laboratory work, dedicated laboratory and office space within the OUCRU Molecular Epidemiology group, and access to shared laboratory spaces, computational resources, and equipment needed to complete the project. His research will be aided by existing laboratory and analytical expertise in the Molecular Epidemiology group, and a research assistant with experience in viral screening and sequencing will be employed to assist in sample processing and data management. As a member of the Molecular Epidemiology group, Cuong will participate in biweekly lab meetings, with regular opportunities to present and receive feedback on his work. He will also benefit from participation in extensive unit-wide academic and training programs.

I believe that the CREID pilot program will provide Cuong with a unique platform from which to develop veterinary epidemiology research within Viet Nam and across Southeast Asia. He would utilize the

knowledge and experience gained under this program to encourage and mentor future cohorts of scientists, veterinarians, and animal health officials in the pursuit of emerging infectious disease and OneHealth research and interventions to improve human and animal health in Viet Nam. He has my complete support.

Yours Faithfully,

(b) (6)

Professor Guy Thwaites FRCP FRCPath FMedSci

Director, Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam;

Professor of Infectious Diseases, University of Oxford

Letter of Collaboration from CREID Research Center PI

Dr. Nguyen Van Cuong
Project Coordinator
Oxford University Clinical Research Unit
Ho Chi Minh City, Viet Nam

Dear Dr. Nguyen,

The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH) at EcoHealth Alliance is extremely interested in working with you and your collaborators at the Oxford University Clinical Research Unit (OUCRU) in Viet Nam on the proposed research: "*Multi-species farming, viral diversity, and risks of cross-species transmission in the Central Highlands of Viet Nam*".

This collaboration will significantly contribute to the goal of the CREID Network and EID-SEARCH to develop in-country research capacity and build the research network for emerging infectious diseases in Southeast Asia. It will also form the foundation for new partnerships among EID-SEARCH, OUCRU and other in-country stakeholders in Viet Nam, which will strengthen international and multi-sectoral cooperation for emerging infectious disease research in Southeast Asia. Further, the networking, training, and mentorship opportunities embedded within the Pilot Research Program will develop your own skills in mentorship, project management, and supervision within a supportive framework, and grow your network both within and outside of Viet Nam.

Beyond capacity and network building, the proposed research strongly aligns with the core of EID-SEARCH's scientific goals by exploring links between wildlife farming and several determinants of spillover risk, including farming practices and wildlife species diversity. By using an unbiased approach to virus surveillance and targeting a high-risk human-animal interface (wildlife farming), the proposed work has the potential to make a significant contribution to our understanding of the drivers behind cross-species transmission and infectious disease emergence. I am confident that this work will lead to at least one high-quality publication on the highly relevant topic of wildlife and emerging infectious diseases, particularly given the links between hunting, farming, and trading wildlife and the emergence of new zoonotic diseases, as highlighted by the ongoing COVID-19 pandemic.

I have high expectations for this collaborative research project and expect that this will only be the beginning of our work together. Members of EID-SEARCH (including myself and Dr Kevin Olival as PI and CI of EID-SEARCH, respectively) are committed to working closely with you to develop the research project and support the efforts needed for the success of this application and project implementation. Our Senior Research Scientist, Dr. Cadhla Firth, has enthusiastically joined the project as your Mentor to advise on study design, data collection, and analysis throughout the project. Dr Firth is committed to providing relevant and timely career advice to assist in your professional development, and brings a well-established network across Southeast Asia, Australia, and North America, which includes world leaders in the ecology and evolution of emerging infectious diseases. You will be invited to join all trainings conducted by EID-SEARCH and EcoHealth Alliance on emerging infectious disease field surveillance and statistical analysis to build your skillset, and Dr Firth will work closely with you to develop your skills in pathogen genetics and genomics. EID-SEARCH will also commit funding and arrange exchange opportunities for you to visit our partners in Singapore to expand your skill set in

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sequencing and serology. You will be supported to present the results of this research at international conferences, as well as meetings with CREID Network and external partners, and to produce high-quality publications from this research.

I am confident that the proposed research collaboration represents a genuine opportunity for your professional development, particularly as you will have successfully defended your PhD on March 15 2021, and are ready to transition to a role with more scientific independence. I am particularly impressed with your extensive work history at the human-animal interface of emerging infectious disease, including as a Program Coordinator with the VIZIONS project, and am confident that you have already begun to emerge as a future leader in this field in Viet Nam.

This letter conveys my strong interest and commitment to making this application a success, and I look forward to working with you on this research project.

Sincerely,

Peter Daszak
Principal Investigator, EID-SEARCH
President, EcoHealth Alliance

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Letter from Mentor

EcoHealth Alliance

Dr. Nguyen Van Cuong
Project Coordinator
Oxford University Clinical Research Unit
Ho Chi Minh City, Viet Nam

Dear Dr. Nguyen,

This letter is to express my support as your Mentor for your application to the CREID Pilot Research Program, entitled "*Mixed-species farming, viral diversity, and risks of cross-species transmission in the Central Highlands of Viet Nam*."

I am extremely excited that you have chosen me to be your Mentor for the proposed project. The research question is timely and important, and your work has the potential to have a strong impact on our understanding of potential disease risks associated with wildlife farming. Given what we understand to be the likely origins of SARS-CoV-2, there has never been a more pressing need to examine the links between our interactions with wildlife and emerging zoonotic diseases.

I would be hard-pressed to think of a more appropriate applicant to lead this project, given your background in veterinary medicine, epidemiology, and project management. The skills you have acquired during your previous work on the VIZIONS project and throughout your PhD will be enhanced by the Mentorship plan we have developed together. It has become clear to me as we have worked on the Pilot Project application that you are ready to make the transition from a junior scientist to an independent researcher capable of driving your own research projects, and I look forward to the opportunity to support you in this process. Towards this aim, I commit to supporting your professional development in the following ways:

- I will actively work to maintain the healthy, open, and informal dialogue we have established while drafting the project proposal, and I will seek feedback from you on a regular basis to ensure that our communication plan continues to meet your needs as the project progresses.
- I will engage with your current and future in-country collaborators in a supportive and inclusive manner.
- I will create opportunities for you to interact with scientists across my network through your research project to widen your exposure to people with a range of expertise.
- I will give you timely feedback on all material in the manner that works best for you.
- I will provide you with opportunities to present your work to my colleagues at EcoHealth Alliance during our academic meetings and include you in any relevant trainings.
- I will share my scientific and technical expertise with you to help ensure the success of the project. When skills outside my area are required, I will assist you in accessing this expertise from scientists within my network.
- I will assist you in career planning, providing advice and support as required to maximize your opportunity to succeed.

EcoHealth Alliance
520 Eighth Avenue, Suite 1200
New York, NY 10018
212.380.4460
EcoHealthAlliance.org

EcoHealth Alliance

One of the likely outcomes from the proposed research project will be the generation of pilot data that can form the basis for additional funding applications. The President of EcoHealth Alliance, Peter Daszak, has already expressed his support for a joint funding application that will follow on from the proposed project, with you as the lead PI. This will present the perfect opportunity for you to complete the transition to an independent scientist, and I am committed to assisting you through the process of writing your first funding applications as lead investigator.

There is significant overlap between your research interests and skills, the overall goals of OUCRU, and those of EcoHealth Alliance and the EID-SEARCH team. This pilot project presents a real opportunity for you to become a liaison between these organizations, and to help drive forward future collaborative research projects in Viet Nam. I am thrilled to be able to support you at this critical stage in your career and am genuinely convinced that you have the potential to become a science leader in Southeast Asia in the very near future.

Sincerely,

(b) (6)

Cadhla Firth, PhD
Senior Scientist and Program Coordinator
EcoHealth Alliance

EcoHealth Alliance
520 Eighth Avenue, Suite 1200
New York, NY 10018
212.380.4460
EcoHealthAlliance.org

Applicant(s) Name (Last, first, middle): Siegers, Jurre, Ynze and Heang, Vireak

Proposal Cover Sheet

Project Title	**In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance**
Principal Investigator Name	Jurre SIEGERS
Position/Title	Postdoctoral scientist
Department	Virology Unit
Institution Name	Institute Pasteur du Cambodge
Street	5 Monivong Blvd
City, State, Zip Code	Phnom Penh
Country	Cambodia
Email	jsiegers@pasteur-kh.org
Phone	+855 (0) 61 234 433
Country(ies) where work will be conducted	Cambodia
Pathogen(s) focus	Emerging respiratory viruses (influenza virus, coronavirus)
Co-Principal Investigator Name (*If applicable*)	Vireak HEANG
Position/Title	Research Engineer
Department	Sequencing Mini-Platform
Institution Name	Institute Pasteur du Cambodge
Street	5 Monivong Blvd
City, State, Zip Code	Phnom Penh
Country	Cambodia
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Contact information for institution(s) that would financially manage the award *(please note: changing the managing institution upon award may result in substantial funding delays)*	
Contractual Contact, Title	Christophe MOUSSET, Director of Administration and Finance
Institution Name	Institute Pasteur du Cambodge
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For Co-Principal Investigator (If applicable):	
Contractual Contact, Title	
Institution Name	
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Name of Mentor	***Dr. Erik A. Karlsson***
Mentor Institution	Virology Unit and Sequencing Mini-Platform, Institute Pasteur du Cambodge
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Research Center Affiliation	

Name of Mentor	***Dr. Cadhla Firth***
Mentor Institution	EcoHealth Alliance
Institution Address	520 Eighth Avenue, Ste. 1200 New York, NY 10018
Permanent location of Mentor	United States of America
Mentor email	firth@ecohealthalliance.org
Mentor phone	(212)380-4488
Research Center Affiliation	Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH)

Total Budget	***Repeat this table if there are two Co-PIs***
Direct Costs	$136,737.00
Indirect Costs	$10,938.96
Proposed Start Date	01/05/2022
Proposed End Date	30/04/2023

Project Abstract (250 words)

The ability to quickly assess risk of emerging infectious diseases at points of high exposure or contact is paramount for early warning systems and preventative actions. In addition, maintenance of surveillance systems in individual animals is costly and time consuming and prevents widespread coverage. One way to address this issue is to incorporate environmental sampling into surveillance programs to cast a wider net at high-risk interfaces and on a longitudinal basis. To this end, we have begun to incorporate environmental sampling at high-risk human-animal interfaces, including live animal markets, slaughterhouses, and domestic/wild animal interfaces. However, these samples have only been tested for specific pathogens of interest by conventional or real-time PCR, limiting their utility. The ability to use these samples for metagenomic surveillance and pathogen discovery would not only increase surveillance capacity, but also contribute to our understanding of new or emerging pathogens at these high-risk interfaces. Therefore, in the proposed project, we will: (1) utilize existing environmental samples from longitudinal pathogen surveillance to build metagenomic sequencing and bioinformatics capacities and capabilities; (2) assess the suitability of environmental metagenomics as an early warning system for endemic and emerging infectious diseases at high-risk interfaces in Cambodia; and, (3) potentially discover new, emerging, or zoonotic pathogens of concern in high-risk interfaces in Cambodia. Overall, the results from this study will potentially help to improve surveillance systems through rapid and broad pathogen detection, reduced cost, lowered occupational risk, and diminished animal and environmental impact.

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In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance

Study Personnel

Jurre SIEGERS, Ph.D., (Postdoctoral Researcher) is a postdoctoral researcher in the Virology Unit at IPC in charge for planning and conducting research on zoonotic viral diseases, including avian influenza and coronaviruses. Dr. Siegers currently overseas all avian influenza virus surveillance efforts of IPC. Dr. Siegers has been vital in implementing COVID-19 sequencing Institut Pasteur du Cambodge. Dr. Siegers will plan, oversee and conduct sample processing, data collection, and analysis for this proposal. He will also be instrumental in preparing data and publications stemming from this work. He will meet with the mentors and technical personnel on a twice-weekly basis to discuss results, issues and future work. Dr. Siegers's commitment to this project constitutes 25% of his total professional effort for the year (3 calendar months).

Vireak HEANG, MS.c., is a research engineer at sequencing platform at IPC in charge of providing sequencing service to other scientists across the unit at IPC. Mr. Heang has an extensive experience over 15 years on molecular biology technique including PCR, Sanger sequencing and NGS. Prior to move to IPC Mr. Heang was working at US Naval Medical Research Unit. 2 (NAMRU-2), the place where he went through various training programs, on-the-job experiences and finally developed a deep understanding of molecular techniques and how it can be used in detection, characterization, and epidemiological modelling of pathogens. When he moved to IPC July last year, Mr. Heang manage to pull all the resources together to make the Illumina Miseq system functioning and he made this happened through his effort and the result was several runs of COVID-19 and Malaria specimens successfully sequenced. Mr. Heang will handle logistic, laboratory work and data analysis of this project. He will meet with the mentors and technical personnel on a twice-weekly basis of discuss results, issues and future work. Mr. Heang will be allocated 25% of his work to this proposed project.

Erik KARLSSON, Ph.D., is a Senior Research Scientist (Assistant Professor equivalent) and acts as the Deputy Head of the Virology Unit at Institut Pasteur du Cambodge. He is an expert on Respiratory Viruses, including SARS-CoV-2, and will act as the Primary Scientific Mentor for Dr. Siegers and Mr. Heang. He is the Director of the National Influenza Center in Cambodia, Director of the WHO Regional H5 Reference Laboratory, and Coordinator of the WHO Global COVID-19 Reference Laboratory housed in the Virology Unit. He is also Co-PI of the Sequencing Mini-Platform formed at IPC in 2021. He will meet regularly with the co-PIs to review experimental progress, troubleshoot issues, interpret data, and participate in preparation of publications resulting from this research.

Cadhla FIRTH, Ph.D., is part of the CREID EID-SEARCH Research Center and will act as the Research Mentor for Dr. Siegers and Mr. Heang. Dr. Firth is Senior Research Scientist and Program Coordinator at EcoHealth Alliance and holds a position as an Adjunct Senior Research Fellow at James Cook University in Australia. Dr Firth has more than 15 years' experience conducting research on emerging infectious diseases, with a focus on the ecology and evolution of zoonotic pathogens at human-animal interfaces. Dr. Firth's expertise in molecular biology, metagenomic sequencing, and the analysis of genomic and metagenomic data match well the goals of the proposed project. Dr. Firth will provide methodological and analytical guidance, mentoring and career advice, and networking opportunities that support the co-PIs progression toward independence, as well as assisting in the preparation of publications or future funding applications that may arise from this work.

Research Aims & Objectives

The ability to quickly assess zoonotic disease risk at potential points of high exposure or contact is of paramount importance for effective early warning systems and the initiation of preventative actions. Therefore, **this pilot project aims to answer the question: "Can metagenomic pathogen discovery on environmental samples improve the speed, comprehensiveness, and cost of existing pathogen surveillance programs?"**

In Cambodia, current pathogen surveillance systems rely primarily on sampling and testing individual animals – a practice that is both costly and time consuming, and prevents widespread coverage of all high-risk areas. **One way to address this issue is the incorporation of environmental sampling (ES) into surveillance programs**. ES includes samples or swabs taken from soil, water sources (drinking, carcass wash, lakes and ponds), feeding sources, feathers, air, and surfaces such as cages, chopping boards, and defeathering machines. As such, **environmental pathogen surveillance casts a wide net at high-risk interfaces, potentially improving surveillance coverage and supporting expanded sampling on a longitudinal basis**. After collection, samples are currently tested using a series of molecular diagnostics targeting a range of pathogens, another cost- and time-limiting factor. We hypothesize that utilizing metagenomic pathogen discovery with ES can: **(i)** improve, expand and simplify existing methods of pathogen surveillance; **(ii)** reduce the cost of pathogen surveillance programs; **(iii)** reduce direct contact between people and large numbers of animals, thereby improving biosafety, animal welfare, and reducing occupational exposure risks; and **(iv)** set a precedent for lower-middle income countries (LMICs) to conduct broad pathogen surveillance cost-effectively.

To test these hypotheses, we will utilize a **large and unique biobank** from Cambodia that includes previously collected **paired samples** from individual animals and ES. These samples originate from ongoing longitudinal surveillance studies for: **(i)** avian influenza viruses in live bird markets (LBMs); **(ii)** swine influenza and Nipah virus in swine abattoirs **(iii)** bat-borne viruses from caves, roosts, and guano farms.

The proposed project has *three aims*:

AIM 1: To assess the suitability of environmental metagenomics as an early warning system for endemic and emerging infectious diseases at high-risk interfaces in Cambodia.

AIM 2: To utilize biobank environmental samples from longitudinal pathogen surveillance programs in Cambodia to build metagenomic sequencing and bioinformatics capacities and capabilities in Cambodia.

AIM 3: To discover new, emerging, or zoonotic pathogens of concern at high-risk interfaces in Cambodia.

The proposed project has *four objectives*:

Objective 1: Characterize and compare the viral diversity present in samples from individual animals (poultry, swine, bat guano/urine) and environmental samples (wash water, air samples, cage swabs), using a combination of metagenomics and polymerase chain reaction (PCR)-based screening methods. **Endpoint:** Comparison of 129 animal samples with 207 ES across 3 species for 3000+ viral targets

Objective 2: Assess whether a surveillance system that incorporates both environmental sampling and virus detection through metagenomics is sensitive and specific enough to replace or support conventional virus surveillance programs**. Endpoint:** Comparative sensitivity (detecting the same virus) in metagenomic versus previously detected pathogens by RT-PCR.

Objective 3: Discover previously undetected, (re)-emerging, and/or zoonotic pathogens of concern at high-risk interfaces in Cambodia. **Endpoint:** Sequencing 2M reads/sample to ensure coverage and maintain cost-effectiveness.

Objective 4: Build capacity and capability in metagenomic sequencing and bioinformatics at Institute Pasteur du Cambodge. **Endpoint**: Successful implementation of a user-friendly metagenomic sequencing and analysis platform.

Study Rationale/Research Gap/Impact

Emerging infectious diseases are a major and ongoing threat to global health and the economy as illustrated by the ongoing SARS-CoV-2 pandemic and a record-breaking avian influenza outbreak across Asia, Europe, the Middle East, and North America. Asia, especially Southeast Asia, is a hotspot of endemic and emerging infectious diseases, including avian influenza viruses (AIVs), and bat-borne viruses such as coronaviruses and henipaviruses with pandemic potential[1-4].

Cambodia is a resource poor, lower-middle income country in tropical Southeast Asia with a large socio-economic dependence on agriculture[5]. Since 2011, Institut Pasteur du Cambodge (IPC) has maintained active longitudinal pathogen surveillance programs at key live bird markets (LBMs), farms/slaughterhouses/storage facilities (poultry and pigs), and at locations where a diverse range of Cambodian bat species roost. These locations represent high-risk human-animal interfaces, where known zoonotic pathogens circulate and novel disease emergence events are likely to occur[6,7]. Sentinel and research pathogen surveillance systems are conducted by IPC for the early detection/warning of known emerging infectious disease to support and inform Cambodian public health systems and the global virus community. Currently, these surveillance programs use PCR-based pathogen detection, which relies on specific or pan-virus primers to amplify conserved genomic targets from an individual virus family. While this approach has led to the successful identification of novel viruses in the past, reliance on conserved genomic targets limits detection of sufficiently divergent pathogens, including those that arise through recombination or reassortment[8,9]. These surveillance efforts are also labor intensive, expensive, limited to known hotspots, and highly species-specific (both animal and viral family). To this end, we have begun to supplement our traditional surveillance methods, which depend on sampling directly from animals, with ES at high-risk human-animal interfaces. These samples include water, swabs, and air samples, which are collected alongside samples taken from individual animals, enabling a direct comparison of pathogens identified from environmental samples with those collected from individuals.

Despite the clear risks associated with Cambodian LBMs, pig farms/slaughterhouses, bat guano farms and caves, there is very limited understanding of virus diversity at these sites. However, the hunt for the origin of SARS-CoV-2 has led to a renewed interest in the viruses present at live animal markets and across the wildlife supply chain, as well as in viruses carried by bats. Hence, improved surveillance of poultry, pigs, bats and other wildlife (as putative reservoirs and intermediate hosts of zoonotic viruses) will be key for the development of early and effective disease prevention and intervention measures by identifying viruses with known or predicted zoonotic potential at high-risk interfaces, where pathogen spillover is most likely to occur. The ability to use environmental samples for metagenomic pathogen surveillance and discovery would not only increase surveillance capacity, but also contribute to our understanding of new or emerging pathogens at these high-risk interfaces. Environmental sampling has several benefits compared to individual animal sampling: 1) **Biosafety benefits**. Handling animals for sampling is risky, both for the animal (discomfort and distress) and the handler (increased exposure to zoonotic infections, trauma from bites/scratches, etc.); 2) **Economic benefits**. Sampling individual animals requires increased sample numbers, trained personnel, more personal protective equipment, increased number of molecular tests, and time which are costly. Short-term and long-term storage of individual samples requires additional space in -80 freezers and/or liquid nitrogen. 3) **Increased comprehensiveness**. Environmental sampling expands the source of sampling to include animal excretions from multiple animals and/or species (urine, feces, saliva), contact material, and aerosolized particles. As many zoonotic diseases are effectively transmitted through the environment (i.e., respiratory viruses, enteric viruses), sampling from these sources may more directly represent human disease risk at key interfaces.

Significance and Approach

Backyard livestock farms, LBMs, and wildlife farms often have limited biosafety measures in place despite representing potentially risky human-livestock-wildlife interfaces, and as a result, have repeatedly been associated with the (re-)emergence of zoonotic diseases[1-4]. Therefore, developing early warning systems at these high-risk locations will be critical for monitoring and preventing zoonotic diseases from generating the next pandemic. Previous pathogen surveillance and virus diversity studies at human-livestock-wildlife interfaces in Cambodia have relied heavily on consensus PCR-based virus detection, and/or focused on only a single animal species[10]. These methods are time consuming and costly and may result in delayed disease detection and reporting. Every second counts between first detection and response. Indeed, the total cost of an outbreak grows exponentially as time from detection increases, making identification critical at emergence or early stages of spread[11]. It is critical to get "left of sneeze." **To this end, the proposed project will use and evaluate cutting-edge metagenomic approaches for multiple viral pathogens on broad coverage samples as a modern alternative to pathogen surveillance at high-risk interfaces**. Comparison to standard methods will be conducted under a hypothesis-driven framework to reveal the diversity, abundance, and zoonotic potential of viruses circulating in high-risk locations for emergence. To our knowledge, this is the first data-driven project with the goal of providing a foundation for the role of metagenomic ES in monitoring virus prevalence and emergence in Cambodia.

Like much of South/Southeast Asia, Cambodia is a hotspot of endemic and emerging infectious diseases. However, despite active, longitudinal surveillance efforts, outbreaks still frequently occur and data on virus diversity at high-risk human-animal interfaces are still lacking[12-14]. Additionally, as a Least Developed Country, the economic impacts of endemic agricultural and human seasonal pathogens are high, making surveillance for novel emerging pathogens at the animal-human interface a costly lower priority. Furthermore, it is increasingly difficult to obtain samples and/or data from some countries in the region, increasing the importance of Cambodia as a critical location for monitoring zoonotic disease emergence. The unique biobank of samples we have collected as part of active, longitudinal surveillance programs represents a remarkable opportunity to compare standard pathogen surveillance using individual animals and PCR with metagenomic ES to determine viral diversity and viral abundance.

Outcomes from this pilot study will provide invaluable data on improved and cost-effective surveillance practices within a "One Health" perspective. This is critical, as viruses will likely jump to new hosts from the water they drink, the surfaces they touch, and the air they breathe - less likely because they are kissing a chicken. **Environmental sampling reduces occupational risk, improves animal welfare, and can have non-invasive or reduced impact on market trade and wildlife habitats.** Improving our ability to monitor multiple pathogens in the environment will increase our capacity to develop and communicate key biosecurity practices and guidelines to reduce risk, and influence policy. Environmental sampling with multiple pathogen detection will also reduce the overall cost of surveillance systems, which is a key consideration in resource-limited settings. Expanded pathogen detection with reduced sampling means being able to conduct surveillance over a broader area and/or more frequently, greatly enhancing the ability to detect a potentially pandemic-level pathogen at a high-risk interface. Indeed, rough estimations from the budget of this pilot project alone predict surveillance could double in length or breadth for a single pathogen itself, let alone the 3000+ targets included in each enhanced metagenomic run.

Thus, in line with the goals of the CREID network and the EID-SEARCH team (to improve the detection and surveillance of important emerging viruses), we will use previously collected samples from high-risk human-animal interfaces in Cambodia to systematically characterize and compare viral diversity within and between environmental and animal samples from the same locations. We will evaluate the benefits of incorporating ES into existing surveillance programs as a means to broaden pathogen

surveillance efforts without increasing the cost of these programs at high-risk interfaces and on a longitudinal basis. We will develop and utilize user-friendly protocols and analysis pipelines **allowing for broad implementation in a resource-limited country.**

Research Methods

A total of 334 (129 animal pools and 205 ES pools/individual) will be analyzed in this pilot research project.

Poultry Surveillance – 224 samples (120 animal pools/104 ES pooled and individual)

Our metagenomic analysis will use environmental and animal samples previously collected as part of AIV surveillance programs at Cambodian LBMs and farms/slaughterhouses/storage facilities for poultry and pigs, which occurred from 2018 to April, 2022[9]. Sampling was performed at two major LBMs – Orussey Market in Phnom Penh province and Takeo Market in Takeo province (**Figure 1**). The LBM at Orussey Market is the largest LBM in Cambodia and the most extensively studied. The LBM at Takeo market is often associated with the emergence of new influenza subtypes, possibly due to its proximity to Viet Nam and the absence of biosafety and biosecurity measures[5,15]. Sampling at these markets occurred 16 times/year, and wash water samples (N=152), and cage swabs (N=55) were collected along with a multitude of individual animal samples from chickens (N=2240), ducks (N=2240), and other poultry. Air sampling using the Thermo Scientific™ AerosolSense™ Sampler was also conducted at three locations in and around the LBMs (N=33). **From this biobank we will select 10 paired ES and animal sampling sessions for metagenomic sequencing and analysis**. Together, this provides us with a unique sample set to observe virus diversity in animal versus ES samples in heavily sampled LBMs.

Figure 1. Overview live bird market (LBM) at Orussey Market, Phnom Penh province. A) Defeathering machines. **B)** Paired throat and cloacal sample collection. **C)** Air sampling machine at the poultry holding facility. **D)** Air sampling machine at poultry slaughter site.

Pig Surveillance – 54 samples (9 animal pooled/45 ES pooled and individual)

Ongoing pig surveillance studies are funded by NIAID/NIH - Centers for Excellence in Influenza Research and Response (CEIRR; 2021-2028) and include individual animal samples and environmental samples (air, cage swabs, and water) collected in three different provinces across Cambodia. Sampling locations were pig farms at high-risk interfaces (slaughterhouses) with minimal biosafety measures in place (**Figure 2**). A total of 45 individual pigs were sampled over three sessions, along with air samples, cage swabs, and wash water samples.

Bat Surveillance – 56 samples (35 animal ES individual/21 air ES individual)

Bat sampling sites for this proposal were chosen based on previous research efforts by IPC in Phnom Penh, Kampong Cham, Stung Treng, Kampot, Kandal, and Battambang provinces (**Figure 2**) [10,16,17]. Our standard pathogen surveillance protocol for bats focuses on the collection of urine and guano; here, these will be referred to as animal samples, although it is worth bearing in mind that they may be representative of multiple animals in some cases. Sample collection for this project is performed under the FAO-USAID and includes air, guano, and urine samples collected in and around seven locations of bat caves, roosting sites, and/or guano farms. Air samplers (N = 21) are deployed at cave entrances, roosting trees, and bat guano farms where large groups of individual bats congregate, maximizing the capacity to sample many individuals collectively. In parallel, air samplers were fitted with a sterile plastic cover (2m x 2m) to collect urine and feces (N = 35) to compare the viruses identified in air samples with those from bat excretions.

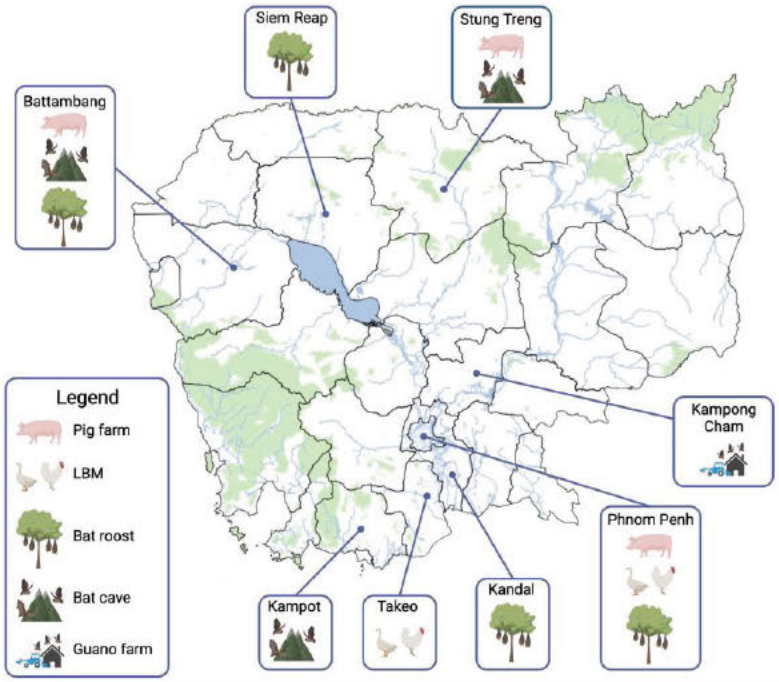


Figure 2. Location and type of sample previously collected in Cambodia and readily available for the proposed study.

Metagenomic Sequencing. While the advent of metagenomic sequencing was a significant step forward as a tool for pathogen detection and discovery, it remains relatively expensive and lacks the analytical sensitivity required for a true pathogen surveillance platform. Target enrichment panels were developed in response to this issue, but, until recently, were unable to facilitate the detection of highly divergent strains or novel viruses[10,18]. In this project, we will use a cutting-edge hybridization-based target enrichment approach - The Twist Comprehensive Viral Research Panel (CVRP) – that was designed to explicitly address this need[19]. The CVRP contains more than a million probes covering the genomes of 15,488 strains from 3,153 unique viruses (including animal viruses), and is tolerant to mismatches between virus and probe, supporting the detection of novel zoonoses. These include ssRNA, dsRNA, ssDNA, and dsDNA viruses belonging to every virus family capable of infecting people. Thus, target enriched metagenomics represents an ideal balance between the promise of unbiased virus detection and the feasibility of the standard tool of public health surveillance programs everywhere – quantitative PCR.

Prior to preparation for metagenomic sequencing, individual animal samples will be pooled by sampling session: 10 samples will be pooled for chickens and ducks per market (2 markets) per visit (10 selected visits), respectively; five samples will be pooled from pigs. Bat guano/urine will be run individually (**Table 1**). Wash water samples will be pooled by sampling session (N=2/session), as will cage swabs (N=5/session), while all air samples will be processed individually. Wash water samples will be pelleted and concentrated using ultrafilters[20] prior to RNA extraction.

In this project, we will follow the manufacturer's recommended protocol for the generation of hybridization-enriched sequencing libraries, under the guidance of Prof. Linfa Wang at Duke-NUS, who

has been involved in the development and implementation of target enriched metagenomic sequencing for several years (see attached letter of support). Briefly, high quality total RNA will be extracted from

Table 1. Sample types and numbers (pools) from paired sampling locations selected for target enriched metagenomic sequencing.

System	Animal sample type	Animal sample number	Environmental sample type	Environmental sample number
Poultry	Cloacal swab	Chicken: 600 (60)	Wash water	120 (60)
	Cloacal swab	Duck: 600 (60)	Cage swabs	55 (11)
			Air samples	33
Pigs	Nose/snout swab	45 (9)	Wash water	18 (9)
			Cage swabs	45 (9)
			Air samples	27
Bats	Urine/Guano	35	Air samples	21

each (pooled) environmental and animal sample using the RNeasy PowerMicrobiome Kit (Qiagen). RNA will be quantified and converted to cDNA using ProtoScript II First Strand cDNA Synthesis kit and random primers (New England Biolabs). The NEB Next Ultra II Non-Directional RNA second Strand Synthesis kit (New England Biolabs) will be subsequently used to convert single-stranded cDNA to dsDNA, and Illumina TruSeq-compatible libraries will be generated using the Twist Library Preparation Enzymatic Fragmentation kit (Twist Bioscience). These libraries will be hybridized for 16 hours with the set of 120 base pair biotinylated probes contained within the CVRP (Twist Bioscience) and sequenced with 2x75 bp paired-end reads on an Illumina MiSeq platform housed at IPC. Twelve target enriched libraries will be sequenced per run to achieve approximately 2 million reads/sample.

Metagenomic data analysis. For target enriched metagenomics to have utility as a public health surveillance tool, data analysis must be user-friendly and understandable to a non-expert in bioinformatics. Towards this aim, we will assess the performance and user-experience of two leading cloud-based metagenomics platforms for virus detection and compare the results to those generated by our bespoke bioinformatics pipeline[21]. Analysis of data generated by the CVRP is supported by One Codex software, a cloud-based platform for microbial genomics that will be used for preliminary data analysis and visualization[22]. One Codex uses a rapid, highly sensitive k-mer classification algorithm that maps sequencing reads to their custom database, which consists of more than 115k microbial genomes. The raw classification results are filtered through several statistical post-processing steps designed to eliminate false positive results caused by contamination or sequencing artifacts. We will analyze the same data using Genome Detective, a web-based platform that sorts sequence reads into bins prior to *de novo* assembly and compares the assembled contigs to the NCBI RefSeq and Swissprot UniRef90 databases, respectively, for virus identification[23]. Each of these platforms is contained within a point-and-click, web-based interface and reports results using easy-to-understand visualizations, with click-through options for additional details should the user wish to perform analyses beyond pathogen detection. Finally, we will analyze these same data with our bespoke bioinformatics pipeline (representing the gold standard approach), which uses Trinity RNA-Seq for *de novo* assembly[21], followed by comparisons with the complete non redundant nucleotide and protein databases available through GenBank using E-value cutoffs of 1E-10 and 1E-4, respectively, and the program Diamond[24]. Additional full genome assembly will be attempted for all known or potentially zoonotic viruses identified by any of the three analysis approaches using either metaSPAdes (for *de novo assembly* of highly divergent viruses), or BWA MEM for reference mapping of known viruses[25,26]. Sequences representing viral genomes will be further

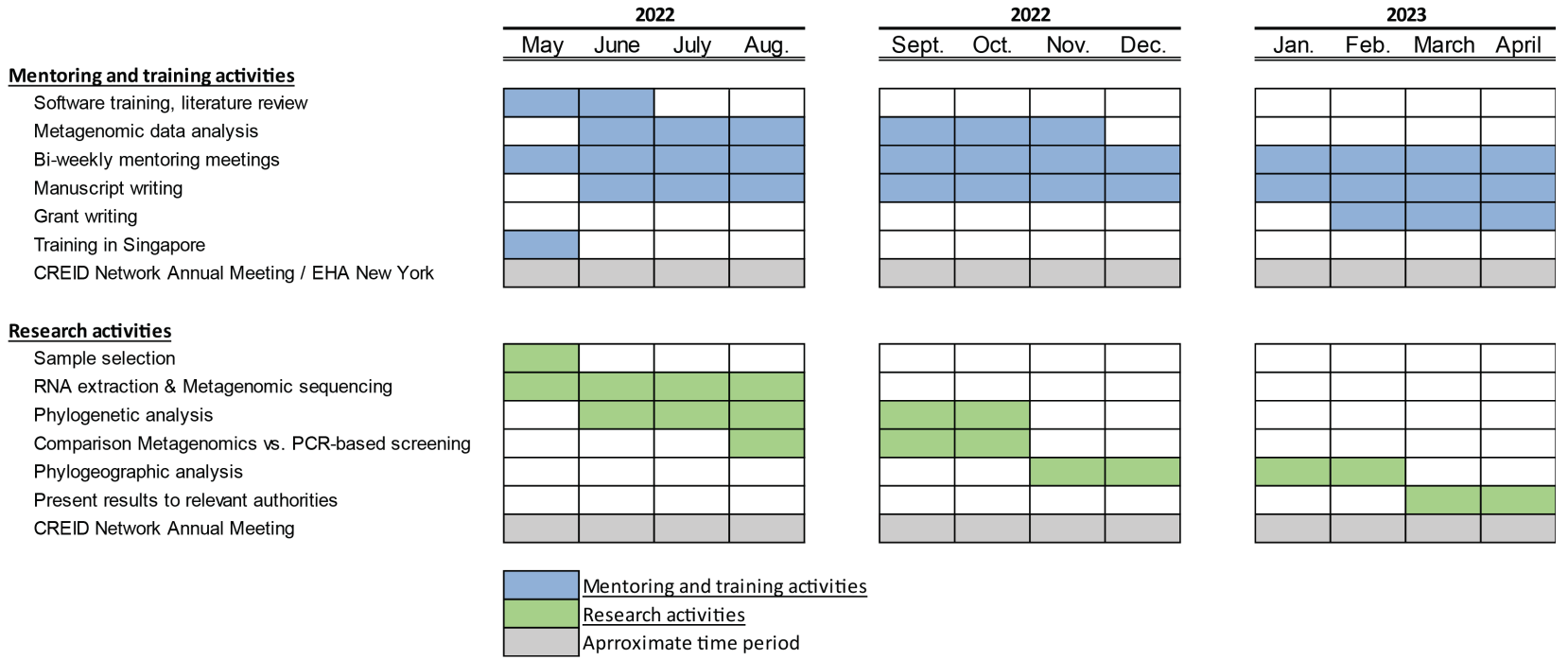
characterized using phylogenetic analysis targeting conserved domains (e.g, the RNA polymerase) that will include related viruses from GenBank to estimate evolutionary relationships and infer potential zoonotic disease risk. Phylogenies will be estimated using the maximum likelihood method implemented in IQ-Tree 2, employing the best-fit amino acid substitution models for each virus family[27]. The resultant phylogenies will be used along with the Virus-Host DB[28] to identify putative host species for viruses found in environmental samples, where genetic material from multiple host species may be present.

Assessing the utility of target-enriched environmental metagenomics for pathogen surveillance. First, we will interrogate the utility of ES as a tool for pathogen surveillance by comparing metagenomic data from environmental samples with metagenomic data from animal samples collected at the same time and location. From each sequencing library, we will calculate the following metrics: number (and identity) of virus families identified, number (and identity) of unique viruses identified, and percent genome coverage for each virus. These will be combined by sample type and sampling session (as replicates) to provide a snapshot of circulating viral diversity at each time/place. The metrics associated with each environmental sample type will be compared to those from the corresponding animal samples from the same time and location. This will allow us to assess what proportion of virus diversity in a host population is detectable from the environment, as well as to identify any taxon-specific biases in environmental sampling, if present. We will also compare viral community structure across sample types using using standard measures from community ecology, including species richness, alpha diversity, and beta diversity, which will be calculated using a range of R packages[29]. Permutational multivariate analysis of variance tests (permanova) will be used to assess differences in virome composition between sample types.

Next, we will examine the utility of target enriched metagenomics as an alternative to traditional molecular diagnostics (qRT-PCR and consensus PCR). As part of our routine pathogen surveillance efforts, individual animal samples from these same sampling sessions have previously been tested for the following pathogens: **Poultry** – AIV and Newcastle disease virus; **Pigs** – influenza, African swine fever virus, classical swine fever virus, Nipah virus, and porcine reproductive and respiratory syndrome virus; **Bats** – coronaviruses, Nipah virus, orthomyxoviruses. The results of these tests will be compared to the metagenomic results from environmental and animal samples to evaluate how well this approach recapitulates the results from standard pathogen surveillance efforts.

Potential Pitfalls and Alternative Plans: Overall, this is a straight forward study using novel and readily accessible samples. Any resultant data will be extremely useful in understanding the benefits of ES over animal sampling for pathogen surveillance. No issues are foreseen with utilizing retrospective samples for metagenomic sequencing; however, storage and collection issues can affect sample quality. If insufficient samples are available, IPC plans to continue surveillance activities throughout the period of this project and fresh samples can be collected for analysis. In addition, given the need to maintain cost-effectiveness, only 2M reads will be collected per sample using CVRP. A series of spike-in experiments will be conducted to ensure sequencing reads and dept is adequate for known viruses before conducting sequencing on precious samples to ensure detectability (not funded through this project). At the current time, no internationally recognized guidelines exist on the use of environmental sampling (ES) and data management. It is hopeful these studies will contribute to future guidelines surrounding the use of ES in surveillance sampling.

Project Timeline



Research Performance Sites (*1-page limit*)

The Institut Pasteur in Cambodia, established in 1953, is a non-profit research institution operating under the auspice of the Cambodian Ministry of Health (MoH) and the Institut Pasteur in Paris, France. IPC missions are: **(i)** to conduct innovative life science and health research on infectious and emerging disease in Cambodia; **(ii)** to provide public health services; **(iii)** to contribute to training and capacity building in the field of biomedical and life science.

IPC constitutes a strong team and a state-of-the-art facility with modern, secured and standardized laboratories appropriate for experiment with hazardous pathogens. These laboratories include biosafety level (BSL) 2 and 3. The BSL3 facility comprises four modules dedicated to different and separate activities including a safety cabinet with gloves box allowing BSL3+ safety conditions. The proposed project will take place within The Virology Unit at IPC, which serves as the reference laboratory for COVID-19, Influenza, Arboviruses, and Rabies for Cambodia, and conducts surveillance and research on endemic, emerging, and zoonotic pathogens. To respond to the COVID-19 pandemic, the Virology Unit at IPC has recently increased its sequencing capacity to include both Oxford Nanopore (GridION and MinION) and Illumina MiSeq technologies.

The Virology Unit works closely with the Epidemiology/Public Health, Entomology, Bacteriology, and Malaria Units at IPC, as well as national and international partners, to conduct One Health-focused surveillance and studies in Cambodian people, domestic animals, and wildlife. As such, IPC is continually searching for new and innovative ways to expand and improve surveillance activities. IPC also serves as a model for state-of-the-art research in a Least Developed Country/Low-Middle Income Country and successful studies at IPC can inform future work in the region.

All samples needed for this work have been maintained in IPC laboratory freezers at -80°C from the time of collection and can be easily linked to collection and pathogen metadata. These unique samples and data are available only at IPC, which is committed to facilitating the proposed research. Please see “12.10 - Facilities and Resources” for a more detailed description of infrastructure and equipment available at IPC to ensure the success of this project.

CREID Research Center Collaboration (*1-page limit*)

The goals and objectives of the proposed research project, "In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance", are fully consistent with the specific aims of EID-SEARCH. EID-SEARCH takes a One Health approach to understanding zoonotic disease emergence at high-risk human animal interfaces. EID-SEARCH aims to understand the frequency and causes of virus spillover in high-risk communities across Southeast Asia and identify strategies and tools to reduce this risk. As the proposed research focuses on improving, expanding and simplifying existing surveillance efforts to cast a wider net for pathogen surveillance and monitor virus diversity at key human-animal interfaces and on a longitudinal basis we expect the results of the project to make a significant contribution towards the overall goals of EID-SEARCH.

The proposed project is well-designed, leverages cutting-edge technology, uses archival samples, and includes a robust individual development and capacity building component that together corresponds to the overall research strategy of CREID and EID-SEARCH. The EID-SEARCH team is highly motivated to support the application of applying metagenomics to pathogen surveillance and assist in the analysis of the unique dataset that will be generated from this research. With the heightened focus on robust surveillance networks to timely identifying emerging zoonotic threat at key human-animal interfaces the proposed research is both timely and likely to yield highly-cited, impactful publications and policy recommendations for EID-SEARCH and its IPC partners.

The EID-SEARCH team includes a number of experts in study design, laboratory-based diagnostics, genetic, genomic, and bioinformatics analyses, who will contribute to project implementation, data analysis, and manuscript preparation as required. Communication and coordination mechanisms will be set up between the IPC research team and scientists at the EID-SEARCH research sites for resource sharing, training, knowledge exchange, and mentorship. In particular, EID-SEARCH will facilitate and encourage relationships between Dr. Jurre Siegers and Vireak Heang and our partners in Southeast Asia (based in Singapore, Australia and the US) that will facilitate Dr. Jurre Siegers and Vireak Heang professional development and strengthen research capacity in the region. Dr. Cadhla Firth, Senior Scientist at EID-SEARCH and the project Mentor, has an established relationship with both Dr. Jurre Siegers and his supervisor, Dr. Erik Karlsson, and will act as the liaison between EID-SEARCH and the IPC research team. An expected outcome of this research project will be pilot data to support a joint funding application in the future, led by Dr. Jurre Siegers.

Mentoring Plan

The co-PIs, Vireak Heang and Jurre Siegers, are at different stages of their respective careers with discrete professional development goals. This Mentoring Plan allows the co-PIs to pursue independent professional goals while working as a team to achieve the learning and development objectives associated with the proposed project.

1. Developing Leadership and Independence

Vireak Heang: My long-term goal is to train the next generation of Cambodian scientists. I can pass on my knowledge in my native language, Khmer, providing greater educational opportunity to all Cambodians. The opportunity for future scientists to learn in their native language is a critical step towards rebuilding the scientific infrastructure of my country. Prior to joining IPC, much of my work was technical, with limited experience in project management. However, at IPC, my Scientific Mentor, Dr. Karlsson, took an immediate interest in my professional development and is actively involving me in the entire scientific process – from conception, to study design, implementation, and analysis. As a result, we have developed a strong mentoring relationship built on trust and mutual respect that will continue across this project to improve my scientific career. My Research Center Mentor, Dr. Firth, has a broad scientific network, and regularly interacts with scientists across Southeast Asia in her role as a Project Coordinator for EID-SEARCH. To strengthen my ability to communicate complex scientific topics, both Mentors will actively provide opportunities for me to interact with and present my research to other scientists across Southeast Asia and through their respective global networks of research partners.

Jurre Siegers: My overall research goal is to improve the health of people, animals, and ecosystems using a "One Health" approach that will allow me to maximize my academic training, and scientific and personal interests. I wish to develop my skills in novel diagnostic tools, such as metagenomics, to contribute to improved surveillance and research capacity in the field of emerging viruses at human-animal interfaces. However, my aspirations are not just limited to the generation of scientific knowledge, methods, and policies. Instead, I wish to actively use my knowledge in virology (Ph.D. training) and emerging infectious diseases (Postdoctoral) to train the next generation of Cambodian scientists that will one day be local leaders in the field of emerging infectious diseases. As my Scientific Mentor, Dr. Karlsson has already demonstrated his commitment to my scientific progress by providing me with opportunities to develop project management skills and improve my scientific writing. Both Mentors will continue to mentor me in this capacity. Both Mentors have also been actively guiding me in grant writing (including with this proposal) and will continue to provide hands-on instruction. Both Mentors will support my attendance and participation at (inter)national conferences on emerging zoonotic diseases and provide opportunities to present my work to scientists at IPC, EcoHealth Alliance (EHA), and across EID-SEARCH/CREID. Both Mentors will also provide me with networking opportunities from across their respective networks as they support my development to become a more independent leader in the field of emerging infectious disease research.

2. Building Expertise in Metagenomics

Vireak Heang: My extensive experience and training in laboratory-based sciences has developed a deep understanding of molecular techniques and how they can be used in the detection, characterization, and epidemiological modeling of pathogens. While my work has primarily dealt with shotgun and amplicon-based sequencing, metagenomics is a critical tool for pathogen detection and discovery. In Cambodia, this

capacity is very limited; however, we recently set up the IPC Sequencing Mini-Platform (Illumina MiSeq and ONT technologies) and are exploring the full potential of this system. Given the opportunity to strengthen my hands-on and analytical skills to include metagenomics, will broaden my skillset, and further transition me to scientific independence in Cambodia, capable of using cutting-edge genomics techniques to solve locally relevant problems. Dr. Firth has more than 15 years' experience in the generation and analysis of genomic and metagenomic data in the context of zoonotic disease detection, discovery, and characterization (including BSL3), and is experienced in training and mentoring scientists from LMICs in Southeast Asia. She is therefore an ideal mentor in this area and will work closely with me to develop laboratory SOPs and workflows for metagenomics that are fit-for-purpose at IPC. In addition, Dr. Firth has arranged for me and Dr. Siegers to visit Prof Linfa Wang's laboratory at Duke-NUS Medical School in Singapore for training in cutting-edge molecular techniques, as well as to interact directly with members of his lab.

Jurre Siegers: In Cambodia and other LMICs, there is a pressing need for increased capacity in the field of (meta)genomics, which is becoming increasingly common in emerging infectious disease surveillance and research. Given the opportunity to further strengthen my analytical skills to include genomic and metagenomic data analysis, I will be in possession of a skillset that will allow me to make significant career steps as a junior scientist, capable of initiating and delivering hypothesis-driven research at the forefront of virology and emerging diseases. I will also actively work to disseminate this knowledge to local scientists and staff to ensure that these methods and tools are available to Cambodian scientists, and to support national and international surveillance efforts. Dr. Firth has a wealth of experience in viral sequence analysis, phylogenetics, and phylodynamics, and a broad network of experts in this field. She will work closely with me to develop my technical skills in this area and will facilitate interactions across her network as needed to achieve the analytical goals of the project and build my expertise in this area.

3. Communication Plan

Through the process of writing the application for the CREID Pilot Research Program, the four of us have established the foundation for an effective communication plan that will continue throughout the project. We have already created a WhatsApp group used to communicate in a real-time, informal setting. This has been invaluable for sharing ideas, asking questions, and facilitating development of good interpersonal relationships - the foundations of effective mentorship. We have also agreed to regularly scheduled Zoom meetings (every two weeks) that are agenda-driven, and focused on a topic or issue of direct relevance to the research project. Both the Co-PIs and the Mentors will set the agenda and follow-up on action items. Co-PIs will meet weekly in-person with Dr. Karlsson to discuss project developments, troubleshoot, and develop skills in project and budget management. In addition, the Co-PIs will attend weekly academic meetings at IPC in person and at EHA (remote), which will expose them to the breadth of research and broaden their knowledge base.

Finally, we have scheduled two in-person visits between Dr. Firth and the Co-PIs. The first will coincide with the CREID annual meeting and will include a visit to EHA's headquarters in New York City. This visit will focus on learning relevant analytical techniques, performing preliminary data analysis, and networking with EHA's scientists and staff. Towards the end of the project, Dr. Firth will join us in Cambodia to assist in preparing and analyzing our data for publication and will discuss future directions and plans for future funding following on from this project.

Vertebrate Animals Section Requirements

Projects involving animal samples were collected (2019-2021) under a protocol approved by National Ethics Committee for Health Research under the Ministry of Health, Cambodia (NECHR143, NECHR149, and NECHR320); however, Cambodia did not have a specific animal ethics review board at the time the collections were conducted. Continuing ethics approvals (NECHR013) for projects involving further animal sample collections in 2022 are in revision and projected to be approved in February 2022.

Human Subjects Research

This proposal will not involve experimental procedures with or on human subjects and is therefore not applicable.

Applicant Name (Last, first, middle): Siegers, Jurre, Ynze and Heang, Vireak

Research and Related Other Project Information

1. Are Human Subjects Involved?	Yes		No	X												
1.a. If YES to Human Subjects																
Is the Project Exempt from Federal regulations?	Yes		No													
If yes, check appropriate exemption number	1		2		3		4		5		6		7		8	
If no, is the IRB review Pending?	Yes		No													
IRB Approval Date:																
Human Subject Assurance Number																

2. Are Vertebrate Animals Used?	Yes		No	X
2.a. If YES to Vertebrate Animals				
Is the IACUC review Pending?	Yes		No	
IACUC Approval Date				
Animal Welfare Assurance Number				

3. Is proprietary/privileged information included in the application?	Yes		No	X

4.a. Does this project have an Actual or Potential impact -- positive or negative -- on the environment?	Yes		No	X
4.b. If yes, please explain				
4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed?	Yes		No	
4.d. If yes, please explain				

5. If the research performance site designated, or eligible to be designated, as a historic place?	Yes		No	X
5.a. If yes, please explain				

6. Does this project involve activities outside of the United States or partnership with international collaborators?	Yes	X	No	
6.a. If yes, identify countries	Cambodia			
6.b. Optional explanation	Please see Foreign Site Justification			

BIOGRAPHICAL SKETCH

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

NAME: Vireak Heang

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

POSITION TITLE: Research Engineer

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)*	Start Date MM/YYYY	Completion Date MM/YYYY	FIELD OF STUDY
Technical School for Medical Care, University of Health Science	Associate Degree	01/1999	03/2001	Medical Laboratory Technician
National University of Management	BA	04/2003	08/2006	Management
University of Florida	N/A	05/2010	07/2011	One Health
National University of Management	MBA	03/2009	03/2012	Management

A. Personal Statement

I first began conducting research in 2006 when I was hired by the US Naval Medical Research Unit No. 2 (NAMRU-2) in Phnom Penh Cambodia and I am now working at Institute Pasteur of Cambodia as a Research Engineer for the sequencing platform. My first position at NAMRU-2 was as a Molecular Biology Laboratory Technician. In this role, I was introduced to the exciting world of infectious disease surveillance in one of the global hotspots of these diseases. Over the next 15 years, I rose to become the Head of Molecular Diagnostics where I oversaw a diverse team of 10 people that conducted molecular detection and characterization of pathogens. Utilizing technologies such as RT-PCR, Sanger sequencing, and Next Generation Sequencing (NGS), we collected and analyzed data on infectious pathogens that was critical not only for the US Department of Defense, but also for the Cambodian Ministry of Health, and the Cambodian Armed Forces. Through various training programs and on-the-job experiences, I have developed a deep understanding of molecular techniques and how they can be used in the detection, characterization and epidemiological modeling of pathogens. In 2017, the NAMRU-2 laboratory was chosen by the Global Emerging Infectious Surveillance and Response System (GEIS) to be one of the NGS and Bioinformatics (BI) Consortiums. The goal was to rapidly detect and characterize known, emerging, and novel infectious agents through the establishment of a harmonized Department of Defense laboratory capability that uses data from NGS and BI to inform armed forces health protection decision making. As part of the consortium, each laboratory was sent a set of blinded specimens and we were to run, analyze, and send the report back to the consortium. My role was to set up and manage the plan for running and analyzing those blinded specimens using our in-house techniques, and we correctly identified 85% of the pathogens that the consortium sent us. I have since managed to sequence a variety of specimens - from bacteria isolates to clinical specimens - using Illumina Miseq, including Escherichia coli, Sa*l*monella spp., Shigella spp., influenza virus, Chikungunya virus, and Dengue viru*s*. In my current role, I am a designated expert on Illumina sequencing and assist scientists from across all different units at IPC, regardless of discipline. Recently, I managed to successfully sequence 96 respiratory specimens for SARS-CoV-2 using the Illumina Miseq ARTIC protocol.

My work has encompassed all aspects of infectious diseases from human-specific diseases to analyzing zoonotic transmission at the animal-human interface. From these experiences, I have come to understand the "whole" of health as it applies to animal and environmental reservoirs and the dynamics of transmission. I also have experience across multiple levels of research – from hands-on work at the bench, to team lead, to leading an entire department executing multiple projects. Through these varied responsibilities, I have developed a keen knowledge of how to manage both projects and teams, excellent communication skills, and programmatic expertise. My personal goals are to continue to expand my knowledge and abilities in molecular science. I am keenly interest in the dynamic relationship that microorganisms, especially pathogens, have with their hosts, including natural reservoirs and spillover hosts. By understanding these interactions, I feel we can develop better protections against disease, whether those be through enhanced detection, novel prophylactics, or targeted therapeutics.

B. Positions, Scientific Appointments and Honors

2020-Present	Research Engineer, Institute Pasteur of Cambodia.
2013-2020	Molecular Biology Project Manager, US Naval Medical Research Unit.2
2010-2013	Laboratory Technician, US Naval Medical Research Unit.2
2006-2008	Laboratory Technician, US Naval Medical Research Unit.2, Contractor
2001-2006	Laboratory/X-ray Technician, International SOS clinic.

C. Contributions to Science

1. My early publications focused on the reemergence of Zika virus in Cambodia. My work first began by detecting and describing a case report of Zika infection in a Cambodian citizen in 2010. The identification of this index case signaled that ZIKV was still circulating in Cambodia. Continuing the investigation, we sequenced the Cambodian isolate with four other isolates from across the globe, expanding the number of characterized isolates in Cambodia from two to seven. Our results demonstrated that there appeared to be two distinct lineages of ZIKV circulating, one originating in Africa and one from Asia.

    a. **Heang V**, Yasuda CY, Sovann L, Haddow AD, Travassos da Rosa AP, Tesh RB, Kasper MR. Zika virus infection, Cambodia, 2010. Emerg Infect Dis. 2012 Feb;18(2):349-51. doi: 10.3201/eid1802.111224. PMID: 22305269; PMCID: PMC3310457.

    b. Haddow AD, Schuh AJ, Yasuda CY, Kasper MR, **Heang V**, Huy R, Guzman H, Tesh RB, Weaver SC. Genetic characterization of Zika virus strains: geographic expansion of the Asian lineage. PLoS Negl Trop Dis. 2012;6(2):e1477. doi: 10.1371/journal.pntd.0001477. Epub 2012 Feb 28. PMID: 22389730; PMCID: PMC3289602

    c. Ladner JT, Wiley MR, Prieto K, Yasuda CY, Nagle E, Kasper MR, Reyes D, Vasilakis N, **Heang V**, Weaver SC, Haddow A, Tesh RB, Sovann L, Palacios G. Complete Genome Sequences of Five Zika Virus Isolates. Genome Announc. 2016 May 12;4(3):e00377-16. doi: 10.1128/genomeA.00377-16. PMID: 27174284; PMCID: PMC4866861.

2. A second focus of my work has been the detection and characterization of drug resistance in bacterial pathogens circulating in Cambodia. Cambodia lacks regulation in the use of antibiotics and thusly the use of incorrect antibiotics and/or improper dosing is common. This has led to a rise in multi-drug resistant strains of bacteria across the country. My worked has helped detect the presence of antibiotic resistance in Cambodia and characterize the mechanisms of resistance using molecular techniques.

    a. **Heang V**, Hout B, Prouty MG, Supraprom C, Ford GW, Newell SW, Leski TA, Vora GJ, Taitt CR. Detection of qnrVC and rmtB genes from a multidrug-resistant Ralstonia pickettii wound infection

isolate in Cambodia. Int J Antimicrob Agents. 2014 Jul;44(1):84-5. doi: 10.1016/j.ijantimicag.2014.04.003. Epub 2014 May 2. PMID: 24888871.

b. Hout B, Oum C, Men P, Vanny V, Supaprom C, **Heang V**, Rachmat A, Prouty M, Newell S, Harrison D, Noor S, Gollogly J, Tho L, Kim YJ, Ford G. Drug resistance in bacteria isolated from patients presenting with wounds at a non-profit Surgical Center in Phnom Penh, Cambodia from 2011-2013. Trop Dis Travel Med Vaccines. 2015 Jul 31;1:4. doi: 10.1186/s40794-015-0006-5. PMID: 28883936; PMCID: PMC5526368.

c. Taitt CR, Leski TA, **Heang V**, Ford GW, Prouty MG, Newell SW, Vora GJ. Antimicrobial resistance genotypes and phenotypes from multidrug-resistant bacterial wound infection isolates in Cambodia. J Glob Antimicrob Resist. 2015 Sep;3(3):198-204. doi: 10.1016/j.jgar.2015.05.006. Epub 2015 Jul 9. PMID: 27873709.

d. Taitt CR, Leski TA, Prouty MG, Ford GW, **Heang V**, House BL, Levin SY, Curry JA, Mansour A, Mohammady HE, Wasfy M, Tilley DH, Gregory MJ, Kasper MR, Regeimbal J, Rios P, Pimentel G, Danboise BA, Hulseberg CE, Odundo EA, Ombogo AN, Cheruiyot EK, Philip CO, Vora GJ. Tracking Antimicrobial Resistance Determinants in Diarrheal Pathogens: A Cross-Institutional Pilot Study. Int J Mol Sci. 2020 Aug 18;21(16):5928. doi: 10.3390/ijms21165928. PMID: 32824772; PMCID: PMC7460656.

3. Finally, I have also contributed to broad topics including drug resistance in malaria and observational studies into the causes of sepsis. Cambodia has experienced malaria treatment failures to multiple frontline drugs, creating great concern should these resistant strains continue to proliferate. My work helped to evaluate the efficacy of new combinations of drugs in the treatment of malaria. Sepsis continues to be a significant cause of morbidity and mortality, especially in areas with a rudimentary health care system such as Cambodia. I contributed to the seminal description of the causes of sepsis in Cambodia through the use of next generation sequencing techniques for pathogen detection and characterization.

   a. Wojnarski M, Lon C, Vanachayangkul P, Gosi P, Sok S, Rachmat A, Harrison D, Berjohn CM, Spring M, Chaoratanakawee S, Ittiverakul M, Buathong N, Chann S, Wongarunkochakorn S, Waltmann A, Kuntawunginn W, Fukuda MM, Burkly H, **Heang V**, Heng TK, Kong N, Boonchan T, Chum B, Smith P, Vaughn A, Prom S, Lin J, Lek D, Saunders D. Atovaquone-Proguanil in Combination With Artesunate to Treat Multidrug-Resistant P. falciparum Malaria in Cambodia: An Open-Label Randomized Trial. Open Forum Infect Dis. 2019 Sep 4;6(9):ofz314. doi: 10.1093/ofid/ofz314. PMID: 31660398;

   b. Rozo M, Schully KL, Philipson C, Fitkariwala A, Nhim D, Som T, Sieng D, Huot B, Dul S, Gregory MJ, **Heang V**, Vaughn A, Vantha T, Prouty AM, Chao CC, Zhang Z, Belinskaya T, Voegtly LJ, Cer RZ, Bishop-Lilly KA, Duplessis C, Lawler JV, Clark DV. An Observational Study of Sepsis in Takeo Province Cambodia: An in-depth examination of pathogens causing severe infections. PLoS Negl Trop Dis. 2020 Aug 17;14(8):e0008381. doi: 10.1371/journal.pntd.0008381. PMID: 32804954; PMCID: PMC7430706.

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: Jurre Ynze Siegers

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

POSITION TITLE: Postdoctoral 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 Applied Sciences van Hall Larenstein, Leeuwarden the Netherlands	BASc	08/2011	Biotechnology
Erasmus University Rotterdam, the Netherlands	MSc	08/2013	Infection & Immunity
Erasmus University Rotterdam, the Netherlands	PhD	03/2022	Virology
Institute Pasteur du Cambodia, Phnom Penh, Cambodia	Postdoctoral		Virology, Global Health

A. Personal Statement

I am virologist who has been working in the field of emerging infectious diseases since 2013. During my PhD training in the Department of Viroscience at the Erasmus Medical Center in Rotterdam, the Netherlands, I studied the extra-respiratory tract complications of (zoonotic) influenza A virus infection using a wide range of disciplines that included virology, immunology, molecular biology and pathology. Following completion of my Ph.D., I began to pursue a research career focused on emerging respiratory viruses at the human-animal interface in Cambodia that incorporates surveillance, viral evolution, host-pathogen interactions, and pathogenesis. As a postdoctoral scientist at Institut Pasteur du Cambodge (IPC) in Phnom Penh, Cambodia, I coordinate and oversee all daily activities related to seasonal human and avian influenza viruses. These include molecular surveillance for influenza-like illness (ILI-surveillance), avian influenza surveillance in Cambodian live bird markets and wild birds, phylogenetics, cell culture work at BSL-2 and BSL-3 level for virus isolation, and serology. Furthermore, I am in charge of implementing novel virus characterization methods at the BSL-2 and BSL-3 lab facilities. Of particular relevance to the proposed project, I also oversee all management, development, and training related to routine sequencing at IPC Virology Unit using Oxford Nanopore technologies.

My research portfolio has a strong multi-disciplinary component that includes virology, immunology, pathology, pathogen genetics and genomics, field work, and public health, and has required me to become adept at combining and analyzing multiple types of data, including experimental, environmental, and genomic data. I also have a strong track record in the discovery/identification and characterization of emerging viruses, with 6 publications in this field, four of which have been cited more than 10 times since 2013 (total citation: 161). Adding pathogen metagenomics to my portfolio would advance my career significantly by becoming an expert in the field of emerging viruses, capable of performing initial pathogen discovery as well as primary virus characterization. As such, my experience, skills, and training are ideally suited to the proposed research project, which builds naturally on my previous work. Further, I am strongly committed to supporting diversity and equity both within the scientific community and in the communities we serve by improving science education and good/safe farming practices for underserved communities in rural Cambodia. I am also committed to providing training and mentorship for low- and middle-income country scientists and scholars.

I. Novel avian-origin influenza A (H7N9) virus attaches to epithelium in both upper and lower respiratory tract of humans. van Riel D, Leijten LME, de Graaf M, **Siegers JY**, Short KR, Spronken MIJ, Schrauwen EJA, Fouchier RAM, Osterhaus ADME, Kuiken T. **Am J Pathol**. 2013 Oct;183(4):1137-1143. doi: 10.1016/j.ajpath.2013.06.011. Epub 2013 Sep 10.

II. Novel avian-origin influenza A (H7N9) virus attachment to the respiratory tract of five animal models. **Siegers JY**, Short KR, Leijten LM, de Graaf M, Spronken MI, Schrauwen EJ, Marshall N, Lowen AC, Gabriel G, Osterhaus AD, Kuiken T, van Riel D. **J Virol**. 2014 Apr;88(8):4595-9. doi: 10.1128/JVI.03190-13. Epub 2014 Jan 29.

III. Phenotypic Differences between Asian and African Lineage Zika Viruses in Human Neural Progenitor Cells. Anfasa F, **Siegers JY**, van der Kroeg M, Mumtaz N, Stalin Raj V, de Vrij FMS, Widagdo W, Gabriel G, Salinas S, Simonin Y, Reusken C, Kushner SA, Koopmans MPG, Haagmans B, Martina BEE, van Riel D. **mSphere**. 2017 Jul 26;2(4):e00292-17. doi: 10.1128/mSphere.00292-17. eCollection 2017 Jul-Aug.

IV. Human Infection with Avian Influenza A(H9N2) Virus, Cambodia, February 2021. Um S, Siegers JY, Sar B, Chin S, Patel S, Bunnary S, Hak M, Sor S, Sokhen O, Heng S, Chau D, Sothyra T, Khalakdina A, Mott JA, Olsen SJ, Claes F, Sovann L, Karlsson EA. **Emerg Infect Dis**. 2021 Oct;27(10):2742-2745. doi: 10.3201/eid2710.211039.

B. Positions and Honors

Positions and Employment

2020- Postdoctoral Scientist, Institut Pasteur du Cambodge, Phnom Penh, Cambodia
2015-2020 PhD Candidate, Erasmus Medican Center, Rotterdam, the Netherlands
2015 Volunteer. Clinical Laboratory Technician (Ebola Virus Diagnostics). Koidu, Sierra Leone
2014 Research Asissant, Murdoch Children's Research Institute, Melbourne, Australia

Other Experience, Commissions of Trust and Professional Memberships

2018- Membership. American Society for Virology
2014- Ad Hoc Reviewer. Peer reviewer for different journals including Neuron, the Lancet, Journal of Virology.

Honors

2021 Young Scientist Fund, The eight ESWI conference, Virtual
2020 Young Scientist Fund, The seventh ESWI conference, Virtual
2019 Travel grant award. CADDE Genomic Epidemiology Workshop, Sao Paulo, Brazil
2018 Travel grant award. Visiting Scientist, University of Queensland, Australia
2017 Young Scientist Fund, The sixth ESWI conference, Riga, Latvia
2017 Travel grant award, European Seminar in Virology, Bertinoro, Italy
2016 Travel grant award, Options XI for the control of Influenza, Chicago, US
2015 Best poster award, Young Predigone (Predemix/Antigone), Rotterdam, the Netherlands
2015 Best abstract award, 7th Orthomyxovirus Research Conference, Toulouse, Frane
2015 Travel grant award, 7th Orthomyxovirus Research Conference, Toulouse, France
2013 Travel grant award, 100th American Association for Immunologist (AAI), Hawaii, US

C. Contributions to Science

I have authored 18 publications (published or in press) in the fields of influenza, respiratory viruses, infectious disease, and immunology, nine of which (50%) are first author publications. The primary theme of my work is understanding the emergence, prevalence, transmission, and pathogenesis of viruses at the human-animal interface. *As of this dossier, Google Scholar**: Total citations**: 374, **h-index**: 10, **i10-index**: 11

1. **Immunologic consequences of chronic diseases in humans.** The start of my scientific career began during my bachelor's degree internship at the Murdoch Children's Research Institute in Melbourne Australia where I started a new line of research within the Cell & Gene Therapy group. This research shed light on the mechanism that underlies the increased susceptibility to bacterial infections observed in patients suffering from the genetic blood disorder beta-thalassemia. I identified that neutrophils of beta-thalassemia patients are functionally abnormal due to a block in neutrophil maturation. My interest in the field of immunology and infectious disease grew during this internship and led me to pursue a MSc degree in infection & immunity. During one of my MSc internships at the department of Hematology, Erasmus Medical Center Rotterdam, the Netherlands, I examined the role of the cytokine IL-22 and the IL-22 receptor in rheumatoid arthritis and identified IL-22 as an important enhancer of the germinal center reaction, which are essential for the production of autoantibody-secreting plasma cells.

   V. Reduced PU.1 expression underlies aberrant neutrophil maturation and function in β-thalassemia mice and patients. Siwaponanan P, **Siegers JY**, Ghazali R, Ng T, McColl B, Ng GZ, Sutton P, Wang N, Ooi I, Thiengtavor C, Fucharoen S, Chaichompoo P, Svasti S, Wijburg O, Vadolas J. **Blood**. 2017 Jun 8;129(23):3087-3099. doi: 10.1182/blood-2016-07-730135. Epub 2017 Mar 21.

   VI. Loss of IL-22 inhibits autoantibody formation in collagen-induced arthritis in mice. Corneth OB, Reijmers RM, Mus AM, Asmawidjaja PS, van Hamburg JP, Papazian N, **Siegers JY**, Mourcin F, Amin R, Tarte K, Hendriks RW, Cupedo T, Lubberts E. **Eur J Immunol**. 2016 Jun;46(6):1404-14. doi: 10.1002/eji.201546241. Epub 2016 May 12.

2. **Characterization of emerging (respiratory) viruses.** After exploring immunology, it was time to pursue my biggest interest, the world of emerging viruses. I was able to contribute scientific knowledge and advancements related to the novel avian-origin H7N9 virus, where I first reported on the attachment pattern of this potentially pandemic virus to the respiratory tract of five different animal models frequently used in influenza virus research. In addition, I showed that in humans, H7N9 viruses attach to cells in both the upper and lower respiratory tract, something never before observed for an avian-origin influenza virus. In terms of virus discovery, I described the first identification and genetic characterization of a dolphin rhabdovirus and during the Zika virus outbreak, a re-emerging virus, I contributed by describing fundamental intrinsic *in vitro* differences between the African and Asian lineage Zika viruses in neuronal cells. Finally, I contributed to the identification of zinc-embedded polyamide fabrics as a suitable "passive" inactivation of SARS-CoV-2 virus that could be used in personal protective equipment.

   I. Novel avian-origin influenza A (H7N9) virus attaches to epithelium in both upper and lower respiratory tract of humans. van Riel D, Leijten LME, de Graaf M, **Siegers JY**, Short KR, Spronken MIJ, Schrauwen EJA, Fouchier RAM, Osterhaus ADME, Kuiken T. **Am J Pathol**. 2013 Oct;183(4):1137-1143. doi: 10.1016/j.ajpath.2013.06.011. Epub 2013 Sep 10.

   II. Novel avian-origin influenza A (H7N9) virus attachment to the respiratory tract of five animal models. **Siegers JY**, Short KR, Leijten LM, de Graaf M, Spronken MI, Schrauwen EJ, Marshall N, Lowen AC, Gabriel G, Osterhaus AD, Kuiken T, van Riel D. **J Virol**. 2014 Apr;88(8):4595-9. doi: 10.1128/JVI.03190-13. Epub 2014 Jan 29.

   III. Phenotypic Differences between Asian and African Lineage Zika Viruses in Human Neural Progenitor Cells. Anfasa F, **Siegers JY**, van der Kroeg M, Mumtaz N, Stalin Raj V, de Vrij FMS, Widagdo W, Gabriel G, Salinas S, Simonin Y, Reusken C, Kushner SA, Koopmans MPG, Haagmans B, Martina BEE, van Riel D. **mSphere**. 2017 Jul 26;2(4):e00292-17. doi: 10.1128/mSphere.00292-17. eCollection 2017 Jul-Aug.

3. **Pathogenesis of seasonal, pandemic, and zoonotic influenza A virus associated extra-respiratory complications.** My PhD project focused on the extra-respiratory complications of influenza virus infection where I made significant contributions to our understanding of the pathogenesis of extra-respiratory disease. First, I described the pathogenesis and extra-respiratory complications of pandemic 1918 H1N1 virus in a ferret animal model and provided new insights into the significant pathogenicity of this virus. Second, I provided new insights into the impact of chronic metabolic diseases (excess weight/obesity) on the

development of influenza virus-associated extra-respiratory disease. Third, I described the intrinsic differences - viral and host factors - in the ability of seasonal, pandemic, and zoonotic influenza A viruses to replicate in cells of the central nervous system, and proposed a potential mechanism. Finally, I evaluated the efficacy of vaccination and pre-exposure prophylaxis antivirals in the development of extra-respiratory tract disease associated with highly pathogenic avian influenza H5N1 virus infection in ferrets.

I. 1918 H1N1 Influenza Virus Replicates and Induces Proinflammatory Cytokine Responses in Extrarespiratory Tissues of Ferrets.de Wit E, **Siegers JY**, Cronin JM, Weatherman S, van den Brand JM, Leijten LM, van Run P, Begeman L, van den Ham HJ, Andeweg AC, Bushmaker T, Scott DP, Saturday G, Munster VJ, Feldmann H, van Riel D. **J Infect Dis**. 2018 Mar 28;217(8):1237-1246. doi: 10.1093/infdis/jiy003.

II. Mini viral RNAs act as innate immune agonists during influenza virus infection. Te Velthuis AJW, Long JC, Bauer DLV, Fan RLY, Yen HL, Sharps J, **Siegers JY**, Killip MJ, French H, Oliva-Martín MJ, Randall RE, de Wit E, van Riel D, Poon LLM, Fodor E. **Nat Microbiol**. 2018 Nov;3(11):1234-1242. doi: 10.1038/s41564-018-0240-5. Epub 2018 Sep 17.

III. A High-Fat Diet Increases Influenza A Virus-Associated Cardiovascular Damage. **Siegers JY**, Novakovic B, Hulme KD, Marshall RJ, Bloxham CJ, Thomas WG, Reichelt ME, Leijten L, van Run P, Knox K, Sokolowski KA, Tse BWC, Chew KY, Christ AN, Howe G, Bruxner TJC, Karolyi M, Pawelka E, Koch RM, Bellmann-Weiler R, Burkert F, Weiss G, Samanta RJ, Openshaw PJM, Bielefeldt-Ohmann H, van Riel D, Short KR. **J Infect Dis**. 2020 Aug 4;222(5):820-831. doi: 10.1093/infdis/jiaa159.

IV. Viral Factors Important for Efficient Replication of Influenza A Viruses in Cells of the Central Nervous System. **Siegers JY**, van de Bildt MWG, Lin Z, Leijten LM, Lavrijssen RAM, Bestebroer T, Spronken MIJ, De Zeeuw CI, Gao Z, Schrauwen EJA, Kuiken T, van Riel D. **J Virol**. 2019 May 15;93(11):e02273-18. doi: 10.1128/JVI.02273-18. Print 2019 Jun 1.

4. **Emerging respiratory viruses at human-animal interface.** My current postdoctoral training is focused on emerging (respiratory) viruses at the human-animal interface in Cambodia and includes - but is not limited to - influenza, coronaviruses, and paramyxoviruses. Using a “One Health” approach I contribute to understanding the dynamics of avian influenza at Cambodian live bird markets and in wild birds, the epidemiology of seasonal influenza, and the emergence of zoonotic viruses in humans. Thus far, I have identified the first human A/H9N2 infection in Cambodia and performed genetic and antigenic characterization of an influenza A(H3N2) outbreak in Cambodia during the COVID-19 pandemic.

   I. Human Infection with Avian Influenza A(H9N2) Virus, Cambodia, February 2021. Um S, **Siegers JY**, Sar B, Chin S, Patel S, Bunnary S, Hak M, Sor S, Sokhen O, Heng S, Chau D, Sothyra T, Khalakdina A, Mott JA, Olsen SJ, Claes F, Sovann L, Karlsson EA. **Emerg Infect Dis**. 2021 Oct;27(10):2742-2745. doi: 10.3201/eid2710.211039.

   II. Genetic and Antigenic Characterization of an Influenza A(H3N2) Outbreak in Cambodia and the Greater Mekong Subregion during the COVID-19 Pandemic, 2020. **Siegers JY**, Dhanasekaran V, Xie R, Deng YM, Patel S, Ieng V, Moselen J, Peck H, Aziz A, Sarr B, Chin S, Heng S, Khalakdina A, Kinzer M, Chau D, Raftery P, Duong V, Sovann L, Barr IG, Karlsson EA. **J Virol**. 2021 Nov 23;95(24):e0126721. doi: 10.1128/JVI.01267-21. Epub 2021 Sep 29.

Complete List of Published Work:

https://pubmed.ncbi.nlm.nih.gov/?term=siegers+jy

*Name of Individual: Heang, V.
Commons ID: N/A

Other Support – Project/Proposal

PREVIOUS

None

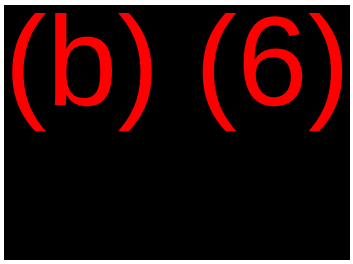
ACTIVE

None

PENDING

None

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.



*Signature: Heang, Vireak

Date: January 30, 2022

*Name of Individual: Siegers, J. Y.
Commons ID: N/A

Other Support – Project/Proposal

PREVIOUS

*Title: The role of obesity on extra-respiratory tract complications of Influenza A virus infection.

Major Goals: The major goals of this project is to describe the pathogenesis and transcriptome of extra-respiratory tract infection in obesity in mice.

*Status of Support: Ended

Project Number: N/A

Name of PD/PI: Dr. Debby van Riel, Dr. Kirsty Short

*Source of Support: Erasmus Trustfonds

*Primary Place of Performance: University of Queensland, Australia

Project/Proposal Start and End Date: (MM/YYYY) (if available): 12/2017 – 05/2018

* Total Award Amount (including Indirect Costs): €1696.51

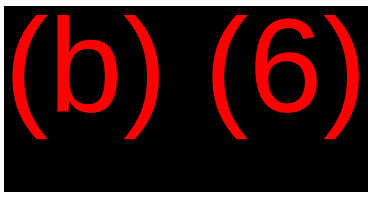
* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2018]	5 calendar

ACTIVE

PENDING

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.



*Signature: Siegers, Jurre

Date: January 30, 2022

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: Firth, Cadhla

eRA COMMONS USER NAME: (b) (6)

POSITION TITLE: Senior Research Scientist and Program Coordinator

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	Completion Date	FIELD OF STUDY
University of Guelph, Guelph, CAN	BS	06/2003	Zoology
University of Guelph, Guelph, CAN	MS	06/2005	Evolutionary Ecology
The Pennsylvania State University, State College, PA	PHD	05/2010	Biology
Columbia University, New York, NY	Postdoctoral	12/2012	Infectious Diseases & Public Health

A. Personal Statement

I am a molecular and evolutionary biologist who has been working on emerging infectious diseases for 15 years. In my current research, I explore the ecological and evolutionary dynamics of emerging zoonoses at the human-animal interface, with a focus on rapidly changing environments in Southeast Asia. I have more than eight years' experience leading independent research projects in this field, including collaborations with industry and local government. Over time, I have been responsible for the successful completion of every aspect of these studies, including project design, funding acquisition, implementation, and reporting/publishing. In 2015 I initiated a research program to investigate the response of zoonotic pathogens to urbanization in Malaysian Borneo using metagenomics, landscape ecology, and population genomics. This research has a strong multi-disciplinary component that includes evolutionary biology, molecular ecology, virology, pathogen genetics and genomics, field biology, and public health, and has required me to become adept at combining and analyzing multiple types of data, including environmental, ecological, and genomic data. This work has already resulted in three last-author publications, with a fourth currently in revisions at *Proceedings of the National Academy of Sciences of the USA.* As such, my experience, skills, and training are ideally suited to the proposed research project, which builds naturally on my previous work. I also have a strong track record in the generation and use of genetic and genomic data to explore the transmission dynamics and evolution of emerging viruses, with more than 20 publications in this field, nine of which have been cited more than 100 times since 2010. The mastery of genomic, phylogenetic, and phylodynamic methods I have developed throughout my career are directly applicable to the proposed research. Further, I am strongly committed to supporting diversity and equity both within the scientific community and in the communities we serve and have led several projects and initiatives aimed at improving science education and health service delivery for underserved communities in tropical northern Australia. I am also committed to providing training and mentorship for low- and middle-income country scientists and scholars, and in my previous role worked to develop protocols and bioinformatics pipelines to enable portable metagenomic sequencing in rural and remote regions in Australia and the Pacific. I am currently supervising two PhD students (James Cook University, AUS) and formally mentor a postdoctoral scientist, Dr. Nguyen Van Cuong, at the Oxford University Clinical Research Unit (OUCRU) in Viet Nam as part of my activities within the Centres for Research in Emerging Infectious Diseases (CREID) network.

B. Positions, Scientific Appointments, and Honors

Positions and Employment

2020-Present	Senior Research Scientist and Program Coordinator, EcoHealth Alliance, New York, NY
2020-Present	Adjunct Senior Research Fellow, College of Public Health, Medical & Vet Sciences, James Cook University, Cairns, AUS
2018-2020	HOT North Career Development Fellow, Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, AUS
2017-2018	Australian Research Council (ARC) Discovery Early Career Research Fellow, School of BioSciences, The University of Melbourne, Parkville, AUS
2013-2017	Research Scientist/ Australian Research Council (ARC) Discovery Early Career Research Fellow, Health and Biosecurity, The Commonwealth Scientific and Industrial Research Organization, Geelong, AUS
2013	Associate Research Scientist, Center for Infection and Immunity, Columbia University, New York, NY
2010-2012	Postdoctoral Fellow, Center for Infection and Immunity, Columbia University, New York, NY
2006-2010	PhD Scholar, The Pennsylvania State University, State College, PA
2001-2006	Research Assistant, Department of Zoology, The University of Guelph, Guelph, CAN

Honors

2019-2021	HOT North Career Development Fellowship, Northern Australia Tropical Disease Collaborative Research Programme, National Health and Medical Research Council (Australia)
2015-2018	Discovery Early Career Researcher Award, The Australian Research Council
2009-2010	Jeanette Ritter Mohnkern Graduate Student Scholarship in Biology, The Pennsylvania State University
2006-2009	Postgraduate Scholarship, Natural Science and Engineering Research Council of Canada
2006-2008	Braddock Graduate Fellowship, The Pennsylvania State University
2003-2005	Ontario Graduate Scholarship, The Province of Ontario (Canada)
1999	Undergraduate Entrance Scholarship, The University of Guelph

C. Contributions to Science

Throughout my career, my research has focused on many aspects of infectious disease emergence and has encompassed evolutionary biology, molecular ecology, virology, pathogen genetics and genomics, field biology, and public/veterinary health. My most significant contributions include:

1. **The use of genomic and metagenomic sequencing techniques to discover new pathogens, study their evolution, and explore microbial diversity.** I have successfully developed and used cutting-edge metagenomic techniques and bespoke bioinformatics pipelines for: (i) pathogen discovery, including a case of acute encephalitis in an immune-suppressed patient, (ii) studies of viral diversity, including coronaviruses and flaviviruses in bats; and (iii) genomics, including the epizoology of bluetongue virus, the evolution of rhabdoviruses, and the characterization of Australian bunyaviruses. I have also co-authored two perspectives on the utility of next-generation sequencing for pathogen discovery in people and animals. These included an invited submission that described an updated framework for proof of causation in the genomics era and discussed the emerging integration of on-the-ground metagenomics-based diagnostics with molecular epidemiology. In 2013, I initiated a study that used metagenomics to characterize the viruses and bacteria carried by New York City rats across a range of habitats, with a focus on the built environment. At the time, little was known about the range of potential pathogens carried by urban rodents or the risk factors associated with zoonotic transmission in a city environment. To begin to address this, I designed the first comprehensive molecular survey of rodent-borne microbes in a U.S. city, and demonstrated that urban rats frequently carry a range of zoonotic pathogens. We also found evidence of substantial heterogeneity in pathogen distribution within and between cities, suggesting that the associated risks of human disease may be similarly heterogeneous.

    a. Quan PL, **Firth C**, Conte JM, Williams SH, Zambrana-Torrelio CM, Anthony SJ, Ellison JA, *et al*. (2013). Bats are a major natural reservoir for hepaciviruses and pegiviruses. **Proceedings of the National Academy of Sciences of the USA** 110: 8194-9. PMCID: PMC3657805

b. **Firth C**, Lipkin WI (2013). The genomics of emerging pathogens. **Annual Review of Genomics and Human Genetics** 14: 281-300. PMID: 24003855

c. **Firth C**, Bhat M, Firth MA, Williams SH, Frye MJ, Simmonds P, Conte JM, *et al*. (2014). Detection of zoonotic pathogens and characterization of novel viruses carried by commensal Rattus norvegicus in New York City. **mBio** 5: e01933-14. PMCID: PMC4102142

d. Steinig E, Duchêne S, Aglua I, Greenhill A, Ford R, Yoannes M, Jaworski J, Drekore J, Urakoko B, Poka H, Wurr C, Ebos E, Nangen D, Laman M, Manning L, **Firth C**, Smith S, Pomat W, Tong SYC, Coin L, McBryde E, Horwood P. *et al*. (2022). Phylodynamic modelling of bacterial outbreaks using nanopore sequencing. **Molecular Biology and Evolution**. *In Press*.

Related Pre-print available: https://doi.org/10.1101/2021.04.30.442218

2. **I have helped develop the emerging field of urban zoonotic disease ecology.** In 2015 I pioneered an ongoing research program to investigate the response of zoonotic pathogens to urbanization using metagenomic sequencing, landscape ecology, and population genomics. Using a multi-disciplinary approach, this research explores how changes in the environment (e.g., land-use changes, microclimates, etc.), and host community and population structure (e.g., biodiversity, connectivity, density), influence pathogen community composition and zoonotic potential. Initial results of this work indicate that while mammalian diversity decreases with increasing urbanization, connectivity between populations increases, as does microbial species richness and the prevalence of known zoonotic pathogens. This suggests that some features of the built environment may inadvertently support pathogen persistence and spread, and as a result, some zoonotic pathogens may be more likely to emerge with intensifying urbanization. My reputation as an emerging researcher in this field has led to multiple invitations to present as a plenary or symposium speaker at national and international meetings, including the Ecological Society of Australia's Annual Conference (2019) and the Annual Meeting of the American Society of Mammalogists (2019), and the Joint Conference of the Asian Society of Conservation Medicine and the Wildlife Disease Association Australasia (2018).

a. Frye MJ, **Firth C**, Bhat M, Firth MA, Che X, Lee D, Williams SH, Lipkin WI (2015). Preliminary survey of ectoparasites and associated pathogens from Norway rats in New York City. **Journal of Medical Entomology** 52: 253-9. PMCID: PMC4481720

b. Blasdell KR, Perera D, **Firth C** (2018). High prevalence of rodent-borne *Bartonella* spp. in urbanizing environments in Sarawak, Malaysian Borneo. **American Journal of Tropical Medicine and Hygiene** 100: 506-9. PMCID: PMC6402934

c. Blasdell KR, Morand S, Perera D, **Firth C** (2019). Association of rodent-borne *Leptospira* spp. with urbanizing environments in Sarawak, Malaysian Borneo. **PloS Neglected Tropical Diseases** 13: e0007141. PMCID: PMC6411199

d. Blasdell KR, Morand S, Laurance SGW, Doggett SL, Hahs A, Perera D, **Firth C** (2021) Rats in the city: implications for zoonotic disease risk in an urbanizing world. bioRxiv 2021.03.18.436089 [**Preprint**] March 19, 2021 [cited 2022 Jan 30]. Available from: https://doi.org/10.1101/2021.03.18.436089
*Revised manuscript Under Review at Proceedings of the National Academy of Sciences of the USA.

3. **I challenged established beliefs about the ecological and evolutionary processes linked to viral disease emergence, and the timescale over which they occur.** During my PhD, I was able to make significant contributions to this field by questioning the idea that viruses evolve either by cross-species transmission (spillover) or by co-evolution with their hosts, but rarely both. My work demonstrated that viruses with many genomic structures are capable of jumping species barriers, and that evolution through both co-divergence and cross-species transmission may be general trend of many virus systems. This has since been supported by numerous subsequent studies that have highlighted the complex evolutionary dynamics of both RNA and DNA viruses.

a. **Ramsden C***, Holmes EC, Charleston MA (2009). Hantavirus evolution in relation to its rodent and insectivore hosts: no evidence for co-divergence. **Molecular Biology and Evolution** 26: 143-53. PMID: 18922760
* Name changed from Ramsden to Firth in 2009

b. Pagan I, **Firth C**, Holmes EC (2010). Phylogenetic analysis reveals rapid evolutionary dynamics in the plant RNA virus genus tobamovirus. **Journal of Molecular Evolution** 71: 298-307. PMID: 20838783

c. **Firth C**, Kitchen A, Shapiro B, Suchard MA, Holmes EC, Rambaut A (2010). Using time-structured data to estimate evolutionary rates of double-stranded DNA viruses. **Molecular Biology and Evolution** 27: 2038-51. PMCID: PMC3107591

d. Sali AA, Faye O, Diallo M, **Firth C**, Kitchen A, Holmes EC (2010). Yellow fever virus exhibits slower evolutionary dynamics than dengue virus. **Journal of Virology** 84: 765-72. PMCID: PMC2798388

4. **I was among the first to apply novel phylodynamic approaches to reconstruct the spatiotemporal processes behind disease emergence events.** Phylodynamic methods enable the synthesis of epidemiological, geographic, and phylogenetic data to reconstruct the processes behind infectious disease emergence and spread through time and space. These methods are of particular use for investigating the emergence and spread of viruses, as they have a rapid evolutionary rate that closely matches the timescale of virus transmission. I began to explore these approaches during my PhD and have now successfully used them to: (i) explore the genetics underpinning the rapid emergence and spread of human enterovirus 68; (ii) examine the patterns and processes that influence the diversity and geographic distribution of New World hantaviruses; (iii) explore the epizoology of porcine circovirus 2; iv) understand the evolutionary and ecological drivers behind the distribution of bluetongue virus in Australia; (v) assess the likely origins of hepatitis C virus; and (vi) understand the global emergence of community-associated MRSA and MSSA.

   a. Kapoor A, Simmonds P, Gerold G, Qaisar N, Jain K, Henriquez JA, **Firth C**, *et al*. (2011). Characterization of a canine homolog of hepatitis C virus. **Proceedings of the National Academy of Sciences of the USA** 108: 11608-13. PMCID: PMC3136326

   b. **Firth C,** Tokarz R, Simith DB, Nunes MR, Bhat M, Rosa ES, Medeiros DB, Palacios G, Vasconcelos PF, Lipkin WI (2012). Diversity and distribution of hantaviruses in South America. **Journal of Virology** 86: 13756-66. PMCID: PMC3503106

   c. Tokarz R, **Firth C**, Madhi SA, Howie SR, Wu W, Sall AA, Haq S, Briese T, Lipkin WI (2012). Worldwide emergence of multiple clades of enterovirus 68. **Journal of General Virology** 93: 1952-8. PMCID: PMC3542132

   d. Steinig E, Aglua I, Duchene S, Meehan MT, Yoannes M, **Firth C**, et al. (2021). Phylodynamic signatures in the emergence of community-associated MRSA. BioRxiV 2021.04.30.442212 [**Preprint**] April 30, 2021 [cited 2022 Jan 30]. Available from: https://doi.org/10.1101/2021.04.30.442212
   *Under Review at Lancet Microbe.

Complete List of Published Work in MyBibliography:
https://www.ncbi.nlm.nih.gov/myncbi/1pcDCsap4HMk2/bibliography/public/

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: Erik Albert KARLSSON

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6)

POSITION TITLE: Deputy Head of Unit, Director of National Influenza Center of Cambodia, Director of WHO Regional H5 Reference Laboratory, Coordinator of WHO Global COVID-19 Reference Laboratory, Virology Unit, Institut Pasteur du Cambodge

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 North Carolina, Chapel Hill	B.S.	05/2005	Biochemistry
University of North Carolina, Chapel Hill	Ph.D.	05/2010	Nutrition
St. Jude Children's Research Hospital	Postdoctoral	06/2016	Virology

A. Personal Statement

During my doctoral work in the Department of Nutrition at the Gilling's School of Global Public Health, University of North Carolina at Chapel Hill, I studied the effect of nutrition, especially diet-induced obesity, on immune responses to viral pathogens. Following completion of my Ph.D., I began to pursue a research career focused on understanding surveillance, viral evolution, host-pathogen interactions, and immune responses. I began working as a Postdoctoral Research Associate with Dr. Stacey Schultz-Cherry and collaborators at St Jude Children's Research Hospital to expand my knowledge of virology, viral pathogenesis, co-infection, immunology (primary and vaccine-induced), and infectious disease surveillance. I became a Staff Scientist in Dr. Schultz-Cherry's laboratory, focusing further on the interaction of nutrition and infectious disease as well as helping to oversee influenza surveillance at the animal-human interface worldwide. I currently serve as a Senior Researcher (Assistant Professor equivalent) at Institut Pasteur du Cambodge (IPC) in Phnom Penh, Cambodia. I am the Deputy Head of the Virology Unit and in charge of all ongoing activities related to respiratory viruses, including human seasonal and zoonotic viruses, and am the Director of the National Influenza Center and the Regional WHO H5 Reference lab. I am integral in the COVID-19 response in Cambodia and worldwide and serve as the Coordinator for the WHO Global COVID-19 Reference Laboratory in the Virology Unit. I also serve as the Co-PI of the newly developed Sequencing Platform at IPC. My work at IPC focuses on surveillance of endemic and emerging viruses in Cambodia, including developing Early Warning systems against the next pandemic. I also frequently consult for UN organizations (FAO and WHO) and am the founder of a research think-tank named: ''CANARIES: Consortium of Animal market Networks to Assess Risks of emerging Infectious diseases through Enhanced Surveillance."

B. Positions and Honors

Positions and Employment

2016-2017 *Staff Scientist*, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN
2017-2020 *Senior Research*, Virology Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia
2020- *Deputy Head*, Virology Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia

Other Experience, Commissions of Trust, and Professional Memberships (since 2011)

2011- American Society for Virology
2011- **Ad Hoc Reviewer**. Peer reviewer for 60+ journals Full review record available on Publons: https://publons.com/author/737520/erik-albert-karlsson#profile
2012-2013 **Secretary**. Nutritional Immunology RIS. American Society for Nutrition
2013-2015 **Chair Elect.** Nutritional Immunology RIS. American Society for Nutrition.
2015-2016 **Chair.** Nutritional Immunology and Inflammation RIS. American Society for Nutrition
2015- **Editorial Board Member**. Journal of Nutritional Biochemistry
2017- **Editorial Board Member**. Virus Genes
2019- **Founder.** Consortium of Animal market Networks to Assess Risks of emerging Infectious diseases through Enhanced Surveillance (CANARIES; http://www.canarieshmhp.org).
2020- **Consultant, Laboratory Technical Advisor**, Emergency Centre for Transboundary Animal Diseases, Food and Agriculture Organization of the United Nations, Regional Office for Asia and the Pacific.
2020-2021 **Consultant, Scientific Expert**. World Health Organization. Global Influenza Surveillance and Response System.
2021- **Consultant, Technical Expert – Genomic Surveillance.** World Health Organization, European Region, Turkey.

Honors Since 2011

2011 **ASV 2011 Postdoctoral Fellow Travel Award**. American Society for Virology General Meeting 2011, Minneapolis, MN
2011 **Travel Award**. ISIRV-AVG Conference: Influenza Antivirals: Efficacy and Resistance. Rio de Janiero, Brazil. November, 2011
2012 **Travel Award**. Nutritional Immunology Research Interest Section. American Society for Nutrition. Experimental Biology 2012, San Diego, CA
2012 **Young Investigator Travel Award**. Palm Beach Infectious Disease Research Institute. PBIDI Symposium 2012, Palm Beach, FL
2012 **Travel Award**. 6 h Orthomyxovirus Research Conference. Bromont, Quebec, Canada. September, 2012
2012 **Travel Award**. Obesity Research Interest Section. American Society for Nutrition. Experimental Biology 2012, San Diego, CA
2013 **Promising Young Investigator Scholarship**. Options for the Control of Influenza VII. Cape Town, South Africa. September 2013.
2015 **Young Investigator Travel Award**. 3rd ISIRV International Symposium on Neglected Influenza Viruses. Athens, Gerogia, USA. April, 2015.
2015 **Merck Junior Investigator Award - Best Speaker**. 3rd ISIRV International Symposium on Neglected Influenza Viruses. Athens, Gerogia, USA. April, 2015.
2018 **Francis Crick Institute Best Poster Award**. ISIRV Symposium – Influenza 2018: Centenary of the 1918 Pandemic. London, UK. June, 2018.

C. Contributions to Science

As of this submission, I have authored 70+ publications (published or in press) in the fields of nutrition, influenza, respiratory viruses, and infectious disease: 28 (40%) first author publications and 7 (10%) senior author publications. Full list available at: https://scholar.google.com/citations?user=lq1f7K0AAAAJ&hl=en
*As of this dossier**: Total citations**: 3,395, **h-index**: 31, **i10-index**: 53

1. **Global avian influenza virus surveillance and response in domestic poultry and wild birds**. Avian influenza virus is a major concern worldwide. Aside from seasonal epidemics and occasional pandemics, avian influenza viruses continue to cause human infections. I have been involved in influenza virus surveillance in wild and domestic poultry in Africa, South America, and Southeast Asia. These studies have defined new hotspots of influenza, determined seasonal patterns, and even helped to define policy recommendations. Selected References:

a) **Karlsson, EA**, Ciuoderis K, Freiden PJ, Seufzer B, Jones JC, Johnson J, Parra R, Gongora A, Cardenas D, Barajas D, Osorio JE, and Schultz-Cherry S. 2013. Prevalence and characterization of influenza viruses in diverse species in Los Llanos, Colombia. Emerging Microbes & Infection. 2, e20; doi:10.1038/emi.2013.20 PMID: 26038461
b) Jiménez-Bluhm P, **Karlsson EA**, Freiden P, Sharp B, Di Pillo F, Osorio JE, Hamilton-West C and Schultz-Cherry S. 2018. *Wild birds in Chile harbor diverse avian influenza A viruses*. Emerg Microbes Infect. Mar 29;7(1):44. doi: 10.1038/s41426-018-0046-9. PMID: 29593259
c) C G Monamele, P Y, **E A Karlsson**, M-A Vernet, A Wade, S Yann, N R Mohamadou, S Kenmoe, G M Yonga, J M Feussom, G Djonwe, C Ndongo, V S Horm, P F Horwood, S Ly, R Njouom and P Dussart. *Outbreak of avian influenza A(H5N1) among poultry in Cameroon and evidence of sub-clinical human infection* Emerg Microbes Infect. 2019 Jan 22; 8(1):186. doi: 10.1080/22221751.2018.1564631
d) **E A Karlsson***, S Tok, S V Horm, S Sorn, D Holl, S Tum, F Claes, K Osbjer and Philippe Dussart. Avian influenza virus detection, temporality and co-infection in poultry in Cambodian border provinces, 2017-2018. Emerg Microbes Infect. 2019; 8(1):637-639. doi: 10.1080/22221751.2019.1604085. PMID: 30999819 *Corresponding Author
e) Vijaykrishna D, Y-M Deng, M L Grau, M Kay, A Suttie, P F Horwood, W Kalpravidh, F Claes, K Osbjer, P Dussart, I G Barr, and **E A Karlsson***. *Emergence of Influenza A(H7N4) Virus, Cambodia*. Emerg Infect Dis. 2019 25(10):1988-1991. doi: 10.3201/eid2510.190506. PMID: 31310233 *Corresponding Author

2. **Seasonal and avian influenza surveillance and response in humans**. Human seasonal and avian strains continue to circulate endemically (seasonal) and cause zoonotic spillover (avian) in humans. I have been involved in both human seasonal and avian influenza surveillance in humans for a number of years. Selected References:

a) Horwood P F, **E A Karlsson**, S V Horm, S Ly, S Chin, D Saunders, S Rith, P Y, B Sar, A Parry, R Tsuyouka, Y-M Deng, A Hurt, I Barr, N Komadina, P Buchy and P Dussart. *Circulation and characterization of human influenza infections in Cambodia, 2012-2015*. Influenza Other Repir Viruses. 2019; 3(5): 465-476. doi: 10.1111/irv.12647. PMID: 31251478
b) Siegers J Y, V Dhanasekaran, R Xie, Y-M Deng, S Patel, V Ieng, J Moselen, H Peck, A Aziz, B Sarr, S Chin, S Heng, A Khalakdina, M Kinzer, D Chau, P Raftery, V Duong, L Sovann, I G Barr, **E A Karlsson*.** *Genetic and antigenic characterization of an influenza A(H3N2) outbreak in Cambodia and the Greater Mekong Subregion during the COVID-19 pandemic, 2020*. Journal of Virology. 2021. 23; 95(24): e0126721. doi: 10.1128/JVI.01267-21 *Corresponding Author
c) L Sovann, B Sar, V Kab, S Yann, M Kinzer, P Raftery, S Patel, P L Hay, H Seng, S Um, S Chin, D Chau, A Khalakdina, **E A Karlsson**, S Olsen, J Mott*. An influenza A(H3N2) virus outbreak during the COVID-19 pandemic, Kingdom of Cambodia, 2020*. International Journal of Infectious Disease. 2021. 103: 352-357. doi: 10.1016/j.ijid.2020.11.178
d) Um S, J Y Siegers, B Sar, S Chin, S Patel, S Bunnary, M Hak, S Sor, O Sokhen, S Heng, D Chau, T Sothyra, A Khalakdina, S J Olsen, J A Mott, F Claes, L Sovann, and **E A Karlsson***. A *Human Infection with Avian Influenza A(H9N2) Virus in Cambodia, February 2021.* Infectious Diseases. 2021. 27(10): 2742-2745. doi: 10.3201/eid2710.211039 *Corresponding Author

3. **Emerging and endemic viral discovery and surveillance in humans, domestic animals, and wild animals**. Aside from avian and human seasonal influenza, I have also contributed to a number of studies on other emerging viral diseases in Southeast Asia, including SARS-CoV-2. Selected References:

a) **Karlsson E A**, C T Small, P Freiden, M M Feeroz, F A Matsen IV , S San, M K Hasan, D Wang, G Engel, L Jones-Engel and S Schultz-Cherry. *Nonhuman primates harbor a diversity of mammalian and avian astroviruses including those associated with human infections.* PLoS Pathog. 2015 Nov 16;11(11):e1005225. doi: 10.1371/journal.ppat.1005225. PMID: 26571270. PMCID: PMC4646697
b) Delaune D*, V Hul*, **E A Karlsson***, A Hassanin, P O Tey, A Baidaliuk, F Gámbaro, Vuong T Tu, L Keatts, J Mazet, C Johnson, P Buchy, P Dussart, T Goldstein, E Simon-Lorière, Duong. *A novel SARS-CoV-2 related coronavirus in bats from Cambodia*. Nature Communications. 2021. 12; 6563. https://doi.org/10.1038/s41467-021-26809-4 *Co-first Author
c) **Karlsson E A**, V Duong. *The continuing search for the origins of SARS-CoV-2*. Cell. 2021. 184(17): 4373-4374. doi: 10.1016/j.cell.2021.07.035

d)

4. **Defining the risk of emerging viruses and the bottlenecks overcome to cross species barriers**. A major concern with zoonotic viruses, especially avian influenza virus, is the risk of zoonotic transmission. These studies seek to define factors (host-related, bacteriological, and virological) which can help to predict risk of zoonotic transmission and severity of viruses and emerging diseases. Selected References:

    a) **Karlsson, EA**, Ip HS, Hall J, Yoon S-W, Johnson J, Beck MA, Webby RJ and Schultz-Cherry S 2014. Respiratory transmission of an avian H3N8 influenza virus isolated from a harbor seal. Nat Commun 5:4791. PMID: 25183346
    b) Zaraket H, Baranovich T, Kaplan B, Carter R, Song M-S, Paulson J, Rehg F, Bahl J, Crumpton J, Seiler P, Edmonson M, Wu G, **Karlsson EA,** Fabrizio II T, Zhu H, Guan Y, Husain M, Schultz-Cherry S, Krauss S, McBride R, Webster RG, Govorkova E, Zhang J, Russell C, and Webby RJ. 2015. Mammalian adaptation of influenza A(H7N9) virus is limited by a narrow genetic bottleneck. Nat Commun 6:6553. PMID:25850788
    c) **Karlsson EA**, Meliopoulos VA, Savage C, Livingston B, Mehle A and Schultz-Cherry S**.** 2015. Visualizing Real-time influenza virus infection, transmission and protection in ferrets. Nat Commun 6:6378. PMID:25744559
    d) Rowe H M[#], **E A Karlsson**, H Echlin, T-C Chang, L Wei, T van Opijnen, S Pounds, S Schultz-Cherry and J W Rosch. *Bacterial factors required for transmission of* Streptococcus pneumoniae *in mammalian hosts.* Cell Host Microbe. 2019 Jun 12;25(6):884-891.e6. doi: 10.1016/j.chom.2019.04.012. PMID: 3112675
    e) Moncla L H, T Bedford, P Dussart, S V Horm, S Rith, P Buchy, **E A Karlsson**, L Li, Y Liu, H Zhu, Y Guan, T C Friedrich, and P F Horwood. *Quantifying within-host evolution of H5N1 influenza in humans and poultry in Cambodia.* 2020 Jan 17;16(1):e1008191. doi: 10.1371/journal.ppat.1008191 PMID: 31951644

D. Additional Information: Research Support and/or Scholastic Performance (Selected since 2019)

1. 2019 - **Grand Challenges Research Fund Networking Grant**. **GCRFNGR3\1497.** CANARIES: Consortium of Animal market Networks to Assess Risks of emerging Infectious diseases through Enhanced Surveillance (**PI**)
2. 2019 - **World Health Organization – LoA 2019/923423-0 -** Testing of ILI specimens for influenza A and B viruses; and testing of SARI specimens for influenza A/H5N1, influenza A/H7N9, other subtypes, and MERS-CoV from June – October 2019 (**PI**)
3. 2019 - **Wellcome Trust Multi-User Equipment Grant – 218310/Z/19/Z** - Advancing flow cytometry for the on-site study of tropical infectious diseases (**Co-PI**)
4. 2019-2024 - **DHHS ASIDE2 Project** - Detect threats early in Cambodia, including real-time biosurveillance, detection, characterization and reporting of emerging influenza strains and other emerging/co-emerging infectious respiratory disease pathogens. (**Co-PI**)
5. 2020 - **World Health Organization – LoA 2020/1027266-0 -** Agreement to Provide laboratory Diagnosis and Confirmation for COVID-19 Surveillance Specimens to Support the Public Health Response (**PI**)
6. 2020 - **World Health Organization – LoA 2020/1049713-0 -** Testing of ILI Specimens for Influenza A and B viruses; and Testing of SARI Specimens for Influenza A/H5N1, influenza A/H7N9, other subtypes, RSV, PIV, and human seasonal CoV from September 4th to September 29th, 2020 (**PI**)
7. 2020 - **FAO – USAID LoA** - **LOA/RAP/2020/09** – Avian and human influenza surveillance activities in Cambodia (**PI**)
8. 2021 – 2022 – **FAO – USAID LoA** - **LOA/RAP/2021/22** – Avian and human influenza surveillance activities in Cambodia and genetic analysis using novel sequencing techniques (**PI**)
9. 2021-2028 - **NIAID Centres of Excellence for Influenza Research and Response – UPenn CEIRR** - Influenza Surveillance, Risk Assessment, and Response Project 1: Swine Influenza Surveillance and Risk Assessment: Cambodia (**Country PI**)
10. 2021 – 2022 – **NIAID Centers for Research in Emerging Infectious Diseases – PICREID** – COVID Supplement: COVID-19 Sequencing Support in Cambodia (**PI**)
11. 2021 – 2022 – **DTRA STEP TD04-001** - Into the Wild: Prevalence of AIV in Wild Birds in the Kingdom of Cambodia (**PI**)

*Name of Individual: Firth, C.
Commons ID: (b) (6)

Other Support – Project/Proposal

ACTIVE

*Title: Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of South East Asia

Major Goals: This project aims to identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife, identify key risk pathways for zoonotic transmission, and identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts focusing in Thailand and Malaysia.

*Status of Support: Active

Project Number: 1U01AI15179

Name of PD/PI: Daszak, Peter

*Source of Support: NIH/NIAID

*Primary Place of Performance: EcoHealth Alliance, New York

Project/Proposal Start and End Date: (MM/YYYY) (if available): 06/2020 – 05/2025

* Total Award Amount (including Indirect Costs): $7,573,721

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	3.0 calendar
2. [2023]	3.0 calendar
3. [2024]	3.0 calendar
4. [2025]	3.0 calendar
5. [2026]	3.0 calendar

*Title: Understanding the establishment and persistence of community associated methicillin-resistant Staphylococcus aureus (CA-MRSA) in resource-limited, high-burden settings: How can we reduce the burden of disease?

Major Goals: To determine the clinical impact of MRSA-related acute hematogenous osteomyelitis (AHO) in the PNG highlands and identify interventions to reduce the burden and impact of this infection. We will evaluate the feasibility of point-of-care diagnosis of MRSA, determine the persistence and transmission pathways of CA-MRSA, and determine the diversity and phylodynamics of MRSA strains circulating in PNG highland communities and their association with AHO cases.

*Status of Support: Active

Project Number:

Name of PD/PI: Horwood, Paul

*Source of Support: National Health and Medical Research Council (Australia)

*Primary Place of Performance: James Cook University, Australia and Papua New Guinea

Project/Proposal Start and End Date: (MM/YYYY) (if available): 01/2022 – 12/2024

* Total Award Amount (including Indirect Costs): $619,987

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	0.5 calendar
2. [2023]	0.5 calendar
3. [2024]	0.5 calendar
4. [2025]	0.5 calendar
5. [2026]	0.5 calendar

PENDING

*Title: Revealing the Determinants of Virus Diversity and Cross-Species Transmission on Wildlife Farms in Southeast Asia

Major Goals: This project will characterize viral diversity, assess the rate of cross-species transmission, and evaluate zoonotic disease risk. Using innovative mathematical models, this project will estimate key epidemiological parameters for cross-species transmission within farms, simulate the impact of different farming practices, and assess the associated profit-risk trade-offs.

*Status of Support: Pending

Project Number: 2207955

Name of PD/PI: Firth, Cadhla

*Source of Support: National Science Foundation

*Primary Place of Performance: EcoHealth Alliance, New York

Project/Proposal Start and End Date: (MM/YYYY) (if available): 07/2022 – 06/2027

* Total Award Amount (including Indirect Costs): $2,999,887

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	2.0 calendar
2. [2023]	2.0 calendar
3. [2024]	2.0 calendar
4. [2025]	2.0 calendar
5. [2026]	2.0 calendar

*Title: Analyzing the Potential for Future Bat Coronavirus Emergence in Myanmar, Laos, & Vietnam

Major Goals: This project will conduct community-based surveys and biological sampling of people frequently exposed to wildlife in Myanmar, Laos, and Vietnam, to find serological evidence of spillover and assess disease spread risk; sampling and PCR screening of bats and other wildlife at community surveillance sites to identify viruses and hosts related to the human

infections; and syndromic surveillance in clinics to identify ‘cryptic’ cases or case clusters caused by bat-CoVs and assess the spread risk..

*Status of Support: Pending

Project Number:

Name of PD/PI: Daszak, Peter

*Source of Support: NIH/NIAID

*Primary Place of Performance: EcoHealth Alliance, New York

Project/Proposal Start and End Date: (MM/YYYY) (if available): 07/2022 – 04/2027

* Total Award Amount (including Indirect Costs): $3,361,851

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	3.0 calendar
2. [2023]	3.0 calendar
3. [2024]	3.0 calendar
4. [2025]	3.0 calendar
5. [2026]	3.0 calendar

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

*Signature: Firth, Cadhla

Date: January 30, 2022

*Name of Individual: Karlsson, E. A.
Commons ID: (b) (6)

Other Support – Project/Proposal

ACTIVE

*Title: Avian and human influenza surveillance activities in Cambodia and genetic analysis using novel sequencing techniques

Major Goals: The major goals of this project are continued longitudinal surveillance of avian influenza virus at the poultry-human interface in Cambodia and integration of new sequencing technologies.

*Status of Support: Active

Project Number: LOA/RAP/2021/22

Name of PD/PI: Karlsson, Erik

*Source of Support: FAO-USAID

*Primary Place of Performance: Institut Pasteur du Cambodge

Project/Proposal Start and End Date: (MM/YYYY) (if available): 06/2021 – 05/2022

* Total Award Amount (including Indirect Costs): $199,970

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	1.8 calendar

*Title: NIAID Centres of Excellence for Influenza Research and Response – UPenn CEIRR - Influenza Surveillance, Risk Assessment, and Response Project 1: Swine Influenza Surveillance and Risk Assessment: Cambodia

Major Goals: The major goals of this project are longitudinal influenza virus surveillance in swine and humans to detect, characterize, and analyze the evolution, disease dynamics, and risk profile of IAVs at the human-swine interface in Cambodia. We will monitor the evolutionary trajectories of swine IAVs currently circulating in backyard and commercial swine holdings in key border regions of Cambodia

*Status of Support: Active

Project Number:

Name of PD/PI: Karlsson, Erik (Country PI), Hensley, Scott (Center PI)

*Source of Support: NIH/NIAID

*Primary Place of Performance: Institut Pasteur du Cambodge

Project/Proposal Start and End Date: (MM/YYYY) (if available): 06/2021 – 03/2028

* Total Award Amount (including Indirect Costs): $1,503,684

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	3.6 calendar
2. [2023]	3.6 calendar
3. [2024]	3.6 calendar
4. [2025]	3.6 calendar
5. [2026]	3.6 calendar
6. [2027]	3.6 calendar
7. [2028]	1.8 calendar

*Title: Into the Wild: Prevalence of AIV in Wild Birds in the Kingdom of Cambodia

Major Goals: The major goals of this project are to obtain preliminary information on avian influenza virus prevalence in wild birds in Cambodia, develop preliminary data for building an Early Warning System (including the use of novel techniques) and to provide capacity building to Cambodian Veterinary Epidemiologists

*Status of Support: Active

Project Number: STEP TD04-001

Name of PD/PI: Karlsson, Erik

*Source of Support: DTRA

*Primary Place of Performance: Institut Pasteur du Cambodge

Project/Proposal Start and End Date: (MM/YYYY) (if available): 10/2021 – 05/2022

* Total Award Amount (including Indirect Costs): $282,154

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	1.8 calendar

*Title: CREID COVID Supplement: Support for COVID Sequencing in Cambodia. PICREID.

Major Goals: The major goals of this project are to further support sequencing and analysis of SARS-CoV-2 genomes in Cambodia as part of the national respoinse to COVID-19.

*Status of Support: Active

Project Number:

Name of PD/PI: Karlsson, Erik

*Source of Support: NIH/NIAID

*Primary Place of Performance: Institut Pasteur du Cambodge

Project/Proposal Start and End Date: (MM/YYYY) (if available): 11/2021 – 05/2022

* Total Award Amount (including Indirect Costs): $107,266

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	0.5 calendar

PENDING

*Title: Novel detection and sampling techniques for surveillance of bat-borne zoonoses in Cambodia

Major Goals: This project will utilize novel field technologies (RT-PCR, sequencing) in conjunction with novel sampling strategies (air, environment) to develop better ways to sample bats across Cambodia.

*Status of Support: Pending LoA

Project Number:

Name of PD/PI: Karlsson, Erik

*Source of Support: FAO-USAID

*Primary Place of Performance: Institut Pasteur du Cambodge

Project/Proposal Start and End Date: (MM/YYYY) (if available): 01/2022 – 11/2022

* Total Award Amount (including Indirect Costs): $77,000

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	1.8 calendar

*Title: One Health understanding of small-holder swine value chain in Cambodia

Major Goals: This study aims to enhance the health and livelihood of small-holder swine value chain stakeholders through cost-effective intervention measures following a One-Health approach. We believe that small-holder pig producers play a key role in the spread of several important zoonotic diseases, including food borne diseases. Applying cost-effective interventions and risk-based surveillance along the value chain will reduce risk and enhance the livelihood of the stakeholders. The project will tackle gender-related inequalities in the roles assigned along the pig-value chain in risk awareness, mitigation and exposure, and identify solutions to empower women in this system.

*Status of Support: Pending

Project Number:

Name of PD/PI: Karlsson, Erik

*Source of Support: ACIAR (Australia), IDRC (Canada)

*Primary Place of Performance: Institut Pasteur du Cambodge

Project/Proposal Start and End Date: (MM/YYYY) (if available): 07/2022 – 07/2025

* Total Award Amount (including Indirect Costs): $698,700

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	1.0 calendar
2. [2023]	2.0 calendar
3. [2024]	2.0 calendar
4. [2025]	1.0 calendar

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

*Signature: Karlsson, Erik

Date: January 30, 2022

Co-PI Plan (only needed if applying as Co-PIs):

As stated in the Mentoring Plan, the co-PIs, Vireak Heang and Jurre Siegers, are at different stages of their respective careers and have discrete professional development goals. This Co-PI Plan was developed to maximize the strengths of each PI, provide opportunities to learn from each other, and create a supportive environment where they can pursue their independent professional goals while working as a team to achieve the project objectives.

Overall, the proposed research project will be jointly and equally managed by Dr. Siegers and Mr. Heang. Dr. Siegers will plan, oversee, and conduct sample management and processing. He will also lead the data analysis aspects of the work. Mr. Heang will be responsible for the technical aspects of metagenomic sequencing, including logistics and protocol development, laboratory work, and initial data curation and quality control. He will work closely with the Research Center Mentor, Dr. Cadhla Firth, to develop these aspects of the project. As the project progresses, Dr. Siegers will be exposed to the technical aspect of metagenomics, including how to prepare and load samples onto the sequencing platform, by learning from Mr. Heang. Likewise, Mr. Heang will develop skills in upstream sample preparation and handling and will have the opportunity to participate in downstream data analysis, as he learns from Dr. Siegers. This collaboration will ensure that both individuals gain technical skills in areas where they have less experience, and each will become a more well-rounded scientist as a result of their joint efforts. While Mr. Heang will be more involved with the technical aspects of sequencing and data generation, Dr. Siegers will take the lead to work with EcoHealth Alliance's modeling experts to explore the virological data and the meaning for potential surveillance efforts.

Both PIs will jointly manage data curation, analysis, and dissemination for this proposal. In addition, the Co-PIs will jointly manage the project budget with oversight from their Scientific Mentor, Dr. Erik Karlsson. The PIs will receive a budget code in the IPC system on which they can charge reagents and supplies, as well as manage spending, and purchases will be co-signed by Dr. Karlsson. In terms of specific reagents, Mr. Heang will oversee the ordering of supplies needed for sequencing, and Dr. Siegers will be responsible charges related to sample preparation. Both PIs will jointly book and conduct travel. While Mr. Heang will be more involved in generating metagenomic sequencing data in the lab, Dr. Siegers will lead the initial drafting of reports and publications that will result from this research. However, both authors will contribute to the writing and publication of any manuscripts and will jointly share authorship. The Co-PIs will work closely with both Mentors on all aspects of the project and will regularly attend IPC and EcoHealth Alliance scientific meetings to present work and progress.

References

1 Allen, T. *et al.* Global hotspots and correlates of emerging zoonotic diseases. *Nat Commun* **8**, 1124, doi:10.1038/s41467-017-00923-8 (2017).

2 Coker, R. J., Hunter, B. M., Rudge, J. W., Liverani, M. & Hanvoravongchai, P. Emerging infectious diseases in southeast Asia: regional challenges to control. *Lancet* **377**, 599-609, doi:10.1016/S0140-6736(10)62004-1 (2011).

3 Yek, C. *et al.* The Pandemic Experience in Southeast Asia: Interface Between SARS-CoV-2, Malaria, and Dengue. *Frontiers in Tropical Diseases* **2**, doi:10.3389/fitd.2021.788590 (2021).

4 Ziegler, S. & Engel, K. *Pandora's box - a report on the human zoonotic disease risk in Southeast Asia with a focus on wildlife markets*. (2020).

5 Karlsson, E. A. *et al.* Avian influenza virus detection, temporality and co-infection in poultry in Cambodian border provinces, 2017-2018. *Emerg Microbes Infect* **8**, 637-639, doi:10.1080/22221751.2019.1604085 (2019).

6 Wille, M., Geoghegan, J. L. & Holmes, E. C. How accurately can we assess zoonotic risk? *PLoS Biol* **19**, e3001135, doi:10.1371/journal.pbio.3001135 (2021).

7 Kelly, T. R. *et al.* One Health proof of concept: Bringing a transdisciplinary approach to surveillance for zoonotic viruses at the human-wild animal interface. *Prev Vet Med* **137**, 112-118, doi:10.1016/j.prevetmed.2016.11.023 (2017).

8 Firth, C. & Lipkin, W. I. The genomics of emerging pathogens. *Annu Rev Genomics Hum Genet* **14**, 281-300, doi:10.1146/annurev-genom-091212-153446 (2013).

9 Wilson, M. R. *et al.* Actionable diagnosis of neuroleptospirosis by next-generation sequencing. *N Engl J Med* **370**, 2408-2417, doi:10.1056/NEJMoa1401268 (2014).

10 Cappelle, J. *et al.* Nipah virus circulation at human-bat interfaces, Cambodia. *Bull World Health Organ* **98**, 539-547, doi:10.2471/BLT.20.254227 (2020).

11 Bank, W. People, Pathogens and Our Planet : The Economics of One Health. (2012).

12 Um, S. *et al.* Human Infection with Avian Influenza A(H9N2) Virus, Cambodia, February 2021. *Emerg Infect Dis* **27**, 2742-2745, doi:10.3201/eid2710.211039 (2021).

13 Suttie, A. *et al.* Diversity of A(H5N1) clade 2.3.2.1c avian influenza viruses with evidence of reassortment in Cambodia, 2014-2016. *PLoS One* **14**, e0226108, doi:10.1371/journal.pone.0226108 (2019).

14 Suttie, A. *et al.* Avian influenza in the Greater Mekong Subregion, 2003-2018. *Infect Genet Evol* **74**, 103920, doi:10.1016/j.meegid.2019.103920 (2019).

15 Vijaykrishna, D. *et al.* Emergence of Influenza A(H7N4) Virus, Cambodia. *Emerg Infect Dis* **25**, 1988-1991, doi:10.3201/eid2510.190506 (2019).

16 Cappelle, J. *et al.* Longitudinal monitoring in Cambodia suggests higher circulation of alpha and betacoronaviruses in juvenile and immature bats of three species. *Sci Rep* **11**, 24145, doi:10.1038/s41598-021-03169-z (2021).

17 Delaune, D. *et al.* A novel SARS-CoV-2 related coronavirus in bats from Cambodia. *Nat Commun* **12**, 6563, doi:10.1038/s41467-021-26809-4 (2021).

18 Briese, T. *et al.* Virome Capture Sequencing Enables Sensitive Viral Diagnosis and Comprehensive Virome Analysis. *mBio* **6**, e01491-01415, doi:10.1128/mBio.01491-15 (2015).

19 Biosciences, T. *Comprehensive Viral Research Panel*, <https://www.twistbioscience.com/products/ngs/fixed-panels/comprehensive-viral-research-panel> (

20 Izquierdo-Lara, R. *et al.* Monitoring SARS-CoV-2 Circulation and Diversity through Community Wastewater Sequencing, the Netherlands and Belgium. *Emerg Infect Dis* **27**, 1405-1415, doi:10.3201/eid2705.204410 (2021).

21 Haas, B. J. *et al.* De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat Protoc* **8**, 1494-1512, doi:10.1038/nprot.2013.084 (2013).
22 Minot, S. S., Krumm, N. & Greenfield, N. B. One Codex: A Sensitive and Accurate Data Platform for Genomic Microbial Identification. *bioRxiv*, 027607, doi:10.1101/027607 (2015).
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List of abbreviations, acronyms, symbols:

AIV: Avian Influenza Virus
BA: Bachelor of Business Administration
BI: Bioinformatics Consortiums
BS: Bachelor of Science
MBA: Master of business administration
BSL2: Biological Safety level 2
BSL3: Biological Safety level 3
CAN: Canada
CEIRR: Centers of Excellence for Influenza Research and Response
CREID: Centers for Research in Emerging Infectious Diseases
COVID-19: Coronavirus Disease 2019
Co-PI: Co-principal investigator
CVRP: Twist Comprehensive Viral Research Panel
DNA: deoxyribonucleic acid
dsDNA: double stranded deoxyribonucleic acid
ssDNA: single stranded deoxyribonucleic acid
Duke-NUS: Duke-National University of Singapore
EHA: EcoHealth Alliance
EID: emerging infectious disease
EID-SEARCH: The Emerging Infectious Disease – South East Asia Research Collaboration Hub
ES: Environmental Sample
FAO: Food and Agriculture Organization
GEIS: Emerging Infectious Surveillance and Response System
GISRS: Global Influenza Surveillance and Response System
IPC: Institut Pasteur du Cambodge
IT: information technology
LBM: live bird market
LMIC: Lover and middle income country
MoH: Ministry of Health, Cambodia
MSc: Master of Science
NAMRU-2: US Naval Medical Research Unit.2, Cambodia
NCBI: National Center for Biotechnology Information
NEB: New England Biolabs
NGS: next-generation sequencing
NIAID: National Institute of Allergy and Infectious Diseases
NIH: National Institutes of Health
NY: New York State
OFFLU: Joint OIE/FAO worldwide scientific network for the control of animal influenzas
OIE: World organization for animal health
ONT: Oxford Nanopore Technologies
PA: Pennsylvania State
cPCR: Conventional PCR
PCR: polymerase chain reaction
qRT-PCR: Quantitative Real-Time PCR
PhD: Doctor of Philosophy
PI: principal investigator

PREDICT: Pandemic Preparedness for Global Health Security
RNA: ribonucleic acid
SARS-CoV-2: Severe Acute Respiratory Coronavirus 2
ssRNA: single stranded ribonucleic acid
RT-PCR: real-time PCR
USAID: U.S. Agency for International Development
WHO: World Health Organization

Facilities and Resources

Laboratory:

The Institut Pasteur in Cambodia (IPC) is an institution of public utility engaged with the Royal Government of Cambodia by a convention and under the patronage of the Ministry of Health to meet the challenges of the future in the areas of scientific research, public health and education. It is a member of the Institut Pasteur International Network with which it develops ongoing collaborations, with 33 member institutes spread across the globe, which facilitates collaboration and scientific exchanges. Visiting scientists from Institut Pasteur Paris can provide expertise to setup specific assays and collaborations with Institut Pasteur Paris enable access state-of-the-art equipment if needed for the proposed research.

The Virology Unit at the Institut Pasteur du Cambodge (IPC) has been instrumental in public health responses in Cambodia, especially against respiratory viruses such as avian influenza. As such, IPC has served as the National Influenza Centre (NIC) for Cambodia since 2006 and was designated as a WHO H5 Reference Laboratory in October 2014 (H5RL), as part of the WHO Global Influenza Surveillance and Response System (GISRS). In addition, IPC serves as a reference laboratory for a number of other pathogens. Programs and procedures are also in place to identify, diagnose, and sequence numerous other emerging and zoonotic pathogens, such as Coronavirus (CoV). The IPC Virology Unit was designated by the Cambodian Ministry of Health as first line laboratory for diagnosis of COVID-19 and currently acts as the National Reference Laboratory for COVID-19. In April 2020, IPC Virology Unit was recently named a WHO Global Reference Laboratory for COVID-19.

The virology laboratory space covered by the Virology Unit contains all equipment needed for virology research. Biological Level 1 and 2 space (200 m^2) is dedicated for sample acquisition, processing and storage, serology, extraction, post-amplification procedures, molecular biology and PCR. Separate BSL2 spaces are also dedicated to human samples (23 m^2), animal samples (24 m^2), cell maintenance (12 m^2) and cell culture work (35 m^2). In response to A/H5N1 circulation in Cambodia the Virology Unit was upgraded in 2008 to include a BSL3 facility (142 m^2). The BSL3 facility comprises four modules dedicated to different and separate activities: arboviruses, emerging viruses (including a safety cabinet with gloves box allowing BSL3+ safety conditions) and mycobacterium (activity handled by the IPC clinical laboratory). The last module includes isolators for animal experiments.

Equipment includes: CO_2 incubators (n=3 dedicated to cell maintenance and cell culture work in a dedicated BSL2 spaces and n=2 dedicated to cell culture work in BSL3), automated extraction (n=4), real time qRT-PCR machines (n=7 BioRad CFX96), conventional PCR machines (n=8 BioRad T100), a Sanger sequencing (ABI 3500xL Genetic analyzer), 2x MinION next generation sequencer (Oxford Nanopore), 1x GridION next generation sequencer (Oxford Nanopore), 1x MiSeq next generation sequencer (Illumina), a gel imaging system (BioRad ChemiDoc WRS+), a capillary electrophoresis device (Qiagen QIAxcel advanced system), light microscopes (n=2 direct light; n=4 inverted light), a fluorescent microscope, bench top centrifuges, three ELISA washers (BioRad), two ELISA readers (BioRad and ThermoFisher Scientific) and Qubit fluorometer.

The sequencing mini-platform at IPC is a newly established platform intended to provide the sequencing services to all scientists within the institute regardless of their discipline. The sequencing mini-platform is situated in the Regional Research Platform-ASIA (PRR) building and contains all the equipment needed to perform sequencing. We have 20m2 dedicated for library preparation and another 20m2 for the instruments. Equipment includes: 1 Illumina Miseq, 1 Tapestation 4150, 1 Qubit 4.0, 1 Thermocycler.

Clinical:

The medical analysis laboratory of IPC has received ISO NF 15189 accreditation for all its applications for the analysis of clinical biology, biochemistry, hematology, and microbiology in 2018.

Animal:

A conventional mouse facility and dedicated BSL2 and BSL3 level space for animal experiments are available at IPC; however, they will not be utilized in this proposal.

Computer:

Information technology access: Since the Institute is located in a low-income country, we are eligible for the Hinari Access to Research for Health Programme of the world health organization (WHO), providing us online access to all major journals in biomedical research. Both the laboratories and PI's office are equipped with MacIntosh and PC computers and printers. Computers are protected by enterprise antivirus software. Email solution is Microsoft Exchange 2010 server. Local network is managed with Cisco catalyst switches, linked with fiberoptics or network cables, with redundant paths. VLANs are used to segregate research units, enabling more confidentiality. Two telco links provide access to the internet: primary/nominal with fiberoptics Online operator, with 8 MBbit/s bandwidth and 24/7 support. IPC has a reliable internet connection with 35Mbs download and 35Mbs upload speeds.Backup link is operated by OpenNet with 4 Mbit/s bandwidth.

The computational resources for generating, analyzing, storing and managing sequencing data are available locally. IPC acquired two Ubuntu server compute nodes (each equipped with 64 cores, 128GB of RAM, and a high-end NVIDIA GPU) connected over 10Gbs switches to 222 TB of shared networked storage.

Office:

Office space is separated from the laboratory space with dedicated offices for postdoctoral researchers, students, PI's and technicians.

Other:

Biological sample storage and access: All samples will be stored in -80°C freezers and liquid nitrogen tanks housed within the IPC biobanking system. The IPC biobank is a multisite biological resource center (BRC), which collects, transforms, analyses, stores and provides resources according to specific pre-requirements and manages associated data. All the activities of preparation, analysis and biological resource (BR) processing are realized according to stringent quality requirements. For long-term security and confidentiality, Biobank Management Software has been implemented and established and used to manage the BR and associated data from different laboratories and research units at IPC. Only the PI and relevant key personnel has access to the samples. The biobank includes human, animal and pathogenic samples.

Foreign Site Information

If the Pilot Award will have multiple foreign sites, complete this form for EACH research site

Principal Investigator Name:	Dr. Jurre SIEGERS and Mr. Vireak HEANG
Project Title:	*In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance*.
Institution:	Institut Pasteur du Cambodge
Foreign Research Site:	Phnom Penh, Cambodia
Point of Contact for Research Site:	Dr. Jurre SIEGERS Virology Unit, Institut Pasteur du Cambodge 5 Monivong Blvd, PO Box #983 Phnom Penh, Cambodia email: jsiegers@pasteur-kh.org

Introduction (3-4 sentences):
Provide a simple description of the overall goals of the project including the work that will be done at the foreign site. Indicate if any of the NIAID grant funds will be sent to the foreign site.

The goals of this project are: (1) to assess the suitability of environmental metagenomics as an early warning system for endemic and emerging infectious diseases at high-risk interfaces in Cambodia; (2) to utilize biobank environmental samples from longitudinal pathogen surveillance programs in Cambodia to build metagenomic sequencing and bioinformatics capacities and capabilities in Cambodia; and, (3) To discover new, emerging, or zoonotic pathogens of concern at high-risk interfaces in Cambodia.

To achieve these goals, investigators and primary Scientific Mentor on site will collaborate with EcoHealth Alliance and their CREID partners as part of the Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH). The EID-SEARCH team will support this project by helping the Institut Pasteur du Cambodge (IPC) team conduct and analyze metagenomic sequencing on Cambodian samples.

$147,675.96 of grant funds will be sent to IPC for these studies.

Foreign component (3-4 sentences):
Describe the specific role of the foreign site (i.e. conduct critical experiments?, conduct data analyses?, provide consultations?, provide samples?, study human and/or animal populations?, conduct observational or interventional clinical trials?, collect samples and/or data to be brought to the US?).

The site will coordinate and/or perform all laboratory work and data analyses necessary for the proposed project (including metagenomic sequencing).

Human Subjects (1 word or 1 sentence per bullet):

- **Parent Study IRB approval**
  - **IRB approval number for parent study:** Not Applicable
  - **IRB approval date:**
  - **Human Subject Assurance Number:**
- **Does this study require a modification to the IRB approval of a parent study (Yes or No)?**
  - No

- **Will existing samples from human subjects will be used: (Yes or No)?**
  - No
  - **How many subjects provided the existing samples to be used?** Not Applicable

- **Will human subjects be recruited (Yes or No)**
  - No
  - **Number of human subjects that will be recruited:** Not Applicable

- **Population parameters:** Not Applicable
  - **Gender:**
  - **Age Group:**
  - **Race/Ethnicity:**

- **Sample collection will include:** Not Applicable
  - **Blood:**
  - **Urine:**
  - **Tissues:**
  - **Other samples (describe):**

- **Sample collection will be completed in how many visits:** Not Applicable

- **Will samples be de-identified (Yes or No)? If No, describe how they will be protected.**
  - Not Applicable

- **Will local IRB approval be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals being sought or state why local IRB approval is not required.**
  - Not Applicable

- **Will informed consent be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals/consents being sought or state why informed consent is not required.**
  - Not Applicable

- **Will samples be brought back to the US (Yes or No)?**
  - Not Applicable

- **Will data be brought back to the US (Yes or No)?**
  - Not Applicable

Animal Subjects (1 word or 1 sentence per bullet):

- **Parent study IACUC approval**
  - **IACUC approval number for parent study:** o Projects involving animal samples were collected (2019-2021) under a protocol approved by National Ethics Committee for Health Research under the Ministry of Health, Cambodia (NECHR143, NECHR149, and NECHR320); however, Cambodia did not have a specific animal ethics review board at the time the collections were conducted. Continuing ethics approvals (NECHR013) for projects involving further animal sample collections in 2022 are in revision and projected to be approved in February 2022.
  - **IACUC approval date:** Not Applicable
    Animal Welfare Assurance Number: Not Applicable
- **Does this study require a modification to the IACUC approval of a parent study (Yes or No)?**
  - **No**

- **Will existing samples from animal subjects will be used: (Yes or No)?**
  - **Yes**
  - **How many animal subjects provided the existing samples to be used?** 2445
- **Will vertebrate animals be collected (Yes or No)?**
  - **No**

- **Species of animals (e.g. rats, mice, rabbits, monkeys):** Not Applicable
- **Animal parameters:**
  - **Total number of animals:**
  - **Gender:**
  - **Age range:**
  - **Lab strain (e.g. Sprague-Dawley rats, Balb/C mice):**
  - **Wild animals procured in country (e.g. Rhesus monkeys from a reserve):**

- **What will be done to them or with them and how often?**
  - Not Applicable

- **What are the follow-ups?**
  - Not Applicable

- **What will be their fate at the end of the experiments – will they be euthanized?**
  - Not Applicable

- **Will in-country clearances be obtained prior to engaging in any animal research (Yes or No)? If No, describe alternate approvals or state why in-country clearances are not required.**
  - Not Applicable
- **Will samples be brought back to the US (Yes or No)?**
  - No
- **Will data be brought back to the US (Yes or No)?**
  - Yes, some of the data will be shared with EcoHealth Alliance/EID-SEARCH for relevant analysis.

ROYAUME DU CAMBODGE
NATION RELIGION ROI

Institut Pasteur du Cambodge

Le Directeur

Phnom Penh, January 31, 2022

O/Ref.: N°034/IPC/DIR/2022

Dr. Jurre Siegers & Mr. Vireak Heang
Institut Pasteur du Cambodge
Phnom Pehn, Cambodia

Object: Institutional Support for your application to the CREID Pilot Research Program, entitled "In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance."

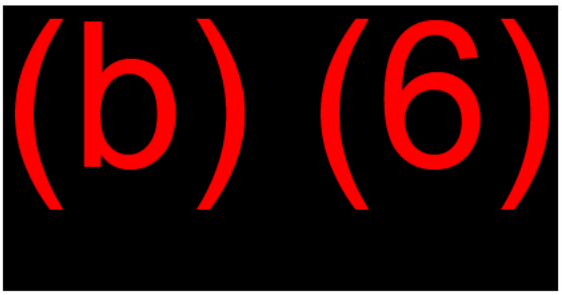
Dear Dr. Siegers & Mr. Heang,

The Institut Pasteur du Cambodge (IPC) is pleased to support your application to the **CREID Pilot Research Program**, entitled "In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance." We are fully committed in executing the statement of work utilizing next-generation metagenomics techniques for emerging zoonoses and potentially developing novel methods of expanding our surveillance in Cambodia.

The work proposed in this project will be given the highest priority and complements nicely to all of the other endemic and emerging virus research being conducted at IPC. In addition, it serves very nicely to bring together our long-standing Virology Unit with the newly created Sequencing Mini-Platform to build capacity and capability not only in our institution, but also for the next generation of Cambodian scientists.

As such, IPC asserts that all necessary time, facilities, and resources required for the project will be made fully available to you to successfully complete the work proposed in this project.

Sincerely,



Prof. André SPIEGEL
Director

Prof André SPIEGEL
Directeur
5, Boulevard Monivong
BP 983 – Phnom Penh
Téléphone : 855 (0) 12 222 659
aspiegel@pasteur-kh.org

EID-SEARCH
Emerging Infectious Diseases
South East Asia Research Collaboration Hub

Dr. Jurre Siegers, Mr. Vireak Heang
Institut Pasteur du Cambodge
No. 5 Monivong Boulevard
P.O Box. 983
Phnom Penh, Cambodia

Dear Jurre and Vireak,

The Emerging Infectious Disease-Southeast Asia Research Collaboration Hub (EID-SEARCH) at EcoHealth Alliance is highly interested in working with you and your collaborators at the Institut Pasteur du Cambodge in Cambodia on the proposed project titled: "*In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance.*"

This project will significantly contribute to the goals of the CREID Network and EID-SEARCH to build in-country research capacity and an international research network for emerging infectious diseases in Southeast Asia. It will develop a foundation for new collaborations among EID-SEARCH, Institut Pasteur du Cambodge, and other in-country stakeholders, strengthening the multi-sectoral cooperation for emerging infectious diseases in Cambodia. Furthermore, the process to develop and implement this project will help develop your skills in project management, provide training and networking opportunities with scientists from EID-SEARCH and the CREID Network, and most importantly, expand your skills in metagenomics, to prepare you to be independent researchers leading emerging infectious disease research both within and outside of Cambodia.

Beyond capacity and network building, the proposed project strongly aligns with the EID-SEARCH's scientific objectives and research strategies by conducting surveillance among humans and animals to identify emerging pathogens with zoonotic potential at the high-risk human-animal interfaces and develop an early-warning system for zoonotic disease emergence. The research sites of this project - live animal markets, bat caves, slaughterhouses, animal farms – represent scenarios where zoonotic spillover are mostly concerned that require further in-depth investigation to reveal the spillover pathways. In addition, the innovative approach to conduct surveillance among environmental samples will not only expand our knowledge in viral discovery from the environment but also promote the One Health surveillance by establishing a validated sampling and analysis method that will significantly contribute to the disease surveillance and prevention strategies in Cambodia through collaborative and coordinative cooperation among a variety of stakeholders.

I am optimistic that this collaborative research project will bring valuable insights to advance disease surveillance and early warning systems for endemic and emerging infectious diseases in Cambodia. Members of EID-SEARCH are committed to working closely with you to develop the research project and support the efforts necessary for the success of this project. Our Senior Research Scientist, Dr. Cadhla Firth, has enthusiastically joined the project as your Mentor to advise on the study design, data collection, and analysis throughout the project. Dr. Firth will work closely with Dr. Erik Karlsson, a mentor from your IPC Virology Unit, to provide pertinent and timely career advice to assist in your professional development and brings a well-established network with leaders and experts in the ecology and evolution of infectious diseases across Southeast Asia, North America, and Australia. You will be invited to join all training conducted by EID-SEARCH and EcoHealth Alliance regarding emerging infectious disease fields, surveillance, and statistical analysis to build your skillset. In addition, Dr. Linfa

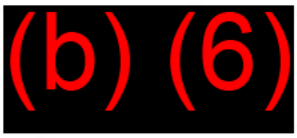
EID-SEARCH
Emerging Infectious Diseases
South East Asia Research Collaboration Hub

Wang, Co-Investigator from the EID-SEARCH, will support you on the metagenomics analysis by advising on the project and providing training or exchange opportunities at the Duke-NUS Medical School. You will be supported to present the findings of this research at international conferences, CREID Network meetings, and external partners and produce high-quality publications from this research.

I am confident that this proposed project will bring genuine opportunities for your professional development. The interdisciplinary expertise, a hallmark of "One Health", in virology, genomics, public health, extensive experience working with sequencing platforms from Dr. Siegers and Mr. Heang, and the improved environmental metagenomics methods and skills through this research will make this a successful and purposeful project to bring public health impacts in a broad region with the similar social and cultural context.

This letter conveys my strong interest and commitment to making this application a success. I look forward to collaborating with you and your team on all phases of this proposed project. I wish you success in the CREID Pilot Research Program application.

Sincerely,



Peter Daszak
Principal Investigator, EID-SEARCH
President, EcoHealth Alliance

January 25, 2022

Jurre Siegers, Vireak Heang
Institut Pasteur du Cambodge
No. 5 Monivong Boulevard
P.O Box. 983
Phnom Penh Cambodia

Dear Jurre and Vireak,

I am writing this letter in support of your CREID Network Pilot Research Program application “In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance” conducted at the Institut Pasteur du Cambodge.

I and my group at Duke-NUS Medical School have long experience working on the emerging infectious diseases associated with bats and other animals. Your current proposal complements and expands this scope to identify the at-risk groups and the risk factors of viral spillover from wildlife to human populations.

I also work with the Emerging Infectious Diseases - South East Asia Research Collaboration Hub (EID-SEARCH) to develop serological tests and conduct virus characterization. I am very much looking forward to participating in this research to contribute my expertise, advise and provide relevant laboratory training.

This letter conveys my strong interest and commitment to making this application a success. I am excited to be part of the research to build scientific evidence to better predict zoonotic disease emergence, and I look forward to working with you on this research.

Yours sincerely,

(b) (6)

Linfa (Lin-Fa) WANG, PhD FTSE
Professor in Programme in Emerging Infectious Diseases

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EcoHealth Alliance

30th January, 2022

Dr. Jurre Siegers & Mr. Vireak Heang
Institut Pasteur du Cambodge
Phnom Pehn, Cambodia

Dear Dr. Siegers & Mr. Heang,

This letter is to express my support as Research Center Mentor for your application to the CREID Pilot Research Program, entitled "*In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance*."

I am extremely excited that you have chosen me to be one of your Mentors for the proposed project. The research question is timely and important, and your work has the potential to have a strong impact on our approach to pathogen surveillance at high-risk human-animal interfaces. Given what we understand to be the likely origins of SARS-CoV-2, there has never been a more pressing need to improve the efficiency, sensitivity, and cost-effectiveness of zoonotic disease surveillance in emerging infectious disease hotspots.

I would be hard-pressed to think of two more appropriate applicants to co-lead this project, given your combined expertise in pathogen surveillance, molecular diagnostics, next-generation sequencing, and emerging respiratory viruses. Although you each have unique longer-term personal and professional goals, you also have a shared vision of using your scientific knowledge and expertise to train the next generation of Cambodian scientists, while building intellectual and scientific capacity around emerging infectious diseases in the country. I believe that increased access to the CREID Network, and to the Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH), in particular, will greatly help facilitate these goals by increasing your access to other scientists working on similar research questions across Southeast Asia. Many Southeast Asian countries face similar challenges in preventing and mitigating the impacts of emerging zoonotic diseases, and stronger regional partnerships have the potential to really drive forward innovations in this area. These include the implementation of novel, cost-effective approaches to pathogen surveillance, as proposed in your pilot project application.

The well-established relationship you each have with your chosen Primary Scientific Mentor for this project, Dr. Erik Karlsson, will provide an excellent foundation for the proposed work that will be enhanced by the joint Mentorship Plan we have developed together. I believe that Dr. Karlsson and I have distinct but complementary scientific skills, expertise, and networks that will maximize your professional and scientific development throughout the duration of the project, and I look forward to working more closely with all of you. I can see multiple opportunities for this new collaboration between the four of us to develop into a fruitful, long-term scientific partnership. Towards that aim, I am committed to supporting your professional development in the following ways:

- I will actively work to maintain the healthy, open, and informal dialogue we have established while drafting the project proposal, and I will seek feedback from you on a regular basis to ensure that our communication plan continues to meet your needs as the project progresses.

EcoHealth Alliance
520 Eighth Avenue, Suite 1200
New York, NY 10018
212.380.4460
EcoHealthAlliance.org

- I will engage with your current and future in-country collaborators in a supportive and inclusive manner.
- I will create opportunities for you to interact with scientists across my network through your research project to widen your exposure to people with a range of expertise.
- I will give you timely feedback on all material in the manner that works best for you.
- I will provide you with opportunities to present your work to my colleagues at EcoHealth Alliance and across EID-SEARCH during our academic meetings and will include you in any relevant training or networking opportunities.
- I will share my scientific and technical expertise with you to help ensure the success of the project. When skills outside my area are required, I will assist you in accessing this expertise from scientists within my network.
- I will assist you in career planning, providing advice and support as required to maximize your opportunity to succeed.

Finally, I believe that the data you will collect about the potential zoonotic pathogens circulating in live bird markets, swine abattoirs, and bat roosts and guano farms in Cambodia will lead to a wealth of new research questions and hypotheses that could form the basis for additional research projects. This will present the perfect opportunity for you to continue the transition towards scientific independence, and I am committed to assisting you through the process of conceptualizing and writing your first funding applications as lead investigators.

There is significant overlap between your research interests and skills, the overall goals of the Institut Pasteur du Cambodge, and those of EcoHealth Alliance and the EID-SEARCH team. This pilot project presents a real opportunity for you to increase collaboration between these organizations, and to help drive forward future research projects on emerging zoonoses at high-risk interfaces in Cambodia. I am thrilled to be able to support you at these stages in your careers and am genuinely convinced that you both have the potential to become scientific leaders in Southeast Asia in the very near future.

Sincerely,

(b) (6)

Cadhla Firth, PhD
Senior Scientist and Program Coordinator
EcoHealth Alliance

INSTITUT PASTEUR
DU CAMBODGE
VIROLOGY UNIT

30th January, 2022

Dr. Jurre Siegers & Mr. Vireak Heang
Institut Pasteur du Cambodge
Phnom Pehn, Cambodia

Object: Support as Primary Scientific Mentor for your application to the CREID Pilot Research Program, entitled "*In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance*."

Dear Dr. Siegers & Mr. Heang,

This letter is to express my support as Primary Scientific Mentor for your application to the CREID Pilot Research Program, entitled "*In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance*."

I am proud to be able to continue to mentor both of you as one of your Mentors for the proposed project. I serve as both your mentor in the Institut Pasteur du Cambodge (IPC) Virology Unit (Dr. Siegers) and in the IPC Sequencing Mini-Platform (Mr. Heang). You are both in the top 1% of individuals I have ever had the pleasure to mentor/supervise. I have observed you both as extremely organized, productive, proactive, and responsive. You have never failed in any challenge placed in front of you and always deliver extra in your work. You are always willing to learn and adapt to new situations, and you both have extremely good leadership qualities, which will make your excellent PIs in the future.

As you well know, IPC serves as a major institution in the Institut Pasteur International Network and has been involved in research and diagnosis of human and animal infectious diseases in the Kingdom of Cambodia since 1953. IPC serves as a research institute, non-profit foundation for vaccination, diagnosis, and treatment, and is part of the Cambodian Ministry of Health (MoH). The Virology Unit at IPC was opened in 1996 and serves as the reference laboratory for Influenza, Arboviruses, and Rabies for Cambodia, as well as conducting surveillance and research on endemic, emerging, and zoonotic pathogens. The unit opened the first, and only, BSL3+ laboratory in the country in 2008. The Virology Unit works in close collaboration with the Epidemiology/Public Health, Entomology, Bacteriology, and Malaria Units at IPC as well as national and international partners to conduct One Health-focused surveillance and studies in Cambodian humans, domestic animals, and wildlife. In 2021, we opened the IPC Sequencing Mini-Platform to further increase these studies through advances in next generation sequencing equipment and support.

The research question proposed in this pilot program is both timely and important, and the work has great potential for increasing capacity and capability at IPC and in Cambodia as a whole. Indeed, the research proposed has significant potential to impact our approach to pathogen surveillance at high-risk human-animal interfaces. Indeed, the project will also serve to further link the Virology Unit and the Sequencing Mini-Platform at IPC with scientists in the CREID Network, specifically the Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH), which will allow future work across Southeast Asia and positively influence your networking and future careers. There is significant overlap between this proposal, the overall mission of the Institut Pasteur du Cambodge, and the goals of EcoHealth Alliance and the EID-SEARCH team. Therefore, this pilot project presents a real opportunity for you to increase

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collaboration, and to spearhead future research projects on emerging zoonoses at high-risk interfaces in Cambodia. In addition, I am completely supportive of your vision to utilize the knowledge you gain from this proposal to train the next generation of Cambodian scientists, a critical part of our mission at IPC.

I fully support your choice of Research Center Mentor for this project, Dr. Cadhla Firth. Dr. Firth and I feel that our distinct but complementary scientific skills, expertise, and networks will maximize your professional and scientific development throughout the duration of the project. In the future, I see multiple opportunities for this new collaboration to develop into long-term scientific partnership. Indeed, I believe the data collected in this proposal regarding new ways to survey potential zoonotic pathogens circulating in Cambodia will lead to scientific independence and further lead investigator applications for you on novel research questions and hypotheses.

Therefore, I am committed to supporting your professional development in the following ways:

- I will continue to actively maintain the healthy, open, and informal mentoring relationship we have established as your mentor at IPC
- I will continue to meet with you on scheduled and *ad hoc* basis to support whatever needs you might have regarding this project, other ongoing projects, and future projects
- I will continue to provide (and ensure the institution provides) the time and resources needed to complete all research activities proposed in this pilot project
- I will continue to engage with our current and future in-country collaborators in a supportive and inclusive manner and promote your engagement with them
- I will continue to give you timely feedback on all material in the manner that works best for you
- I will continue to support and promote opportunities to interact with other scientists through your research project to widen your exposure to people with a range of expertise.
- I will continue to share my scientific and technical expertise with you to help ensure the success of the project. When skills outside my area are required, I will continue to assist (and encourage) you in accessing this expertise from scientists within my network.
- I continue to assist you in career planning, providing advice, and any other support as needed to maximize your opportunity to succeed.

Overall, I am thrilled you have chosen me as Primary Scientific Mentor on this project and I look forward to working with you on this project and throughout your careers.

Best Regards,

(b) (6)

Erik A. Karlsson, Ph.D.
Deputy Head, Virology Unit
Director, National Influenza Center of C[illegible]i
Director, WHO H5 Regional Reference Laboratory
Coordinator, WHO Global COVID-19 Referral Laboratory
Institut Pasteur du Cambodge
5 Monivong Blvd. P.O. Box 983
Phnom Penh, Cambodia 12201
Phone: +855 (0) 70 297 804
Email: ekarlsson@pasteur-kh.org

Pilot Program Application Submission Checklist – Final Application Submission

Title of Application: In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance

Submitted by: Siegers, Jurre, Ynze and Heang, Vireak

Submission Date: 1/31/2022

Full Application Submission Requirements

- ☒ Proposal Cover Sheet
- ☒ Title and Table of Contents
- ☒ Study Personnel *(1-page limit)*
- Main Application Body Section Requirements *(7-page limit):*
  - ☒ Research Aims & Objectives
  - ☒ Study Rationale/Research Gap/Impact
  - ☒ Significance and Approach
  - ☒ Research Methods
- ☒ Project Timeline (*1-page limit*)
- ☒ Research Performance Sites *(1-page limit)*
- ☒ CREID Research Center Collaboration *(1-page limit)*
- ☒ Mentoring Plan *(2-page limit)*
- Vertebrate Animals Section Requirements *(3-page limit):*
  - ☐ Description of Procedures
  - ☐ Justifications
  - ☐ Minimization of Pain and Distress
  - ☐ Method of Euthanasia (Cover Page Supplement / PHS Fellowship Supplemental Form)
- Human Subjects Research *(3-page limit):*
  - ☐ Summary of the parent study and IRB approval information for the study
  - ☐ Risks to the subjects
  - ☐ Adequacy of protection against these risks
  - ☐ Potential benefits of the research to the subjects and others
  - ☐ Importance of the knowledge gained or to be gained
  - ☐ Country / institution-specific ethics / IRB regulations addressed
- Research, Related Project Information, and Budget/Budget Justification
  - ☒ R&R Other Project Information Form
  - ☒ Full budget, with total costs of no more than $150,000
  - ☒ Budget justification which describes the labor and other direct costs
  - ☐ If your institution does not have adequate funds for a cost-reimbursement award and requires pre-payment of funds during the award year, please note this in your budget justification and outline a payment schedule that will function for your project.

Supporting Documentation

- Biographical Sketch and Other Support. All applications must include:
  - ☒ Applicant PI Biographical Sketch (*4-page limit*)
  - ☒ Applicant PI Previous/Current/Pending Support (Include funding amounts, *no page limit*)
  - ☒ Mentor Biographical Sketch (*4-page limit*)
  - ☒ Mentor Current/Pending Support (*no page limit*)
  - ☐ Key Personnel Biographical Sketches (*4-page limit each*)
  - ☐ Key Personnel Current/Pending Support (*no page limit*)

Pilot Program Application Submission Checklist – Final Application Submission
Title of Application: In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance
Submitted by: Siegers, Jurre, Ynze and Heang, Vireak
Submission Date: 1/31/2022

- Co-PI Plan (only needed if applying as Co-PIs) (*1-page limit*)
  - ☒ What each Co-PI will contribute to the proposed research study
  - ☒ How the Co-PIs will jointly work with the affiliated Research Center
  - ☒ How the Co-PIs will jointly manage the proposed study

☒ References Cited (*no page limit*)
☒ List of Abbreviations, Acronyms, and Symbols
☒ Facilities, Existing Equipment, and Other Resources (*template provided*)
☒ NIH Foreign Clearance form (*template provided*)
☒ Letters of Organizational Support *(2-page limit per letter)*
☒ Letter of Collaboration from CREID Research Center PI *(2-page limit per letter)*
☒ Letter from Research Center Mentor (*2-page limit)*
☒ Letter from Primary Scientific Mentor (if different than Research Center mentor) (*2-page limit)*

February 23, 2021

Nguyen Van Cuong, DVM, MSc
Project Coordinator & PhD candidate (viva date: 15 March 2021)
Oxford University Clinical Research Unit
Ho Chi Minh City, Viet Nam
(+84) 91 9574976
cuongnv@oucru.org

Re: Letter of Intent, CREID Network Pilot Research Program

Dear Review Committee:

This letter is to inform you that I, Nguyen Van Cuong, intend to submit an application for the CREID Network Pilot Research Program by the 12th of March, 2021. The title of my application will be: '**Multi-species farming, viral diversity, and risks of cross-species transmission in the Central Highlands of Viet Nam**'.

The project team will include myself, my long-term collaborator, Dr. Maia Rabaa (Group Head, Molecular Epidemiology, Oxford University Clinical Research Unit, Viet Nam), and my chosen Mentor, Dr. Cadhla Firth (Senior Research Scientist and Program Coordinator, EcoHealth Alliance). I will collaborate closely with the Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH), who will provide training and exchange opportunities within its network to achieve the research goals. This project will form the foundation of a new collaboration between the CREID Research Center, EID-SEARCH, and the Oxford University Clinical Research Unit in Viet Nam, which will focus on zoonotic diseases and the animal-human interface whilst building the emerging infectious disease research collaborative network in Southeast Asia.

In the proposed project, I will use samples and metadata previously collected (but not analyzed) as part of a three-year high-risk cohort study that has now come to an end. Animal samples were collected from more than 15 species present in farms and slaughterhouses across Dak Lak province in the Central Highlands of Viet Nam between 2012 and 2015. Serum samples from 300 people in contact with these animals were collected simultaneously, along with questionnaire data to assess risk. Using sequence data generated from these animal samples, we will explore how species diversity in Central Highland farms contributes to viral diversity and viral sharing between animal species (i.e., cross-species transmission). If time and resources permit, human questionnaire data and serum samples will also be analyzed to identify potential risk factors for zoonotic transmission associated with the viruses circulating on multi-species farms.

The proposed project will have three aims:

1. Estimate the frequency of viral sharing between animals of different species and identify viruses that commonly cross the species barrier under mixed-farming conditions.
2. Identify environmental and ecological factors associated with variations in viral prevalence and diversity, or rates of cross-species transmission, of zoonotic viruses on multi-species farms.
3. Identify risk factors for zoonotic infection on multi-species farms (time permitting).

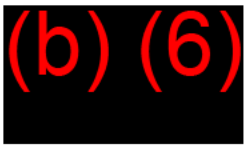
The proposed project will have three objectives:

1. Characterize the viral diversity present in samples from animals housed on multi-species farms using a combination of metagenomics and PCR-based screening.
2. Identify viruses with known or predicted zoonotic potential using ecological and phylogenetic methods. Estimate the distribution, prevalence and relatedness of these viruses across animal species and farms.
3. Correlate human behavioral and demographic characteristics from existing questionnaire data with evidence of zoonotic infection from targeted serological analyses (time permitting).

All samples for this project have been previously collected under approved Research Ethics and IRB protocols. No additional samples or engagements with individuals will be required for the successful completion of this project.

All work required for completion of the project will be performed at the Oxford University Clinical Research Unit in Ho Chi Minh City, Viet Nam. The samples were previously collected from Dak Lak province and are all currently stored and available for use in the Oxford University Clinical Research Unit laboratories in Ho Chi Minh City.

Sincerely,



Nguyen Van Cuong

Institut Pasteur du Cambodge

13th December, 2018

Object: Letter of Intent, CREID Network Pilot Research Program

Dear Review Committee:

This letter is to inform you that we, Vireak Heang and Jurre Siegers, intend to submit an application as **co-PIs** for the CREID Network Pilot Research Program by 31st of January 2022. The title of our application will be: '*In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance*'.

The project team will include ourselves (Vireak Heang, a Category 1 LMIC applicant with a master's degree and 15 years of experience; and Jurre Siegers, a Category 2 postdoctoral applicant with a PhD in Virology), and two Mentors: Dr. Erik Karlsson (Deputy Head, Virology Unit, Institut Pasteur du Cambodge) and Dr. Cadhla Firth (Senior Research Scientist, EcoHealth Alliance). We will collaborate closely with the CREID Research Center 'Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)', who will provide training and exchange opportunities within its network to achieve the research goals. This project will form the foundation of a new collaboration between EID-SEARCH, and the Institut Pasteur du Cambodge (IPC), which will focus on zoonotic disease research at high-risk animal-human interfaces.

The proposed project will take place within The Virology Unit at IPC, which serves as the reference laboratory for COVID-19, Influenza, Arboviruses, and Rabies for Cambodia, and conducts surveillance and research on endemic, emerging, and zoonotic pathogens. The Virology Unit works closely with the Epidemiology/Public Health, Entomology, Bacteriology, and Malaria Units at IPC, as well as national and international partners, to conduct One Health-focused surveillance and studies in Cambodian people, domestic animals, and wildlife. As such, IPC is continually searching for new and innovative ways to expand and improve surveillance activities. **The ability to quickly assess risk at points of high exposure or contact is paramount for early warning systems and preventative actions**. In addition, maintenance of surveillance systems in individual animals is costly and time consuming and prevents widespread coverage in all high-risk areas. **One way to address this issue is the incorporation of environmental sampling into surveillance programs to cast a wider net for pathogen surveillance at high-risk interfaces and on a longitudinal basis.**

To this end, we have begun to incorporate environmental sampling at high-risk human-animal interfaces, including live animal markets, slaughterhouses (poultry/swine), and domestic/wild animal interfaces (lakes, bat caves, farms). These samples include water (environmental and direct wash water), swabs (surfaces and other contact areas), and air samples (live animal markets, slaughterhouses, bat caves), which have been collected along with swabs from individual animals, enabling a direct comparison of pathogens identified from environmental samples with those collected from individuals. However, these samples have only been tested for specific pathogens of interest by conventional or real-time PCR, limiting their utility for pathogen surveillance. The ability to use these samples for metagenomic surveillance and pathogen discovery would not only increase surveillance capacity, but also contribute to our understanding of new or emerging pathogens at these high-risk interfaces. To respond to the COVID-19

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pandemic, the Virology Unit at IPC has recently increased its sequencing capacity to include both Oxford Nanopore (GridION and MinION) and Illumina MiSeq technologies. We now have the capability to perform metagenomic surveillance; however, these systems are still primarily used for amplicon-based sequencing. As such, their full potential as tools for pathogen surveillance has not yet been realized.

In the proposed project, we will use previously and prospectively collected environmental and animal samples from high-risk human-animal interfaces across Cambodia (e.g., live animal markets, slaughterhouses, farms, and bat caves) to assess the utility of environmental metagenomics as a tool for pathogen surveillance, while building capacity in this area at IPC.

The proposed project will have three aims:

1) To utilize existing and prospective environmental samples from longitudinal pathogen surveillance to build metagenomic sequencing and bioinformatics capacities and capabilities.
2) To assess the suitability of environmental metagenomics as an early warning system for endemic and emerging infectious diseases at high-risk interfaces in Cambodia.
3) To discover new, emerging, or zoonotic pathogens of concern in high-risk interfaces in Cambodia.

This project will use previously and prospectively collected environmental samples and PCR data from swabs taken from animals collected under approved protocols from the National Ethics Committee for Health Research (NECHR) of Cambodia: NECHR143, NECHR149, and NECHR320. No new animal sampling will occur as part of this project. No human sampling or engagement with individuals will be performed as part of this project.

The contact information of our Mentors is as follows:

Dr. Erik A. Karlsson
Deputy Head, Virology Unit
Institut Pasteur du Cambodge
5 Monivong Blvd.
PO Box #983
Phnom Penh, Cambodia
+855 (0) 70 297 804
ekarlsson@pasteur-kh.org

Dr. Cadhla Firth
Sr. Research Scientist
EcoHealth Alliance/EID-SEARCH
20 Eighth Avenue, Ste. 1200
New York, NY 10018
1(212) 380-4488
firth@ecohealthalliance.org

The contact information for the institutional signing official responsible for executing this award:

Christophe Mousset
Director of Administration and Finance
Institut Pasteur du Cambodge
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Phnom Penh, Cambodia
+855 (0) 12 802 977
cmousset@pasteur-kh.org

Sincerely,

(b) (6)

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jsiegers@pasteur-kh.org

(b) (6)

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hvireak@pasteur-kh.org

From: Hongying Li on behalf of Hongying Li <li@ecohealthalliance.org>
To: Krongkan Srimuang
Cc: Sasiprapa Ninwattana; Sterling, Spencer; Supaporn Wacharapluesadee; eric.laing_usuhs; Peter Daszak
Subject: CREID Network pilot research proposal 2023
Date: Wednesday, November 23, 2022 11:45:42 AM
Attachments: Final - CREIDApplication.NguyenVanCuong.Proposal.pdf
CREID_Pilot_Program_Application_SIEGERS_HEANG_FINAL_PDF_FOR_SUBMISSION.pdf
LOI.Cuong.EID-SEARCH.pdf
LOI final signed Vireak Heang Jurre Siegers.pdf

Dear Krongkan and Spencer,

Thanks for sharing the proposal abstract!

Peter and Kevin reviewed all proposed ideas and think this one perfectly aligns with EID-SEARCH's research objectives and will help answer some important questions.

However, can we meet to further discuss the title and writing regarding the significance of this work? The current text sounds a bit like adding additional lab procedures that have already been covered by current EID-SEARCH work, so we need to make it sound more significant...And please read through the instruction here for the proposal preparation https://creid-network.org/documents/pilot-program/2023/CREIDPilotProgram_CallforApplications_2023.pdf to see the research and objective priorities (e.g., well-defined hypothesis, and human subject research in LOI, etc.)

Please let me know when you will be available for a quick *call anytime next Monday or Tuesday.*

I'm re-attaching two successful applications, as well as some LOIs examples for your reference.

Best,
Hongying

On Sat, Nov 19, 2022 at 8:42 AM Krongkan Srimuang <krongkan5678@gmail.com> wrote:

> Dear Hongying,
>
> Please find attached the abstract for applying to the CREID Pilot Research Program 2023 from Emerging Infectious Diseases Clinical Center (EIDCC), Thailand.
> If you require any further information, please feel free to contact us.
>
> Thank you very much
>
> Best Regards,
> Krongkan Srimuang
>
> On Wed, Nov 16, 2022 at 5:08 PM Hongying Li <li@ecohealthalliance.org> wrote:
>
> > Thank you, Bow!
> >
> > Kio and Spencer,
> >
> > Attached are the two successful applications for 2021 and 2022 for your reference.

And can you please quickly write up one paragraph of your proposed idea (like the abstract) **before Monday next week**? We have received eight requests to collaborate this year, so Kevin, Peter, and I need to review all of them together on Monday to decide which three to support, and it would be great if you can send something written-down.

Best,
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

On Wed, Nov 16, 2022 at 4:01 PM Sasiprapa Ninwattana <sasiprapa.n@outlook.com> wrote:

> Dear All,
>
> Attached please find the proposal that we submitted for last year's pilot research program for CREID network for your reference.
> Please note that the letter of intent needs to be submitted by December 5, 2022.
>
> Please feel free to ask me or Hongying any questions you may have.
>
> 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

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 Gurley[4], M. Jahangir Hossein, Hume. E. Field[1], Gary Crameri[7], Lin-Fa Wang[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. Johns Hopkins School Bloomberg School of Public Health, 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, AUS
8. Programme in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore
9. Stanford University, Stanford California, USA
10. Department of Microbiology and Immunology, Uniformed Services University, Bethesda, Maryland, USA

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

Commented [NR1]: Please check that your affiliation is as it should be!

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].

Commented [EL2]: Cara Brook has a nice article about bats and virulent viruses

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].

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, a lethal neutropic zoonotic virus ~~which~~that 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

Commented [EL3]: Only 2/5 listed are lethal, only 3 have been isolated. Add in Gamak, Daeryong, Angovkeely, LayV.

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.

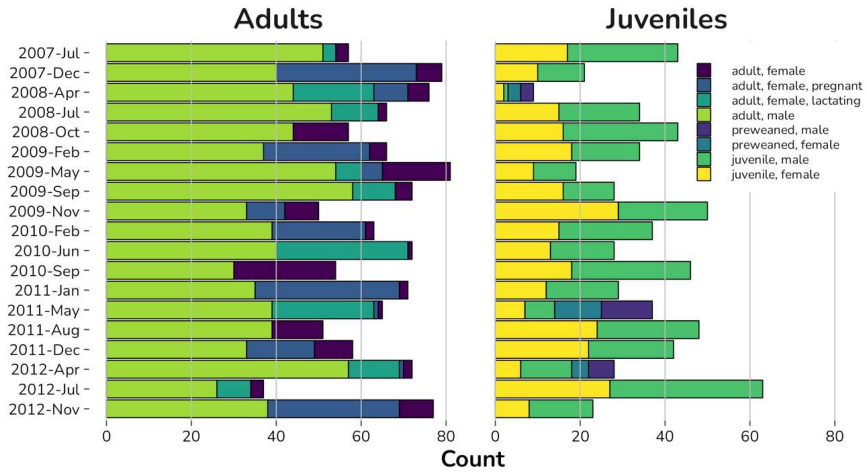

Figure 1. Demographics of bats captured in longitudinal sampling. Pregnant females were captured in months from November to June, lactating females were captured in months from April to July. Females with pre-weaning juveniles attached were found from April to May.

Patterns of Immunity and Co-immunity

We found bats were seropositive for antibodies against the Nipah virus (NiVsero+), a filovirus (EBOVsero+), 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 EBOVsero+. Among the 921 juveniles, 192 were MenVsero+, 311 were NiVsero+ and 348 were EBOVsero+. Of the 122 pre-weaned juveniles, 43 were MenVsero+, 52 were NiVsero+, and 32 were EBOVsero+.

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).

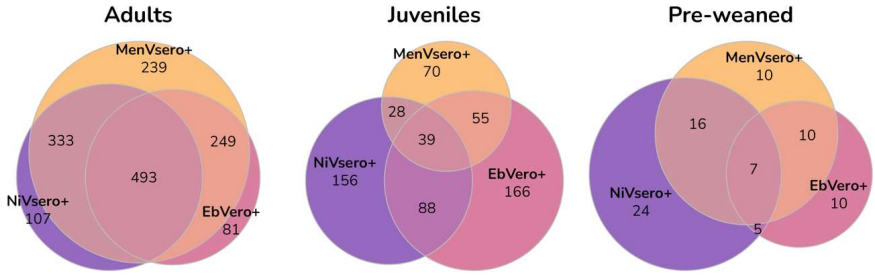

Figure 2. Detection of IgG antibodies against multiple viruses in individual *Pteropus medius*: Venn diagrams for adult, juvenile, and pre-weaned bats testing positive for antibodies against Nipah virus, filovirus, and rRubulavirus. Numbers under labels are counts of bats with only those viruses, numbers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

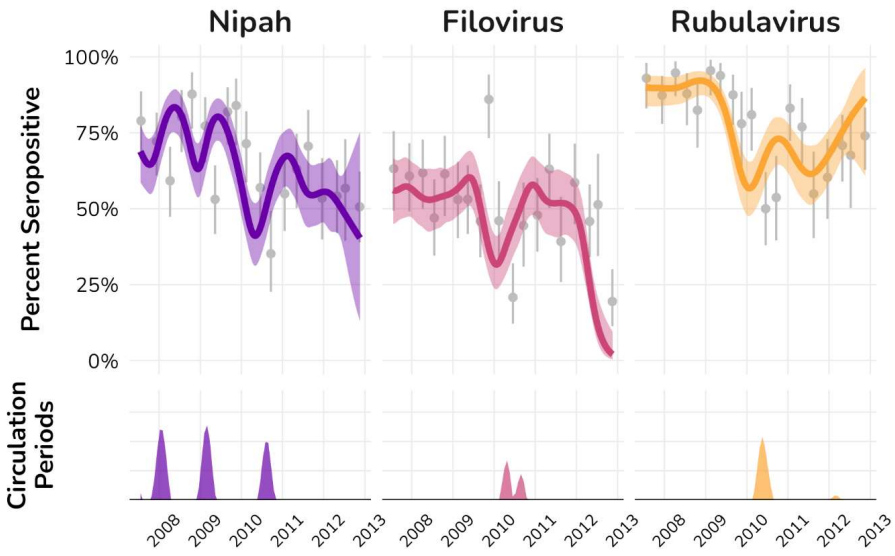

Figure 3. Inter-annual serodynamics in adult bats. Top: Measured and modeled population seroprevalence in adults over the term of the study. X-axis ticks mark the first day of the year. Grey points and bars represent measured population seroprevalence from individual sampling events on and 95% exact 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.

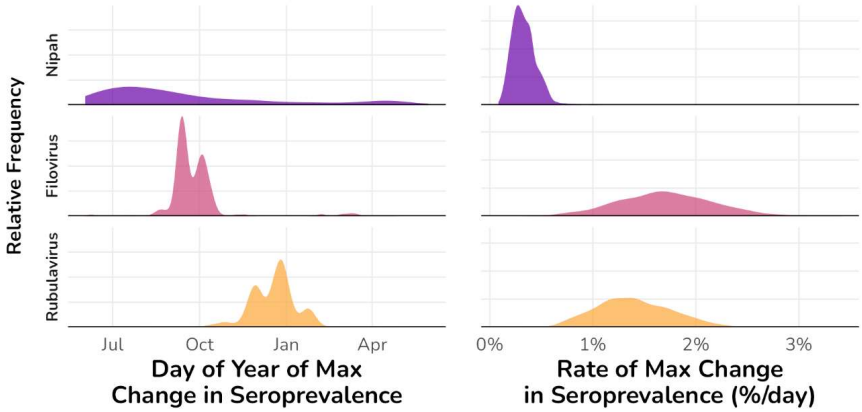

Figure 4. Timing and strength of seasonality of viral circulation in adults. Plots show posterior densities of the date for which increase in seroprevalence is greatest (left), and the rate of increase (right), for adult bats, drawn from GAMM models of seasonality. Densities are of these quantities derived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

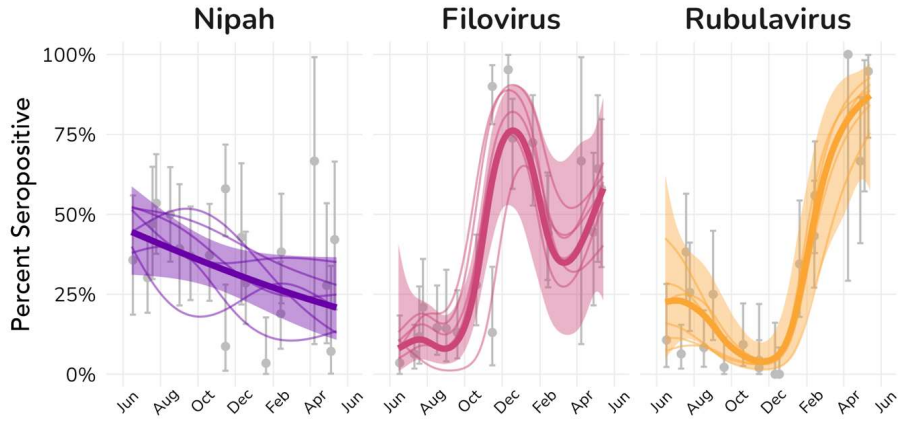

Figure 5. Serodynamics in juvenile bats in five-year study. Measured and modeled population seroprevalence in juvenile bats caught over their first year of life. Sampling events from multiple years are transposed onto a single year starting June (the earliest month free-flying young-of-the-year juveniles 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.

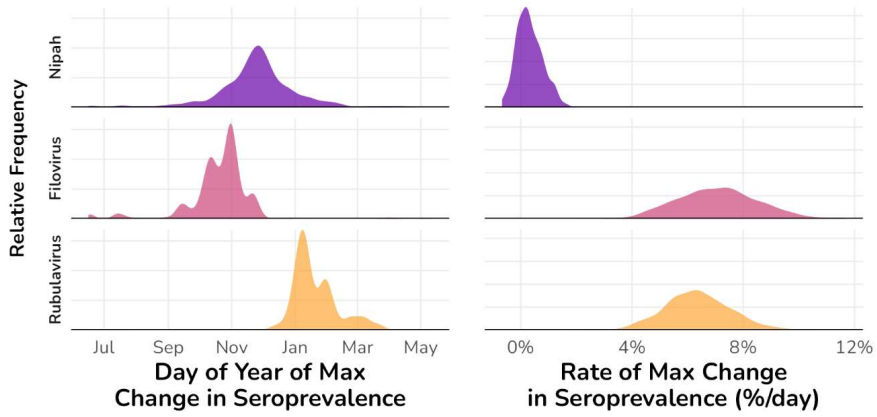

Figure 6. Timing and strength of seasonality of viral circulation in juveniles over the five-year study. Plots show posterior densities of the date for which increase in seroprevalence is greatest (left), and the rate of increase (right), for juvenile bats. Densities are of these quantities derived from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

Filovirus seroprevalence exhibited different patterns. Among adults, seroprevalence against filovirus was lower than for Nipah virus or rRubulavirus. 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). Ffilovirus 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.

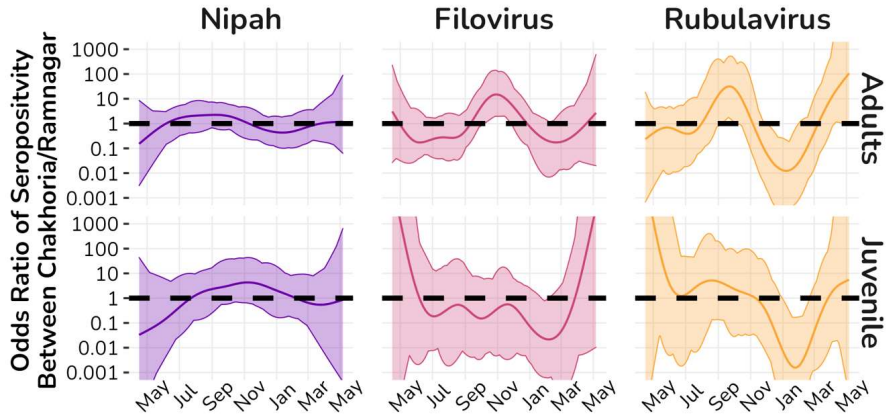

Figure 7. Comparison of splines for seroprevalence trends between locations in adults and juveniles over one-year study. Thick lines and shaded errors represent the estimated difference in trends and their associated 95% confidence intervals. The horizontal dashed line is set at zero. Where the confidence 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~~ antibody assays (see Methods). This relationship varied between viruses (Figure 7). For

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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.

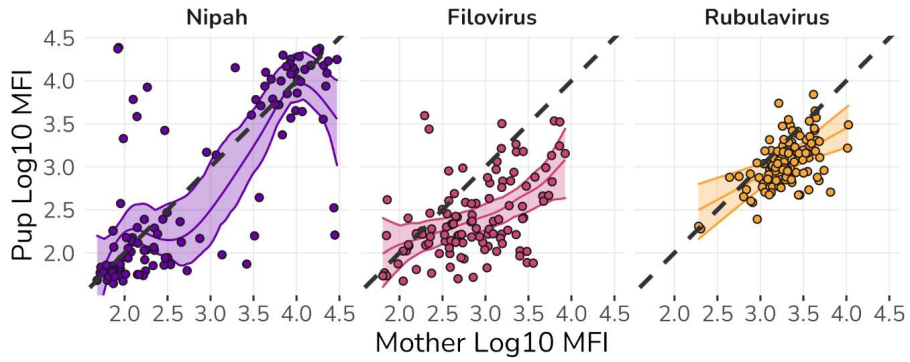

Figure 8. Maternal inheritance of antibodies. In each plot, points are measured log-MFI values for adults and juveniles in mother-pup pairs for each of the three antibody tests. Lines represent the predicted mean relationship between the two and their associated 95% confidence intervals.

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 rRubulavirus. 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 rRubulavirus, 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 an 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 Australian *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 rRubulaviruses 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 rRubulaviruses, 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]. On the other hand, ~~Human~~ Nipah virus infections of humans ~~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~~–~~, including ~~These include~~ Bombali virus in Sierra Leone[50] and Kenya [cite]~~,~~ 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], India [cite], 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

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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 antibody ~~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 IgG levels ~~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

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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~~multiplex microsphere-based immunassay that specifically detects antibodies to native-like ~~the~~ recombinant envelope ~~s~~oluble attachment glycoprotein ectodomains s[57,63,64] ~~for~~ from a panel of viruses. Glycoprotein-coupled microspheres~~.~~ ~~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 antigen-antibody complexes. Beads were interrogated by lasers in a BioRad BioPlex 200 multiplexing system ~~machine~~ and the results recorded as the m~~M~~edian f~~F~~luorescent i~~I~~ntensity (MFI) of 100 beads. We report here serology results for Nipah virus, Ebola ~~Zaire~~ virus, and Menangle virus, 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 virus test more~~is well~~ established[66], the Ebola ~~Zaire~~virus and Menangle virus are less qualified and ~~tests are~~ cross-reacti~~ons~~ve with other Asiatic ~~Ebola~~ filoviruses and rubulavirus ~~species~~es remains possible [67,68]~~.~~, t~~T~~hus we refer to these as tests for filovirus and r~~R~~ubulavirus.

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

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From: Laing, Eric on behalf of Laing, Eric <eric.laing@usuhs.edu>
To: Noam Ross
Subject: Re: NIH R01 on serology bioinformatics
Date: Wednesday, November 23, 2022 9:42:40 AM
Attachments: Ross-et-al_bangladesh-bats-cocirculation-serology_2022-08-11-EDL.docx

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Hey Noam,

I made some edits on a recent flight but completely lost track of the document. If you are still working on this my edits are attached.

- Eric

Eric D. Laing, Ph.D.
Assistant Professor
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On Wed, Nov 2, 2022 at 12:46 PM Laing, Eric <eric.laing@usuhs.edu> wrote:

> Excellent!
>
> Danny Doueck presented PREMISE to the CREID Lab Group - so cool to see a whole program around serology. Not sure whether I like "dark matter" more than "virus shadows"
>
> Eric D. Laing, Ph.D.
> Assistant Professor
> Department of Microbiology and Immunology
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>
> eric.laing@usuhs.edu
>
> On Wed, Nov 2, 2022 at 12:35 PM Noam Ross <ross@ecohealthalliance.org> wrote:
>
> > Already cited! I have some writing time reserved next week, expect an updated outline.
> >
> > Best,

Noam
--

Dr. Noam Ross
Principal Scientist, Computational Research

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

On Wed, Nov 2, 2022 at 10:12 AM Laing, Eric <eric.laing@usuhs.edu> wrote:

> Here's an interesting article
>
> https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7292646/
>
> Eric D. Laing, Ph.D.
> Assistant Professor
> Department of Microbiology and Immunology
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>
> eric.laing@usuhs.edu
>
> On Tue, Sep 6, 2022 at 8:41 AM Noam Ross <ross@ecohealthalliance.org> wrote:
>
> > Whoops! Fixed.
> >
> > On Tue, Sep 6, 2022, 8:28 AM Laing, Eric <eric.laing@usuhs.edu> wrote:
> >
> > > Hey Noam,
> > >
> > > The invite is showing up for Monday (yesterday) Sept 5.
> > >
> > > On Mon, Sep 5, 2022 at 8:55 AM Noam Ross <ross@ecohealthalliance.org> wrote:
> > >
> > > > Great, I've sent a Zoom/Calendar invite for Sep 6 at 1:30PM EST. Talk to you soon!

--

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

On Fri, Sep 2, 2022 at 3:23 PM Laing, Eric <eric.laing@usuhs.edu> wrote:

> Hi Noam,
>
> Apologies for my absence, you have my focus now.
>
> Here are my follow-up items:
>
> ***- I'd like to have a conversation with you about nuts and bolts and budgeting.*** I'm headed out for vacation Friday afternoon through Labor day. ***If you want to chat tomorrow or Friday pick a time*** at this link, if not I will write when I return: https://calendly.com/noamross/60-minute-chat-extended-hours
> Can we touch base next Tuesday Sept 6th? I'm free after 1pm EST. I'd like to go over some of the assays mentioned in the pdf you attached.
>
> - ***I'll get you a draft*** with a bulleted section on this stuff that you and Spencer can make sound like a virologist who knows what they are talking about. I expect this ***around September 12.***
> Sounds good.
>
> - I think it would be good for me to ***come down to USU for a day or two in early-mid September*** for us to work through some of this stuff if you agree and have the time. If so, let's schedule that.
> Yes, I'll be out of town from Sept 18 - 22, but in town until that date.
>
> - Eric
>
> Eric D. Laing, Ph.D.
> Assistant Professor
> Department of Microbiology and Immunology
> Uniformed Services University

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On Wed, Aug 24, 2022 at 4:58 PM Noam Ross <ross@ecohealthalliance.org> wrote:

> Hi Eric,
>
> Hi! I hope you are well. I had a call with Spencer yesterday about this grant as I know you've been away for family reasons. (CC'ing him to chat with you about this when he has the chance). We discussed things that we could write into the grant on your side, notably validation/calibration testing using the Filovirus standard coming out of Oxford, and also running tests on the sera from experimental infections that are at BSL-4 labs at RMN, UTMB, and maybe South Africa. These could be used to model how we expect real responses to look in related virus and host species.
>
> Here are my follow-up items:
>
> ***- I'd like to have a conversation with you about nuts and bolts and budgeting.*** I'm headed out for vacation Friday afternoon through Labor day. ***If you want to chat tomorrow or Friday pick a time*** at this link, if not I will write when I return: https://calendly.com/noamross/60-minute-chat-extended-hours
>
> - ***I'll get you a draft*** with a bulleted section on this stuff that you and Spencer can make sound like a virologist who knows what they are talking about. I expect this ***around September 12.***
>
> - I think it would be good for me to ***come down to USU for a day or two in early-mid September*** for us to work through some of this stuff if you agree and have the time. If so, let's schedule that.
>
> Best,
> Noam
> --
>
> Dr. Noam Ross
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EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.

On Mon, Aug 8, 2022 at 8:51 AM Noam Ross <ross@ecohealthalliance.org> wrote:

> Hi Eric,
>
> After many false starts, I really am planning to submit an R01 on bioinformatics and statistical methods for Luminex serology in the next cycle (due Oct 6). Everyone came back from the BatID conference really jazzed about this. I still think it works best with you and I as co-PIs. If you are still game, want to have a call about it? I'd still like to come down to USU for a day in the next month, too, if you'd like to spend a day working on it and other related issues.
>
> Schedule a call on my calendar here: https://calendly.com/noamross/60-min-chat
>
> Last draft of specific aims attached.
>
> Best,
>
> Noam

--
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– DETAILED SCIENTIFIC AGENDA –

FINAL AGENDA

DAY 1 | WEDNESDAY, SEPTEMBER 21

Scientific Meeting Zoom Link

Time (UMT -4)	Session	Location
13:00-14:00	**Opening, Priorities, and Vision** ▪ Welcome from DMID | Emily Erbelding (NIH/NIAID/DMID) ▪ CREID Network Priorities | Jean Patterson (NIH/NIAID/DMID) ▪ Welcome from the CREID Network | Nikos Vasilakis (CREATE-NEO), Christine Johnson (EEIDI) ▪ CREID Network Shared Vision | Njenga Kariuki (CREID-ECA), Eva Harris (A2CARES)	Fitzgerald B/C & Zoom
14:00-14:15	Break	
14:15-15:15	**Collaboration for Outbreak Research Response & Tabletop Introduction** ▪ Richard Reithinger (CREID CC) ▪ Jessica Vanhomwegen, Richard Njouom (PICREID) ▪ Scott Weaver (WAC-EID)	Fitzgerald B/C & Zoom
15:15-16:15	**CREID Network Stakeholders: Existing Collaborations** Session Chairs: Sara Woodson (DMID), Peter Daszak (EID-SEARCH) ▪ PREMISE | Peter Daszak (EID-SEARCH) ▪ WRCEVA | Scott Weaver (WAC-EID) ▪ TGHN/CSPH Virtual Biorepository | Tony Moody (CREID CC) ▪ NIBSC | Greg Sempowski (CREID CC) ▪ Abbott Pandemic Defense Coalition | Tony Moody (CREID CC), Ambroise Ahouidi (UWARN) ▪ Benefit sharing and preparing for downstream translation | Sara Woodson (DMID), Peter Daszak (EID-SEARCH)	Fitzgerald B/C & Zoom
16:15-16:30	Break	
16:30-16:45	**CREID Network Stakeholders: Potential Collaborations** Session Chairs: Tony Moody (CREID CC), Jean Patterson (DMID) ▪ CEPI, Fogarty, BV-BRC	Fitzgerald B/C & Zoom
16:45-17:00	**Day 1 Takeaways** David Wang (CREID-ESP), Sara Woodson (DMID)	Fitzgerald B/C & Zoom

Acronyms (p7)

DAY 2 | THURSDAY, SEPTEMBER 22

Scientific Meeting Zoom Link

Time (UMT -4)	Session (Virtual Presentation)	Location
11:00-12:00	**CREID Research Center Y2 Review Panels** Session Chair: Greg Sempowski (CREID CC) ▪ EID-SEARCH | Peter Daszak ▪ UWARN | Wes Van Voorhis ▪ CREID-ESP | David Wang ▪ WARN-ID | Kristian Andersen ▪ EEIDI | Christine Johnson	Fitzgerald B/C & Zoom
12:00-13:00	Lunch *Participants obtain on their own.*	
13:00-14:00	**CREID Research Center Y2 Review Panels (continued)** Session Chair: Tony Moody (CREID CC) ▪ PICREID | Anavaj Sakuntabhai ▪ CREATE-NEO | Nikos Vasilakis ▪ CREID-ECA | Njenga Kariuki ▪ WAC-EID | Scott Weaver ▪ A2CARES | Eva Harris	Fitzgerald B/C & Zoom
14:00-15:00	**Oral Presentations 1: Identifying and Characterizing Emerging Pathogens** Session Chair: Njenga Kariuki (CREID-ECA) Session Co-Chair: Betania Drummond (2021 Pilot Awardee, CREATE-NEO) ▪ Rift Valley Fever and Crimean-Congo Hemorrhagic Fever in Senegal: animal seroprevalence as indicator of virus circulation in nature | Déthié Ngom (PICREID) ▪ Evidence of co-circulation of multiple endemic arboviruses based on syndromic sentinel surveillance in Senegal | Gamou Fall (PICREID) ▪ Multi-RC Arbovirus Active Surveillance in Mosquito Enzootic Vectors and Potential Host in Panama | Nikos Vasilakis (CREATE-NEO) ▪ From malaria to fevers of unknown origins: genomic surveillance in Senegal | Aida Badiane (WARN-ID) ▪ Retrospective investigation of horses with encephalitis reveals unnoticed circulation of West Nile Virus in Northeastern Brazilian states | Luiz Alcantara (UWARN) ▪ Dengue-2 Cosmopolitan genotype detection and emergence in South America | Marta Giovanetti (UWARN)	Fitzgerald B/C & Zoom
15:10-15:30	Break	
15:30-16:40	**Oral Presentations 2: Ecology, Environs and Entomology** Session Chair: Mariana Leguía (EEIDI) Session Co-Chair: Janin Nouhin (2021 Pilot Awardee, PICREID) ▪ High burden of Arbovirus in Remote Rural Villages under environmental change in Ecuador | Paulina Andrade (A2CARES) ▪ Social constructs of place and their relevance in locating dengue fever outbreaks | James Trostle (A2CARES) ▪ Ecology of Aedes-transmitted arboviruses and their vectors in sylvatic and urban settings of Senegal: entomogical findings | Diawo Diallo (WAC-EID) ▪ Multi-RC Implications of land used and land coverage in the emergence of Madariaga Encephalitis in an endemic region of Venezuelan Equine Encephalitis Virus in Eastern Panama | Jean-Paul Carrera (CREATE-NEO) ▪ Multi-RC Ecological features of potential Madariaga and Venezuelan equine encephalitis virus enzootic hosts in Panama | Jean-Paul Carrera (CREATE-NEO) ▪ Larval microbiome by Aedes aegypti genotype interactions drive susceptibility to Zika virus | Laura Dickson (WAC-EID)	Fitzgerald B/C & Zoom
16:40-17:00	**Day 2 Takeaways** Njenga Kariuki (CREID-ECA), Sara Woodson (DMID)	Fitzgerald B/C & Zoom

Acronyms (p7)

DAY 3 | FRIDAY, SEPTEMBER 23

Scientific Meeting Zoom Link

Time (UMT -4)	Session (Virtual Presentation)	Location
11:00-12:00	**2021 Pilot Awardee Presentations** Session Chairs: (Tony Moody), Nikos Vasilakis (CREATE-NEO) ▪ Investigation of the spatiotemporal dynamics and ecological drivers of enzootic arbovirus circulation in non-human primates in Minas Gerais State/Southeast Brazil | Betania Drumond, 2021 Pilot Awardee (CREATE-NEO) ▪ Hantavirus detection and characterization in humans and rodents from Cambodia | Janin Nouhin, 2021 Pilot Awardee (PICREID) ▪ Defining antiviral humoral immunity against SARS-CoV-2 in Kenya | Bronwyn Gunn and Robert Langat, 2021 Pilot Awardees (CREID-ECA) ▪ Multi-species farming, viral diversity, and risks of cross-species transmission in the Central Highlands of Viet Nam | Cuong Van Nguyen, 2021 Pilot Awardee (EID-SEARCH) ▪ Revealing vector species with potential to mediate pathogen spillover from wildlife to livestock in the Pantanal | Daniel Aguiar, 2021 Pilot Awardee (CREATE-NEO) ▪ Development of a real-time pathogen surveillance system in Jordan | Issa Abu-Dayyeh, 2021 Pilot Awardee, WARN-ID	Fitzgerald B/C & Zoom
12:00-13:00	Lunch *Participants obtain on their own.*	
13:00-14:30	**Lightning Talk Concurrent Sessions** – *see pp4-6 for details* A: Ecology, Entomology and Field Methods B: One Health and Zoonotic Surveillance C: Clinical and Laboratory Science D: Epidemiology and Surveillance Across the Americas E: Epidemiology and Surveillance Across Africa and Asia	 Fitzgerald B/C & Zoom Fitzgerald A North & Zoom Fitzgerald A South & Zoom Warfields & Zoom Grason & Zoom
14:30-14:50	Break	
14:50-16:00	**Oral Presentations 3: Advanced Tools, Techniques and Late Breakers** Session Chair: Kathryn Hanley (CREATE-NEO) Session Co-Chair: Issa Abu-Dayyeh, 2021 Pilot Awardee (WARN-ID) ▪ An enrichment method for capturing SARS-CoV-2-related whole genome sequences directly from bat samples | Sininat Petcharat (EID-SEARCH) ▪ Multi-RC: Real-time RT-PCR for Venezuelan equine encephalitis complex, Madariaga and Eastern equine encephalitis viruses: application in clinical diagnostic and mosquito surveillance | Sandra Lopez-Verges (CREATE-NEO) ▪ Simple and economical extraction of viral RNA and storage at ambient temperature | Jesse Waggoner (A2CARES) ▪ Late breaker: Serological evidence of significant Middle East Respiratory Syndrome coronavirus transmission to humans among camel-owning households in Northern Kenya | Isaac Ngere (CREID-ECA) ▪ Late breaker: Concurrent non-human primate, bat, mosquito, and human One Health surveillance in the Peruvian Amazon 2021-2022 | Amy Morrison (EEIDI)	Fitzgerald B/C & Zoom
16:00-16:15	**Closing Remarks** Mark Challberg (DMID)	Fitzgerald B/C & Zoom

2022 Pilot Awardee ePosters

(*see Between Session slides*)

- Investigation of ecological drivers of sarbecoviruses spillover in Myanmar and Nepal | Ohnmar Aung (EEIDI)
- Vector surveillance in context of urban transmission and spread of Crimean-Congo hemorrhagic fever virus (CCHFV), Karachi Pakistan | Najia Ghanchi (UWARN)
- In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance | Jurre Siegers and
- Vireak Heang (EID-SEARCH)
- Pathogenic Mammarenaviruses and Orthohantaviruses in Argentina | María Martin and Carina Sen (WAC-EID)
- Surveillance for known and novel viruses with zoonotic potential at the interface between humans and livestock in Kenya | Stephanie Seifert and Isaac Ngere (CREID-ECA)
- Characterization of the mosquito microbiome and its role in arbovirus emergence and maintenance in Senegal | Laura Dickson and Alioune Gaye (WAC-EID)

Lightning Talk Concurrent Session Details

Day 3 | Friday, September 23 | 13:00-14:30

Concurrent Session A: Ecology, Entomology and Field Methods Location: Fitzgerald B/C & Zoom

Session Chair: Peter Dascak (EID-SEARCH)

Session Co-Chair: Tierra Smiley (EEIDI)

- Building capacity for ecological surveillance and molecular diagnostics of mosquito-borne viruses in Sierra Leone | Robert Cross on behalf of Aiah Lebbie (WAC-EID)
- Active surveillance to detect low-level Rift Valley Fever Virus transmission in humans in East and Central Africa | Jeanette Dawa (CREID-ECA)
- The use of satellite imagery to create robust geospatial sampling designs and examine landscape dynamics across an urban to rural gradient in Managua, Nicaragua | Kathryn Hacker (A2CARES)
- Higher infestation indices of the vector Aedes aegypti in rural areas than in urban areas in Managua, Nicaragua | Jose Victor Zambrana (A2CARES)
- Rainfall and the prevalence of Aedes aegypti in northern coastal Ecuador | Jessica Uruchima (A2CARES)
- Setting the standard for field biosafety at the human-animal interface | Marc Valitutto (EID-SEARCH)
- Effective Recruitment Strategies during an Infectious Disease Surveillance Study at Rural Health Facilities in Liberia; WARN-ID Liberia Team Experience | Bode Shobayo (WARN-ID)
- Environmental surveillance of informal sewage systems reveals community SARS-CoV-2 transmission dynamics | Joshua Levy (WARN-ID)
- Mosquito ecology and risk of arboviral infectious disease spillover in southwestern Uganda | Jalika Joyner (EEIDI)
- Report of arbovirosis surveillance prospective cohort as model of early detection of viral circulation | Mauricio Noguiera (CREATE-NEO)
- Towards the laboratory maintenance of Haemagogus janthinomys, the major neotropical vector of sylvatic yellow fever | Adam Hendy (CREATE-NEO)

Lightning Talk Concurrent Session Details, continued

Concurrent Session B: One Health and Zoonotic Surveillance Location: Fitzgerald A North & Zoom

Session Chair: Christine Johnson (EEIDI)

Session Co-Chair: Isaac Ngere (2022 Pilot Awardee, CREID-ECA)

- One Health Pathogen Surveillance in the Bwindi Impenetrable Forest Region of Uganda | John Kayiwa (EEIDI)
- Use of molecular epidemiological, serological and experimental approaches to study COVID-19 transmission in Hong Kong | Leo Poon (CREID-ESP)
- Evidence of Bourbon virus in ticks and humans in St. Louis Missouri, USA | Ishmael D. Aziati (CREID-ESP)
- Potential reservoirs hosts zoonotic pathogens in Senegal including emerging viruses such arboviruses, Lassa, Ebola, coronaviruses and henipaviruses | Mawlouth Diallo (WAC-EID)
- A One Health investigation framework for zoonotic and vector-borne disease outbreaks | Soledad Colombe (CREID-ECA)
- Coronavirus Circulation in Peridomestic Rodent Populations in Sierra Leone | Allison Smither (WAC-EID)
- One Health surveillance of Lassa fever in rodents and human close contacts in North Central Nigeria | Nathan Shehu on behalf of Pam Luka (WAC-EID)
- Plasmodium vivax infection in two non-human primates in the Amazon | Marcus Lacerda (CREATE-NEO)
- YF-neutralizing antibodies in pied tamarins (Saguinus bicolor) captured in Amazon Rainforest fragments in the urban area of Manaus, Brazil | Marcus Lacerda (CREATE-NEO)
- CREATE-NEO arboviral surveillance in mosquitoes, febrile humans and non-human primates in transition zones in Panama and Darien | Sandra Lopez-Verges (CREATE-NEO)
- Late Breaker: Predicting the zoonotic capacity of mammals to transmit SARS-CoV-2 | Barbara Han (CREATE-NEO)
- Late Breaker: Coronavirus surveillance among farmed collared peccaries (Dicotyles tajacu) and caretakers in the Peruvian Amazon | Carlos Calvo-Mac (EEIDI)
- Late Breaker: SARS-CoV-2 Genomic Variant Surveillance in Human and Non-Human Primates in Peru | Mariana Leguía (EEIDI)

Concurrent Session C: Clinical and Laboratory Science Location: Fitzgerald A South & Zoom

Session Chair: Rob Brieman (CREID-ECA)

Session Co-Chair: Bronwyn Gunn (2021 Pilot Awardee, CREID-ECA)

- Acute Neurologic Syndromes associated with Chikungunya virus infections in Salvador, Brazil | Lorena Martins on behalf of Mateus Santana do Rosário (UWARN)
- Predictors of severity in dengue-suspected pediatric patients during 2019 dengue epidemic in Brazil | Mauricio Noguiera (CREATE-NEO)
- Influence of previous Zika virus exposure on Brazilian dengue outbreak in 2019 | Cassia Fernanda Estofolete (CREATE-NEO)
- Immune escape mutations in the Spike protein of an endemic SARS-CoV-2 variant in Panama | Sandra Lopez-Verges (CREATE-NEO)
- The highly conserved stem-loop II motif is important for the lifecycle of astroviruses but dispensable for SARS-CoV-2 | David Wang (CREID-ESP)
- Antibody fucosylation predicts disease severity in secondary dengue infection | Tineke Cantaert (PICREID)
- Unexpected Acute Viral Fever Mimicking Dengue-Like Illness in Major City of Pakistan | Najeeha Talat Iqbal (UWARN)
- Comparison of the Immunogenicity of five COVID-19 vaccines in Sri Lanka | Chandima Jeewandara (A2CARES)
- SARS-CoV-2 Variant Detection and Surveillance with an Economical and Scalable Molecular Protocol | Jesse Waggoner (A2CARES)
- Multiplexed detection of respiratory viruses and SARS-CoV-2 variants with mCARMEN | Nicole Welch (WARN-ID)
- Developing Rapid Antigen Diagnostics for Emerging Viruses using Antibodies Cloned from Sorted Single Memory B-cells | Lee Gehrke (CREATE-NEO)

- A simplified Cas13-based assay for the identification of SARS-CoV-2 and its variants | Jon Artizti Sanz (WARN-ID)
- Late Breaker: Dynamics of infectious virus neutralization from convalescent and vaccine cohorts across global SARS-CoV-2 variant lineages reveals boost protection and novel monoclonal antibody efficacy against Omicron strains | Michael Gale (UWARN)

Concurrent Session D: Epidemiology and Surveillance Across the Americas Location: Warfields & Zoom

Session Chair: Wes Van Voorhis (UWARN)

Session Co-Chair: Daniel Aguiar, 2021 Pilot Awardee (CREATE-NEO)

- A Dengue outbreak in Panama, 2022 | Luis Felipe Rivera (CREATE-NEO)
- Seroprevalence of Zika, Dengue, and Chikungunya viruses in a rural area in Brazil | Marcos Vinicius Lima de Oliveira Francisco (UWARN)
- Zika virus infection surveillance in Manaus, Amazonas state | Marcus Lacerda (CREATE-NEO)
- Real-time genomic surveillance of DENV-1 and DENV-2 in Brazil: improving public health outbreaks response | Luiz Alcantara (UWARN)
- Surveillance and evolutionary analysis of Dengue viruses to understand the epidemiological dynamics of dengue outbreaks, São José do Rio Preto, São Paulo, Brazil | Lívia Sacchetto (CREATE-NEO)
- Monitoring the genetic diversity of reemerging chikungunya virus in Brazil | Luiz Alcantara (CREATE-NEO)
- Genomic and epidemiological monitoring of YFV reemergence in Brazil: unveiling the corridor of spread and the geographic hot spots for predicting and preventing other possible spillover events | Marta Giovanetti (UWARN)
- Genomic surveillance of SARS-CoV-2 in symptomatic vaccinated and unvaccinated asymptomatic patients in Brazil | Luiz Alcantara (UWARN)
- SARS-CoV-2 genomic surveillance and the impact of different lineages circulation in the epidemiological landscape of São José do Rio Preto, São Paulo, Brazil | Cecília Artico Banho (CREATE-NEO)
- Genomic epidemiology reveals the impact of national and international restrictions on the SARS-CoV-2 epidemic in Brazil | Marta Giovanetti (UWARN)
- Occurrence of SARS-CoV-2 reinfections at regular intervals in Ecuador | Paul Cardenas (A2CARES)
- Genomic characterization of SARS-CoV-2 during the COVID-19 pandemic in Nicaragua | Cristhiam Cerpas (A2CARES)

Concurrent Session E: Epidemiology and Surveillance Across Africa and Asia Location: Grason & Zoom

Session Chair: Anavaj Sakuntabhai (PICREID)

Session Co-Chair: Robert Langat, 2021 Pilot Awardee (CREID-ECA)

- Flaviviruses and Lassa fever in febrile patients in North Central Nigeria: A cross-sectional study | Nathan Shehu (WAC-EID)
- Double Stigma and discrimination: A qualitative study of Lassa fever and hearing loss in Northern Nigeria | Kachollom Best (WAC-EID)
- A brief chronicle of SARS-CoV-2 genomic surveillance in Cambodia | PICREID Representative on behalf of Erik Karlsson (PICREID)
- Identification of Genetic Variations of SARS-CoV-2 Omicron Strain and their Clinical Significance in Karachi, Pakistan | Aqsa Khalid (UWARN)
- Genetic characterization of Norovirus strains from an unusual diarrheal outbreak in the community in Thailand | Krongkan Srimuang (EID-SEARCH)
- Factors associated with changing Dengue case numbers during the COVID-19 pandemic in Sri Lanka | Dinuka Ariyarantne (A2CARES)
- SARS-CoV-2 genomic epidemiology in Sierra Leone | John Demby Sandi (WARN-ID)
- Yellow Fever outbreak in eastern Senegal, 2020–2021 | Moussa Diagne (WAC-EID)
- The evolving SARS-CoV-2 epidemic in Africa: Insights from rapidly expanding genomic surveillance | Houriiyah Tegally (UWARN)

- Detection and Characterization of Variants of Concern: Insights from the South African Epidemic | James Emmanuel San (UWARN)
- Emergence of novel combinations of SARS-CoV-2 spike receptor binding domain variants in Senegal | Amroise Ahouidi (UWARN)
- COVID-19 laboratory surveillance at IRESSEF in Senegal | Cheikh Ibrahim Lo (UWARN)
- Dynamics of Variants of Concern (VOC) during the different waves of COVID-19 in Senegal | Abdou Padane (UWARN)

Acronyms and Abbreviations

A2CARES	American and Asian Centers for Arboviral Research and Enhanced Surveillance
BV-BRC	Bacterial and Viral Bioinformatics Resource Center
CEPI	Coalition for Epidemic Preparedness Innovations
CREATE-NEO	Coordinating Research on Emerging Arboviral Threats Encompassing the Neotropics
CREID	Centers for Research in Emerging Infectious Diseases
CREID CC	CREID Coordinating Center
CREID-ECA	Center for Research in Emerging Infectious Diseases – East and Central Africa
CREID-ESP	Center for Research in Emerging Infectious Diseases – Epidemiology, Surveillance and Pathogenesis
CSPH	Colorado School of Public Health
DMID	Division of Microbiology and Infectious Diseases
EEIDI	EpiCenter for Emerging Infectious Disease Intelligence
EID-SEARCH	Emerging Infectious Diseases: South East Asia Research Collaboration Hub
NIAID	National Institute of Allergy and Infectious Diseases
NIBSC	National Institute for Biological Standards and Controls
NIH	National Institutes of Health
PICREID	Pasteur International Center for Research on Emerging Infectious Diseases
PREMISE	Pandemic Response Repository through Microbial and Immunological Surveillance and Epidemiology
RC	Research Center
TGHN	The Global Health Network
UWARN	United World Antiviral Research Network
WAC-EID	West African Center for Emerging Infectious Diseases
WARN-ID	West African Research Network for Infectious Diseases
WRCEVA	World Reference Center for Emerging Viruses and Arboviruses

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Subject:	RE: CREID Scientific Meeting Day 1
Date:	Wednesday, September 21, 2022 1:26:52 PM
Attachments:	CREIDAgenda_Detailed_Scientific_v2.pdf

Hi all,

Please find an updated agenda with scientific meeting zoom link attached.

Thank you,

Aaron

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-----Original Appointment-----
From: Macoubray, Aaron
Sent: Friday, September 16, 2022 3:58 PM
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Cc: Pickett, Thames (NIH/NIAID) [E]; Sempowski, Greg; ssebide@gmail.com; Tineke CANTAERT; Vireak HEANG; alhajiboumaloljalloh@gmail.com; sini.petcharat@gmail.com; Bohlja@nih.gov; alvillas@utmb.edu; Golovko, Georgiy; lorenacmartins@hotmail.com; Oscar Ortega; nicole.lukovsky@nih.gov; Smither, Allison R.
Subject: CREID Scientific Meeting Day 1
When: Wednesday, September 21, 2022 1:00 PM-5:00 PM (UTC-05:00) Eastern Time (US & Canada).
Where:

Hello CREID Scientific Meeting Attendees,

Please plan to join the **Scientific Meeting on Day 1** of the Annual Meeting. Final agenda and materials will be shared on Monday, September 19.

In-person attendees, please go to **Fitzgerald B/C.**
Virtual attendees, please use the following zoom link for this meeting:
https://explorepsa.zoomgov.com/j/1619281154?pwd=T0JlL1FHK3p2cU9nQ3h2TzFLaHdhQT09

Thank you,

Aaron
CREID Coordinating Center Project Coordinator

Application Cover Sheet

Project Title	Mixed-species farming, viral diversity, and risks of cross-species transmission in the Central Highlands of Viet Nam
Principal Investigator Name	Nguyen Van Cuong
Position/Title	Project Coordinator and PhD candidate (viva data: 03/15/21)
Department	Centre for Tropical Medicine
Institution Name	Oxford University Clinical Research Unit
Street	764 Vo Van Kiet, Ward 1, District 5
City, State, Zip Code	Ho Chi Minh City, TP Ho Chi Minh, 700000
Country	Viet Nam
Email	cuongnv@oucru.org
Phone	+84 8 39237954
Country(ies) where work will be conducted	Viet Nam
Pathogen(s) focus	Zoonoses, Coronaviruses, Paramyxoviruses

Collaborating CREID Research Center	The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH)
Contact PI	Peter Daszak
CREID RC Primary Point of Contact	Hongying Li
CREID RC Primary Point of Contact Location or Institution	EcoHealth Alliance (EHA)

Name of Mentor	Cadhla Firth
Mentor Institution	EcoHealth Alliance
Institution Address	520 Eighth Avenue, Ste. 1200 New York, NY 10018
Permanent location of Mentor	New York, NY
Mentor email	firth@ecohealthalliance.org
Mentor phone	+61 447 361 669

Total Budget	**$149,970.58**
Direct Costs	$138,861.64
Indirect Costs	$11,041.17
Proposed Start Date	06/01/2021
Proposed End Date	05/31/2021

Project Abstract

Viet Nam sits within a critical Southeast Asian emerging infectious disease hotspot with diverse wildlife and a rapidly increasing human population. In this region, wildlife farming is a growing but inadequately regulated industry that exhibits many features thought to contribute to increased risks of cross-species transmission and zoonotic disease emergence. We will focus on a key zoonotic disease pathway that was involved in the emergence of Nipah virus, SARS-CoV, and potentially SARS-CoV-2 – the spillover of viruses from wildlife to multi-species farms, and subsequently to people. We will test a key hypothesis: viral diversity and cross-species transmission will increase with species

diversity on mixed-species farms, and this pattern will be further amplified by the presence of wildlife species. We will access a previously untouched sample set from 64 mixed-species farms in a biodiverse region of central Viet Nam where wildlife and domestic species are farmed both together and separately. We will characterize viral diversity in single- and mixed-species farms using metagenomics and PCR, identify their zoonotic potential using ecological and phylogenetic methods, and estimate risk of emergence by analyzing distribution and prevalence. To inform the local community of our results, we will develop a two-way dialogue with farmers and government stakeholders in Viet Nam regarding potential disease risks associated with wildlife and mixed-species farming. Importantly, this project builds capacity for emerging infectious disease research in Southeast Asia by supporting the in-country development of analytically robust, hypothesis-driven research into the ecology and evolution of emerging infectious diseases.

Applicant Name (Last, first, middle): Nguyen, Cuong, Van

Title

Mixed-species farming, viral diversity, and risks of cross-species transmission in the Central Highlands of Viet Nam.

Study Personnel

The PI on this project, Dr. Cuong Nguyen, is ideally suited to conduct and manage this project. Dr. Nguyen is trained as a veterinarian and will be defending his PhD thesis (Open University, United Kingdom (UK)) focused on veterinary epidemiology and antimicrobial usage on farms in Viet Nam on 15 March 2021. Dr. Nguyen possesses experience in performing both laboratory- and field-based research, as well as translating research outcomes into locally-relevant interventions and policy recommendations. These efforts have resulted in 20 peer-reviewed publications since 2018. Dr. Nguyen has worked with the Oxford University Clinical Research Unit (OUCRU) since 2011, during which time he acted as a coordinator of veterinary fieldwork for the Wellcome Trust-funded Vietnamese Initiative for Zoonotic Infections (VIZIONS) project, which ended in 2016, and under which the samples for the proposed project were collected. Dr. Nguyen will be responsible for project coordination, data analysis, public engagement, and presentation/publication of the research conducted under the proposed project. He will be allocated 100% protected time to work on this project and his professional development over the one-year period. To ensure that the proposed research can be accomplished on time and within budget, all laboratory work will be completed by a Senior Research Assistant who will be supervised directly by Dr. Nguyen within the Molecular Epidemiology group at OUCRU. Several members of this research group were part of the VIZIONS team and are familiar with the sampling and laboratory protocols required to ensure the completion of this study in a timely manner.

As co-lead of OUCRU's Molecular Epidemiology research group, Dr. Maia Rabaa will be a collaborator on this project and will supervise Dr. Nguyen's research throughout the proposed project. Dr. Rabaa is a molecular epidemiologist with eight years of experience working at OUCRU, and contributed to the design, implementation, and analysis of data related to the VIZIONS project. Dr. Rabaa and her team have access to the relevant samples, were involved in the design and conduct of the VIZIONS project, have significant expertise in sequencing and phylogenetic analysis, and will provide additional support and advice during Dr. Nguyen's laboratory work, analysis, and writing. As the group lead, Dr. Rabaa will also supervise Dr. Nguyen's progress and administrative tasks and assist in the further development of his budget and human resources management skills as he progresses toward scientific independence.

Dr. Cadhla Firth will act as Mentor for Dr. Nguyen under the Centers for Research in Emerging Infectious Diseases (CREID) Pilot Research Program. Dr. Firth is Senior Research Scientist and Program Coordinator at EcoHealth Alliance (EHA), which houses the Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH) program. Dr Firth has more than 15 years' experience conducting research on emerging infectious diseases, with a focus on the ecology and evolution of viruses in animal populations. Dr. Firth's expertise in study design, molecular biology, metagenomic sequencing, and the analysis of metagenomic and ecological data match well with Dr. Nguyen's interests and the proposed project. Dr. Firth will provide scientific guidance, analytical training, mentoring, and career advice, as well as networking opportunities that support Dr. Nguyen's progression toward independence.

As colleagues on the VIZIONS project, Dr. Nguyen and Dr. Rabaa have built a strong collaborative foundation that complements the peer mentorship that Dr. Rabaa and Dr. Firth established in 2008 during their respective PhD studies under Prof Edward Holmes, and which continues to this day. With Dr. Firth's recent move to EHA, Dr. Rabaa's promotion to Group Head and subsequent collaboration with EHA, and the completion of Dr. Nguyen's PhD work, this team is now in an advantageous position to deliver a strong research project with tangible outcomes under the CREID Pilot Research Program.

Research Aims and Objectives

This project aims to answer the question: "How does species diversity influence viral diversity and the risk of cross-species transmission on mixed-species farms?".

We hypothesize that: (i) viral diversity and cross-species transmission will increase with species diversity on mixed-species farms, (ii) the presence of both wildlife and livestock species on a farm will be associated with increased viral diversity, and (iii) the diversity of viruses with zoonotic potential will increase with species diversity on mixed-species farms. To test these hypotheses, we will access a unique sample set from farms in the Central Highlands of Viet Nam, where a variety of wildlife (e.g., deer, bamboo rats, civets, wild boars, rabbits, porcupines) and domestic species (e.g., chickens, ducks, cats, pigs, goats) are farmed both together and separately. Mixed-species farms create new interfaces between wildlife, domestic animals, and people that may provide opportunities for cross-species transmission of viruses to occur (**Figure 1**).

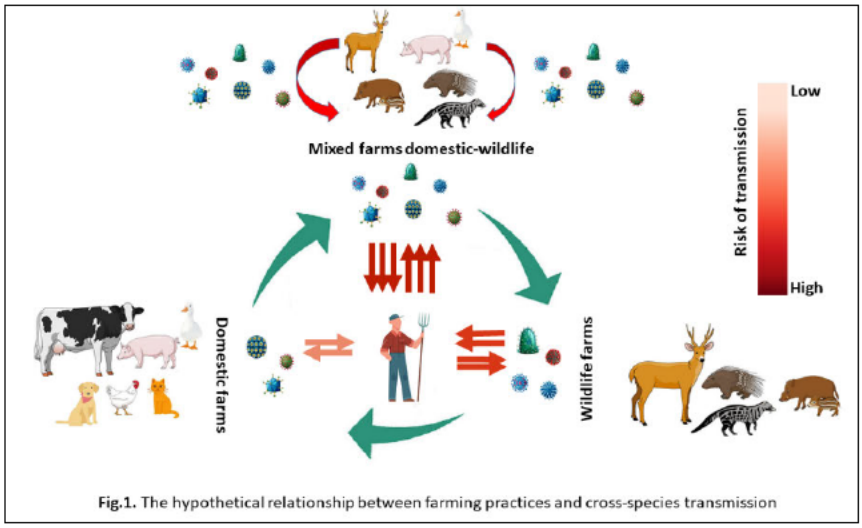


Fig.1. The hypothetical relationship between farming practices and cross-species transmission

The proposed project has three aims:

AIM 1: Estimate the frequency of viral sharing between animals of different species under mixed- and single-species farming conditions and identify 'risky' viruses that commonly cross the species barrier under each of these conditions.

AIM 2: Identify environmental and ecological factors associated with variations in viral prevalence, diversity, or rates of cross-species transmission of 'risky' viruses.

AIM 3: Identify farm management policies and practices to increase awareness and reduce the risk of cross-species transmission of viruses associated with mixed-species farms.

The proposed project has three objectives:

OBJ 1: Characterize the viral diversity present in samples from animals housed on single and mixed-species farms using a combination of metagenomics and polymerase chain reaction (PCR)-based screening.

OBJ 2: Identify viruses with known or predicted zoonotic potential using ecological and phylogenetic methods. Estimate the distribution, prevalence, and relatedness of these viruses across animal species and farms.

OBJ 3: In collaboration with the policy and public engagement departments at OUCRU, engage in two-way dialogues with government stakeholders and the farming community in the Central Highlands about potential disease risks associated with wildlife and mixed-species farming.

Study Rationale/Research Gap/Impact

On July 23, 2020, the Prime Minister of Viet Nam issued a new directive (Directive 29/CT-TTg) [1] calling for increased enforcement of legislation regarding the control of wildlife hunting and trade, which has been widely interpreted as a move toward a complete ban on wildlife trading [2]. This follows similar legislation passed in China in February 2020, which introduced new regulations banning almost all consumption and trade of terrestrial wild animals in the country, and which is expected to cause knock-on policy effects across Asia [3]. Commercial wildlife farming has been proposed as a potential solution to problems caused by a ban on wildlife hunting and trade by creating a regulated and monitored source for wildlife products, while supporting conservation efforts and addressing cultural, economic, and food security issues that may otherwise drive these practices into unregulated marketplaces [4–6]. However, wildlife have also been associated with the emergence of many zoonotic diseases, and several features of wildlife or mixed-species farms suggest that they may pose a greater risk to people than domestic farms alone [7–10].

More than 175 wildlife species are farmed in Viet Nam, including those previously associated with zoonotic transmission risks (e.g., civets, primates, rodents) [11]. However, a recent survey of more than 4000 wildlife farms in southern Viet Nam revealed that 70% of farms also raised domestic species (including livestock) [11]. Critically, pathogens with broad host ranges are significantly more likely to emerge in human populations, which suggests that the presence of multiple species (both wild and domestic) on the same farms may be cause for concern due to the potential for cross-species transmission and viral amplification [12–15]. The risks posed by wildlife farming are further complicated by the high frequency of wildlife laundering, which was the dominant form of wildlife production on 26 Vietnamese farms surveyed between 2014 and 2015 [4]. The frequent movement of wildlife from the natural environment into a domestic setting creates a conduit for the emergence of novel pathogens, including those with zoonotic potential. In addition, the majority of farmed wildlife are raised for food, yet there is a lack of veterinary oversight and infection control measures on Vietnamese wildlife farms that is not mirrored in the farming of domestic species in the region. In a census of more than 4000 wildlife farms in southern Viet Nam, an absence of veterinary care was noted in nearly all cases, along with high numbers of sick and dead animals from unknown causes [11].

Despite the clear risks associated with wildlife farming in Viet Nam, there is very limited understanding of the impacts of wildlife and mixed-species farming practices on viral diversity, virus sharing, and zoonotic transmission [15]. However, the renewed interest in wildlife hunting, farming, and trading that has resulted from the hunt for the origin of SARS-CoV-2 has led to a resurgence in effort to understand the impacts of the policies and practices around wildlife farm management. Although a stated aim of this work is to reduce human and animal disease risk, and zoonotic transmission in particular, there remains an extreme paucity of data related to the risks posed by wildlife farming, or specific farming practices that contribute to this risk. For the first time in the proposed project, we will explicitly test the relationships between species diversity and composition, viral diversity, and cross-species transmission on wildlife and mixed-species farms.

Thus, in line with the goals of the CREID network and the EID-SEARCH team, we aim to use previously collected samples from Vietnamese farms to systematically characterize the diversity and transmission of viruses within animal populations on mixed-species farms. We will assess the risk these viruses may pose to human and animal populations, and use these data to assist in the development of farm management practices and policies to mitigate such risks.

Significance and approach

Wildlife farming, hunting, and trade have repeatedly been associated with the emergence of new zoonotic diseases, including SARS-CoV, SARS-CoV-2, monkeypox virus, and Ebola and Marburg filoviruses [16]. However, strikingly little is known about the drivers of disease emergence from wildlife, particularly with respect to the food chain, including wildlife farms. Previous studies on virus diversity and viral sharing along wildlife supply chains have relied exclusively on consensus PCR-based virus detection or have focused on only a single animal species, even on mixed-species farms [15,17,18]. In contrast, this project will use cutting-edge genomic approaches in ecology and evolution within a hypothesis-driven framework to reveal the determinants of viral diversity, abundance, and transmission within mixed-species farms. To our knowledge, this will be the first data-driven project with the goal of providing a foundation for understanding the roles of mixed-species farming, wildlife farming, and species-species interactions as drivers of disease emergence.

Data collected as part of the VIZIONS project represents a unique opportunity to build a holistic picture of the links between wildlife farming practices, viral diversity, and cross-species transmission. VIZIONS, which ended in 2016, involved international research institutions collaborating with Vietnamese governmental organizations and hospitals to collect samples at three sites across Viet Nam to study clinical illness, epidemiology, pathogen diversity, and social and behavioral factors within a One Health framework. The VIZIONS project had two fundamental components: 1) a hospital disease surveillance program to characterize endemic infections, novel infections, and diseases of unknown origin in humans, and 2) a high-risk human sentinel cohort (HRSC) to assess zoonotic disease incidence and cross-species transmission through sampling of both HRSC members and the animals with which they had contact [19]. Most HRSC cohort members (72.8%) were individuals living on farms, with 53.7% of members in Dak Lak Province reporting exposure to farmed wildlife, including deer, bamboo rats, civets, wild boars, rabbits, and porcupines [20]. We have access to untouched animal samples from this unique dataset from Dak Lak Province, which are linked to publicly available metagenomic data [21,22] from co-located samples from farm workers, and to questionnaire data designed to assess zoonotic disease risk. This provides an unprecedented opportunity to examine multiple dimensions of risk in a complex and highly topical system.

This study also provides an opportunity to link zoonotic disease research back to the communities most at risk of novel disease emergence, including the farmers and government health officials who make daily decisions that directly influence disease risk. Community and government engagement activities were key components of the VIZIONS project and these relationships have been maintained by the OUCRU public engagement team. This will enable a dialogue between the project team and a network of key stakeholders (i.e., farmers, provincial and regional animal health officers, public health officials, and representatives of the Departments of Agriculture and Forestry), through which we can identify potential risks associated with mixed-species farming and wildlife trade in these communities. The result of this engagement activity will be an understanding of how policy is implemented at the local level, and the identification of avenues for future collaborative research and engagement that will benefit the community and expand the CREID and EID-SEARCH networks to improve our understanding and response to the risk of zoonotic virus emergence in Viet Nam and across the region.

Research Methods

Site and sample selection.

Our metagenomic analysis will use animal samples previously collected as part of a three-year high-risk cohort study conducted across Dak Lak Province in Viet Nam's Central Highlands (**Figure 2**). From March 2012 to September 2015, human and animal samples were collected from 64 farms across Dak Lak at multiple time points (median of five visits per farm), including during reported episodes of human illness. Human samples included nasopharyngeal swabs, rectal swabs, and sera, and were associated with questionnaire data to assess human-animal contact and potential risk factors for zoonotic transmission. Human samples collected during illness have been previously analyzed by metagenomic analysis [21,22], and the data are available for comparison with the results of this study. Animal samples primarily included feces and/or rectal swabs (N = 3675), nasal and/or throat swabs (N = 2499), and sera (N = 1245), which were collected from 2251 live animals, comprised of 19 species (12 domestic species, seven wildlife species). These uninterrogated animal samples have been stored at OUCRU in -80°C freezers since the time of collection and are available for metagenomic sequencing in this study.

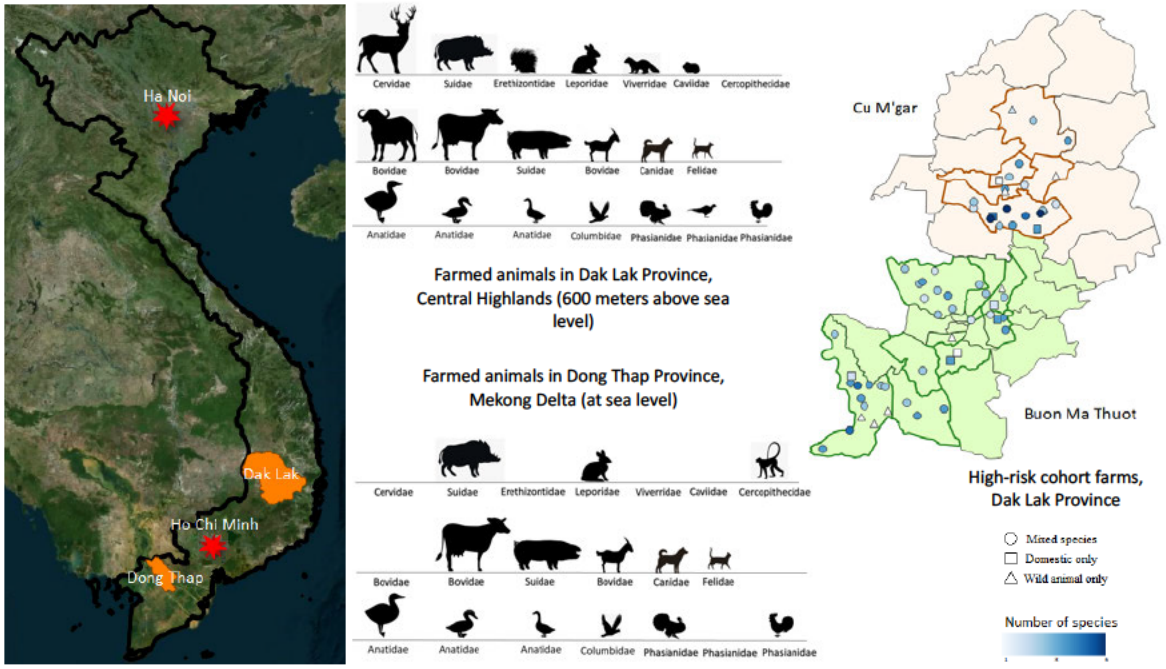


Figure 2. Summary of farmed animals sampled within the VIZIONS project (Dak Lak & Dong Thap Provinces), and distribution of domestic, wildlife, and mixed farms in Dak Lak Province, Viet Nam.

Of the 64 farms sampled as part of the cohort study, 67% contained both domestic and wildlife species, 27% contained domestic species only, and 6% contained wildlife only. The number of species per farm ranged from one to nine, with a median value of five species/farm. To explore the relationships between mixed-species farming, viral diversity, and cross-species transmission, we will select a subset of sampled farms for metagenomics that best capture a gradient of farming intensity in this region. These will include farms with low, medium and high species diversity, as well as farms with and without wildlife (**Figure 3**). Farms will also be selected to achieve maximum overlap in species composition, whereby the species sampled on low diversity farms are also represented on high diversity farms. As such, we will initially target the most frequently sampled domestic (e.g., chickens, ducks, pigs, cats) and wildlife species (e.g., porcupines, rabbits, wild boar, deer) across farms.

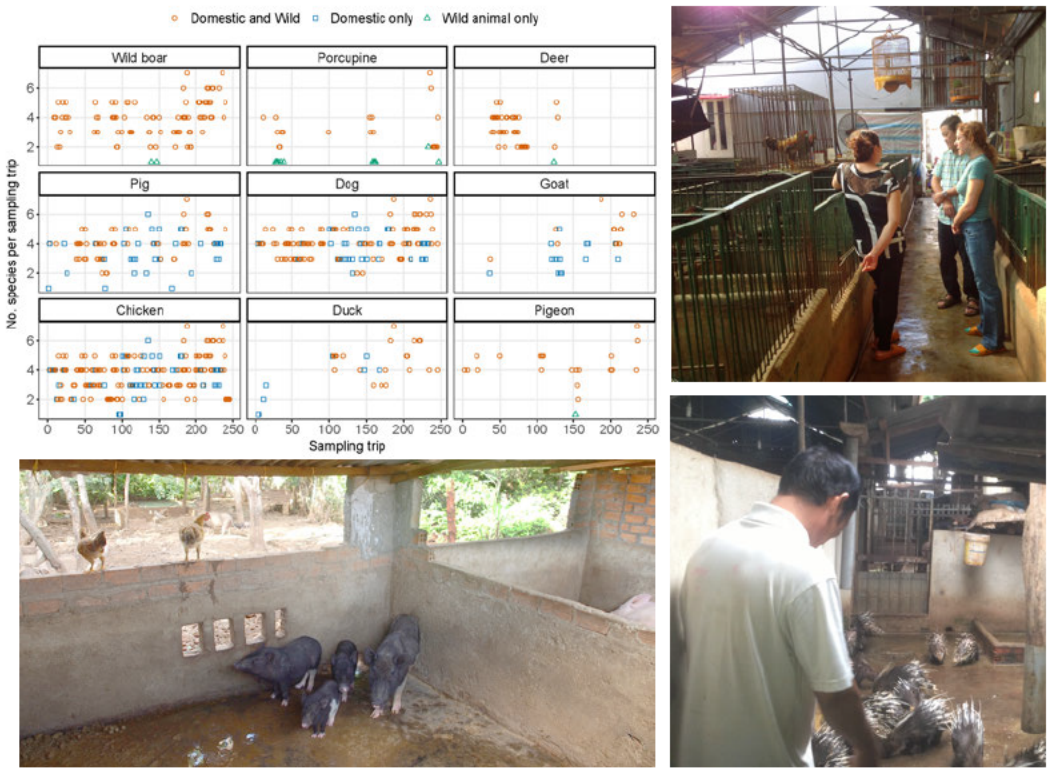


Figure 3. The number of animal species sampled per sampling trip, according to species (select data shown). For example, in the top left corner, wild boar were the only species present on a farm at two sampling events, while one or more other species was sampled on all other sampling trips involving wild boar. Different shapes and colors differentiate farm types (domestic, wild, and mixed wild/domestic). Additional images illustrate farming practices and species diversity on Dak Lak farms.

RNA isolation and metagenomic sequencing.

It has been estimated that only a very small fraction of viral diversity on earth has been described, such that our ability to contextualize disease emergence and cross-species transmission is strongly skewed towards viral families that have already been well-characterized [23]. This bias is further amplified by the widespread use of 'consensus PCR' assays as a mechanism of virus discovery, as this approach attempts to detect new viruses by using sequences from conserved regions of the genome (i.e., primers) of known viruses. As such, this method is likely to be unable to detect viruses that are significantly divergent from those which are already known. In contrast, many recent studies have demonstrated that a metagenomic approach has the ability to reveal the entire virus composition of a sample (i.e., the virome), as well as the relative abundances of each virus [23,24]. We will use this approach to provide a comprehensive view of all viruses present in our dataset.

To comprehensively characterize viromes at both the farm and species level, high quality total ribonucleic acid (RNA) will be extracted from 1000 fecal samples and/or rectal swabs, and 1000 nasal and/or throat swabs (henceforth respiratory swabs) from animals sampled from the subset of farms described above. Fecal samples were selected due to their association with high viral diversity (e.g., coronaviruses, picornaviruses) and the potential importance of fecal-oral transmission in viral emergence and spread [25–27]. Respiratory swabs were selected due to the potential to detect respiratory viruses, which have been associated with multiple significant emerging viral diseases (e.g., SARS, COVID-19, influenza) in the past [28,29]. Extracted RNA will be quantified and samples will be pooled by sample type, species, and farm to achieve a total of 120 pools with a maximum of 10

samples/pool. When animal numbers on a farm are low, pooling will be by taxonomic order (e.g., Rodentia, Carnivora, Artiodactyla), rather than by species. Sequencing libraries will be generated from each pool using the Stranded Total RNA Prep with Ribo-Zero Plus kit (Illumina), which includes a ribosomal RNA depletion step to reduce the amount of animal and bacterial RNA sequenced. Libraries will be assessed for quality on the Agilent 2100 Bioanalyzer and sequenced using the Illumina HiSeq-2500 platform at Macrogen (South Korea).

Sequence analysis.
Raw sequencing reads from each pool will be filtered for quality and assembled *de novo* using Trinity RNA-Seq [30]. Assembled sequences will be identified and annotated by comparing them to the complete nonredundant nucleotide and protein databases available through GenBank using E-value cutoffs of 1E-10 and 1E-4, respectively, and the program Diamond [31]. Sequences representing viral genomes will be further characterized using phylogenetic analysis targeting conserved domains (e.g., the RNA polymerase) that will include related viruses from GenBank to estimate evolutionary relationships. Phylogenies will be inferred using the maximum likelihood method implemented in IQ-Tree, employing the best-fit amino acid substitution models for each virus family [32]. The resultant phylogenies will be used along with the Virus-Host DB [33] to identify putative host species for viruses found in feces or rectal swabs, which commonly also contain plant and invertebrate viruses ingested as part of an animal's diet. Only viruses predicted or known to infect vertebrate animals will be investigated in downstream analyses.

PCR-based screening.
A subset of viruses identified from the metagenomic data will be selected for additional analyses based on phylogenetic relationships and host associations, as described above. As a priority, we will select: (i) viruses with known or predicted zoonotic potential (e.g., coronaviruses, paramyxoviruses), and (ii) viruses found at high prevalence and in multiple species that will facilitate the analysis needed to achieve AIM 2. Primers will be designed from the metagenomic data and used to screen relevant individual samples (i.e., not pools). An effort will be made to target regions of the genome likely to contain enough genetic diversity to support phylogenetic resolution at the farm and species level (e.g., the capsid gene, glycoprotein genes). Positive PCRs will be sequenced in both forward and reverse directions using Sanger sequencing technology (Macrogen) and assembled *de novo* using Geneious [34].

Assessing viral sharing.
To address AIM 1, the prevalence of each known or putative vertebrate virus will be estimated for all pools using the reference mapping software BWA, with RNA polymerase sequences from the metagenomic data as a reference [35]. The abundance of each vertebrate virus within a pool will be estimated from the sequencing data using RNA-Seq by Expectation-Maximization (RSEM) [36], implemented in Trinity. These data will be used to compare viral community structure across farms and host species using standard measures from community ecology, including species richness, alpha diversity, and beta diversity, which will be calculated using a range of R packages [37]. Permutational multivariate analysis of variance tests (permanova) will be used to assess differences in virome composition between farms and species.

For a more detailed analysis of the processes underlying viral sharing, the sequence data generated from our PCR-based screening on individual samples will be used to infer virus-specific phylogenetic trees, using maximum likelihood methods (as above) as well as Bayesian inference where phylogenetic resolution is high. Unlike our previous analysis, these phylogenies will contain only samples from this study and will reflect viral genetic variation across individuals, host species, and farms. To assess phylogenetic clustering and cross-species transmission at the farm and species level, we will utilize statistical frameworks developed for the phylogenetic analysis of discrete character states. These will

include association indices [38], parsimony scores [39], and maximum exclusive single-state clade sizes [40]. Further, we will impose models of discrete trait evolution [41], often utilized in phylogeographical analyses, to investigate viral sharing and the directionality of cross-species transmission between host species and between farms.

For AIM 2, we will use non-metric multidimensional scaling (NMDS) to ordinate each farm based on a set of ecological and environmental variables using R [37]. This analysis will be performed at two scales – local and regional. The local analysis will include data about each farm that was acquired during sampling or from questionnaires, such as the abundance and diversity of animal species, the presence of sick animals (and type of illness), and human activities previously associated with increased disease risk (e.g., butchering/slaughtering, selling wild animals, eating raw meat, etc.) [42]. In addition, the regional analysis will include variables representing the environmental and ecological context of each farm, which will be estimated from publicly available databases. Examples of these variables include average rainfall, average temperature, the mean normalized difference vegetation index (using LANDSAT data [43]), distance to an urban area, distance to water, and an index of wild mammal diversity (using International Union for Conservation of Nature [IUCN] data). Critically, the regional analysis will also include virus data previously generated and published from farms in Dong Thap Province (**Figure 2**) in the Mekong Delta, which were sampled under the same protocols (ENA accession numbers PRJEB6505, PRJEB26687, PRJEB27881) [18,44,45]. The addition of this second sampling location will allow us to separate the effects of farming practices on viral sharing (local scale) from the impacts of the surrounding environment (regional scale). The ordination axes resulting from the NMDS performed at each scale will be used as predictor variables in independent generalized linear models (GLMs) to examine their influence on virus presence and abundance for the subset of prioritized viruses using R. Model fit will be assessed using the Hosmer-Lemeshow goodness of fit test. The resulting models will be used to identify behavioral, environmental, and ecological factors that best predict the presence of 'risky' viruses on mixed-species farms.

Community and policy engagement.
To address AIM 3, we will utilize the results from the above analyses to identify potentially 'risky' farming practices, such as high farm-level species diversity, specific host-virus interactions, or interactions between farming practices and the surrounding environment, to engage stakeholders in discussions about the perception and management of risk in mixed-species farming. We will first organize meetings to engage stakeholders responsible for animal health, human health, and farm management within Dak Lak Province. These include provincial and regional animal and human health officials, and provincial representatives of the Departments of Agriculture (overseeing domestic farm management) and Forestry (overseeing wildlife farm management). We will use these meetings to create a two-way dialogue around these issues and better understand how recommendations on farm health and management are disseminated through public policy and farm management structures. Where recommendations on farm management can be made from our findings, we (with the OUCRU public engagement and policy teams) will work within the identified structures to determine the best practices with which to disseminate these findings to animal health workers and farming communities.

Working with these government stakeholders, we will identify opportunities to engage former members of the Dak Lak HRSC from the VIZIONS project in order to share our findings and better understand current perceptions of the risks associated with wildlife farming, particularly in light of potential links to the COVID-19 pandemic. With the OUCRU public engagement team, we will design surveys to initiate future discussions (extending beyond the scope of this proposal) that consider questions related to bans on wildlife farming and consumption at the policy level and their potential economic impacts, as well as perceived risks and approaches to protecting animal and human health at the farm level.

Research Performance Sites

OUCRU in Ho Chi Minh City (HCMC) was the primary site of the VIZIONS project, which ran from 2012-2016. This project involved two components: 1) hospital-based surveillance of pathogens causing selected human illnesses and 2) three cohorts (~300 humans/cohort) of individuals considered at high occupational risk of contracting zoonotic infections. These cohorts were sampled over three years, along with the animals with which they were in contact. The proposed project will focus on animal samples collected under component 2 of the VIZIONS project in Dak Lak Province. All samples needed for this work have been maintained in OUCRU HCMC laboratory freezers at -80°C from the time of collection and can be easily linked to human questionnaire, serological, and pathogen data collected from the human cohorts within OUCRU's in-house data management system (CliRes).

These unique samples and data are available only at OUCRU, which is committed to facilitating the proposed research. Leadership and several staff members within OUCRU's Molecular Epidemiology group (including Dr. Nguyen) were involved in the design and conduct of the VIZIONS project, and will facilitate access to samples and data, as well as assist in sample processing and analysis. Expertise in molecular biology, viral surveillance, phylogenetics, and molecular epidemiology within the research group will ensure that Dr. Nguyen has the support necessary to complete the proposed project.

OUCRU have established state-of-the-art clinical diagnostics, as well as basic and applied research laboratories that will facilitate successful completion of all aspects of the proposed project. Laboratory facilities include: clinical laboratories (ISO 15189:2012 certified), Biosafety level (BSL) 2 and BSL3 laboratories, and a bio-archiving facility. The laboratories are equipped with safety cabinets (fume hoods, biosafety category II and III), shaking & stationary incubators, refrigerated centrifuges, thermocyclers, real-time quantitative PCR machines, a gel imaging platform (chemiluminescence, ultraviolet, and visible light), gel boxes, water baths, mixing devices, capillary DNA sequencers, Illumina MiSeq sequencers, Oxford Nanopore MinION sequencers, an ultra-centrifuge, a Luminex Flexmap 3D, -80°C freezers, -20°C freezers, refrigerators, liquid nitrogen tanks, ice machines, and autoclaves.

Molecular epidemiology research at OUCRU is conducted in facilities across the laboratories, including a specimen processing facility, molecular diagnostic facility, genomics facility, and an immune-biology facility with serology and cell culture capabilities. High-risk laboratory processes are conducted in the BSL3 laboratory. OUCRU maintains a bio-archiving facility (-20°C, -86°C, liquid nitrogen storage facility) containing 2.5 million samples with continuous temperature monitoring and sample tracking (Lab guard system, Freezerworks system, and biometric access control). Dr. Nguyen will have access to all of the infrastructure and support associated with these facilities to complete the proposed project.

All OUCRU employees have around-the-clock access to computer servers, a virtual private network (VPN), encryption software, information technology (IT) support, and all necessary software including: Git and Github (hosted software revision/audit service), Oracle Virtualbox virtual machines, Google Apps (hosted email and collaboration web-based software), Python, NodeJS, and R programming languages, Meteor (Javascript framework), Jenkins (Continuous Integration server), Microsoft Office, and Adobe CS6. OUCRU also has a dedicated 150+ core Linux server with 8 terabyte (TB) hard drives, and the Molecular Epidemiology group has two dedicated 16-core Mac Pro Servers with 4TB hard drives. Either server individually or in combination may be used for intensive sequence analysis, computational modeling and/or database processing by Dr. Nguyen to ensure successful completion of the project.

CREID Research Center Collaboration

The goals and objectives of the proposed research project, "*Mixed-species farming, viral diversity, and risks of cross-species transmission in the Central Highlands of Viet Nam"*, are fully consistent with the specific aims of EID-SEARCH. EID-SEARCH takes a One Health approach to understanding zoonotic disease emergence, with a focus on viral diversity in wildlife at high-risk human animal interfaces. EID-SEARCH aims to understand the frequency and causes of virus spillover in high-risk communities across Southeast Asia and identify strategies and tools to reduce this risk. As the proposed research focuses on the links between farming practices, wildlife diversity, virus diversity, and cross-species transmission in Viet Nam, we expect the results of the project to make a significant contribution towards the overall goals of EID-SEARCH.

The proposed project is well-designed, leverages cutting-edge technology, and includes a robust ecological component that corresponds to the overall research strategy of EID-SEARCH. The EID-SEARCH team is highly motivated to support the application of metagenomics to ecological questions and assist in the analysis of the unique dataset that will be generated from this research. With the heightened focus on identifying and limiting risks associated with the complex interactions between humans, wildlife, and domestic mammals that has resulted from the emergence of SARS-CoV-2 from a wet market in China, the proposed research is both timely and likely to yield highly-cited, impactful publications for EID-SEARCH and its OUCRU partners.

The EID-SEARCH team includes a number of experts in study design, laboratory-based diagnostics, genetic and genomic analyses, and ecological modelling who will contribute to project implementation, data analysis, and manuscript preparation as required. Communication and coordination mechanisms will be set up between the OUCRU research team and scientists at the EID-SEARCH research sites for resource sharing, training, knowledge exchange, and mentorship. In particular, EID-SEARCH will facilitate and encourage relationships between Dr. Nguyen and our partners in Southeast Asia (based in Malaysia, Singapore, and Thailand) that will facilitate Dr. Nguyen's professional development and strengthen research capacity in the region. Dr. Cadhla Firth, Senior Scientist at EID-SEARCH and the project Mentor, has an established relationship with both Dr. Nguyen and his supervisor, Dr. Maia Rabaa, and will act as the liaison between EID-SEARCH and the OUCRU research team. An expected outcome of this research project will be pilot data to support a joint funding application in the future, led by Dr. Nguyen.

Furthermore, we expect this research to not only improve scientific understanding of zoonotic disease emergence, but also to raise risk awareness among government and local stakeholders and help inform local policies. EID-SEARCH has a team with a strong policy and social science background and extensive experience working with communities and engaging local governments. The EID-SEARCH team will support this project by helping the OUCRU team to develop locally-relevant evidence-based messages, and will strengthen multi-sectoral collaboration in developing practical risk-mitigation strategies. Dr. Nguyen has nearly ten years of experience engaging with local officials and farming communities to develop locally-acceptable research and One Health interventions on the ground in Viet Nam. These relationships Dr. Nguyen has cultivated during his time at OUCRU will facilitate the establishment of a sustainable scientific platform for ongoing research in emerging infectious diseases in Southeast Asia, as well as provide a direct pathway for policy impacts under the EID-SEARCH program.

Mentoring Plan

Although I am scheduled to complete my PhD on March 15, 2021 (viva date), I have been actively involved in research as a veterinarian and Program Coordinator at OUCRU since 2011. As a result, I have a broad understanding of the challenges involved in developing and implementing a research project, and a clear idea of how I would like to progress my career. My overall research goal is to improve the health of people in Viet Nam using a One Health approach that will allow me to maximize the impacts of both my veterinary and academic training. However, my aspirations are not just limited to the creation of scientific knowledge; instead, I wish to actively use this knowledge to address social issues in Viet Nam, as demonstrated by the focus of my PhD research on antimicrobial stewardship on poultry farms.

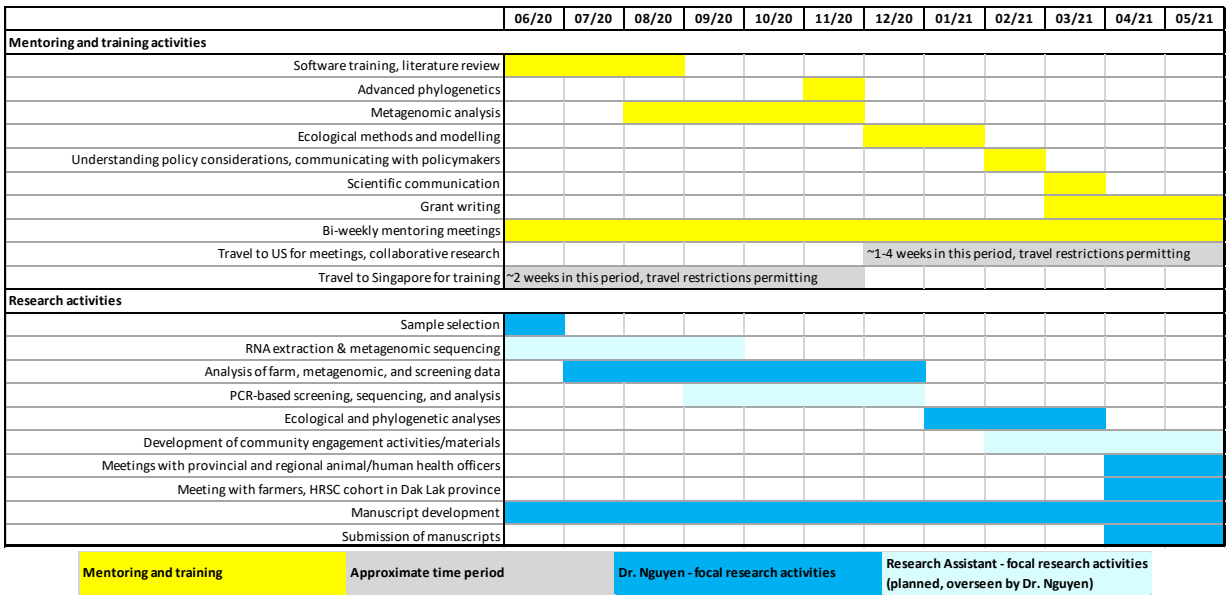
My previous community engagement experiences have focused primarily on highlighting the need for data-driven policy, and I have had only limited experience working with political agencies to translate scientific evidence into laws and regulations. Through the public engagement aim of the proposed project, I will be able to strengthen my relationships with government and local stakeholders, grow my network across Viet Nam, and enable my long-term success using data-driven, community-led research to generate real-world impacts. By working closely with members of EHA, who have a strong background in community engagement and policy development, and the recently formed Policy Engagement group at OUCRU, I feel that this project will provide the perfect opportunity for me to develop my career vision as an independent scientist.

Throughout my career, I have had experience and training in veterinary medicine, laboratory-based science, program coordination, and epidemiology. However, it is clear that genomics (and metagenomics) is now an increasingly common aspect of emerging infectious disease research. There is a pressing need for increased capacity in this field in Viet Nam, as well as in many other lower- and middle-income countries, to ensure that we have access to the best methods and analytical tools available to support a thriving science industry. I believe that, given the opportunity to further strengthen my analytical skills to include metagenomic data analysis, I will be in possession of a comprehensive skillset that will allow me to transition from a junior scientist to an independent science leader in Viet Nam, capable of initiating and delivering hypothesis-driven research to solve locally relevant problems.

With more than 15 years' experience working on emerging infectious diseases, Dr. Firth (my Mentor) has built a research program with a strong multi-disciplinary focus that is only enhanced by her recent appointment as Senior Scientist with EHA. Her expertise includes nearly 10 years' experience in the generation and analysis of metagenomic sequence data, which she has used in both clinically relevant and veterinary contexts, as well as to address a range of ecological and evolutionary questions [25,46–50]. Dr. Firth also has significant experience with field and laboratory science and has led or participated in projects in multiple countries, including Malaysia, Viet Nam, Australia, Brazil, The United States, and Canada. As a result, she has a broad scientific network that involves global leaders in infectious disease research, including those within Southeast Asia (e.g., Prof. Linfa Wang, Director, Emerging Infectious Diseases Program at Duke-NUS Medical School, Singapore; Prof. David Perera, Director, Institute of Health & Community Medicine, UNIMAS). Dr. Firth will actively create opportunities for me to interact with Prof. Wang and other leading scientists through the proposed research project, which will greatly increase my research network. A two-week visit to Prof. Wang's laboratory has been budgeted as part of the proposed project and will provide me with the opportunity to learn cutting-edge laboratory techniques, as well as interact directly with members of his lab. Dr. Firth has also committed to creating opportunities for me to discuss my metagenomic data directly with Prof. Edward Holmes (a world leader in virus discovery and metagenomics), who is highly supportive of the proposed project.

In addition to her scientific skills and network, Dr. Firth has previous experiences training and mentoring scientists in lower- and middle-income countries. This should increase the effectiveness of her mentorship since I am also a junior scientist from one of these countries in Southeast Asia. Dr. Firth also has a close personal relationship with my in-country project supervisor and long-term collaborator, Dr. Maia Rabaa, who is co-lead of OUCRU's Molecular Epidemiology research group. Drs. Firth and Rabaa completed their PhD degrees together and have maintained a strong relationship focused on peer mentorship since that time. As a result of their joint commitment to both this research project and my professional development, I will have access to an extremely high level of support and mentorship both locally and internationally. The combined mentoring, experience, and expertise I will gain through the CREID Pilot Research Program will assist me in my progression toward scientific independence by providing opportunities to develop my grant writing, budgeting, study management, and networking skills, in addition to the new scientific skills I will gain. The data generated as a result of the proposed project will also provide critical pilot data for future grant applications that will form the foundation of a long-term collaborative relationship with EHA.

Through the process of writing the application for the CREID Pilot Research Program, Dr. Firth and I have already established the foundation for an effective communication plan that will continue throughout the project. We have created a WhatsApp group that also includes Dr. Rabaa, which we use to communicate in an informal setting and in real-time. This has been invaluable for sharing ideas, asking questions, and facilitating the development of a good personal relationship that is a pre-requisite for effective mentorship. We have also agreed to a regularly scheduled Zoom meeting that will occur every two weeks, be agenda-driven, and focused on a topic or issue of direct relevance to the research project. Both Dr. Firth and I will be responsible for setting the agenda and following up on action items. I will also attend weekly academic meetings at OUCRU and EHA (over Zoom), which will expose me to the breadth of research at these institutions, and which I have found to be extremely useful for me in the past. Dr. Firth and I have also agreed to extend my visit to the United States to attend the annual CREID meeting to include a visit to EHA in New York City. The timing of this visit will allow me to bring some metagenomic data from the project with me and work through data analysis with Dr. Firth and other EHA scientists. Finally, Dr. Firth, Dr. Rabaa, and I will travel to Dak Lak province in Viet Nam at the end of the project to present the study outputs to the community members that participated in the VIZIONS study, as part of the public engagement aim.



Vertebrate Animals Section Requirements

No new animal sampling will be conducted in the proposed research study. All samples and data to be used in the proposed research project were previously obtained as part of the VIZIONS project, which concluded in 2016. Samples were collected under a protocol approved by the Oxford Tropical Research Ethics Committee (OxTREC code 157-12), and were locally reviewed and approved by the ethical review board of the Hospital for Tropical Diseases in HCMC, with local review and acceptance at all sites. Viet Nam did not have an animal ethics review board at the time that the VIZIONS project was conducted.

Details on the VIZIONS project are available in a 2015 publication [19].

The principal aims of the VIZIONS project were:

1. To establish a model international collaborative consortium with an integrated approach to human and animal health research.
2. To estimate the burden of viral and zoonotic diseases in Viet Nam.
3. To investigate the disease epidemiology of specified clinical syndromes and infections in a cohort of high-risk individuals occupationally exposed to animals, with targeted sampling from domestic animals and wildlife associated with these individuals.
4. To elucidate the etiology of infectious diseases of unknown origin in the human population and provide a repository of putative pathogens for further study.
5. To characterize genetic diversity within virus populations on either side of the species barrier to understand cross-species transmission and disease emergence.
6. To identify socio-demographic, environmental, and behavioral drivers for disease emergence.
7. To create a platform and resource for further research on zoonotic disease agents.

Key in-country partners on the VIZIONS project included:

- Hospitals (The Hospital for Tropical Diseases, HCMC; Dong Thap General Hospital, Cao Lanh City, Dong Thap Province; Dak Lak General Hospital, Buon Ma Thuot City, Dak Lak Province; Khanh Hoa General Hospital, Nha Trang City, Khanh Hoa Province; Hue Central Hospital, Hue City, Thua Thien Hue Province; National Hospital for Tropical Diseases, Ha Noi; Ba Vi District Hospital, Ha Noi)
- Academic institutions (OUCRU, HCMC; OUCRU, Ha Noi; Hanoi Medical University)
- Regional Animal Health Offices (RAHO5, Buon Ma Thuot City, Dak Lak Province)
- Sub-departments of Animal Health (sDAH) (Dak Lak sDAH, Buon Ma Thuot City, Dak Lak Province; Dong Thap sDAH, Cao Lanh City, Dong Thap Province)
- Preventive Medicine Centers (PMCs) (Dak Lak PMC, Buon Ma Thuot City, Dak Lak Province; Dong Thap PMC, Cao Lanh City, Dong Thap Province; Ba Vi District PMC, Ha Noi)
- Ba Vi District Veterinary Station

Applicant Name (Last, first, middle): Nguyen, Cuong, Van

Human Subjects Research

No human subjects, samples, or data will be involved in this research.

1. Are Human Subjects Involved?			No													
1.a. If YES to Human Subjects																
Is the Project Exempt from Federal regulations?	Yes		No													
If yes, check appropriate exemption number	1		2		3		4		5		6		7		8	
If no, is the IRB review Pending?	Yes		No													
IRB Approval Date:																
Human Subject Assurance Number																
2. Are Vertebrate Animals Used?			No													
2.a. If YES to Vertebrate Animals																
Is the IACUC review Pending?	Yes		No													
IACUC Approval Date																
Animal Welfare Assurance Number																
3. Is proprietary/privileged information included in the application?			No													
4.a. Does this project have an Actual or Potential impact -- positive or negative -- on the environment?			No													
4.b. If yes, please explain																
4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed?	Yes		No													
4.d. If yes, please explain																
5. If the research performance site designated, or eligible to be designated, as a historic place?			No													
5.a. If yes, please explain																
6. Does this project involve activities outside of the United States or partnership with international collaborators?	Yes															
6.a. If yes, identify countries	Viet Nam															
6.b. Optional explanation																

Budget Justification

In total, we request $149,970.57 for Dr. Nguyen for this one-year project, starting 1 June 2021, including: $50,878.00 for personnel, $1,200.00 for regulatory fees, $1,000 for equipment, $76,473.76 for consumables, $9,309.88 for travel, and $11,108.93 for indirect costs. Detailed expenses are calculated as follows:

A. Personnel ($50,878.00)

Dr. Nguyen, PI, will commit 12 months (1 FTE) per year to this project for study design, supervising the laboratory work, training in new analytical methods, and conducting the metagenomic and epidemiological analyses. Dr. Nguyen is a veterinary epidemiologist specializing in pathogen surveillance at the human-animal interface and will have just completed his PhD studies (15 March 2021). We request $28,563.00 total salary for Dr. Nguyen for the period of 12 months.

We also request funding for a senior research assistant who will commit 12 months (1 FTE) per year to this project to perform laboratory work and assist in the development of community engagement activities and materials. The research assistant will have significant experience working with and performing virus surveillance/sequencing on samples collected under the VIZIONS project, and will have experience participating in public engagement projects in Viet Nam. We request $22,315.00 total salary for this research assistant for the period of 12 months.

B. Regulatory fees ($1,200.00)

An import license from the Ministry of Health will be required for importation of any consumables, equipment, reagents, and biological materials related to this project, at the cost of $1,200.00.

C. Equipment ($1,000.00)

No equipment will be purchased with the exception of one laptop computer for Dr. Nguyen. We request budget ($1,000.00) for one laptop computer for use by Dr. Nguyen.

D. Consumables ($76,437.76)

RNA Extractions ($29,170.00). We request ($29,170.00, $14.59 per sample) for RNA extractions. This total covers the cost of all kits, reagents, and consumables (MagNA Pure 96 DNA and Viral NA SV Kits, MagNA Pure 96 Processing Cartridges, MagNA Pure 96 Filter Tips, MagNA Pure 96 System Fluid, MagNA Pure 96 Sealing Foil, MagNA Pure 96 Output Plates, Qubit™ RNA HS Assay Kits, Qubit™ Assay Tubes, and TURBO™ DNase) for 2000 extractions.

Metagenomics ($32,500.00). We request ($32,500.00, $270.83 per 10-sample pool) for metagenomic sequencing. This total covers the cost of reagents and consumables for library prep (Illumina Stranded Total RNA Prep with Ribo-Zero Plus kit, Illumina Sequencing Adapters, Agilent AMPure Beads, RNA Clean XP) and sequencing on a HiSeq-4000 at Macrogen (South Korea), for 120 pools.

Follow-up PCRs ($14,173.56). We request ($14,173.56, $11.81 per sample) for targeted RT-PCR following results of the metagenomic analysis. This total covers the cost of all kits, reagents, and consumables (LightCycler 480 Multiwell Plates, 96–Well Half Skirt PCR Plates, Bubble Strip Caps for PCR Strips, SuperScript III One-Step RT-PCR, Primers, Agarose, SYBR™ Safe DNA Gel Stain, DNA Clean & Concentrator-5, Amplicon Sequencing) to cover 1200 reactions.

Other reagents & consumables ($630.21). We request ($630.21) to cover miscellaneous consumables. This total covers the cost of tubes, tips, personal protective equipment, and other consumables (varied

Axygen™ Aerosol Filter Tips, Water - Molecular Biology Reagent, Absolute Ethanol, LoBind Eppendorf tubes, nitrile gloves) required to process samples in the laboratory.

E. Travel ($9,309.88)

International Travel ($8,229.00). We request: 1) $4,335.00 for one trip to the United States for Dr. Nguyen to the CREID meeting in Rockville, Maryland. This total covers one round-trip flight ($1,600.00), lodging and per diem for 4 days/nights ($250 per day; total $1,000), ground transportation costs ($150.00), insurance and visas ($185.00), and quarantine costs for 14 days on return to Viet Nam ($100.00 per day; total $1,400.00). Additional costs for travel to New York City, lodging, and subsistence during this trip will be covered by EHA. 2) We also request an additional $3,894.00 for one trip to meet and train with EID-SEARCH partners in Singapore. This total covers one round-trip flight ($400.00), lodging and per diem for 14 days/nights ($140.00 per day; total $1,960.00), ground transportation costs ($100.00), insurance ($34.00), and quarantine costs for 14 days on return to Viet Nam ($100.00 per day; total $1,400.00).

Inter-province Travel ($1080.88). We request $1080.88 for Dr. Nguyen and two OUCRU staff members (one from the Molecular Epidemiology group and one from the Public Engagement team) to travel to Buon Ma Thuot city, Dak Lak Province for discussions with stakeholders and engagement planning activities. This total covers three round-trip flights ($200.00 per person; total $600.00), lodging and per diem for 3 people for 3 days ($43.43 per person per day; total $390.88), and ground transportation costs ($30.00). Additional costs for engagement activities will be covered by internal OUCRU funding for Public Engagement activities.

Additional travel

EID-SEARCH (EcoHealth Alliance) will provide additional funding for international travel costs related to additional face-to-face meetings and collaborative work periods for Dr. Nguyen and Dr. Firth.

F. Indirect costs ($11,108.93)

We are requesting 8% indirect costs on all direct costs (total $11,108.93).

BIOGRAPHICAL SKETCH

NAME: Nguyen, Cuong, Van

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

POSITION TITLE: Project coordinator

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	Completion Date	FIELD OF STUDY
Agriculture and Forestry University (VN)	DVM	09/2008	Veterinary Medicine
University of Paris XI (France)	MSc	09/2011	Biotechnology (Genome, Cells)
The Open University (UK) (thesis submitted, viva March 2021)	PhD	03/2021	Life, Health and Chemical Sciences

A. Personal Statement

I have strong motivation to lead a research project characterizing viral diversity across multiple animal species, particularly in wildlife/domestic mixed species farms. I was involved in the implementation within the VIZIONS project from 2012-2016, acting as a research assistant and field coordinator. My research interests are focused on community-based studies investigating high potential zoonotic diseases and antimicrobial resistance at the animal-human interface. My primary research work during the VIZIONS study was coordinating the collection of high-quality data and samples, as well as establishing critical community partnerships in both the human and animal health sectors. Beyond my field coordinator role, I also processed and tested for the presence of known viruses in animal samples from the VIZIONS project. These studies collected and have reported on varied sources of data (i.e. epidemiological, clinical, laboratory, genomic, and behavioral data) to better understand the epidemiology and emergence of viral zoonoses. I currently coordinate an intervention project (ViParc: Vietnamese Platform for Antimicrobial Reduction in Chicken production) that aims to reduce antimicrobial usage in chicken production in southern Viet Nam. I now have nearly completed my PhD work characterizing antimicrobial usage in chicken production. I have delivered a number of high impact publications focused on key issues in veterinary epidemiology. I am also committed to providing training for local veterinary students on statistics and epidemiology, veterinary public health, and animal welfare (visiting university lecturer).

a. **Cuong, NV**, Carrique-Mas J, Thu HTV, Hien ND, Hoa NT, Nguyet LA, Anh PH, Bryant JE (2014). *Serological and virological surveillance for porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, and influenza A viruses among smallholder swine farms of the Mekong Delta, Vietnam*. **Journal Swine Health and Production**; 22(5):224–231.

b. **Van Cuong N**, Carrique-Mas J, Vo Be H, An NN, Tue NT, Anh NL, Anh PH, Phuc NT, Baker S, Voutilainen L, Jääskeläinen A, Huhtamo E, Utriainen M, Sironen T, Vaheri A, Henttonen H, Vapalahti O, Chaval Y, Morand S, Bryant JE (2015). *Rodents and risk in the Mekong Delta of Vietnam: seroprevalence of selected zoonotic viruses in rodents and humans*. **Vector Borne Zoonotic Dis**; 15(1):65-72.

c. **Cuong NV,** Truc VN, Nhung NT, Thanh TT, Chieu TT, Hieu TQ, Men NT, Mai HH, Chi HT, Boni MF, van Doorn HR, Thwaites GE, Carrique-Mas JJ, Hoa NT. (2016). *Highly pathogenic avian influenza virus A/H5N1 infection in vaccinated meat duck flocks in the Mekong Delta of Vietnam*. **Transboundary and Emerging Diseases**; 63(2):127–135.

d. **Van Cuong N**, Nhung NT, Nghia NH, Mai Hoa NT, Trung NV, Thwaites G, Carrique-Mas J (2016). *Antimicrobial consumption in medicated feeds in Vietnamese pig and poultry production*. **Eco Health**; 13(3):490-498.

B. Positions and Honors

YEAR(S)	POSITION
2011-2013	Research Assistant, Oxford University Clinical Research Unit (OUCRU), Viet Nam
2014-2016	Senior Research Assistant, VIZIONs Field Coordinator, OUCRU, Viet Nam
2017-2021	PhD candidate, ViPARC project coordinator, OUCRU, Viet Nam
2020-present	Visiting University Lecturer at the Applied Science Institute, Ho Chi Minh University of Technology (HUTECH), Viet Nam

C. Contributions to Science

1. Zoonotic transmission at the human-animal interface

I played a crucial role in implementing a community-based study of zoonotic infections in Viet Nam under the VIZIONS project. By coordinating high quality data and sample collection, I contributed to the success and unique study design of many VIZIONS-related studies, driving a number of sub-studies investigating pathogen evolution and epidemiology. These studies provided insight into viral, bacterial, and parasitological zoonotic infections presenting in communities in Viet Nam. I played multiple roles on the project, including field work and sampling, laboratory-based screening, and complex epidemiological investigations of pathogen diversity in domestic and wild animal farms, as well as in wild animal populations (capturing bats in the jungle and rats in rice fields).

a. Loan HK, **Van Cuong N,** Takhampunya R, Kiet BT, Campbell J, Them LN, Bryant JE, Tippayachai B, Van Hoang N, Morand S, Hien VB, Carrique-Mas JJ (2015). *How important are rats as vectors of leptospirosis in the Mekong Delta of Vietnam?* **Vector Borne Zoonotic Dis**; 15(1):56-64.

b. Van Dung N, Anh PH, **Van Cuong N**, Hoa NT, Carrique-Mas J, Hien VB, Sharp C, Rabaa M, Berto A, Campbell J, Baker S, Farrar J, Woolhouse ME, Bryant JE, Simmonds P (2016). *Large-scale screening and characterization of enteroviruses and kobuviruses infecting pigs in Vietnam*. **J Gen Virol**; 97(2):378-388.

c. Van Nguyen VC, Le Buu C, Desquenes M, Herder S, Nguyen PHL, Campbell JI, **Nguyen VC**, Yimming B, Chalermwond P, Jittapalapong S, Franco JR, Ngo TT, Rabaa MA, Carrique-Mas JJ, Thanh TPT, Nga TVT, Berto A, Hoa NT, Hoang NVM, Tu NC, Chuyen NK, Wills B, Hien TT, Thwaites GE, Yacoub S, Baker S (2016). *A clinical and epidemiological investigation of the first reported human case of the zoonotic parasite* Trypanosoma evansi *in Vietnam*. **Clin Inf Dis**; 62(8):1002-8.

d. Van Nguyen D, **Van Nguyen C**, Bonsall D, Ngo TT, Carrique-Mas J, Pham AH, Bryant JE, Thwaites G, Baker S, Woolhouse M, Simmonds P (2018). *Detection and Characterization of Homologues of Human Hepatitis Viruses and Pegiviruses in Rodents and Bats in Vietnam.* **Viruses**; 10(3):102.

2. Usage and drivers of antimicrobial resistance in animal production

Antimicrobial resistance (AMR) is a global threat to the health and wealth of nations. The AMR crisis has been attributed to the overuse and misuse of antimicrobials. Excessive use of antimicrobials in animal production is one of the contributing factors to this global threat. In my PhD work, I characterized antimicrobial usage (AMU) in small-scale chicken farms in the Mekong Delta region of Viet Nam. This includes consumption of antimicrobials mixed with water by the farmer as well those included in commercial feeds as antimicrobial growth promoters (AGPs). The epidemiological data that I gathered in

this project was used to investigate the relationship between AMU and disease. Results from this thesis suggest that efforts to promote responsible use of antimicrobials and limit excessive AMU are effective in reducing AMU in animal production, and thus should focus on: 1) educating farmers on good farming practices and simple veterinary diagnostics, and 2) strengthening veterinary systems, including farm audits and provision of effective advice from animal health experts. These studies have also shown that that the probability of effective treatment is often very low. My research concluded that the message 'prophylactic AMU does not reduce the probability of disease in flocks' should be further disseminated to poultry farming communities to reduce unnecessary AMU on farms.

a. **Cuong N**, Padungtod P, Thwaites G, Carrique-Mas JJ (2018). *Antimicrobial Usage in Animal Production: A Review of the Literature with a Focus on Low- and Middle-Income Countries*. **Antibiotics** (Basel); 7(3):75.

b. **Cuong NV**, Phu DH, Van NTB, Dinh Truong B, Kiet BT, Hien BV, Thu HTV, Choisy M, Padungtod P, Thwaites G, Carrique-Mas J (2019). *High-Resolution Monitoring of Antimicrobial Consumption in Vietnamese Small-Scale Chicken Farms Highlights Discrepancies Between Study Metrics*. **Frontiers in Vet Sci**; 6:174.

c. Choisy M, **Van Cuong N**, Bao TD, Kiet BT, Hien BV, Thu HV, Chansiripornchai N, Setyawan E, Thwaites G, Rushton J, Carrique-Mas J (2019) *Assessing antimicrobial misuse in small-scale chicken farms in Vietnam from an observational study*. **BMC Vet Res**; 15(1):206.

d. Carrique-Mas JJ, Choisy M, **Cuong NV**, Thwaites GE, Baker S (2020). *An estimation of total antimicrobial usage in humans and animals in Vietnam*. **Antimicrobial resistance and infection control;** 9:16.

3. Understanding AMR in commensal and pathogenic microorganisms in animal production

A better understanding of AMR in both commensal and pathogenic microorganisms in animal production is vital to improve treatment and control, particularly in the context of preventing those bacterial infections in animals as well as the transfer of their genes into human pathogenic bacteria. Epidemiological data from my studies have shown that high levels of AMU resulted in high levels of AMR in both commensal and pathogenic bacteria. These data have resulted in improved treatment and diagnostic policies for animal production across Viet Nam.

a. Yen NTP, Nhung NT, Van NTB, **Cuong NV**, Kiet BT, Phu DH, Hien VB, Campbell J, Chansiripornchai N, E Thwaites G, Carrique-Mas JJ (2020). *Characterizing Antimicrobial Resistance in Chicken Pathogens: A Step towards Improved Antimicrobial Stewardship in Poultry Production in Vietnam*. **Antibiotics (Basel);** 9(8):499.

b. Nguyen NT, Nguyen HM, **Nguyen CV**, Nguyen TV, Nguyen MT, Thai HQ, Ho MH, Thwaites G, Ngo HT, Baker S, Carrique-Mas J (2016). *Use of Colistin and Other Critical Antimicrobials on Pig and Chicken Farms in Southern Vietnam and Its Association with Resistance in Commensal* Escherichia coli *Bacteria*. **Appl Environ Microbiol**; 82(13):3727-3735.

c. Tu LT, Hoang NV, **Cuong NV**, Campbell J, Bryant JE, Hoa NT, Kiet BT, Thompson C, Duy DT, Phat VV, Hien VB, Thwaites G, Baker S, Carrique-Mas JJ (2015). *High levels of contamination and antimicrobial-resistant non-typhoidal* Salmonella *serovars on pig and poultry farms in the Mekong Delta of Vietnam*. **Epidemiol Infect**; 143(14):3074-86.

d. Nhung NT, **Cuong NV**, Campbell J, Hoa NT, Bryant JE, Truc VN, Kiet BT, Jombart T, Trung NV, Hien VB, Thwaites G, Baker S, Carrique-Mas J (2015). *High levels of antimicrobial resistance among* Escherichia coli *isolates from livestock farms and synanthropic rats and shrews in the Mekong Delta of Vietnam*. **Appl Environ Microbiol**; 81(3):812-20.

Complete List of Published Work in MyBibliography:

https://www.ncbi.nlm.nih.gov/myncbi/1NKsW-nQefwcQc/bibliography/public/

BIOGRAPHICAL SKETCH

NAME: Nguyen, Cuong, Van

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

D. Additional Information: Research Support and/or Scholastic Performance

Completed Research Support

My PhD fellowship was funded by the ViPARC project (12/2016-01/2021). This project was funded by the Wellcome Trust through an Intermediate Clinical Fellowship awarded to Dr. Juan J. Carrique-Mas (Grant Reference Number 110085/Z/15/Z).

BIOGRAPHICAL SKETCH

NAME: Firth, Cadhla

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6)

POSITION TITLE: Senior Research Scientist and Program Coordinator, EcoHealth Alliance

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	Completion Date	FIELD OF STUDY
University of Guelph, Guelph, CAN	BS	06/2003	Zoology
University of Guelph, Guelph, CAN	MS	06/2005	Evolutionary Ecology
The Pennsylvania State University, State College, PA	PhD	05/2010	Biology
Columbia University, New York, NY	Postdoctoral	12/2012	Infectious Diseases & Public Health

A. Personal Statement

I am a molecular and evolutionary biologist who has been working on emerging infectious diseases for almost 15 years. In my current research, I explore the ecological and evolutionary dynamics of emerging zoonoses at the human-animal interface, with a focus on rapidly changing environments. I have more than seven years' experience leading independent research projects in this field, including collaborations with industry and local government. Over time, I have been responsible for the successful completion of every aspect of these studies, including project design, funding acquisition, implementation, and reporting/publishing. In 2015 I initiated a research program to investigate the response of zoonotic pathogens to urbanization in Malaysian Borneo using metagenomics, landscape ecology, and population genomics. This research has a strong multi-disciplinary component that includes evolutionary biology, molecular ecology, virology, pathogen genetics and genomics, field biology, and public health, and has required me to become adept at combining and analyzing multiple types of data, including environmental, ecological, and genomic data. As such, my experience, skills, and training are ideally suited to the proposed research project, which builds naturally on my previous work. I also have a strong track record in the generation and use of genetic and genomic data to explore the transmission dynamics and evolution of emerging viruses, with more than 20 publications in this field, seven of which have been cited more than 100 times since 2010. The mastery of phylogenetic and phylodynamic methods I have developed throughout my career are directly applicable to the proposed research. Further, I am strongly committed to supporting diversity and equity both within the scientific community and in the communities we serve, and have led several projects and initiatives aimed at improving science education and health service delivery for underserved communities in tropical northern Australia. I am also committed to providing training and mentorship for low- and middle-income country scientists and scholars, and in my previous role worked to develop protocols and bioinformatics pipelines to enable portable metagenomic sequencing in rural and remote regions in Australia and the Pacific. I am currently supervising three PhD students and three postdoctoral researchers (James Cook University, AUS).

B. Positions and Honors

Positions and Employment

2001-2006 Research Assistant, Department of Zoology, The University of Guelph, Guelph, CAN
2006-2010 PhD Scholar, The Pennsylvania State University, State College, PA
2010-2012 Postdoctoral Fellow, Center for Infection and Immunity, Columbia University, New York, NY
2013 Associate Research Scientist, Center for Infection and Immunity, Columbia University, New York, NY

2013-2017	Research Scientist/ Australian Research Council (ARC) Discovery Early Career Research Fellow, Health and Biosecurity, The Commonwealth Scientific and Industrial Research Organization, Geelong, AUS
2017-2018	Australian Research Council (ARC) Discovery Early Career Research Fellow, School of BioSciences, The University of Melbourne, Parkville, AUS
2018-2020	HOT North Career Development Fellow, Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, AUS
2021-	Senior Research Scientist and Program Coordinator, EcoHealth Alliance, New York, NY

Honors

2003-2005	Ontario Graduate Scholarship, The Province of Ontario (Canada)
2006-2009	Postgraduate Scholarship, Natural Science and Engineering Research Council of Canada
2015-2018	Discovery Early Career Researcher Award, The Australian Research Council
2019-2021	HOT North Career Development Fellowship, Northern Australia Tropical Disease Collaborative Research Programme, National Health and Medical Research Council (AUS)

C. Contributions to Science

Throughout my career, my research has focused on many aspects of infectious disease emergence and has encompassed evolutionary biology, molecular ecology, virology, pathogen genetics and genomics, field biology, and public/veterinary health. My most significant contributions include:

1. The use of genomic and metagenomic sequencing techniques to discover new pathogens, study their evolution, and explore microbial diversity.

I have successfully developed and used cutting-edge metagenomic techniques and bespoke bioinformatics pipelines for: (i) pathogen discovery, including a case of acute encephalitis in an immune-suppressed patient, (ii) studies of viral diversity, including coronaviruses and flaviviruses in bats; and (iii) genomics, including the epizoology of bluetongue virus, the evolution of rhabdoviruses, and the characterization of Australian bunyaviruses. I have also co-authored two perspectives on the utility of next-generation sequencing for pathogen discovery in people and animals. These included an invited submission that described an updated framework for proof of causation in the genomics era and discussed the emerging integration of on-the-ground metagenomics-based diagnostics with molecular epidemiology.

In 2013, I initiated a study that used metagenomics to characterize the viruses and bacteria carried by New York City rats across a range of habitats, with a focus on the built environment. At the time, little was known about the range of potential pathogens carried by urban rodents or the risk factors associated with zoonotic transmission in a city environment. To begin to address this, I designed the first comprehensive molecular survey of rodent-borne microbes in a U.S. city, and demonstrated that urban rats frequently carry a range of zoonotic pathogens. We also found evidence of substantial heterogeneity in pathogen distribution within and between cities, suggesting that the associated risks of human disease may be similarly heterogeneous.

a. **Firth C**, Bhat M, Firth MA, Williams SH, Frye MJ, Simmonds P, Conte JM, et al. 2014. Detection of zoonotic pathogens and characterization of novel viruses carried by commensal Rattus norvegicus in New York City. mBio 5: e01933-14. PMCID: PMC4102142

b. Quan PL, **Firth C**, Conte JM, Williams SH, Zambrana-Torrelio CM, Anthony SJ, Ellison JA, et al. 2013. Bats are a major natural reservoir for hepaciviruses and pegiviruses. Proceedings of the National Academy of Sciences 110: 8194-9. PMCID: PMC3657805

c. **Firth C**, Lipkin WI. 2013. The genomics of emerging pathogens. Annual Review of Genomics and Human Genetics 14: 281-300. PMID: 24003855

d. Quan PL, **Firth C**, Street C, Henriquez JA, Petrosov A, Tashmukhamedova A, Hutchison SK, et al. 2010. Identification of a severe acute respiratory syndrome coronavirus-like virus in a leaf-nosed bat in Nigeria. mBio 1: e00208-10. PMCID: PMC2975989

e. Quan P-L, Wagner T, Briese T, Kapur RP, Torgerson TR, Hornig M, **Firth C**, et al. 2010. Astrovirus encephalitis in X-linked agammaglobulinemia. Emerging Infectious Diseases 16: 918-25. PMCID: PMC4102142

2. I have helped develop the emerging field of urban zoonotic disease ecology.

In 2015, I pioneered an ongoing research program to investigate the response of zoonotic pathogens to urbanization using metagenomic sequencing, landscape ecology, and population genomics. Using a multi-disciplinary approach, this research explores how changes in the environment (e.g., land-use changes, microclimates, etc.), and host community and population structure (e.g., biodiversity, connectivity, density), influence pathogen community composition and zoonotic potential. Initial results of this work indicate that while mammalian diversity decreases with increasing urbanization, connectivity between populations increases, as does microbial species richness and the prevalence of known zoonotic pathogens. This suggests that some features of the built environment may inadvertently support pathogen persistence and spread, and as a result, some zoonotic pathogens may be more likely to emerge with intensifying urbanization. My reputation as an emerging researcher in this field has led to multiple invitations to present as a plenary or symposium speaker at national and international meetings, including the Ecological Society of Australia's Annual Conference (2019) and the Annual Meeting of the American Society of Mammalogists (2019), and the Joint Conference of the Asian Society of Conservation Medicine and the Wildlife Disease Association Australasia (2018).

a. Blasdell KR, Morand S, Perera D, **Firth C.** 2019. Association of rodent-borne *Leptospira* spp. with urbanizing environments in Sarawak, Malaysian Borneo. PloS Neglected Tropical Diseases 13: e0007141. PMCID: PMC6411199

b. Blasdell KR, Perera D, **Firth C.** 2018. High prevalence of rodent-borne *Bartonella* spp. in urbanizing environments in Sarawak, Malaysian Borneo. American Journal of Tropical Medicine and Hygiene 100: 506-9. PMCID: PMC6402934

c. Peterson AC, Ghersi BM, Alda F, **Firth C**, Frye MJ, Bai Y, Osikowicz LM, et al. 2017. Rodent-borne Bartonella infection varies according to host species within and among cities. EcoHealth 14:771-82. PMID: 29164472

d. Frye MJ, **Firth C**, Bhat M, Firth MA, Che X, Lee D, Williams SH, Lipkin WI. 2015. Preliminary survey of ectoparasites and associated pathogens from Norway rats in New York City. Journal of Medical Entomology 52: 253-9. PMCID: PMC4481720

e. **Firth C**, Bhat M, Firth MA, Williams SH, Frye MJ, Simmonds P, Conte JM, et al. 2014. Detection of zoonotic pathogens and characterization of novel viruses carried by commensal Rattus norvegicus in New York City. mBio 5: e01933-14. PMCID: PMC4102142

3. I challenged established beliefs about the ecological and evolutionary processes linked to viral disease emergence, and the timescale over which they occur.

During my PhD, I was able to make significant contributions to this field by questioning the idea that viruses evolve either by cross-species transmission (spillover) or by co-evolution with their hosts, but rarely both. My work demonstrated that viruses with many genomic structures are capable of jumping species barriers, and that evolution through both co-divergence and cross-species transmission may be a

general trend of many virus systems. This has since been supported by numerous subsequent studies that have highlighted the complex evolutionary dynamics of both RNA and DNA viruses.

a. Pagan I, **Firth C**, Holmes EC. 2010. Phylogenetic analysis reveals rapid evolutionary dynamics in the plant RNA virus genus tobamovirus. Journal of Molecular Evolution 71: 298-307. PMID: 20838783

b. **Firth C**, Kitchen A, Shapiro B, Suchard MA, Holmes EC, Rambaut A. 2010. Using time-structured data to estimate evolutionary rates of double-stranded DNA viruses. Molecular Biology and Evolution 27: 2038-51. PMCID: PMC3107591

c. Sali AA, Faye O, Diallo M, **Firth C**, Kitchen A, Holmes EC. 2010. Yellow fever virus exhibits slower evolutionary dynamics than dengue virus. Journal of Virology 84: 765-72. PMCID: PMC2798388

d. **Ramsden C***, Holmes EC, Charleston MA. Hantavirus evolution in relation to its rodent and insectivore hosts: no evidence for co-divergence. 2009. Molecular Biology and Evolution 26: 143-53. PMID: 18922760

* Name changed from Ramsden to Firth in 2009

4. I was among the first to apply novel phylodynamic approaches to reconstruct the spatiotemporal processes behind disease emergence events.

Phylodynamic methods enable the synthesis of epidemiological, geographic, and phylogenetic data to reconstruct the processes behind infectious disease emergence and spread through time and space. These methods are of particular use for investigating the emergence and spread of viruses, as they have a rapid evolutionary rate that closely matches the timescale of virus transmission. I began to explore these approaches during my PhD and have now successfully used them to: (i) explore the genetics underpinning the rapid emergence and spread of human enterovirus 68; (ii) examine the patterns and processes that influence the diversity and geographic distribution of New World hantaviruses; (iii) explore the epizoology of porcine circovirus 2; iv) understand the evolutionary and ecological drivers behind the distribution of bluetongue virus in Australia; and (v) assess the likely origins of hepatitis C virus.

a. **Firth C**, Blasdell KR, Amos-Ritchie R, Sendow I, Agnihotri K, Boyle DB, Daniels P, Kirkland PD, Walker PJ. 2017. Genomic analysis of bluetongue virus episystems in Australia and Indonesia. Veterinary Research 48: 82. PMCID: PMC5701493

b. **Firth C,** Tokarz R, Simith DB, Nunes MR, Bhat M, Rosa ES, Medeiros DB, Palacios G, Vasconcelos PF, Lipkin WI. 2012. Diversity and distribution of hantaviruses in South America. Journal of Virology 86: 13756-66. PMCID: PMC3503106

c. Tokarz R, **Firth C**, Madhi SA, Howie SR, Wu W, Sall AA, Haq S, Briese T, Lipkin WI. 2012. Worldwide emergence of multiple clades of enterovirus 68. Journal of General Virology 93: 1952-8. PMCID: PMC3542132

d. Kapoor A, Simmonds P, Gerold G, Qaisar N, Jain K, Henriquez JA, **Firth C**, et al. 2011. Characterization of a canine homolog of hepatitis C virus. Proceedings of the National Academy of Sciences 108: 11608-13. PMCID: PMC3136326

e. **Firth C**, Charleston MA, Duffy S, Shapiro B, Holmes EC. 2009. Insights into the evolutionary history of an emerging livestock pathogen: porcine circovirus 2. Journal of Virology 83: 12813-21. PMCID: PMC2786836

Complete List of Published Work in MyBibliography:
https://www.ncbi.nlm.nih.gov/myncbi/1pcDCsap4HMk2/bibliography/public/

BIOGRAPHICAL SKETCH

NAME: Firth, Cadhla

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6)

POSITION TITLE: Senior Research Scientist and Program Coordinator, EcoHealth Alliance

D. Additional Information: Research Support and/or Scholastic Performance

Pending Research Support

National Institute Health (Grant 13220428) Daszak P (PI) 2021-2026

Title: Analyzing the potential for future bat coronavirus emergence in Southeast Asia.

Role: Co-Investigator. My role will include project co-ordination across all country partners, data collection, management and analysis, the preparation of manuscripts, and the communication of results to partners and the public.

Goal: The goal of this project is to build a platform that pinpoints the risk of future coronavirus emergence in the region where SARS-CoV-2 likely originated, informs strategies to intervene and prevent future pandemics, and provides critical reagents, therapeutic interventions and recombinant viruses for public health preparedness against future SARS- and COVID-like events.

Current Research Support

National Institutes of Health (1U01AI151797) Daszak P (PI) 2020-2025

Title: Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of South East Asia.

Role: Key Personnel. My role is to help drive the research-focused agenda of the project, including supporting in-country partners in Southeast Asia and developing new collaborations to support project goals. I am also responsible for assisting in data collection, management and analysis, and the preparation of manuscripts for publication.

Goal: The goals of this project are to identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife; identify evidence of novel virus spillover and key risk pathways for transmission; and identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts.

Tropical Australian Academic Health Centre Research Seed Grant McBride J (PI) 2019-2021

Title: Can portable genome sequencing provide a rapid, comprehensive, point-of-care diagnostic test for Far North Queensland hospitals and healthcare centers?

Role: Co-Principal Investigator. My role is project management and testing of the resultant platform *in situ* at Cairns Hospital. I am also leading data analysis and interpretation, and liaising with the clinical co-investigators throughout the project.

Goal: To trial the clinical metagenomics platform we developed as a point-of-care diagnostic test for fever, sepsis, and pneumonia at Cairns Hospital, and test the hypothesis that this approach will increase the proportion of infections that are diagnosed while also reducing time-to-diagnosis.

Previous Research Support

QIMR Berghofer Coronavirus Research Grant Reid D (PI) 2020

Title: Prognostics, Diagnostics and Therapeutic targets in response to infectious threats to the Australian population; (Phase 1) COVID-19.

Role: Co-Investigator. My role was to sequence the genomes of SARS-CoV-2 from patients and perform relevant analyses.

Goal: To predict clinical outcomes from SARS-CoV-2 infection using a combination of immunological and pathogen data collected from nasopharyngeal swabs, blood, and urine samples.

Tropical Australian Academic Health Centre Research Assistance Brown J (PI) 2020

Title: Building Research Support for Cairns Hospital.

Role: Co-Investigator. My role was to collaborate with and support clinicians engaging in infectious disease research, and help build a culture that fosters scientific research.

Goal: Cairns Hospital is a regional facility serving as a referral hospital for remote communities across Far North Queensland, yet does not have a strong culture of scientific research. This grant supported collaborations between academics and clinicians working in emerging infectious diseases in this region to improve research output for rural/remote Australia.

HOT North Pilot Project Grant Firth C (PI) 2019-2020

Title: Portable genome sequencing as a point-of-care diagnostic test in remote tropical Australia

Role: Principal Investigator. My role was to design the patient and sampling strategies, design a laboratory workflow suitable for uptake in a clinical diagnostic lab, assist in the development of novel bioinformatics pipelines for clinical metagenomics, and validate the approach.

Goal: To develop a clinical metagenomics ID diagnostic platform suitable for use in rural and remote hospitals and health services in tropical Northern Australia.

Australian Research Council Discovery Early Career Researcher Firth C (PI) 2015-2018

Title: Characterizing the impact of urbanization on viral diversity, ecology and disease emergence

Role: Principal Investigator. My role was project design and management, fieldwork and sample collection, metagenomic sequencing, data analysis and interpretation.

Goal: This project examined the viral response to changes in reservoir host and vector population structure and dynamics that occur as a result of urbanization, and identified viral characteristics associated with survival in an urban environment in Southeast Asia.

NIEHS Center for Environmental Health Pilot Project Grant Firth C (PI) 2013

Title: Identification of rodent-borne human pathogens in an urban environment and the features that influence their presence and prevalence

Role: Principal Investigator. My role was project design and management, fieldwork and sample collection, metagenomic sequencing, data analysis, and interpretation.

Goal: To characterize the zoonotic pathogens present in rats and their ectoparasites in New York City.

BIOGRAPHICAL SKETCH

NAME: Rabaa, Maia Anita

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6)

POSITION TITLE: University Research Lecturer in Medicine, Molecular Epidemiology Group Head

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	Completion Date	FIELD OF STUDY
Walsh University	BS	05/2001	Biology
Johns Hopkins Bloomberg School of Public Health	MHS	05/2006	International Health, Disease Epidemiology and Control
The Pennsylvania State University	PhD	12/2012	Biology

A. Personal Statement

I have the experience, expertise, training, and motivation needed to successfully support the proposed research project to analyze viral diversity and cross-species transmission on mixed-species farms in Viet Nam. I am a molecular epidemiologist at OUCRU Viet Nam. I have 8 years of experience in the design and implementation of research in human and animal health in Viet Nam, with a focus on emerging infections, viral zoonoses and antimicrobial resistance. I currently lead the Molecular Epidemiology group at OUCRU: a group of 28 staff (including one postdoctoral scientist, PhD students, clinicians, research assistants and technicians, nurses, and experienced project coordinators).

My primary research interests are laboratory, community, and hospital-based studies investigating the epidemiology, ecology, and evolution of pathogens in human populations and at the animal-human interface. My postdoctoral work focused on the development and implementation of a countrywide study of virus epidemiology and viral zoonoses, called VIZIONS, in which I designed and implemented scientific research and assisted in the management of collaborations across 7 Vietnamese hospitals and 3 local and regional animal health offices. I am now the PI on laboratory work and analyses of samples collected within the VIZIONS study, including identification of pathogens in farmed and wild animal populations. These studies use genomics, epidemiology, clinical, laboratory, and field science to test hypotheses on the emergence of viral zoonoses. Project coordinators currently on my team also worked on the VIZIONS study and maintain valuable links to collaborating institutions focused on human and animal health across the country. My team's previous experience and our nationwide network of collaborators will facilitate a scientifically rigorous study with important buy-in from local communities and health authorities. I have managed various institutional grants and delivered a number of significant and highly visible publications focused on a portfolio of varied pathogens and public health issues.

Further, I am committed to providing training and mentorship for low- and middle-income country researchers. In addition to junior research staff, I have supervised 3 PhD students to completion (University of Oxford [UK], Open University [UK]), and am currently supervising 2 PhD students (University of Oxford [UK]) and 1 postdoctoral scientist.

a. **Rabaa MA**, Ngo TT, Tran MP, Carrique-Mas J, Saylors K, Cotten M, Bryant JE, Ho DTN, Nguyen VC, Ngo TH, Wertheim H, Nadjm B, Monagin C, van Doorn HR, Rahman M, Campbell, Boni MF, Pham TTT, Simmonds P, Rambaut A, Nguyen VVC, Wolfe ND, Kellam P, Farrar J, Tran TH, Thwaites GE, Woolhouse MEJ, Baker S. (2015) The Vietnamese initiative on zoonotic infections (VIZIONS): a strategic approach to studying emerging zoonotic infectious diseases across Vietnam. **EcoHealth**. 12(4):726-35.

b. Pham HA, Nguyen VC, Nguyen ST, Ngo TT, Kosoy M, Woolhouse MEJ, Baker S, Bryant JE, Thwaites G, Carrique-Mas JJ, **Rabaa MA**. (2015) Diversity of *Bartonella* identified in bats in southern Vietnam. **Emerging Infectious Diseases**. 21(7):1266-67.

c. Phan M, Anh PH, Cuong NV, Munnink BO, van der Hoek L, Phuc TM, Tue NT, Bryant JE, Baker S, Thwaites G, Woolhouse ME, Kellam P, **Rabaa MA**, and Cotten M. (2016) Unbiased whole-genome deep sequencing of human and porcine stool samples reveals circulation of multiple rotavirus genogroups and putative zoonotic infection. **Virus Evolution**. 2(2):vew027.

d. Berto A, Pham HA, Carrique-Mas JJ, Simmonds P, Van Nguyen C, Tri TN, Nguyen DV, Woolhouse ME, Smith I, Marsh GA, Bryant JE, Thwaites GE, Baker S, **Rabaa MA**. (2017) The detection of potentially novel paramyxoviruses and coronaviruses in bats and rats in the Mekong Delta region of southern Viet Nam. **Zoonoses and Public Health**. 65(1):30-42.

B. Positions and Honors

YEAR(S)	POSITION
2007-2008	Research Associate at the Fogarty International Center, U.S. National Institutes of Health, Maryland, USA
2008-2009	Campbell Distinguished Graduate Fellowship, The Pennsylvania State University, USA
2009-2012	Graduate Research Fellowship, National Science Foundation, USA
2012-2015	Postdoctoral Research Fellow at the University of Edinburgh, Edinburgh, UK
2012-2020	Lead Molecular Epidemiologist at OUCRU, Ho Chi Minh City, VN
2015-2020	Research Fellow at the Nuffield Department of Medicine, University of Oxford, UK
2020-present	Molecular Epidemiology Research Group Head at OUCRU, Ho Chi Minh City, VN
2020-present	University Research Lecturer at the Nuffield Department of Medicine, University of Oxford, UK

C. Contributions to Science

1. Pathogen diversity and exchange at the human-animal interface

With Southeast Asia considered a hub of zoonotic transfer, I, with colleagues at OUCRU, the University of Edinburgh, and the Sanger Institute, sought to track the frequency and risks for pathogen transfer at the human-animal interface. I played a pivotal role in designing and implementing a national hospital- and community-based study of zoonotic infections in Viet Nam, driving a number of substudies and conducting analyses investigating pathogen evolution and epidemiology. These studies have uncovered frequent zoonotic infections presenting in clinical settings, and I am now the PI for several continuing analyses using archived samples and data to perform virological and serological investigations of zoonotic infections, as well as investigations of pathogen diversity in farmed and wild animal populations, including bats.

a. Van Nguyen VC, Le Buu C, Desquenes M, Herder S, Nguyen PHL, Campbell JI, Nguyen VC, Yimming B, Chalermwond P, Jittapalapong S, Franco JR, Ngo TT, **Rabaa MA**, Carrique-Mas JJ, Thanh TPT, Nga TVT, Berto A, Hoa NT, Hoang NVM, Tu NC, Chuyen NK, Wills B, Hien TT, Thwaites GE, Yacoub S, Baker S. (2016) A clinical and epidemiological investigation of the first reported human case of the zoonotic parasite *Trypanosoma evansi* in Vietnam. **Clinical Infectious Disease**. 62(8):1002-8.

b. Lu L, Dung NV, Ivens A, Bogaardt C, O'Toole A, Bryant JE, Carrique-Mas JC, Cuong NV, Anh PH, **Rabaa MA**, Tue NT, Thwaites GE, Baker S, Simmonds P, Woolhouse ME. (2018) Genetic diversity and cross-species transmission of kobuvirus in Vietnam. **Virus Evolution**. 4(1):vey002.

c. Campbell JI, Lan NPH, Phuong PM, Buu Chau L, Duc TP, Guzmán-Verri C, Ruiz-Villalobos N, Tam PTM, Muñoz Álvaro PM, Moreno E, Thwaites GE, **Rabaa MA**, Vinh Chau NV, Baker S. (2017)

Human *Brucella melitensis* infections in southern Vietnam. **Clinical Microbiology and Infection**. 23(11):788-790.

d. Tra My PV, **Rabaa MA**, Donato C, Cowley D, Vinh PV, Ngoc DT, Pham HA, Bryant JE, Woolhouse ME, Kirkwood CD, Baker S. (2014) Novel porcine-related G26P[19] rotavirus identified in pediatric diarrhea patients in Ho Chi Minh City. **Journal of General Virology**. 95(12):2727-33.

2. Evolution and epidemiology of flaviviruses

In my PhD and postdoctoral work, I performed detailed phylogenetic and epidemiological analyses of flavivirus populations to identify the determinants of novel flavivirus emergence in endemic and epidemic settings. This research showed the utility of connecting epidemiological and genomic data in understanding the spread of vector-borne disease, identifying risk factors (population connectivity, immunity, and climate) for flavivirus emergence at various spatial scales. Genomic studies of viruses sampled during phase III dengue vaccine trials showed that dengue virus genetics play a limited role in determining vaccine efficacy and identified genomic regions of the virus that may be under selection for vaccine escape. In these studies, I designed sampling strategies and conducted analyses of sequence data, clinical and epidemiological surveillance data, and geographical, climatological, and census data.

a. **Rabaa MA**, Girerd-Chambaz Y, Hue KDT, Tuan TV, Wills B, Bonaparte M, van der Vliet D, Langevin E, Cortes M, Zambrano B, Dunod C, Wartel-Tram A, Jackson N, Simmons CP. (2017) Genetic epidemiology of dengue virus populations in phase III trials of the CYD tetravalent dengue vaccine and implications for efficacy. **eLife**. 6:e24196.

b. Ho ZJM, Hapuarachchi HC, Barkham T, Chow A, Ng LC, Lee JMV, Leo YS, Prem K, Lim YHG, de Sessions PF, **Rabaa MA**, Chong CS, Tan CH, Rajarethinam J, Tan J, Anderson DE, Ong X, Cook AR, Chong CY, Hsu LY, Yap G, Lai YL, Chawla T, Pan L, Sim S, Chen IM, Thoon KC, Yung CF, Li JH, Ng HLD, Nandar K, Ooi PL, Lin RTP, Aw P, Uehara A, Pratim De P, Soon W, Hibberd ML, Ng HH, Maurer-Stroh S, Sessions OM. (2017) Outbreak of Zika in Singapore – an epidemiological, entomological, virological and clinical account. **Lancet Infectious Diseases**. 17(8):813-821.

c. **Rabaa MA**, Simmons CP, Fox A, Le MQ, Nguyen TTT, Le HY, Gibbons RV, Nguyen XT, Holmes EC, Aaskov JG. (2013) Dengue virus in sub-tropical northern and central Viet Nam: population immunity and climate shape patterns of viral invasion and maintenance. **PLoS Neglected Tropical Disease**. 7(12):e2581

d. **Rabaa MA**, Hang VTT, Wills B, Farrar J, Simmons CP, Holmes EC. (2010) Phylogeography of recently emerged DENV-2 in southern Viet Nam. **PLoS Neglected Tropical Disease**. 4(7):e766.

3. Mechanisms and determinants of antimicrobial resistance (AMR) emergence

Continual emergence of antimicrobial resistance is among the greatest threats to public health. As lead molecular epidemiologist at OUCRU, I have led various investigations of the genetics of AMR and its global spread. Working with collaborators across Asia, I drive both computational and lab-based experimental evolution studies to determine the drivers of the emergence and maintenance of AMR in human and animal populations. These studies have shown that plasmid transfer from healthy human gut microbiota can drive the emergence of resistance in pathogenic *Shigella*, and that inappropriate antimicrobial usage may enhance the frequency of plasmid transfer. Our studies also identify South and Southeast Asia, where inappropriate antimicrobial usage is rampant, as important hotspots of AMR emergence.

a. Duy PT, Nguyen TNT, Duong VT, Chung The H, Boinett C, Thanh HND, Tuyen HT, Thwaites GE, **Rabaa MA***, Baker S*. (2020) Commensal Escherichia coli are a reservoir for the transfer of XDR plasmids into epidemic fluoroquinolone-resistant *Shigella sonnei*. **Nature Microbiology**. doi:10.1038/s41564-019-0645-9.

b. Chung The H, Boinett C, Duy PT, Jenkins C, Weill F-X, Howden BP, Valcanis M, De Lappe N, Cormican M, Wangchuk S, Bodhidatta L, Mason CJ, Nguyen TNT, Tuyen HT, Phat VV, Duong VT, Lan NPH, Turner P, Wick R, Ceyssens P-J, Thwaites GE, Holt KE, Thomson NR, **Rabaa MA***, Baker S*. (2019) Dissecting the molecular evolution of fluoroquinolone-resistant *Shigella sonnei*. **Nature Communications**. 10:4828.

c. The HC, **Rabaa MA**, Thanh DP, Delappe N, Cormican M, Valcanis M, Howden BP, Wangchuk S, Bodhidatta L, Mason CJ, Nguyen TNT, Thuy DV, Thompson CN, Nguyen PHL, Phat VV, Thanh TH, Turner P, Sar P, Thwaites G, Thomson NR, Holt KE, Baker S. (2016) South Asia as a reservoir for the global spread of ciprofloxacin resistant *Shigella sonnei*. **PLoS Medicine**. 13(8):e1002055.

d. The HC, Karkey A, Thanh DP, Boinett CJ, Cain AK, Ellington M, Baker K, Dongol S, Thompson C, Harris SR, Jombart T, Phuong TLT, Hoang NTD, Joshi S, Basnyat B, Thwaites GE, Thomson NR*, **Rabaa MA***, Baker S*. (2015) A high-resolution genomic cross-sectional analysis of dual multi-drug resistant hospital outbreaks of *Klebsiella pneumoniae* in a low-income setting. **EMBO Molecular Medicine**. 7(3):227-39.

4. Typhoid epidemiology, genomics, and the role of carriage in transmission

A better understanding of the role of genetics in typhoid epidemiology is needed to improve diagnostics, treatment, and control, particularly in the context of introduction of typhoid conjugate vaccine in endemic regions. I have recently led and collaborated on studies that have utilized bacterial genomics to investigate the relationships between treatment failure, AMR, and vaccination, as well as to identify the selective pressures induced by gallbladder carriage and to better characterize the potential roles of typhoid carriers in typhoid transmission following large-scale vaccination. Our studies have shown that novel emerging clades of AMR *Salmonella* Typhi can rapidly undermine accepted treatment regimens; these data have resulted in improved treatment and diagnostic policies across Nepal. Novel studies of gallbladder carriage are expected to inform typhoid surveillance, including the identification and treatment of typhoid carriers, thus aiding elimination efforts.

a. Duy PT, Nga TVT, To NTN, Dan Thanh HN, Dongol S, Karkey A, Carey M, Basnyat B, Thwaites GE, **Rabaa MA***, Baker S*. Gallbladder carriage generates genetic variation and genome degradation in *Salmonella* Typhi. **PLoS Pathogens**. 16(10):e1008998.

b. Duy PT, Dongol S, Giri A, To NTN, Dan Thanh HN, Quynh NPN, Trung ND, Thwaites GE, Basnyat B, Baker S, **Rabaa MA***, Karkey A*. The emergence of azithromycin-resistant *Salmonella* Typhi in Nepal. **JAC-Antimicrobial Resistance**. 2(4):dlaa109.

c. Dyson ZA, Thanh DP, Bodhidatta L, Mason CJ, **Rabaa MA**, Phat VV, Tuyen HT, Thwaites GE, Baker S, Holt KE. (2017) Whole genome sequence analysis of *Salmonella* Typhi isolated in Thailand before and after the introduction of a national immunization program. **PLoS Neglected Tropical Diseases**. 11(1):e0005274.

d. Pham Thanh D, Karkey A, Dongol S, Thi NH, Thompson CN, **Rabaa MA**, Aryjal A, Holt KE, Wong V, Nga TVT, Phat VV, Tuyen HT, Pradhan A, Shrestha SK, Gajurel D, Pickard D, Parry CM, Dougan G, Wolbers M, Dolecek C, Thwaites G, Basnyat B, Baker S. (2016) A novel ciprofloxacin-resistant subclade of H58 *Salmonella* Typhi is associated with fluoroquinolone treatment failure. **eLife**. 5:e14003.

* equal contribution

Complete List of Published Work in MyBibliography:
https://www.ncbi.nlm.nih.gov/myncbi/1292rmtPpBsAK/bibliography/public/

BIOGRAPHICAL SKETCH

NAME: Rabaa, Maia Anita

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6)

POSITION TITLE: University Research Lecturer in Medicine, Molecular Epidemiology Group Head

D. Additional Information: Research Support and/or Scholastic Performance

Pending Research Support

NIAID/NIH (Grant 13220428) Daszak (PI) 07/2021 – 06/2026

Title: Analyzing the potential for future bat coronavirus emergence in Southeast Asia

Goal: The overall goal of this study is to build a platform to pinpoint the risk of future coronavirus emergence in a region where COVID-19 likely originated, inform strategies to intervene and prevent future pandemics, and provide critical reagents, therapeutic interventions, and recombinant viruses for public health preparedness against future SARS- and COVID-like events.

Role: Co-Investigator (country PI)

Pfizer (Investigator Sponsored Research: Pre-clinical/Clinical) Pham (PI) 03/2021 – 03/2023

Title: The bacterial etiology and antimicrobial susceptibility patterns of lower respiratory and intra-abdominal infections in Viet Nam

Goal: The goal of this study is to characterize and compare the microbiology, antimicrobial resistance patterns, and clinical outcomes of lower respiratory tract and intra-abdominal infections in 6 metropolitan hospitals in Viet Nam between 2019 and 2020.

Role: Co-Investigator

Current Research Support

COVID-19 Research Response Fund, University of Oxford Baird (PI) 05/2020 – 03/2021

Title: Validating GeneXpert platform for SARS-CoV-2 diagnosis for remote sites in Eastern Indonesia

Goal: The goal is to validate the GeneXpert platform using various sample types and to estimate the prevalence of SARS-CoV-2 infection among patients seeking treatment for febrile illness in Sumba, Indonesia.

Role: Co-Investigator

Research grant, Oxford University Clinical Research Unit Rabaa (PI) 02/2020 – 12/2021

Title: Molecular epidemiology of K. pneumoniae in bloodstream infections

Goal: The goal is to investigate the epidemiology and evolution of *K. pneumoniae* causing bloodstream infections in southern Viet Nam from 2010-2019.

Role: PI

Research grant, Oxford University Clinical Research Unit Rabaa (PI) 02/2020 – 12/2021

Title: A novel molecular assay for simultaneous detection of pathogen and AMR genes

Goal: The goal is to develop a rapid, inexpensive assay that will allow for rapid screening and pathogen/AMR diagnostics for bloodstream infections in resource-poor hospitals.

Role: PI

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List of abbreviations, acronyms, symbols

AGP: antimicrobial growth promoter
AMR: antimicrobial resistance
AMU: antimicrobial usage
AUS: Australia
BS: Bachelor of Science
BSL: biosafety level
CAN: Canada
CREID: Centers for Research in Emerging Infectious Diseases
DNA: deoxyribonucleic acid
Duke-NUS: Duke-National University of Singapore
DVM: Doctor of Veterinary Medicine
EHA: EcoHealth Alliance
EID: emerging infectious disease
EID-SEARCH: The Emerging Infectious Disease – South East Asia Research Collaboration Hub
ELISA: enzyme-linked immunosorbent assay
FACS: fluorescence-activated cell sorting
GCP: good clinical practices
GLM: generalized linear model
GLP: good laboratory practices
HCMC: Ho Chi Minh City
HPLC: high-performance liquid chromatography
HRSC: high-risk human sentinel cohort
HUTECH: Ho Chi Minh University of Technology
IT: information technology
IUCN: International Union for Conservation of Nature
MHS: Master of Health Science
MoH: Ministry of Health, Viet Nam
MS: Master of Science
MSc: Master of Science
NGS: next-generation sequencing
NIAID: National Institute of Allergy and Infectious Diseases
NIH: National Institutes of Health
NMDS: non-metric multidimensional scaling
NY: New York State
OUCRU: Oxford University Clinical Research Unit
OxTREC: Oxford Tropical Research Ethics Committee
PA: Pennsylvania State
PCR: polymerase chain reaction
PhD: Doctor of Philosophy
PI: principal investigator
PMC: Preventative Medicine Center
RAHO: Regional Animal Health Office
RNA: ribonucleic acid
RSEM: RNA-Seq by expectation-maximization
RT-PCR: real-time PCR
sDAH: sub-departments of Animal Health

SEA: Southeast Asia
TB: terabyte
UK: United Kingdom
UNIMAS: Universiti Malaysia Sarawak
USA: United States of America
ViPARC: Vietnamese Platform for Antimicrobial Reduction in Chicken production
VIZIONS: Vietnamese Initiative for Zoonotic Infections
VN: Viet Nam
VPN: virtual private network
WGS: whole genome sequencing

Facilities, Existing Equipment, and Other Resources

Laboratory:

OUCRU, in partnership with the Hospital for Tropical Diseases, have established state-of-the-art clinical diagnostic, basic, and applied research laboratories. The laboratory facilities include: clinical laboratories (ISO 15189:2012 certified), BSL2 research laboratories (GLP and GCLP compliance), BSL3 laboratories (MoH, Viet Nam certified), and a bio-archiving facility (Biosecurity compliance).

OUCRU laboratories in HCMC and Hanoi are collectively equipped with 29 safety cabinets (fume hoods, biosafety category II and III), 21 shaking and stationary incubators, 36 centrifuges including refrigerated centrifuges that accommodate swinging bucket and fixed angle rotors, six refrigerated microfuges, 12 thermocyclers, five real-time PCR machines, four UV/Visible microplate readers, a gel imaging platform (chemiluminescence, UV, and visible light), gel boxes, water baths, and mixing devices, 11 water-jacketed CO_2 incubators, five inverted microscopes, three fluorescence-activated flow cytometers (one with cell sorting capability), three capillary DNA sequencers, two Illumina MiSeq sequencers, Oxford Nanopore MinION sequencers, two HPLC systems, one GenXpert machine, one ultra-centrifuge, two Chef Mapper XA Systems, one Luminex Flexmap 3D, one Bio-Plex Suspension Array System, 26 -80°C freezers, 77 -20°C freezers, 33 refrigerators, eight liquid nitrogen tanks, a liquid nitrogen shipper, ice machines, autoclaves, and film developers.

Molecular Epidemiology research is conducted in several facilities across the laboratories including a specimen processing facility, a molecular diagnostic facility (GeneXpert and RT-PCR based diagnosis), a genomics facility (NGS facility using Illumina MiSeq and Oxford Nanopore MinION systems), and an immune-biology facility (ELISA, Luminex, FACS Lyrics system, Fluoresce microscopy, and cell culture facility). High-risk laboratory processes are conducted in the BSL3 laboratory. OUCRU maintains a bio-archiving facility (-20°C, -80°C, and liquid nitrogen storage facility) containing 2.5 million samples with continuous temperature monitoring and sample tracking system (Lab guard system, Freezerworks system and biometric access control system).

Clinical:

NA

Animal:

NA

Computer:

OUCRU employees have around-the-clock access to servers, VPN, encryption software, IT support, and all necessary software including: Git and Github (Hosted software revision/audit service), BBEdit text editors, Oracle Virtualbox virtual machines, Google Apps (Hosted email and collaboration web based software), Python, NodeJS, and R programming languages, Meteor (Javascript framework), Bash shell scripts, Jenkins (Continuous Integration server), Microsoft Office and Adobe CS6 running on Apple Mac OS X, Ubuntu Linux, and Windows Operating Systems. OUCRU has a dedicated 150+ core Linux server with 8TB hard drives, and the Molecular Epidemiology group has two dedicated 16-core Mac Pro Servers with 4TB hard drives. Either server individually or in combination may be used for intensive sequence analysis, computational modeling and/or database processing by Dr. Nguyen. Access to supercomputing services is maintained by a dedicated staff member from the IT team.

Office:

The applicant will have a dedicated desk in the office of the Molecular Epidemiology Group under the supervision of Drs. Maia Rabaa and Duy Pham Thanh, with expertise in both wet and dry laboratory research.

Other:

NA

Applicant Name (Last, first, middle): Nguyen, Cuong, Van

Letter of Organizational Support

28th February 2021

Dear CREID pilot research program funding committee,

Proposal from Nguyen Van Cuong

As Director of the Oxford University Clinical Research Unit (OUCRU) in Vietnam I would like to confirm my strongest possible support for the application from Nguyen Van Cuong.

Cuong has worked at OUCRU in Ho Chi Minh City since 2011, first as a Research Assistant and Project Coordinator for the Wellcome Trust funded Vietnam Initiative on Zoonotic infections (VIZIONS) project, and since 2017 as a PhD student and Project Coordinator for the ViPARC project (a Veterinary Intervention to Reduce Antimicrobial Usage in Animal Production). He has submitted an excellent PhD thesis (Open University, UK) and is scheduled to defend it on 15 March, 2021. In Cuong's tenure at OUCRU, he has developed strong skills in veterinary epidemiology, project coordination, and biostatistical analysis, along with varied wet laboratory skills. He will make an excellent post-doctoral researcher.

As Cuong moves into his postdoctoral career, the CREID pilot research program presents an excellent opportunity for him to grow in his independence as a scientist while gaining new scientific and program management skills as principal investigator of the proposed project. The proposed project will allow Cuong to develop additional expertise in study design, metagenomic sequencing, and bioinformatics. Additionally, the built-in mentorship program and regional and global networks that will be established through this collaboration with EcoHealth Alliance and the EID-SEARCH team will aid Cuong in designing and developing future impactful research on emerging infectious diseases at the human-animal interface, a topic that is in line with both Cuong's personal scientific agenda and the mission of OUCRU.

If funding is obtained for Cuong's proposal from the CREID pilot research program, OUCRU is committed to providing sufficient support to complete the project. Under this scheme, Cuong will have 100% protected time to complete the laboratory and analytical work to achieve the aims of this 1-year study, while working as a postdoctoral researcher within the OUCRU Molecular Epidemiology group, jointly led by Drs. Maia Rabaa and Duy Pham Thanh.

Cuong will have access to a remarkable collection of archived samples and data from the VIZIONS project for screening and downstream laboratory work, dedicated laboratory and office space within the OUCRU Molecular Epidemiology group, and access to shared laboratory spaces, computational resources, and equipment needed to complete the project. His research will be aided by existing laboratory and analytical expertise in the Molecular Epidemiology group, and a research assistant with experience in viral screening and sequencing will be employed to assist in sample processing and data management. As a member of the Molecular Epidemiology group, Cuong will participate in biweekly lab meetings, with regular opportunities to present and receive feedback on his work. He will also benefit from participation in extensive unit-wide academic and training programs.

I believe that the CREID pilot program will provide Cuong with a unique platform from which to develop veterinary epidemiology research within Viet Nam and across Southeast Asia. He would utilize the

knowledge and experience gained under this program to encourage and mentor future cohorts of scientists, veterinarians, and animal health officials in the pursuit of emerging infectious disease and OneHealth research and interventions to improve human and animal health in Viet Nam. He has my complete support.

Yours Faithfully,

Professor Guy Thwaites FRCP FRCPath FMedSci

Director, Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam;

Professor of Infectious Diseases, University of Oxford

Letter of Collaboration from CREID Research Center PI

Dr. Nguyen Van Cuong
Project Coordinator
Oxford University Clinical Research Unit
Ho Chi Minh City, Viet Nam

Dear Dr. Nguyen,

The Emerging Infectious Disease – South East Asia Research Collaboration Hub (EID-SEARCH) at EcoHealth Alliance is extremely interested in working with you and your collaborators at the Oxford University Clinical Research Unit (OUCRU) in Viet Nam on the proposed research: "*Multi-species farming, viral diversity, and risks of cross-species transmission in the Central Highlands of Viet Nam*".

This collaboration will significantly contribute to the goal of the CREID Network and EID-SEARCH to develop in-country research capacity and build the research network for emerging infectious diseases in Southeast Asia. It will also form the foundation for new partnerships among EID-SEARCH, OUCRU and other in-country stakeholders in Viet Nam, which will strengthen international and multi-sectoral cooperation for emerging infectious disease research in Southeast Asia. Further, the networking, training, and mentorship opportunities embedded within the Pilot Research Program will develop your own skills in mentorship, project management, and supervision within a supportive framework, and grow your network both within and outside of Viet Nam.

Beyond capacity and network building, the proposed research strongly aligns with the core of EID-SEARCH's scientific goals by exploring links between wildlife farming and several determinants of spillover risk, including farming practices and wildlife species diversity. By using an unbiased approach to virus surveillance and targeting a high-risk human-animal interface (wildlife farming), the proposed work has the potential to make a significant contribution to our understanding of the drivers behind cross-species transmission and infectious disease emergence. I am confident that this work will lead to at least one high-quality publication on the highly relevant topic of wildlife and emerging infectious diseases, particularly given the links between hunting, farming, and trading wildlife and the emergence of new zoonotic diseases, as highlighted by the ongoing COVID-19 pandemic.

I have high expectations for this collaborative research project and expect that this will only be the beginning of our work together. Members of EID-SEARCH (including myself and Dr Kevin Olival as PI and CI of EID-SEARCH, respectively) are committed to working closely with you to develop the research project and support the efforts needed for the success of this application and project implementation. Our Senior Research Scientist, Dr. Cadhla Firth, has enthusiastically joined the project as your Mentor to advise on study design, data collection, and analysis throughout the project. Dr Firth is committed to providing relevant and timely career advice to assist in your professional development, and brings a well-established network across Southeast Asia, Australia, and North America, which includes world leaders in the ecology and evolution of emerging infectious diseases. You will be invited to join all trainings conducted by EID-SEARCH and EcoHealth Alliance on emerging infectious disease field surveillance and statistical analysis to build your skillset, and Dr Firth will work closely with you to develop your skills in pathogen genetics and genomics. EID-SEARCH will also commit funding and arrange exchange opportunities for you to visit our partners in Singapore to expand your skill set in

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sequencing and serology. You will be supported to present the results of this research at international conferences, as well as meetings with CREID Network and external partners, and to produce high-quality publications from this research.

I am confident that the proposed research collaboration represents a genuine opportunity for your professional development, particularly as you will have successfully defended your PhD on March 15 2021, and are ready to transition to a role with more scientific independence. I am particularly impressed with your extensive work history at the human-animal interface of emerging infectious disease, including as a Program Coordinator with the VIZIONS project, and am confident that you have already begun to emerge as a future leader in this field in Viet Nam.

This letter conveys my strong interest and commitment to making this application a success, and I look forward to working with you on this research project.

Sincerely,

Peter Daszak
Principal Investigator, EID-SEARCH
President, EcoHealth Alliance

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Letter from Mentor

EcoHealth Alliance

Dr. Nguyen Van Cuong
Project Coordinator
Oxford University Clinical Research Unit
Ho Chi Minh City, Viet Nam

Dear Dr. Nguyen,

This letter is to express my support as your Mentor for your application to the CREID Pilot Research Program, entitled "*Mixed-species farming, viral diversity, and risks of cross-species transmission in the Central Highlands of Viet Nam*."

I am extremely excited that you have chosen me to be your Mentor for the proposed project. The research question is timely and important, and your work has the potential to have a strong impact on our understanding of potential disease risks associated with wildlife farming. Given what we understand to be the likely origins of SARS-CoV-2, there has never been a more pressing need to examine the links between our interactions with wildlife and emerging zoonotic diseases.

I would be hard-pressed to think of a more appropriate applicant to lead this project, given your background in veterinary medicine, epidemiology, and project management. The skills you have acquired during your previous work on the VIZIONS project and throughout your PhD will be enhanced by the Mentorship plan we have developed together. It has become clear to me as we have worked on the Pilot Project application that you are ready to make the transition from a junior scientist to an independent researcher capable of driving your own research projects, and I look forward to the opportunity to support you in this process. Towards this aim, I commit to supporting your professional development in the following ways:

- I will actively work to maintain the healthy, open, and informal dialogue we have established while drafting the project proposal, and I will seek feedback from you on a regular basis to ensure that our communication plan continues to meet your needs as the project progresses.
- I will engage with your current and future in-country collaborators in a supportive and inclusive manner.
- I will create opportunities for you to interact with scientists across my network through your research project to widen your exposure to people with a range of expertise.
- I will give you timely feedback on all material in the manner that works best for you.
- I will provide you with opportunities to present your work to my colleagues at EcoHealth Alliance during our academic meetings and include you in any relevant trainings.
- I will share my scientific and technical expertise with you to help ensure the success of the project. When skills outside my area are required, I will assist you in accessing this expertise from scientists within my network.
- I will assist you in career planning, providing advice and support as required to maximize your opportunity to succeed.

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One of the likely outcomes from the proposed research project will be the generation of pilot data that can form the basis for additional funding applications. The President of EcoHealth Alliance, Peter Daszak, has already expressed his support for a joint funding application that will follow on from the proposed project, with you as the lead PI. This will present the perfect opportunity for you to complete the transition to an independent scientist, and I am committed to assisting you through the process of writing your first funding applications as lead investigator.

There is significant overlap between your research interests and skills, the overall goals of OUCRU, and those of EcoHealth Alliance and the EID-SEARCH team. This pilot project presents a real opportunity for you to become a liaison between these organizations, and to help drive forward future collaborative research projects in Viet Nam. I am thrilled to be able to support you at this critical stage in your career and am genuinely convinced that you have the potential to become a science leader in Southeast Asia in the very near future.

Sincerely,

(b) (6)

Cadhla Firth, PhD
Senior Scientist and Program Coordinator
EcoHealth Alliance

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Proposal Cover Sheet

Project Title	**In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance**
Principal Investigator Name	Jurre SIEGERS
Position/Title	Postdoctoral scientist
Department	Virology Unit
Institution Name	Institute Pasteur du Cambodge
Street	5 Monivong Blvd
City, State, Zip Code	Phnom Penh
Country	Cambodia
Email	jsiegers@pasteur-kh.org
Phone	+855 (0) 61 234 433
Country(ies) where work will be conducted	Cambodia
Pathogen(s) focus	Emerging respiratory viruses (influenza virus, coronavirus)
Co-Principal Investigator Name (*If applicable*)	Vireak HEANG
Position/Title	Research Engineer
Department	Sequencing Mini-Platform
Institution Name	Institute Pasteur du Cambodge
Street	5 Monivong Blvd
City, State, Zip Code	Phnom Penh
Country	Cambodia
Email	hvireak@pasteur-kh.org
Phone	+855 (0) 12 998 893

Contact information for institution(s) that would financially manage the award *(please note: changing the managing institution upon award may result in substantial funding delays)*	
Contractual Contact, Title	Christophe MOUSSET, Director of Administration and Finance
Institution Name	Institute Pasteur du Cambodge
Street	5 Monivong Blvd
City, State, Zip Code	Phnom Penh
Country	Cambodia
Email	cmousset@pasteur-kh.org
Phone	+855(0) 12802977
For Co-Principal Investigator (If applicable):	
Contractual Contact, Title	
Institution Name	
Street	
City, State, Zip Code	
Country	
Email	
Phone	

Name of Mentor	***Dr. Erik A. Karlsson***
Mentor Institution	Virology Unit and Sequencing Mini-Platform, Institute Pasteur du Cambodge
Institution Address	5 Monivong Blvd, Phnom Penh, Cambodia
Permanent location of Mentor	Cambodia
Mentor email	ekarlsson@pasteur-kh.org
Mentor phone	+855 (0) 70 297 804
Research Center Affiliation	

Name of Mentor	***Dr. Cadhla Firth***
Mentor Institution	EcoHealth Alliance
Institution Address	520 Eighth Avenue, Ste. 1200 New York, NY 10018
Permanent location of Mentor	United States of America
Mentor email	firth@ecohealthalliance.org
Mentor phone	(212)380-4488
Research Center Affiliation	Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH)

Total Budget	***Repeat this table if there are two Co-PIs***
Direct Costs	$136,737.00
Indirect Costs	$10,938.96
Proposed Start Date	01/05/2022
Proposed End Date	30/04/2023

Project Abstract (250 words)

The ability to quickly assess risk of emerging infectious diseases at points of high exposure or contact is paramount for early warning systems and preventative actions. In addition, maintenance of surveillance systems in individual animals is costly and time consuming and prevents widespread coverage. One way to address this issue is to incorporate environmental sampling into surveillance programs to cast a wider net at high-risk interfaces and on a longitudinal basis. To this end, we have begun to incorporate environmental sampling at high-risk human-animal interfaces, including live animal markets, slaughterhouses, and domestic/wild animal interfaces. However, these samples have only been tested for specific pathogens of interest by conventional or real-time PCR, limiting their utility. The ability to use these samples for metagenomic surveillance and pathogen discovery would not only increase surveillance capacity, but also contribute to our understanding of new or emerging pathogens at these high-risk interfaces. Therefore, in the proposed project, we will: (1) utilize existing environmental samples from longitudinal pathogen surveillance to build metagenomic sequencing and bioinformatics capacities and capabilities; (2) assess the suitability of environmental metagenomics as an early warning system for endemic and emerging infectious diseases at high-risk interfaces in Cambodia; and, (3) potentially discover new, emerging, or zoonotic pathogens of concern in high-risk interfaces in Cambodia. Overall, the results from this study will potentially help to improve surveillance systems through rapid and broad pathogen detection, reduced cost, lowered occupational risk, and diminished animal and environmental impact.

Table of contents

In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance

Study Personnel

Jurre SIEGERS, Ph.D., (Postdoctoral Researcher) is a postdoctoral researcher in the Virology Unit at IPC in charge for planning and conducting research on zoonotic viral diseases, including avian influenza and coronaviruses. Dr. Siegers currently overseas all avian influenza virus surveillance efforts of IPC. Dr. Siegers has been vital in implementing COVID-19 sequencing Institut Pasteur du Cambodge. Dr. Siegers will plan, oversee and conduct sample processing, data collection, and analysis for this proposal. He will also be instrumental in preparing data and publications stemming from this work. He will meet with the mentors and technical personnel on a twice-weekly basis to discuss results, issues and future work. Dr. Siegers's commitment to this project constitutes 25% of his total professional effort for the year (3 calendar months).

Vireak HEANG, MS.c., is a research engineer at sequencing platform at IPC in charge of providing sequencing service to other scientists across the unit at IPC. Mr. Heang has an extensive experience over 15 years on molecular biology technique including PCR, Sanger sequencing and NGS. Prior to move to IPC Mr. Heang was working at US Naval Medical Research Unit. 2 (NAMRU-2), the place where he went through various training programs, on-the-job experiences and finally developed a deep understanding of molecular techniques and how it can be used in detection, characterization, and epidemiological modelling of pathogens. When he moved to IPC July last year, Mr. Heang manage to pull all the resources together to make the Illumina Miseq system functioning and he made this happened through his effort and the result was several runs of COVID-19 and Malaria specimens successfully sequenced. Mr. Heang will handle logistic, laboratory work and data analysis of this project. He will meet with the mentors and technical personnel on a twice-weekly basis of discuss results, issues and future work. Mr. Heang will be allocated 25% of his work to this proposed project.

Erik KARLSSON, Ph.D., is a Senior Research Scientist (Assistant Professor equivalent) and acts as the Deputy Head of the Virology Unit at Institut Pasteur du Cambodge. He is an expert on Respiratory Viruses, including SARS-CoV-2, and will act as the Primary Scientific Mentor for Dr. Siegers and Mr. Heang. He is the Director of the National Influenza Center in Cambodia, Director of the WHO Regional H5 Reference Laboratory, and Coordinator of the WHO Global COVID-19 Reference Laboratory housed in the Virology Unit. He is also Co-PI of the Sequencing Mini-Platform formed at IPC in 2021. He will meet regularly with the co-PIs to review experimental progress, troubleshoot issues, interpret data, and participate in preparation of publications resulting from this research.

Cadhla FIRTH, Ph.D., is part of the CREID EID-SEARCH Research Center and will act as the Research Mentor for Dr. Siegers and Mr. Heang. Dr. Firth is Senior Research Scientist and Program Coordinator at EcoHealth Alliance and holds a position as an Adjunct Senior Research Fellow at James Cook University in Australia. Dr Firth has more than 15 years' experience conducting research on emerging infectious diseases, with a focus on the ecology and evolution of zoonotic pathogens at human-animal interfaces. Dr. Firth's expertise in molecular biology, metagenomic sequencing, and the analysis of genomic and metagenomic data match well the goals of the proposed project. Dr. Firth will provide methodological and analytical guidance, mentoring and career advice, and networking opportunities that support the co-PIs progression toward independence, as well as assisting in the preparation of publications or future funding applications that may arise from this work.

Research Aims & Objectives

The ability to quickly assess zoonotic disease risk at potential points of high exposure or contact is of paramount importance for effective early warning systems and the initiation of preventative actions. Therefore, **this pilot project aims to answer the question: "Can metagenomic pathogen discovery on environmental samples improve the speed, comprehensiveness, and cost of existing pathogen surveillance programs?"**

In Cambodia, current pathogen surveillance systems rely primarily on sampling and testing individual animals – a practice that is both costly and time consuming, and prevents widespread coverage of all high-risk areas. **One way to address this issue is the incorporation of environmental sampling (ES) into surveillance programs**. ES includes samples or swabs taken from soil, water sources (drinking, carcass wash, lakes and ponds), feeding sources, feathers, air, and surfaces such as cages, chopping boards, and defeathering machines. As such, **environmental pathogen surveillance casts a wide net at high-risk interfaces, potentially improving surveillance coverage and supporting expanded sampling on a longitudinal basis**. After collection, samples are currently tested using a series of molecular diagnostics targeting a range of pathogens, another cost- and time-limiting factor. We hypothesize that utilizing metagenomic pathogen discovery with ES can: **(i)** improve, expand and simplify existing methods of pathogen surveillance; **(ii)** reduce the cost of pathogen surveillance programs; **(iii)** reduce direct contact between people and large numbers of animals, thereby improving biosafety, animal welfare, and reducing occupational exposure risks; and **(iv)** set a precedent for lower-middle income countries (LMICs) to conduct broad pathogen surveillance cost-effectively.

To test these hypotheses, we will utilize a **large and unique biobank** from Cambodia that includes previously collected **paired samples** from individual animals and ES. These samples originate from ongoing longitudinal surveillance studies for: **(i)** avian influenza viruses in live bird markets (LBMs); **(ii)** swine influenza and Nipah virus in swine abattoirs **(iii)** bat-borne viruses from caves, roosts, and guano farms.

The proposed project has *three aims*:

AIM 1: To assess the suitability of environmental metagenomics as an early warning system for endemic and emerging infectious diseases at high-risk interfaces in Cambodia.

AIM 2: To utilize biobank environmental samples from longitudinal pathogen surveillance programs in Cambodia to build metagenomic sequencing and bioinformatics capacities and capabilities in Cambodia.

AIM 3: To discover new, emerging, or zoonotic pathogens of concern at high-risk interfaces in Cambodia.

The proposed project has *four objectives*:

Objective 1: Characterize and compare the viral diversity present in samples from individual animals (poultry, swine, bat guano/urine) and environmental samples (wash water, air samples, cage swabs), using a combination of metagenomics and polymerase chain reaction (PCR)-based screening methods. **Endpoint:** Comparison of 129 animal samples with 207 ES across 3 species for 3000+ viral targets

Objective 2: Assess whether a surveillance system that incorporates both environmental sampling and virus detection through metagenomics is sensitive and specific enough to replace or support conventional virus surveillance programs**. Endpoint:** Comparative sensitivity (detecting the same virus) in metagenomic versus previously detected pathogens by RT-PCR.

Objective 3: Discover previously undetected, (re)-emerging, and/or zoonotic pathogens of concern at high-risk interfaces in Cambodia. **Endpoint:** Sequencing 2M reads/sample to ensure coverage and maintain cost-effectiveness.

Objective 4: Build capacity and capability in metagenomic sequencing and bioinformatics at Institute Pasteur du Cambodge. **Endpoint**: Successful implementation of a user-friendly metagenomic sequencing and analysis platform.

Study Rationale/Research Gap/Impact

Emerging infectious diseases are a major and ongoing threat to global health and the economy as illustrated by the ongoing SARS-CoV-2 pandemic and a record-breaking avian influenza outbreak across Asia, Europe, the Middle East, and North America. Asia, especially Southeast Asia, is a hotspot of endemic and emerging infectious diseases, including avian influenza viruses (AIVs), and bat-borne viruses such as coronaviruses and henipaviruses with pandemic potential[1-4].

Cambodia is a resource poor, lower-middle income country in tropical Southeast Asia with a large socio-economic dependence on agriculture[5]. Since 2011, Institut Pasteur du Cambodge (IPC) has maintained active longitudinal pathogen surveillance programs at key live bird markets (LBMs), farms/slaughterhouses/storage facilities (poultry and pigs), and at locations where a diverse range of Cambodian bat species roost. These locations represent high-risk human-animal interfaces, where known zoonotic pathogens circulate and novel disease emergence events are likely to occur[6,7]. Sentinel and research pathogen surveillance systems are conducted by IPC for the early detection/warning of known emerging infectious disease to support and inform Cambodian public health systems and the global virus community. Currently, these surveillance programs use PCR-based pathogen detection, which relies on specific or pan-virus primers to amplify conserved genomic targets from an individual virus family. While this approach has led to the successful identification of novel viruses in the past, reliance on conserved genomic targets limits detection of sufficiently divergent pathogens, including those that arise through recombination or reassortment[8,9]. These surveillance efforts are also labor intensive, expensive, limited to known hotspots, and highly species-specific (both animal and viral family). To this end, we have begun to supplement our traditional surveillance methods, which depend on sampling directly from animals, with ES at high-risk human-animal interfaces. These samples include water, swabs, and air samples, which are collected alongside samples taken from individual animals, enabling a direct comparison of pathogens identified from environmental samples with those collected from individuals.

Despite the clear risks associated with Cambodian LBMs, pig farms/slaughterhouses, bat guano farms and caves, there is very limited understanding of virus diversity at these sites. However, the hunt for the origin of SARS-CoV-2 has led to a renewed interest in the viruses present at live animal markets and across the wildlife supply chain, as well as in viruses carried by bats. Hence, improved surveillance of poultry, pigs, bats and other wildlife (as putative reservoirs and intermediate hosts of zoonotic viruses) will be key for the development of early and effective disease prevention and intervention measures by identifying viruses with known or predicted zoonotic potential at high-risk interfaces, where pathogen spillover is most likely to occur. The ability to use environmental samples for metagenomic pathogen surveillance and discovery would not only increase surveillance capacity, but also contribute to our understanding of new or emerging pathogens at these high-risk interfaces. Environmental sampling has several benefits compared to individual animal sampling: 1) **Biosafety benefits**. Handling animals for sampling is risky, both for the animal (discomfort and distress) and the handler (increased exposure to zoonotic infections, trauma from bites/scratches, etc.); 2) **Economic benefits**. Sampling individual animals requires increased sample numbers, trained personnel, more personal protective equipment, increased number of molecular tests, and time which are costly. Short-term and long-term storage of individual samples requires additional space in -80 freezers and/or liquid nitrogen. 3) **Increased comprehensiveness**. Environmental sampling expands the source of sampling to include animal excretions from multiple animals and/or species (urine, feces, saliva), contact material, and aerosolized particles. As many zoonotic diseases are effectively transmitted through the environment (i.e., respiratory viruses, enteric viruses), sampling from these sources may more directly represent human disease risk at key interfaces.

Significance and Approach

Backyard livestock farms, LBMs, and wildlife farms often have limited biosafety measures in place despite representing potentially risky human-livestock-wildlife interfaces, and as a result, have repeatedly been associated with the (re-)emergence of zoonotic diseases[1-4]. Therefore, developing early warning systems at these high-risk locations will be critical for monitoring and preventing zoonotic diseases from generating the next pandemic. Previous pathogen surveillance and virus diversity studies at human-livestock-wildlife interfaces in Cambodia have relied heavily on consensus PCR-based virus detection, and/or focused on only a single animal species[10]. These methods are time consuming and costly and may result in delayed disease detection and reporting. Every second counts between first detection and response. Indeed, the total cost of an outbreak grows exponentially as time from detection increases, making identification critical at emergence or early stages of spread[11]. It is critical to get "left of sneeze." **To this end, the proposed project will use and evaluate cutting-edge metagenomic approaches for multiple viral pathogens on broad coverage samples as a modern alternative to pathogen surveillance at high-risk interfaces**. Comparison to standard methods will be conducted under a hypothesis-driven framework to reveal the diversity, abundance, and zoonotic potential of viruses circulating in high-risk locations for emergence. To our knowledge, this is the first data-driven project with the goal of providing a foundation for the role of metagenomic ES in monitoring virus prevalence and emergence in Cambodia.

Like much of South/Southeast Asia, Cambodia is a hotspot of endemic and emerging infectious diseases. However, despite active, longitudinal surveillance efforts, outbreaks still frequently occur and data on virus diversity at high-risk human-animal interfaces are still lacking[12-14]. Additionally, as a Least Developed Country, the economic impacts of endemic agricultural and human seasonal pathogens are high, making surveillance for novel emerging pathogens at the animal-human interface a costly lower priority. Furthermore, it is increasingly difficult to obtain samples and/or data from some countries in the region, increasing the importance of Cambodia as a critical location for monitoring zoonotic disease emergence. The unique biobank of samples we have collected as part of active, longitudinal surveillance programs represents a remarkable opportunity to compare standard pathogen surveillance using individual animals and PCR with metagenomic ES to determine viral diversity and viral abundance.

Outcomes from this pilot study will provide invaluable data on improved and cost-effective surveillance practices within a "One Health" perspective. This is critical, as viruses will likely jump to new hosts from the water they drink, the surfaces they touch, and the air they breathe - less likely because they are kissing a chicken. **Environmental sampling reduces occupational risk, improves animal welfare, and can have non-invasive or reduced impact on market trade and wildlife habitats.** Improving our ability to monitor multiple pathogens in the environment will increase our capacity to develop and communicate key biosecurity practices and guidelines to reduce risk, and influence policy. Environmental sampling with multiple pathogen detection will also reduce the overall cost of surveillance systems, which is a key consideration in resource-limited settings. Expanded pathogen detection with reduced sampling means being able to conduct surveillance over a broader area and/or more frequently, greatly enhancing the ability to detect a potentially pandemic-level pathogen at a high-risk interface. Indeed, rough estimations from the budget of this pilot project alone predict surveillance could double in length or breadth for a single pathogen itself, let alone the 3000+ targets included in each enhanced metagenomic run.

Thus, in line with the goals of the CREID network and the EID-SEARCH team (to improve the detection and surveillance of important emerging viruses), we will use previously collected samples from high-risk human-animal interfaces in Cambodia to systematically characterize and compare viral diversity within and between environmental and animal samples from the same locations. We will evaluate the benefits of incorporating ES into existing surveillance programs as a means to broaden pathogen

surveillance efforts without increasing the cost of these programs at high-risk interfaces and on a longitudinal basis. We will develop and utilize user-friendly protocols and analysis pipelines **allowing for broad implementation in a resource-limited country.**

Research Methods

A total of 334 (129 animal pools and 205 ES pools/individual) will be analyzed in this pilot research project.

Poultry Surveillance – 224 samples (120 animal pools/104 ES pooled and individual)

Our metagenomic analysis will use environmental and animal samples previously collected as part of AIV surveillance programs at Cambodian LBMs and farms/slaughterhouses/storage facilities for poultry and pigs, which occurred from 2018 to April, 2022[9]. Sampling was performed at two major LBMs – Orussey Market in Phnom Penh province and Takeo Market in Takeo province (**Figure 1**). The LBM at Orussey Market is the largest LBM in Cambodia and the most extensively studied. The LBM at Takeo market is often associated with the emergence of new influenza subtypes, possibly due to its proximity to Viet Nam and the absence of biosafety and biosecurity measures[5,15]. Sampling at these markets occurred 16 times/year, and wash water samples (N=152), and cage swabs (N=55) were collected along with a multitude of individual animal samples from chickens (N=2240), ducks (N=2240), and other poultry. Air sampling using the Thermo Scientific™ AerosolSense™ Sampler was also conducted at three locations in and around the LBMs (N=33). **From this biobank we will select 10 paired ES and animal sampling sessions for metagenomic sequencing and analysis**. Together, this provides us with a unique sample set to observe virus diversity in animal versus ES samples in heavily sampled LBMs.

Figure 1. Overview live bird market (LBM) at Orussey Market, Phnom Penh province. A) Defeathering machines. **B)** Paired throat and cloacal sample collection. **C)** Air sampling machine at the poultry holding facility. **D)** Air sampling machine at poultry slaughter site.

Pig Surveillance – 54 samples (9 animal pooled/45 ES pooled and individual)

Ongoing pig surveillance studies are funded by NIAID/NIH - Centers for Excellence in Influenza Research and Response (CEIRR; 2021-2028) and include individual animal samples and environmental samples (air, cage swabs, and water) collected in three different provinces across Cambodia. Sampling locations were pig farms at high-risk interfaces (slaughterhouses) with minimal biosafety measures in place (**Figure 2**). A total of 45 individual pigs were sampled over three sessions, along with air samples, cage swabs, and wash water samples.

Bat Surveillance – 56 samples (35 animal ES individual/21 air ES individual)

Bat sampling sites for this proposal were chosen based on previous research efforts by IPC in Phnom Penh, Kampong Cham, Stung Treng, Kampot, Kandal, and Battambang provinces (**Figure 2**) [10,16,17]. Our standard pathogen surveillance protocol for bats focuses on the collection of urine and guano; here, these will be referred to as animal samples, although it is worth bearing in mind that they may be representative of multiple animals in some cases. Sample collection for this project is performed under the FAO-USAID and includes air, guano, and urine samples collected in and around seven locations of bat caves, roosting sites, and/or guano farms. Air samplers (N = 21) are deployed at cave entrances, roosting trees, and bat guano farms where large groups of individual bats congregate, maximizing the capacity to sample many individuals collectively. In parallel, air samplers were fitted with a sterile plastic cover (2m x 2m) to collect urine and feces (N = 35) to compare the viruses identified in air samples with those from bat excretions.

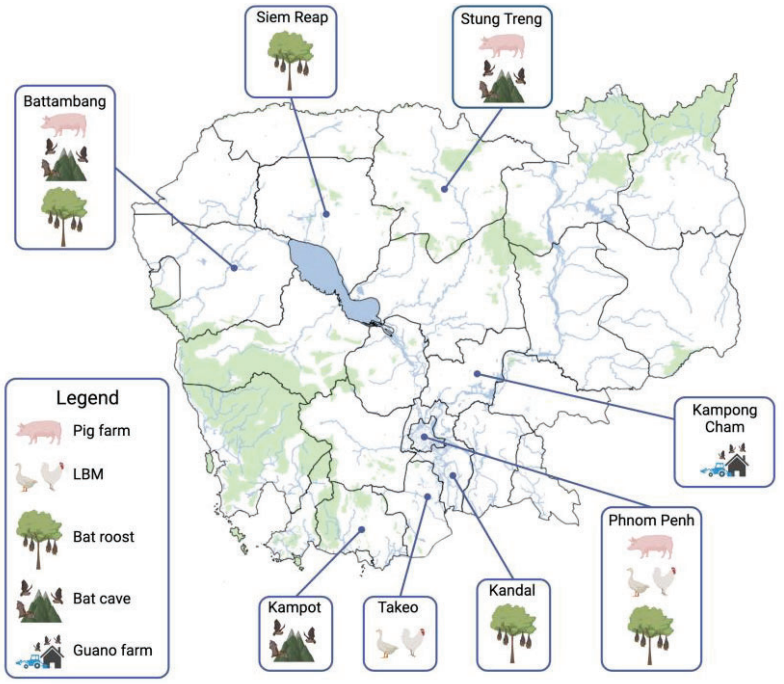


Figure 2. Location and type of sample previously collected in Cambodia and readily available for the proposed study.

Metagenomic Sequencing. While the advent of metagenomic sequencing was a significant step forward as a tool for pathogen detection and discovery, it remains relatively expensive and lacks the analytical sensitivity required for a true pathogen surveillance platform. Target enrichment panels were developed in response to this issue, but, until recently, were unable to facilitate the detection of highly divergent strains or novel viruses[10,18]. In this project, we will use a cutting-edge hybridization-based target enrichment approach - The Twist Comprehensive Viral Research Panel (CVRP) – that was designed to explicitly address this need[19]. The CVRP contains more than a million probes covering the genomes of 15,488 strains from 3,153 unique viruses (including animal viruses), and is tolerant to mismatches between virus and probe, supporting the detection of novel zoonoses. These include ssRNA, dsRNA, ssDNA, and dsDNA viruses belonging to every virus family capable of infecting people. Thus, target enriched metagenomics represents an ideal balance between the promise of unbiased virus detection and the feasibility of the standard tool of public health surveillance programs everywhere – quantitative PCR.

Prior to preparation for metagenomic sequencing, individual animal samples will be pooled by sampling session: 10 samples will be pooled for chickens and ducks per market (2 markets) per visit (10 selected visits), respectively; five samples will be pooled from pigs. Bat guano/urine will be run individually (**Table 1**). Wash water samples will be pooled by sampling session (N=2/session), as will cage swabs (N=5/session), while all air samples will be processed individually. Wash water samples will be pelleted and concentrated using ultrafilters[20] prior to RNA extraction.

In this project, we will follow the manufacturer's recommended protocol for the generation of hybridization-enriched sequencing libraries, under the guidance of Prof. Linfa Wang at Duke-NUS, who

has been involved in the development and implementation of target enriched metagenomic sequencing for several years (see attached letter of support). Briefly, high quality total RNA will be extracted from

Table 1. Sample types and numbers (pools) from paired sampling locations selected for target enriched metagenomic sequencing.

System	Animal sample type	Animal sample number	Environmental sample type	Environmental sample number
Poultry	Cloacal swab	Chicken: 600 (60)	Wash water	120 (60)
	Cloacal swab	Duck: 600 (60)	Cage swabs	55 (11)
			Air samples	33
Pigs	Nose/snout swab	45 (9)	Wash water	18 (9)
			Cage swabs	45 (9)
			Air samples	27
Bats	Urine/Guano	35	Air samples	21

each (pooled) environmental and animal sample using the RNeasy PowerMicrobiome Kit (Qiagen). RNA will be quantified and converted to cDNA using ProtoScript II First Strand cDNA Synthesis kit and random primers (New England Biolabs). The NEB Next Ultra II Non-Directional RNA second Strand Synthesis kit (New England Biolabs) will be subsequently used to convert single-stranded cDNA to dsDNA, and Illumina TruSeq-compatible libraries will be generated using the Twist Library Preparation Enzymatic Fragmentation kit (Twist Bioscience). These libraries will be hybridized for 16 hours with the set of 120 base pair biotinylated probes contained within the CVRP (Twist Bioscience) and sequenced with 2x75 bp paired-end reads on an Illumina MiSeq platform housed at IPC. Twelve target enriched libraries will be sequenced per run to achieve approximately 2 million reads/sample.

Metagenomic data analysis. For target enriched metagenomics to have utility as a public health surveillance tool, data analysis must be user-friendly and understandable to a non-expert in bioinformatics. Towards this aim, we will assess the performance and user-experience of two leading cloud-based metagenomics platforms for virus detection and compare the results to those generated by our bespoke bioinformatics pipeline[21]. Analysis of data generated by the CVRP is supported by One Codex software, a cloud-based platform for microbial genomics that will be used for preliminary data analysis and visualization[22]. One Codex uses a rapid, highly sensitive k-mer classification algorithm that maps sequencing reads to their custom database, which consists of more than 115k microbial genomes. The raw classification results are filtered through several statistical post-processing steps designed to eliminate false positive results caused by contamination or sequencing artifacts. We will analyze the same data using Genome Detective, a web-based platform that sorts sequence reads into bins prior to *de novo* assembly and compares the assembled contigs to the NCBI RefSeq and Swissprot UniRef90 databases, respectively, for virus identification[23]. Each of these platforms is contained within a point-and-click, web-based interface and reports results using easy-to-understand visualizations, with click-through options for additional details should the user wish to perform analyses beyond pathogen detection. Finally, we will analyze these same data with our bespoke bioinformatics pipeline (representing the gold standard approach), which uses Trinity RNA-Seq for *de novo* assembly[21], followed by comparisons with the complete non redundant nucleotide and protein databases available through GenBank using E-value cutoffs of 1E-10 and 1E-4, respectively, and the program Diamond[24]. Additional full genome assembly will be attempted for all known or potentially zoonotic viruses identified by any of the three analysis approaches using either metaSPAdes (for *de novo assembly* of highly divergent viruses), or BWA MEM for reference mapping of known viruses[25,26]. Sequences representing viral genomes will be further

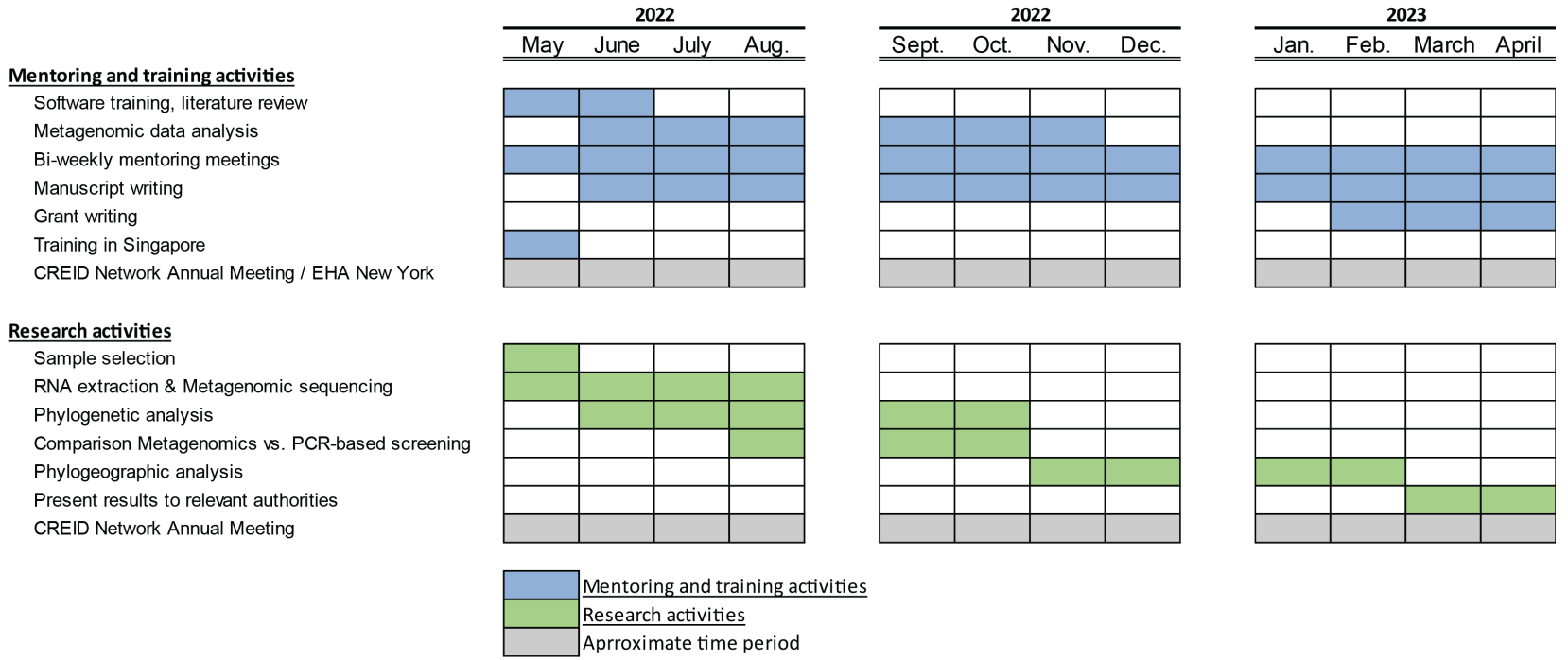
characterized using phylogenetic analysis targeting conserved domains (e.g, the RNA polymerase) that will include related viruses from GenBank to estimate evolutionary relationships and infer potential zoonotic disease risk. Phylogenies will be estimated using the maximum likelihood method implemented in IQ-Tree 2, employing the best-fit amino acid substitution models for each virus family[27]. The resultant phylogenies will be used along with the Virus-Host DB[28] to identify putative host species for viruses found in environmental samples, where genetic material from multiple host species may be present.

Assessing the utility of target-enriched environmental metagenomics for pathogen surveillance. First, we will interrogate the utility of ES as a tool for pathogen surveillance by comparing metagenomic data from environmental samples with metagenomic data from animal samples collected at the same time and location. From each sequencing library, we will calculate the following metrics: number (and identity) of virus families identified, number (and identity) of unique viruses identified, and percent genome coverage for each virus. These will be combined by sample type and sampling session (as replicates) to provide a snapshot of circulating viral diversity at each time/place. The metrics associated with each environmental sample type will be compared to those from the corresponding animal samples from the same time and location. This will allow us to assess what proportion of virus diversity in a host population is detectable from the environment, as well as to identify any taxon-specific biases in environmental sampling, if present. We will also compare viral community structure across sample types using using standard measures from community ecology, including species richness, alpha diversity, and beta diversity, which will be calculated using a range of R packages[29]. Permutational multivariate analysis of variance tests (permanova) will be used to assess differences in virome composition between sample types.

Next, we will examine the utility of target enriched metagenomics as an alternative to traditional molecular diagnostics (qRT-PCR and consensus PCR). As part of our routine pathogen surveillance efforts, individual animal samples from these same sampling sessions have previously been tested for the following pathogens: **Poultry** – AIV and Newcastle disease virus; **Pigs** – influenza, African swine fever virus, classical swine fever virus, Nipah virus, and porcine reproductive and respiratory syndrome virus; **Bats** – coronaviruses, Nipah virus, orthomyxoviruses. The results of these tests will be compared to the metagenomic results from environmental and animal samples to evaluate how well this approach recapitulates the results from standard pathogen surveillance efforts.

Potential Pitfalls and Alternative Plans: Overall, this is a straight forward study using novel and readily accessible samples. Any resultant data will be extremely useful in understanding the benefits of ES over animal sampling for pathogen surveillance. No issues are foreseen with utilizing retrospective samples for metagenomic sequencing; however, storage and collection issues can affect sample quality. If insufficient samples are available, IPC plans to continue surveillance activities throughout the period of this project and fresh samples can be collected for analysis. In addition, given the need to maintain cost-effectiveness, only 2M reads will be collected per sample using CVRP. A series of spike-in experiments will be conducted to ensure sequencing reads and dept is adequate for known viruses before conducting sequencing on precious samples to ensure detectability (not funded through this project). At the current time, no internationally recognized guidelines exist on the use of environmental sampling (ES) and data management. It is hopeful these studies will contribute to future guidelines surrounding the use of ES in surveillance sampling.

Project Timeline



Research Performance Sites (*1-page limit*)

The Institut Pasteur in Cambodia, established in 1953, is a non-profit research institution operating under the auspice of the Cambodian Ministry of Health (MoH) and the Institut Pasteur in Paris, France. IPC missions are: **(i)** to conduct innovative life science and health research on infectious and emerging disease in Cambodia; **(ii)** to provide public health services; **(iii)** to contribute to training and capacity building in the field of biomedical and life science.

IPC constitutes a strong team and a state-of-the-art facility with modern, secured and standardized laboratories appropriate for experiment with hazardous pathogens. These laboratories include biosafety level (BSL) 2 and 3. The BSL3 facility comprises four modules dedicated to different and separate activities including a safety cabinet with gloves box allowing BSL3+ safety conditions. The proposed project will take place within The Virology Unit at IPC, which serves as the reference laboratory for COVID-19, Influenza, Arboviruses, and Rabies for Cambodia, and conducts surveillance and research on endemic, emerging, and zoonotic pathogens. To respond to the COVID-19 pandemic, the Virology Unit at IPC has recently increased its sequencing capacity to include both Oxford Nanopore (GridION and MinION) and Illumina MiSeq technologies.

The Virology Unit works closely with the Epidemiology/Public Health, Entomology, Bacteriology, and Malaria Units at IPC, as well as national and international partners, to conduct One Health-focused surveillance and studies in Cambodian people, domestic animals, and wildlife. As such, IPC is continually searching for new and innovative ways to expand and improve surveillance activities. IPC also serves as a model for state-of-the-art research in a Least Developed Country/Low-Middle Income Country and successful studies at IPC can inform future work in the region.

All samples needed for this work have been maintained in IPC laboratory freezers at -80°C from the time of collection and can be easily linked to collection and pathogen metadata. These unique samples and data are available only at IPC, which is committed to facilitating the proposed research. Please see “12.10 - Facilities and Resources” for a more detailed description of infrastructure and equipment available at IPC to ensure the success of this project.

CREID Research Center Collaboration (*1-page limit*)

The goals and objectives of the proposed research project, "In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance", are fully consistent with the specific aims of EID-SEARCH. EID-SEARCH takes a One Health approach to understanding zoonotic disease emergence at high-risk human animal interfaces. EID-SEARCH aims to understand the frequency and causes of virus spillover in high-risk communities across Southeast Asia and identify strategies and tools to reduce this risk. As the proposed research focuses on improving, expanding and simplifying existing surveillance efforts to cast a wider net for pathogen surveillance and monitor virus diversity at key human-animal interfaces and on a longitudinal basis we expect the results of the project to make a significant contribution towards the overall goals of EID-SEARCH.

The proposed project is well-designed, leverages cutting-edge technology, uses archival samples, and includes a robust individual development and capacity building component that together corresponds to the overall research strategy of CREID and EID-SEARCH. The EID-SEARCH team is highly motivated to support the application of applying metagenomics to pathogen surveillance and assist in the analysis of the unique dataset that will be generated from this research. With the heightened focus on robust surveillance networks to timely identifying emerging zoonotic threat at key human-animal interfaces the proposed research is both timely and likely to yield highly-cited, impactful publications and policy recommendations for EID-SEARCH and its IPC partners.

The EID-SEARCH team includes a number of experts in study design, laboratory-based diagnostics, genetic, genomic, and bioinformatics analyses, who will contribute to project implementation, data analysis, and manuscript preparation as required. Communication and coordination mechanisms will be set up between the IPC research team and scientists at the EID-SEARCH research sites for resource sharing, training, knowledge exchange, and mentorship. In particular, EID-SEARCH will facilitate and encourage relationships between Dr. Jurre Siegers and Vireak Heang and our partners in Southeast Asia (based in Singapore, Australia and the US) that will facilitate Dr. Jurre Siegers and Vireak Heang professional development and strengthen research capacity in the region. Dr. Cadhla Firth, Senior Scientist at EID-SEARCH and the project Mentor, has an established relationship with both Dr. Jurre Siegers and his supervisor, Dr. Erik Karlsson, and will act as the liaison between EID-SEARCH and the IPC research team. An expected outcome of this research project will be pilot data to support a joint funding application in the future, led by Dr. Jurre Siegers.

Mentoring Plan

The co-PIs, Vireak Heang and Jurre Siegers, are at different stages of their respective careers with discrete professional development goals. This Mentoring Plan allows the co-PIs to pursue independent professional goals while working as a team to achieve the learning and development objectives associated with the proposed project.

1. Developing Leadership and Independence

Vireak Heang: My long-term goal is to train the next generation of Cambodian scientists. I can pass on my knowledge in my native language, Khmer, providing greater educational opportunity to all Cambodians. The opportunity for future scientists to learn in their native language is a critical step towards rebuilding the scientific infrastructure of my country. Prior to joining IPC, much of my work was technical, with limited experience in project management. However, at IPC, my Scientific Mentor, Dr. Karlsson, took an immediate interest in my professional development and is actively involving me in the entire scientific process – from conception, to study design, implementation, and analysis. As a result, we have developed a strong mentoring relationship built on trust and mutual respect that will continue across this project to improve my scientific career. My Research Center Mentor, Dr. Firth, has a broad scientific network, and regularly interacts with scientists across Southeast Asia in her role as a Project Coordinator for EID-SEARCH. To strengthen my ability to communicate complex scientific topics, both Mentors will actively provide opportunities for me to interact with and present my research to other scientists across Southeast Asia and through their respective global networks of research partners.

Jurre Siegers: My overall research goal is to improve the health of people, animals, and ecosystems using a "One Health" approach that will allow me to maximize my academic training, and scientific and personal interests. I wish to develop my skills in novel diagnostic tools, such as metagenomics, to contribute to improved surveillance and research capacity in the field of emerging viruses at human-animal interfaces. However, my aspirations are not just limited to the generation of scientific knowledge, methods, and policies. Instead, I wish to actively use my knowledge in virology (Ph.D. training) and emerging infectious diseases (Postdoctoral) to train the next generation of Cambodian scientists that will one day be local leaders in the field of emerging infectious diseases. As my Scientific Mentor, Dr. Karlsson has already demonstrated his commitment to my scientific progress by providing me with opportunities to develop project management skills and improve my scientific writing. Both Mentors will continue to mentor me in this capacity. Both Mentors have also been actively guiding me in grant writing (including with this proposal) and will continue to provide hands-on instruction. Both Mentors will support my attendance and participation at (inter)national conferences on emerging zoonotic diseases and provide opportunities to present my work to scientists at IPC, EcoHealth Alliance (EHA), and across EID-SEARCH/CREID. Both Mentors will also provide me with networking opportunities from across their respective networks as they support my development to become a more independent leader in the field of emerging infectious disease research.

2. Building Expertise in Metagenomics

Vireak Heang: My extensive experience and training in laboratory-based sciences has developed a deep understanding of molecular techniques and how they can be used in the detection, characterization, and epidemiological modeling of pathogens. While my work has primarily dealt with shotgun and amplicon-based sequencing, metagenomics is a critical tool for pathogen detection and discovery. In Cambodia, this

capacity is very limited; however, we recently set up the IPC Sequencing Mini-Platform (Illumina MiSeq and ONT technologies) and are exploring the full potential of this system. Given the opportunity to strengthen my hands-on and analytical skills to include metagenomics, will broaden my skillset, and further transition me to scientific independence in Cambodia, capable of using cutting-edge genomics techniques to solve locally relevant problems. Dr. Firth has more than 15 years' experience in the generation and analysis of genomic and metagenomic data in the context of zoonotic disease detection, discovery, and characterization (including BSL3), and is experienced in training and mentoring scientists from LMICs in Southeast Asia. She is therefore an ideal mentor in this area and will work closely with me to develop laboratory SOPs and workflows for metagenomics that are fit-for-purpose at IPC. In addition, Dr. Firth has arranged for me and Dr. Siegers to visit Prof Linfa Wang's laboratory at Duke-NUS Medical School in Singapore for training in cutting-edge molecular techniques, as well as to interact directly with members of his lab.

Jurre Siegers: In Cambodia and other LMICs, there is a pressing need for increased capacity in the field of (meta)genomics, which is becoming increasingly common in emerging infectious disease surveillance and research. Given the opportunity to further strengthen my analytical skills to include genomic and metagenomic data analysis, I will be in possession of a skillset that will allow me to make significant career steps as a junior scientist, capable of initiating and delivering hypothesis-driven research at the forefront of virology and emerging diseases. I will also actively work to disseminate this knowledge to local scientists and staff to ensure that these methods and tools are available to Cambodian scientists, and to support national and international surveillance efforts. Dr. Firth has a wealth of experience in viral sequence analysis, phylogenetics, and phylodynamics, and a broad network of experts in this field. She will work closely with me to develop my technical skills in this area and will facilitate interactions across her network as needed to achieve the analytical goals of the project and build my expertise in this area.

3. Communication Plan

Through the process of writing the application for the CREID Pilot Research Program, the four of us have established the foundation for an effective communication plan that will continue throughout the project. We have already created a WhatsApp group used to communicate in a real-time, informal setting. This has been invaluable for sharing ideas, asking questions, and facilitating development of good interpersonal relationships - the foundations of effective mentorship. We have also agreed to regularly scheduled Zoom meetings (every two weeks) that are agenda-driven, and focused on a topic or issue of direct relevance to the research project. Both the Co-PIs and the Mentors will set the agenda and follow-up on action items. Co-PIs will meet weekly in-person with Dr. Karlsson to discuss project developments, troubleshoot, and develop skills in project and budget management. In addition, the Co-PIs will attend weekly academic meetings at IPC in person and at EHA (remote), which will expose them to the breadth of research and broaden their knowledge base.

Finally, we have scheduled two in-person visits between Dr. Firth and the Co-PIs. The first will coincide with the CREID annual meeting and will include a visit to EHA's headquarters in New York City. This visit will focus on learning relevant analytical techniques, performing preliminary data analysis, and networking with EHA's scientists and staff. Towards the end of the project, Dr. Firth will join us in Cambodia to assist in preparing and analyzing our data for publication and will discuss future directions and plans for future funding following on from this project.

Vertebrate Animals Section Requirements

Projects involving animal samples were collected (2019-2021) under a protocol approved by National Ethics Committee for Health Research under the Ministry of Health, Cambodia (NECHR143, NECHR149, and NECHR320); however, Cambodia did not have a specific animal ethics review board at the time the collections were conducted. Continuing ethics approvals (NECHR013) for projects involving further animal sample collections in 2022 are in revision and projected to be approved in February 2022.

Human Subjects Research

This proposal will not involve experimental procedures with or on human subjects and is therefore not applicable.

Research and Related Other Project Information

1. Are Human Subjects Involved?	Yes		No	X
1.a. If YES to Human Subjects				
Is the Project Exempt from Federal regulations?	Yes		No	
If yes, check appropriate exemption number	1		2	
If no, is the IRB review Pending?	Yes		No	
IRB Approval Date:				
Human Subject Assurance Number				

2. Are Vertebrate Animals Used?	Yes		No	X
2.a. If YES to Vertebrate Animals				
Is the IACUC review Pending?	Yes		No	
IACUC Approval Date				
Animal Welfare Assurance Number				

3. Is proprietary/privileged information included in the application?	Yes		No	X

4.a. Does this project have an Actual or Potential impact -- positive or negative -- on the environment?	Yes		No	X
4.b. If yes, please explain				
4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed?	Yes		No	
4.d. If yes, please explain				

5. If the research performance site designated, or eligible to be designated, as a historic place?	Yes		No	X
5.a. If yes, please explain				

6. Does this project involve activities outside of the United States or partnership with international collaborators?	Yes	X	No	
6.a. If yes, identify countries	Cambodia			
6.b. Optional explanation	Please see Foreign Site Justification			

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: Vireak Heang

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

POSITION TITLE: Research Engineer

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)*	Start Date MM/YYYY	Completion Date MM/YYYY	FIELD OF STUDY
Technical School for Medical Care, University of Health Science	Associate Degree	01/1999	03/2001	Medical Laboratory Technician
National University of Management	BA	04/2003	08/2006	Management
University of Florida	N/A	05/2010	07/2011	One Health
National University of Management	MBA	03/2009	03/2012	Management

A. Personal Statement

I first began conducting research in 2006 when I was hired by the US Naval Medical Research Unit No. 2 (NAMRU-2) in Phnom Penh Cambodia and I am now working at Institute Pasteur of Cambodia as a Research Engineer for the sequencing platform. My first position at NAMRU-2 was as a Molecular Biology Laboratory Technician. In this role, I was introduced to the exciting world of infectious disease surveillance in one of the global hotspots of these diseases. Over the next 15 years, I rose to become the Head of Molecular Diagnostics where I oversaw a diverse team of 10 people that conducted molecular detection and characterization of pathogens. Utilizing technologies such as RT-PCR, Sanger sequencing, and Next Generation Sequencing (NGS), we collected and analyzed data on infectious pathogens that was critical not only for the US Department of Defense, but also for the Cambodian Ministry of Health, and the Cambodian Armed Forces. Through various training programs and on-the-job experiences, I have developed a deep understanding of molecular techniques and how they can be used in the detection, characterization and epidemiological modeling of pathogens. In 2017, the NAMRU-2 laboratory was chosen by the Global Emerging Infectious Surveillance and Response System (GEIS) to be one of the NGS and Bioinformatics (BI) Consortiums. The goal was to rapidly detect and characterize known, emerging, and novel infectious agents through the establishment of a harmonized Department of Defense laboratory capability that uses data from NGS and BI to inform armed forces health protection decision making. As part of the consortium, each laboratory was sent a set of blinded specimens and we were to run, analyze, and send the report back to the consortium. My role was to set up and manage the plan for running and analyzing those blinded specimens using our in-house techniques, and we correctly identified 85% of the pathogens that the consortium sent us. I have since managed to sequence a variety of specimens - from bacteria isolates to clinical specimens - using Illumina Miseq, including Escherichia coli, Sa*l*monella spp., Shigella spp., influenza virus, Chikungunya virus, and Dengue viru*s*. In my current role, I am a designated expert on Illumina sequencing and assist scientists from across all different units at IPC, regardless of discipline. Recently, I managed to successfully sequence 96 respiratory specimens for SARS-CoV-2 using the Illumina Miseq ARTIC protocol.

My work has encompassed all aspects of infectious diseases from human-specific diseases to analyzing zoonotic transmission at the animal-human interface. From these experiences, I have come to understand the "whole" of health as it applies to animal and environmental reservoirs and the dynamics of transmission. I also have experience across multiple levels of research – from hands-on work at the bench, to team lead, to leading an entire department executing multiple projects. Through these varied responsibilities, I have developed a keen knowledge of how to manage both projects and teams, excellent communication skills, and programmatic expertise. My personal goals are to continue to expand my knowledge and abilities in molecular science. I am keenly interest in the dynamic relationship that microorganisms, especially pathogens, have with their hosts, including natural reservoirs and spillover hosts. By understanding these interactions, I feel we can develop better protections against disease, whether those be through enhanced detection, novel prophylactics, or targeted therapeutics.

B. Positions, Scientific Appointments and Honors

2020-Present	Research Engineer, Institute Pasteur of Cambodia.
2013-2020	Molecular Biology Project Manager, US Naval Medical Research Unit.2
2010-2013	Laboratory Technician, US Naval Medical Research Unit.2
2006-2008	Laboratory Technician, US Naval Medical Research Unit.2, Contractor
2001-2006	Laboratory/X-ray Technician, International SOS clinic.

C. Contributions to Science

1. My early publications focused on the reemergence of Zika virus in Cambodia. My work first began by detecting and describing a case report of Zika infection in a Cambodian citizen in 2010. The identification of this index case signaled that ZIKV was still circulating in Cambodia. Continuing the investigation, we sequenced the Cambodian isolate with four other isolates from across the globe, expanding the number of characterized isolates in Cambodia from two to seven. Our results demonstrated that there appeared to be two distinct lineages of ZIKV circulating, one originating in Africa and one from Asia.

    a. **Heang V**, Yasuda CY, Sovann L, Haddow AD, Travassos da Rosa AP, Tesh RB, Kasper MR. Zika virus infection, Cambodia, 2010. Emerg Infect Dis. 2012 Feb;18(2):349-51. doi: 10.3201/eid1802.111224. PMID: 22305269; PMCID: PMC3310457.

    b. Haddow AD, Schuh AJ, Yasuda CY, Kasper MR, **Heang V**, Huy R, Guzman H, Tesh RB, Weaver SC. Genetic characterization of Zika virus strains: geographic expansion of the Asian lineage. PLoS Negl Trop Dis. 2012;6(2):e1477. doi: 10.1371/journal.pntd.0001477. Epub 2012 Feb 28. PMID: 22389730; PMCID: PMC3289602

    c. Ladner JT, Wiley MR, Prieto K, Yasuda CY, Nagle E, Kasper MR, Reyes D, Vasilakis N, **Heang V**, Weaver SC, Haddow A, Tesh RB, Sovann L, Palacios G. Complete Genome Sequences of Five Zika Virus Isolates. Genome Announc. 2016 May 12;4(3):e00377-16. doi: 10.1128/genomeA.00377-16. PMID: 27174284; PMCID: PMC4866861.

2. A second focus of my work has been the detection and characterization of drug resistance in bacterial pathogens circulating in Cambodia. Cambodia lacks regulation in the use of antibiotics and thusly the use of incorrect antibiotics and/or improper dosing is common. This has led to a rise in multi-drug resistant strains of bacteria across the country. My worked has helped detect the presence of antibiotic resistance in Cambodia and characterize the mechanisms of resistance using molecular techniques.

    a. **Heang V**, Hout B, Prouty MG, Supraprom C, Ford GW, Newell SW, Leski TA, Vora GJ, Taitt CR. Detection of qnrVC and rmtB genes from a multidrug-resistant Ralstonia pickettii wound infection

isolate in Cambodia. Int J Antimicrob Agents. 2014 Jul;44(1):84-5. doi: 10.1016/j.ijantimicag.2014.04.003. Epub 2014 May 2. PMID: 24888871.

b. Hout B, Oum C, Men P, Vanny V, Supaprom C, **Heang V**, Rachmat A, Prouty M, Newell S, Harrison D, Noor S, Gollogly J, Tho L, Kim YJ, Ford G. Drug resistance in bacteria isolated from patients presenting with wounds at a non-profit Surgical Center in Phnom Penh, Cambodia from 2011-2013. Trop Dis Travel Med Vaccines. 2015 Jul 31;1:4. doi: 10.1186/s40794-015-0006-5. PMID: 28883936; PMCID: PMC5526368.

c. Taitt CR, Leski TA, **Heang V**, Ford GW, Prouty MG, Newell SW, Vora GJ. Antimicrobial resistance genotypes and phenotypes from multidrug-resistant bacterial wound infection isolates in Cambodia. J Glob Antimicrob Resist. 2015 Sep;3(3):198-204. doi: 10.1016/j.jgar.2015.05.006. Epub 2015 Jul 9. PMID: 27873709.

d. Taitt CR, Leski TA, Prouty MG, Ford GW, **Heang V**, House BL, Levin SY, Curry JA, Mansour A, Mohammady HE, Wasfy M, Tilley DH, Gregory MJ, Kasper MR, Regeimbal J, Rios P, Pimentel G, Danboise BA, Hulseberg CE, Odundo EA, Ombogo AN, Cheruiyot EK, Philip CO, Vora GJ. Tracking Antimicrobial Resistance Determinants in Diarrheal Pathogens: A Cross-Institutional Pilot Study. Int J Mol Sci. 2020 Aug 18;21(16):5928. doi: 10.3390/ijms21165928. PMID: 32824772; PMCID: PMC7460656.

3. Finally, I have also contributed to broad topics including drug resistance in malaria and observational studies into the causes of sepsis. Cambodia has experienced malaria treatment failures to multiple frontline drugs, creating great concern should these resistant strains continue to proliferate. My work helped to evaluate the efficacy of new combinations of drugs in the treatment of malaria. Sepsis continues to be a significant cause of morbidity and mortality, especially in areas with a rudimentary health care system such as Cambodia. I contributed to the seminal description of the causes of sepsis in Cambodia through the use of next generation sequencing techniques for pathogen detection and characterization.

   a. Wojnarski M, Lon C, Vanachayangkul P, Gosi P, Sok S, Rachmat A, Harrison D, Berjohn CM, Spring M, Chaoratanakawee S, Ittiverakul M, Buathong N, Chann S, Wongarunkochakorn S, Waltmann A, Kuntawunginn W, Fukuda MM, Burkly H, **Heang V**, Heng TK, Kong N, Boonchan T, Chum B, Smith P, Vaughn A, Prom S, Lin J, Lek D, Saunders D. Atovaquone-Proguanil in Combination With Artesunate to Treat Multidrug-Resistant P. falciparum Malaria in Cambodia: An Open-Label Randomized Trial. Open Forum Infect Dis. 2019 Sep 4;6(9):ofz314. doi: 10.1093/ofid/ofz314. PMID: 31660398;

   b. Rozo M, Schully KL, Philipson C, Fitkariwala A, Nhim D, Som T, Sieng D, Huot B, Dul S, Gregory MJ, **Heang V**, Vaughn A, Vantha T, Prouty AM, Chao CC, Zhang Z, Belinskaya T, Voegtly LJ, Cer RZ, Bishop-Lilly KA, Duplessis C, Lawler JV, Clark DV. An Observational Study of Sepsis in Takeo Province Cambodia: An in-depth examination of pathogens causing severe infections. PLoS Negl Trop Dis. 2020 Aug 17;14(8):e0008381. doi: 10.1371/journal.pntd.0008381. PMID: 32804954; PMCID: PMC7430706.

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: Jurre Ynze Siegers

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

POSITION TITLE: Postdoctoral 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 Applied Sciences van Hall Larenstein, Leeuwarden the Netherlands	BASc	08/2011	Biotechnology
Erasmus University Rotterdam, the Netherlands	MSc	08/2013	Infection & Immunity
Erasmus University Rotterdam, the Netherlands	PhD	03/2022	Virology
Institute Pasteur du Cambodia, Phnom Penh, Cambodia	Postdoctoral		Virology, Global Health

A. Personal Statement

I am virologist who has been working in the field of emerging infectious diseases since 2013. During my PhD training in the Department of Viroscience at the Erasmus Medical Center in Rotterdam, the Netherlands, I studied the extra-respiratory tract complications of (zoonotic) influenza A virus infection using a wide range of disciplines that included virology, immunology, molecular biology and pathology. Following completion of my Ph.D., I began to pursue a research career focused on emerging respiratory viruses at the human-animal interface in Cambodia that incorporates surveillance, viral evolution, host-pathogen interactions, and pathogenesis. As a postdoctoral scientist at Institut Pasteur du Cambodge (IPC) in Phnom Penh, Cambodia, I coordinate and oversee all daily activities related to seasonal human and avian influenza viruses. These include molecular surveillance for influenza-like illness (ILI-surveillance), avian influenza surveillance in Cambodian live bird markets and wild birds, phylogenetics, cell culture work at BSL-2 and BSL-3 level for virus isolation, and serology. Furthermore, I am in charge of implementing novel virus characterization methods at the BSL-2 and BSL-3 lab facilities. Of particular relevance to the proposed project, I also oversee all management, development, and training related to routine sequencing at IPC Virology Unit using Oxford Nanopore technologies.

My research portfolio has a strong multi-disciplinary component that includes virology, immunology, pathology, pathogen genetics and genomics, field work, and public health, and has required me to become adept at combining and analyzing multiple types of data, including experimental, environmental, and genomic data. I also have a strong track record in the discovery/identification and characterization of emerging viruses, with 6 publications in this field, four of which have been cited more than 10 times since 2013 (total citation: 161). Adding pathogen metagenomics to my portfolio would advance my career significantly by becoming an expert in the field of emerging viruses, capable of performing initial pathogen discovery as well as primary virus characterization. As such, my experience, skills, and training are ideally suited to the proposed research project, which builds naturally on my previous work. Further, I am strongly committed to supporting diversity and equity both within the scientific community and in the communities we serve by improving science education and good/safe farming practices for underserved communities in rural Cambodia. I am also committed to providing training and mentorship for low- and middle-income country scientists and scholars.

I. Novel avian-origin influenza A (H7N9) virus attaches to epithelium in both upper and lower respiratory tract of humans. van Riel D, Leijten LME, de Graaf M, **Siegers JY**, Short KR, Spronken MIJ, Schrauwen EJA, Fouchier RAM, Osterhaus ADME, Kuiken T. **Am J Pathol**. 2013 Oct;183(4):1137-1143. doi: 10.1016/j.ajpath.2013.06.011. Epub 2013 Sep 10.

II. Novel avian-origin influenza A (H7N9) virus attachment to the respiratory tract of five animal models. **Siegers JY**, Short KR, Leijten LM, de Graaf M, Spronken MI, Schrauwen EJ, Marshall N, Lowen AC, Gabriel G, Osterhaus AD, Kuiken T, van Riel D. **J Virol**. 2014 Apr;88(8):4595-9. doi: 10.1128/JVI.03190-13. Epub 2014 Jan 29.

III. Phenotypic Differences between Asian and African Lineage Zika Viruses in Human Neural Progenitor Cells. Anfasa F, **Siegers JY**, van der Kroeg M, Mumtaz N, Stalin Raj V, de Vrij FMS, Widagdo W, Gabriel G, Salinas S, Simonin Y, Reusken C, Kushner SA, Koopmans MPG, Haagmans B, Martina BEE, van Riel D. **mSphere**. 2017 Jul 26;2(4):e00292-17. doi: 10.1128/mSphere.00292-17. eCollection 2017 Jul-Aug.

IV. Human Infection with Avian Influenza A(H9N2) Virus, Cambodia, February 2021. Um S, Siegers JY, Sar B, Chin S, Patel S, Bunnary S, Hak M, Sor S, Sokhen O, Heng S, Chau D, Sothyra T, Khalakdina A, Mott JA, Olsen SJ, Claes F, Sovann L, Karlsson EA. **Emerg Infect Dis**. 2021 Oct;27(10):2742-2745. doi: 10.3201/eid2710.211039.

B. Positions and Honors

Positions and Employment

2020- Postdoctoral Scientist, Institut Pasteur du Cambodge, Phnom Penh, Cambodia
2015-2020 PhD Candidate, Erasmus Medican Center, Rotterdam, the Netherlands
2015 Volunteer. Clinical Laboratory Technician (Ebola Virus Diagnostics). Koidu, Sierra Leone
2014 Research Asissant, Murdoch Children's Research Institute, Melbourne, Australia

Other Experience, Commissions of Trust and Professional Memberships

2018- Membership. American Society for Virology
2014- Ad Hoc Reviewer. Peer reviewer for different journals including Neuron, the Lancet, Journal of Virology.

Honors

2021 Young Scientist Fund, The eight ESWI conference, Virtual
2020 Young Scientist Fund, The seventh ESWI conference, Virtual
2019 Travel grant award. CADDE Genomic Epidemiology Workshop, Sao Paulo, Brazil
2018 Travel grant award. Visiting Scientist, University of Queensland, Australia
2017 Young Scientist Fund, The sixth ESWI conference, Riga, Latvia
2017 Travel grant award, European Seminar in Virology, Bertinoro, Italy
2016 Travel grant award, Options XI for the control of Influenza, Chicago, US
2015 Best poster award, Young Predigone (Predemix/Antigone), Rotterdam, the Netherlands
2015 Best abstract award, 7th Orthomyxovirus Research Conference, Toulouse, Frane
2015 Travel grant award, 7th Orthomyxovirus Research Conference, Toulouse, France
2013 Travel grant award, 100th American Association for Immunologist (AAI), Hawaii, US

C. Contributions to Science

I have authored 18 publications (published or in press) in the fields of influenza, respiratory viruses, infectious disease, and immunology, nine of which (50%) are first author publications. The primary theme of my work is understanding the emergence, prevalence, transmission, and pathogenesis of viruses at the human-animal interface. *As of this dossier, Google Scholar**: Total citations**: 374, **h-index**: 10, **i10-index**: 11

1. **Immunologic consequences of chronic diseases in humans.** The start of my scientific career began during my bachelor's degree internship at the Murdoch Children's Research Institute in Melbourne Australia where I started a new line of research within the Cell & Gene Therapy group. This research shed light on the mechanism that underlies the increased susceptibility to bacterial infections observed in patients suffering from the genetic blood disorder beta-thalassemia. I identified that neutrophils of beta-thalassemia patients are functionally abnormal due to a block in neutrophil maturation. My interest in the field of immunology and infectious disease grew during this internship and led me to pursue a MSc degree in infection & immunity. During one of my MSc internships at the department of Hematology, Erasmus Medical Center Rotterdam, the Netherlands, I examined the role of the cytokine IL-22 and the IL-22 receptor in rheumatoid arthritis and identified IL-22 as an important enhancer of the germinal center reaction, which are essential for the production of autoantibody-secreting plasma cells.

   V. Reduced PU.1 expression underlies aberrant neutrophil maturation and function in β-thalassemia mice and patients. Siwaponanan P, **Siegers JY**, Ghazali R, Ng T, McColl B, Ng GZ, Sutton P, Wang N, Ooi I, Thiengtavor C, Fucharoen S, Chaichompoo P, Svasti S, Wijburg O, Vadolas J. **Blood**. 2017 Jun 8;129(23):3087-3099. doi: 10.1182/blood-2016-07-730135. Epub 2017 Mar 21.

   VI. Loss of IL-22 inhibits autoantibody formation in collagen-induced arthritis in mice. Corneth OB, Reijmers RM, Mus AM, Asmawidjaja PS, van Hamburg JP, Papazian N, **Siegers JY**, Mourcin F, Amin R, Tarte K, Hendriks RW, Cupedo T, Lubberts E. **Eur J Immunol**. 2016 Jun;46(6):1404-14. doi: 10.1002/eji.201546241. Epub 2016 May 12.

2. **Characterization of emerging (respiratory) viruses.** After exploring immunology, it was time to pursue my biggest interest, the world of emerging viruses. I was able to contribute scientific knowledge and advancements related to the novel avian-origin H7N9 virus, where I first reported on the attachment pattern of this potentially pandemic virus to the respiratory tract of five different animal models frequently used in influenza virus research. In addition, I showed that in humans, H7N9 viruses attach to cells in both the upper and lower respiratory tract, something never before observed for an avian-origin influenza virus. In terms of virus discovery, I described the first identification and genetic characterization of a dolphin rhabdovirus and during the Zika virus outbreak, a re-emerging virus, I contributed by describing fundamental intrinsic *in vitro* differences between the African and Asian lineage Zika viruses in neuronal cells. Finally, I contributed to the identification of zinc-embedded polyamide fabrics as a suitable "passive" inactivation of SARS-CoV-2 virus that could be used in personal protective equipment.

   I. Novel avian-origin influenza A (H7N9) virus attaches to epithelium in both upper and lower respiratory tract of humans. van Riel D, Leijten LME, de Graaf M, **Siegers JY**, Short KR, Spronken MIJ, Schrauwen EJA, Fouchier RAM, Osterhaus ADME, Kuiken T. **Am J Pathol**. 2013 Oct;183(4):1137-1143. doi: 10.1016/j.ajpath.2013.06.011. Epub 2013 Sep 10.

   II. Novel avian-origin influenza A (H7N9) virus attachment to the respiratory tract of five animal models. **Siegers JY**, Short KR, Leijten LM, de Graaf M, Spronken MI, Schrauwen EJ, Marshall N, Lowen AC, Gabriel G, Osterhaus AD, Kuiken T, van Riel D. **J Virol**. 2014 Apr;88(8):4595-9. doi: 10.1128/JVI.03190-13. Epub 2014 Jan 29.

   III. Phenotypic Differences between Asian and African Lineage Zika Viruses in Human Neural Progenitor Cells. Anfasa F, **Siegers JY**, van der Kroeg M, Mumtaz N, Stalin Raj V, de Vrij FMS, Widagdo W, Gabriel G, Salinas S, Simonin Y, Reusken C, Kushner SA, Koopmans MPG, Haagmans B, Martina BEE, van Riel D. **mSphere**. 2017 Jul 26;2(4):e00292-17. doi: 10.1128/mSphere.00292-17. eCollection 2017 Jul-Aug.

3. **Pathogenesis of seasonal, pandemic, and zoonotic influenza A virus associated extra-respiratory complications.** My PhD project focused on the extra-respiratory complications of influenza virus infection where I made significant contributions to our understanding of the pathogenesis of extra-respiratory disease. First, I described the pathogenesis and extra-respiratory complications of pandemic 1918 H1N1 virus in a ferret animal model and provided new insights into the significant pathogenicity of this virus. Second, I provided new insights into the impact of chronic metabolic diseases (excess weight/obesity) on the

development of influenza virus-associated extra-respiratory disease. Third, I described the intrinsic differences - viral and host factors - in the ability of seasonal, pandemic, and zoonotic influenza A viruses to replicate in cells of the central nervous system, and proposed a potential mechanism. Finally, I evaluated the efficacy of vaccination and pre-exposure prophylaxis antivirals in the development of extra-respiratory tract disease associated with highly pathogenic avian influenza H5N1 virus infection in ferrets.

I. 1918 H1N1 Influenza Virus Replicates and Induces Proinflammatory Cytokine Responses in Extrarespiratory Tissues of Ferrets.de Wit E, **Siegers JY**, Cronin JM, Weatherman S, van den Brand JM, Leijten LM, van Run P, Begeman L, van den Ham HJ, Andeweg AC, Bushmaker T, Scott DP, Saturday G, Munster VJ, Feldmann H, van Riel D. **J Infect Dis**. 2018 Mar 28;217(8):1237-1246. doi: 10.1093/infdis/jiy003.

II. Mini viral RNAs act as innate immune agonists during influenza virus infection. Te Velthuis AJW, Long JC, Bauer DLV, Fan RLY, Yen HL, Sharps J, **Siegers JY**, Killip MJ, French H, Oliva-Martín MJ, Randall RE, de Wit E, van Riel D, Poon LLM, Fodor E. **Nat Microbiol**. 2018 Nov;3(11):1234-1242. doi: 10.1038/s41564-018-0240-5. Epub 2018 Sep 17.

III. A High-Fat Diet Increases Influenza A Virus-Associated Cardiovascular Damage. **Siegers JY**, Novakovic B, Hulme KD, Marshall RJ, Bloxham CJ, Thomas WG, Reichelt ME, Leijten L, van Run P, Knox K, Sokolowski KA, Tse BWC, Chew KY, Christ AN, Howe G, Bruxner TJC, Karolyi M, Pawelka E, Koch RM, Bellmann-Weiler R, Burkert F, Weiss G, Samanta RJ, Openshaw PJM, Bielefeldt-Ohmann H, van Riel D, Short KR. **J Infect Dis**. 2020 Aug 4;222(5):820-831. doi: 10.1093/infdis/jiaa159.

IV. Viral Factors Important for Efficient Replication of Influenza A Viruses in Cells of the Central Nervous System. **Siegers JY**, van de Bildt MWG, Lin Z, Leijten LM, Lavrijssen RAM, Bestebroer T, Spronken MIJ, De Zeeuw CI, Gao Z, Schrauwen EJA, Kuiken T, van Riel D. **J Virol**. 2019 May 15;93(11):e02273-18. doi: 10.1128/JVI.02273-18. Print 2019 Jun 1.

4. **Emerging respiratory viruses at human-animal interface.** My current postdoctoral training is focused on emerging (respiratory) viruses at the human-animal interface in Cambodia and includes - but is not limited to - influenza, coronaviruses, and paramyxoviruses. Using a “One Health” approach I contribute to understanding the dynamics of avian influenza at Cambodian live bird markets and in wild birds, the epidemiology of seasonal influenza, and the emergence of zoonotic viruses in humans. Thus far, I have identified the first human A/H9N2 infection in Cambodia and performed genetic and antigenic characterization of an influenza A(H3N2) outbreak in Cambodia during the COVID-19 pandemic.

   I. Human Infection with Avian Influenza A(H9N2) Virus, Cambodia, February 2021. Um S, **Siegers JY**, Sar B, Chin S, Patel S, Bunnary S, Hak M, Sor S, Sokhen O, Heng S, Chau D, Sothyra T, Khalakdina A, Mott JA, Olsen SJ, Claes F, Sovann L, Karlsson EA. **Emerg Infect Dis**. 2021 Oct;27(10):2742-2745. doi: 10.3201/eid2710.211039.

   II. Genetic and Antigenic Characterization of an Influenza A(H3N2) Outbreak in Cambodia and the Greater Mekong Subregion during the COVID-19 Pandemic, 2020. **Siegers JY**, Dhanasekaran V, Xie R, Deng YM, Patel S, Ieng V, Moselen J, Peck H, Aziz A, Sarr B, Chin S, Heng S, Khalakdina A, Kinzer M, Chau D, Raftery P, Duong V, Sovann L, Barr IG, Karlsson EA. **J Virol**. 2021 Nov 23;95(24):e0126721. doi: 10.1128/JVI.01267-21. Epub 2021 Sep 29.

Complete List of Published Work:

https://pubmed.ncbi.nlm.nih.gov/?term=siegers+jy

*Name of Individual: Heang, V.
Commons ID: N/A

Other Support – Project/Proposal

PREVIOUS

None

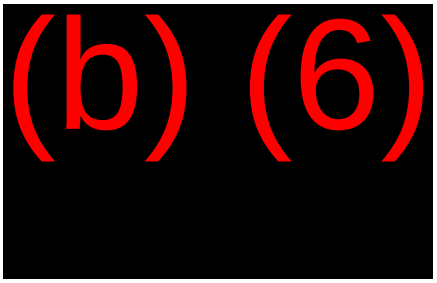
ACTIVE

None

PENDING

None

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.



*Signature: Heang, Vireak

Date: January 30, 2022

*Name of Individual: Siegers, J. Y.
Commons ID: N/A

Other Support – Project/Proposal

PREVIOUS

*Title: The role of obesity on extra-respiratory tract complications of Influenza A virus infection.

Major Goals: The major goals of this project is to describe the pathogenesis and transcriptome of extra-respiratory tract infection in obesity in mice.

*Status of Support: Ended

Project Number: N/A

Name of PD/PI: Dr. Debby van Riel, Dr. Kirsty Short

*Source of Support: Erasmus Trustfonds

*Primary Place of Performance: University of Queensland, Australia

Project/Proposal Start and End Date: (MM/YYYY) (if available): 12/2017 – 05/2018

* Total Award Amount (including Indirect Costs): €1696.51

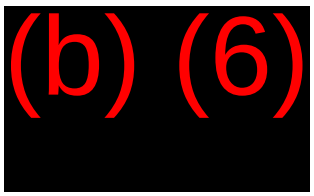
* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2018]	5 calendar

ACTIVE

PENDING

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.



*Signature: Siegers, Jurre

Date: January 30, 2022

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: Firth, Cadhla

eRA COMMONS USER NAME: (b) (6)

POSITION TITLE: Senior Research Scientist and Program Coordinator

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	Completion Date	FIELD OF STUDY
University of Guelph, Guelph, CAN	BS	06/2003	Zoology
University of Guelph, Guelph, CAN	MS	06/2005	Evolutionary Ecology
The Pennsylvania State University, State College, PA	PHD	05/2010	Biology
Columbia University, New York, NY	Postdoctoral	12/2012	Infectious Diseases & Public Health

A. Personal Statement

I am a molecular and evolutionary biologist who has been working on emerging infectious diseases for 15 years. In my current research, I explore the ecological and evolutionary dynamics of emerging zoonoses at the human-animal interface, with a focus on rapidly changing environments in Southeast Asia. I have more than eight years' experience leading independent research projects in this field, including collaborations with industry and local government. Over time, I have been responsible for the successful completion of every aspect of these studies, including project design, funding acquisition, implementation, and reporting/publishing. In 2015 I initiated a research program to investigate the response of zoonotic pathogens to urbanization in Malaysian Borneo using metagenomics, landscape ecology, and population genomics. This research has a strong multi-disciplinary component that includes evolutionary biology, molecular ecology, virology, pathogen genetics and genomics, field biology, and public health, and has required me to become adept at combining and analyzing multiple types of data, including environmental, ecological, and genomic data. This work has already resulted in three last-author publications, with a fourth currently in revisions at *Proceedings of the National Academy of Sciences of the USA.* As such, my experience, skills, and training are ideally suited to the proposed research project, which builds naturally on my previous work. I also have a strong track record in the generation and use of genetic and genomic data to explore the transmission dynamics and evolution of emerging viruses, with more than 20 publications in this field, nine of which have been cited more than 100 times since 2010. The mastery of genomic, phylogenetic, and phylodynamic methods I have developed throughout my career are directly applicable to the proposed research. Further, I am strongly committed to supporting diversity and equity both within the scientific community and in the communities we serve and have led several projects and initiatives aimed at improving science education and health service delivery for underserved communities in tropical northern Australia. I am also committed to providing training and mentorship for low- and middle-income country scientists and scholars, and in my previous role worked to develop protocols and bioinformatics pipelines to enable portable metagenomic sequencing in rural and remote regions in Australia and the Pacific. I am currently supervising two PhD students (James Cook University, AUS) and formally mentor a postdoctoral scientist, Dr. Nguyen Van Cuong, at the Oxford University Clinical Research Unit (OUCRU) in Viet Nam as part of my activities within the Centres for Research in Emerging Infectious Diseases (CREID) network.

B. Positions, Scientific Appointments, and Honors

Positions and Employment

2020-Present	Senior Research Scientist and Program Coordinator, EcoHealth Alliance, New York, NY
2020-Present	Adjunct Senior Research Fellow, College of Public Health, Medical & Vet Sciences, James Cook University, Cairns, AUS
2018-2020	HOT North Career Development Fellow, Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, AUS
2017-2018	Australian Research Council (ARC) Discovery Early Career Research Fellow, School of BioSciences, The University of Melbourne, Parkville, AUS
2013-2017	Research Scientist/ Australian Research Council (ARC) Discovery Early Career Research Fellow, Health and Biosecurity, The Commonwealth Scientific and Industrial Research Organization, Geelong, AUS
2013	Associate Research Scientist, Center for Infection and Immunity, Columbia University, New York, NY
2010-2012	Postdoctoral Fellow, Center for Infection and Immunity, Columbia University, New York, NY
2006-2010	PhD Scholar, The Pennsylvania State University, State College, PA
2001-2006	Research Assistant, Department of Zoology, The University of Guelph, Guelph, CAN

Honors

2019-2021	HOT North Career Development Fellowship, Northern Australia Tropical Disease Collaborative Research Programme, National Health and Medical Research Council (Australia)
2015-2018	Discovery Early Career Researcher Award, The Australian Research Council
2009-2010	Jeanette Ritter Mohnkern Graduate Student Scholarship in Biology, The Pennsylvania State University
2006-2009	Postgraduate Scholarship, Natural Science and Engineering Research Council of Canada
2006-2008	Braddock Graduate Fellowship, The Pennsylvania State University
2003-2005	Ontario Graduate Scholarship, The Province of Ontario (Canada)
1999	Undergraduate Entrance Scholarship, The University of Guelph

C. Contributions to Science

Throughout my career, my research has focused on many aspects of infectious disease emergence and has encompassed evolutionary biology, molecular ecology, virology, pathogen genetics and genomics, field biology, and public/veterinary health. My most significant contributions include:

1. **The use of genomic and metagenomic sequencing techniques to discover new pathogens, study their evolution, and explore microbial diversity.** I have successfully developed and used cutting-edge metagenomic techniques and bespoke bioinformatics pipelines for: (i) pathogen discovery, including a case of acute encephalitis in an immune-suppressed patient, (ii) studies of viral diversity, including coronaviruses and flaviviruses in bats; and (iii) genomics, including the epizoology of bluetongue virus, the evolution of rhabdoviruses, and the characterization of Australian bunyaviruses. I have also co-authored two perspectives on the utility of next-generation sequencing for pathogen discovery in people and animals. These included an invited submission that described an updated framework for proof of causation in the genomics era and discussed the emerging integration of on-the-ground metagenomics-based diagnostics with molecular epidemiology. In 2013, I initiated a study that used metagenomics to characterize the viruses and bacteria carried by New York City rats across a range of habitats, with a focus on the built environment. At the time, little was known about the range of potential pathogens carried by urban rodents or the risk factors associated with zoonotic transmission in a city environment. To begin to address this, I designed the first comprehensive molecular survey of rodent-borne microbes in a U.S. city, and demonstrated that urban rats frequently carry a range of zoonotic pathogens. We also found evidence of substantial heterogeneity in pathogen distribution within and between cities, suggesting that the associated risks of human disease may be similarly heterogeneous.

    a. Quan PL, **Firth C**, Conte JM, Williams SH, Zambrana-Torrelio CM, Anthony SJ, Ellison JA, *et al*. (2013). Bats are a major natural reservoir for hepaciviruses and pegiviruses. **Proceedings of the National Academy of Sciences of the USA** 110: 8194-9. PMCID: PMC3657805

b. **Firth C**, Lipkin WI (2013). The genomics of emerging pathogens. **Annual Review of Genomics and Human Genetics** 14: 281-300. PMID: 24003855

c. **Firth C**, Bhat M, Firth MA, Williams SH, Frye MJ, Simmonds P, Conte JM, *et al*. (2014). Detection of zoonotic pathogens and characterization of novel viruses carried by commensal Rattus norvegicus in New York City. **mBio** 5: e01933-14. PMCID: PMC4102142

d. Steinig E, Duchêne S, Aglua I, Greenhill A, Ford R, Yoannes M, Jaworski J, Drekore J, Urakoko B, Poka H, Wurr C, Ebos E, Nangen D, Laman M, Manning L, **Firth C**, Smith S, Pomat W, Tong SYC, Coin L, McBryde E, Horwood P. *et al*. (2022). Phylodynamic modelling of bacterial outbreaks using nanopore sequencing. **Molecular Biology and Evolution**. *In Press*.

Related Pre-print available: https://doi.org/10.1101/2021.04.30.442218

2. **I have helped develop the emerging field of urban zoonotic disease ecology.** In 2015 I pioneered an ongoing research program to investigate the response of zoonotic pathogens to urbanization using metagenomic sequencing, landscape ecology, and population genomics. Using a multi-disciplinary approach, this research explores how changes in the environment (e.g., land-use changes, microclimates, etc.), and host community and population structure (e.g., biodiversity, connectivity, density), influence pathogen community composition and zoonotic potential. Initial results of this work indicate that while mammalian diversity decreases with increasing urbanization, connectivity between populations increases, as does microbial species richness and the prevalence of known zoonotic pathogens. This suggests that some features of the built environment may inadvertently support pathogen persistence and spread, and as a result, some zoonotic pathogens may be more likely to emerge with intensifying urbanization. My reputation as an emerging researcher in this field has led to multiple invitations to present as a plenary or symposium speaker at national and international meetings, including the Ecological Society of Australia's Annual Conference (2019) and the Annual Meeting of the American Society of Mammalogists (2019), and the Joint Conference of the Asian Society of Conservation Medicine and the Wildlife Disease Association Australasia (2018).

a. Frye MJ, **Firth C**, Bhat M, Firth MA, Che X, Lee D, Williams SH, Lipkin WI (2015). Preliminary survey of ectoparasites and associated pathogens from Norway rats in New York City. **Journal of Medical Entomology** 52: 253-9. PMCID: PMC4481720

b. Blasdell KR, Perera D, **Firth C** (2018). High prevalence of rodent-borne *Bartonella* spp. in urbanizing environments in Sarawak, Malaysian Borneo. **American Journal of Tropical Medicine and Hygiene** 100: 506-9. PMCID: PMC6402934

c. Blasdell KR, Morand S, Perera D, **Firth C** (2019). Association of rodent-borne *Leptospira* spp. with urbanizing environments in Sarawak, Malaysian Borneo. **PloS Neglected Tropical Diseases** 13: e0007141. PMCID: PMC6411199

d. Blasdell KR, Morand S, Laurance SGW, Doggett SL, Hahs A, Perera D, **Firth C** (2021) Rats in the city: implications for zoonotic disease risk in an urbanizing world. bioRxiv 2021.03.18.436089 [**Preprint**] March 19, 2021 [cited 2022 Jan 30]. Available from: https://doi.org/10.1101/2021.03.18.436089
*Revised manuscript Under Review at Proceedings of the National Academy of Sciences of the USA.

3. **I challenged established beliefs about the ecological and evolutionary processes linked to viral disease emergence, and the timescale over which they occur.** During my PhD, I was able to make significant contributions to this field by questioning the idea that viruses evolve either by cross-species transmission (spillover) or by co-evolution with their hosts, but rarely both. My work demonstrated that viruses with many genomic structures are capable of jumping species barriers, and that evolution through both co-divergence and cross-species transmission may be general trend of many virus systems. This has since been supported by numerous subsequent studies that have highlighted the complex evolutionary dynamics of both RNA and DNA viruses.

a. **Ramsden C***, Holmes EC, Charleston MA (2009). Hantavirus evolution in relation to its rodent and insectivore hosts: no evidence for co-divergence. **Molecular Biology and Evolution** 26: 143-53. PMID: 18922760
* Name changed from Ramsden to Firth in 2009

b. Pagan I, **Firth C**, Holmes EC (2010). Phylogenetic analysis reveals rapid evolutionary dynamics in the plant RNA virus genus tobamovirus. **Journal of Molecular Evolution** 71: 298-307. PMID: 20838783

c. **Firth C**, Kitchen A, Shapiro B, Suchard MA, Holmes EC, Rambaut A (2010). Using time-structured data to estimate evolutionary rates of double-stranded DNA viruses. **Molecular Biology and Evolution** 27: 2038-51. PMCID: PMC3107591

d. Sali AA, Faye O, Diallo M, **Firth C**, Kitchen A, Holmes EC (2010). Yellow fever virus exhibits slower evolutionary dynamics than dengue virus. **Journal of Virology** 84: 765-72. PMCID: PMC2798388

4. **I was among the first to apply novel phylodynamic approaches to reconstruct the spatiotemporal processes behind disease emergence events.** Phylodynamic methods enable the synthesis of epidemiological, geographic, and phylogenetic data to reconstruct the processes behind infectious disease emergence and spread through time and space. These methods are of particular use for investigating the emergence and spread of viruses, as they have a rapid evolutionary rate that closely matches the timescale of virus transmission. I began to explore these approaches during my PhD and have now successfully used them to: (i) explore the genetics underpinning the rapid emergence and spread of human enterovirus 68; (ii) examine the patterns and processes that influence the diversity and geographic distribution of New World hantaviruses; (iii) explore the epizoology of porcine circovirus 2; iv) understand the evolutionary and ecological drivers behind the distribution of bluetongue virus in Australia; (v) assess the likely origins of hepatitis C virus; and (vi) understand the global emergence of community-associated MRSA and MSSA.

   a. Kapoor A, Simmonds P, Gerold G, Qaisar N, Jain K, Henriquez JA, **Firth C**, *et al*. (2011). Characterization of a canine homolog of hepatitis C virus. **Proceedings of the National Academy of Sciences of the USA** 108: 11608-13. PMCID: PMC3136326

   b. **Firth C,** Tokarz R, Simith DB, Nunes MR, Bhat M, Rosa ES, Medeiros DB, Palacios G, Vasconcelos PF, Lipkin WI (2012). Diversity and distribution of hantaviruses in South America. **Journal of Virology** 86: 13756-66. PMCID: PMC3503106

   c. Tokarz R, **Firth C**, Madhi SA, Howie SR, Wu W, Sall AA, Haq S, Briese T, Lipkin WI (2012). Worldwide emergence of multiple clades of enterovirus 68. **Journal of General Virology** 93: 1952-8. PMCID: PMC3542132

   d. Steinig E, Aglua I, Duchene S, Meehan MT, Yoannes M, **Firth C**, et al. (2021). Phylodynamic signatures in the emergence of community-associated MRSA. BioRxiV 2021.04.30.442212 [**Preprint**] April 30, 2021 [cited 2022 Jan 30]. Available from: https://doi.org/10.1101/2021.04.30.442212
   *Under Review at Lancet Microbe.

Complete List of Published Work in MyBibliography:
https://www.ncbi.nlm.nih.gov/myncbi/1pcDCsap4HMk2/bibliography/public/

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: Erik Albert KARLSSON

eRA COMMONS USER NAME (credential, e.g., agency login): (b) (6)

POSITION TITLE: Deputy Head of Unit, Director of National Influenza Center of Cambodia, Director of WHO Regional H5 Reference Laboratory, Coordinator of WHO Global COVID-19 Reference Laboratory, Virology Unit, Institut Pasteur du Cambodge

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 North Carolina, Chapel Hill	B.S.	05/2005	Biochemistry
University of North Carolina, Chapel Hill	Ph.D.	05/2010	Nutrition
St. Jude Children's Research Hospital	Postdoctoral	06/2016	Virology

A. Personal Statement

During my doctoral work in the Department of Nutrition at the Gilling's School of Global Public Health, University of North Carolina at Chapel Hill, I studied the effect of nutrition, especially diet-induced obesity, on immune responses to viral pathogens. Following completion of my Ph.D., I began to pursue a research career focused on understanding surveillance, viral evolution, host-pathogen interactions, and immune responses. I began working as a Postdoctoral Research Associate with Dr. Stacey Schultz-Cherry and collaborators at St Jude Children's Research Hospital to expand my knowledge of virology, viral pathogenesis, co-infection, immunology (primary and vaccine-induced), and infectious disease surveillance. I became a Staff Scientist in Dr. Schultz-Cherry's laboratory, focusing further on the interaction of nutrition and infectious disease as well as helping to oversee influenza surveillance at the animal-human interface worldwide. I currently serve as a Senior Researcher (Assistant Professor equivalent) at Institut Pasteur du Cambodge (IPC) in Phnom Penh, Cambodia. I am the Deputy Head of the Virology Unit and in charge of all ongoing activities related to respiratory viruses, including human seasonal and zoonotic viruses, and am the Director of the National Influenza Center and the Regional WHO H5 Reference lab. I am integral in the COVID-19 response in Cambodia and worldwide and serve as the Coordinator for the WHO Global COVID-19 Reference Laboratory in the Virology Unit. I also serve as the Co-PI of the newly developed Sequencing Platform at IPC. My work at IPC focuses on surveillance of endemic and emerging viruses in Cambodia, including developing Early Warning systems against the next pandemic. I also frequently consult for UN organizations (FAO and WHO) and am the founder of a research think-tank named: ''CANARIES: Consortium of Animal market Networks to Assess Risks of emerging Infectious diseases through Enhanced Surveillance."

B. Positions and Honors

Positions and Employment

2016-2017 *Staff Scientist*, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN
2017-2020 *Senior Research*, Virology Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia
2020- *Deputy Head*, Virology Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia

Other Experience, Commissions of Trust, and Professional Memberships (since 2011)

2011- American Society for Virology
2011- **Ad Hoc Reviewer**. Peer reviewer for 60+ journals Full review record available on Publons: https://publons.com/author/737520/erik-albert-karlsson#profile
2012-2013 **Secretary**. Nutritional Immunology RIS. American Society for Nutrition
2013-2015 **Chair Elect.** Nutritional Immunology RIS. American Society for Nutrition.
2015-2016 **Chair.** Nutritional Immunology and Inflammation RIS. American Society for Nutrition
2015- **Editorial Board Member**. Journal of Nutritional Biochemistry
2017- **Editorial Board Member**. Virus Genes
2019- **Founder.** Consortium of Animal market Networks to Assess Risks of emerging Infectious diseases through Enhanced Surveillance (CANARIES; http://www.canarieshmhp.org).
2020- **Consultant, Laboratory Technical Advisor**, Emergency Centre for Transboundary Animal Diseases, Food and Agriculture Organization of the United Nations, Regional Office for Asia and the Pacific.
2020-2021 **Consultant, Scientific Expert**. World Health Organization. Global Influenza Surveillance and Response System.
2021- **Consultant, Technical Expert – Genomic Surveillance.** World Health Organization, European Region, Turkey.

Honors Since 2011

2011 **ASV 2011 Postdoctoral Fellow Travel Award**. American Society for Virology General Meeting 2011, Minneapolis, MN
2011 **Travel Award**. ISIRV-AVG Conference: Influenza Antivirals: Efficacy and Resistance. Rio de Janiero, Brazil. November, 2011
2012 **Travel Award**. Nutritional Immunology Research Interest Section. American Society for Nutrition. Experimental Biology 2012, San Diego, CA
2012 **Young Investigator Travel Award**. Palm Beach Infectious Disease Research Institute. PBIDI Symposium 2012, Palm Beach, FL
2012 **Travel Award**. 6th Orthomyxovirus Research Conference. Bromont, Quebec, Canada. September, 2012
2012 **Travel Award**. Obesity Research Interest Section. American Society for Nutrition. Experimental Biology 2012, San Diego, CA
2013 **Promising Young Investigator Scholarship**. Options for the Control of Influenza VII. Cape Town, South Africa. September 2013.
2015 **Young Investigator Travel Award**. 3rd ISIRV International Symposium on Neglected Influenza Viruses. Athens, Gerogia, USA. April, 2015.
2015 **Merck Junior Investigator Award - Best Speaker**. 3rd ISIRV International Symposium on Neglected Influenza Viruses. Athens, Gerogia, USA. April, 2015.
2018 **Francis Crick Institute Best Poster Award**. ISIRV Symposium – Influenza 2018: Centenary of the 1918 Pandemic. London, UK. June, 2018.

C. Contributions to Science

As of this submission, I have authored 70+ publications (published or in press) in the fields of nutrition, influenza, respiratory viruses, and infectious disease: 28 (40%) first author publications and 7 (10%) senior author publications. Full list available at: https://scholar.google.com/citations?user=lq1f7K0AAAAJ&hl=en
*As of this dossier**: Total citations**: 3,395, **h-index**: 31, **i10-index**: 53

1. **Global avian influenza virus surveillance and response in domestic poultry and wild birds**. Avian influenza virus is a major concern worldwide. Aside from seasonal epidemics and occasional pandemics, avian influenza viruses continue to cause human infections. I have been involved in influenza virus surveillance in wild and domestic poultry in Africa, South America, and Southeast Asia. These studies have defined new hotspots of influenza, determined seasonal patterns, and even helped to define policy recommendations. Selected References:

a) **Karlsson, EA**, Ciuoderis K, Freiden PJ, Seufzer B, Jones JC, Johnson J, Parra R, Gongora A, Cardenas D, Barajas D, Osorio JE, and Schultz-Cherry S. 2013. Prevalence and characterization of influenza viruses in diverse species in Los Llanos, Colombia. Emerging Microbes & Infection. 2, e20; doi:10.1038/emi.2013.20 PMID: 26038461
b) Jiménez-Bluhm P, **Karlsson EA**, Freiden P, Sharp B, Di Pillo F, Osorio JE, Hamilton-West C and Schultz-Cherry S. 2018. *Wild birds in Chile harbor diverse avian influenza A viruses*. Emerg Microbes Infect. Mar 29;7(1):44. doi: 10.1038/s41426-018-0046-9. PMID: 29593259
c) C G Monamele, P Y, **E A Karlsson**, M-A Vernet, A Wade, S Yann, N R Mohamadou, S Kenmoe, G M Yonga, J M Feussom, G Djonwe, C Ndongo, V S Horm, P F Horwood, S Ly, R Njouom and P Dussart. *Outbreak of avian influenza A(H5N1) among poultry in Cameroon and evidence of sub-clinical human infection* Emerg Microbes Infect. 2019 Jan 22; 8(1):186. doi: 10.1080/22221751.2018.1564631
d) **E A Karlsson***, S Tok, S V Horm, S Sorn, D Holl, S Tum, F Claes, K Osbjer and Philippe Dussart. Avian influenza virus detection, temporality and co-infection in poultry in Cambodian border provinces, 2017-2018. Emerg Microbes Infect. 2019; 8(1):637-639. doi: 10.1080/22221751.2019.1604085. PMID: 30999819 *Corresponding Author
e) Vijaykrishna D, Y-M Deng, M L Grau, M Kay, A Suttie, P F Horwood, W Kalpravidh, F Claes, K Osbjer, P Dussart, I G Barr, and **E A Karlsson***. *Emergence of Influenza A(H7N4) Virus, Cambodia*. Emerg Infect Dis. 2019 25(10):1988-1991. doi: 10.3201/eid2510.190506. PMID: 31310233 *Corresponding Author

2. **Seasonal and avian influenza surveillance and response in humans**. Human seasonal and avian strains continue to circulate endemically (seasonal) and cause zoonotic spillover (avian) in humans. I have been involved in both human seasonal and avian influenza surveillance in humans for a number of years. Selected References:

a) Horwood P F, **E A Karlsson**, S V Horm, S Ly, S Chin, D Saunders, S Rith, P Y, B Sar, A Parry, R Tsuyouka, Y-M Deng, A Hurt, I Barr, N Komadina, P Buchy and P Dussart. *Circulation and characterization of human influenza infections in Cambodia, 2012-2015*. Influenza Other Repir Viruses. 2019; 3(5): 465-476. doi: 10.1111/irv.12647. PMID: 31251478
b) Siegers J Y, V Dhanasekaran, R Xie, Y-M Deng, S Patel, V Ieng, J Moselen, H Peck, A Aziz, B Sarr, S Chin, S Heng, A Khalakdina, M Kinzer, D Chau, P Raftery, V Duong, L Sovann, I G Barr, **E A Karlsson*.** *Genetic and antigenic characterization of an influenza A(H3N2) outbreak in Cambodia and the Greater Mekong Subregion during the COVID-19 pandemic, 2020*. Journal of Virology. 2021. 23; 95(24): e0126721. doi: 10.1128/JVI.01267-21 *Corresponding Author
c) L Sovann, B Sar, V Kab, S Yann, M Kinzer, P Raftery, S Patel, P L Hay, H Seng, S Um, S Chin, D Chau, A Khalakdina, **E A Karlsson**, S Olsen, J Mott*. An influenza A(H3N2) virus outbreak during the COVID-19 pandemic, Kingdom of Cambodia, 2020*. International Journal of Infectious Disease. 2021. 103: 352-357. doi: 10.1016/j.ijid.2020.11.178
d) Um S, J Y Siegers, B Sar, S Chin, S Patel, S Bunnary, M Hak, S Sor, O Sokhen, S Heng, D Chau, T Sothyra, A Khalakdina, S J Olsen, J A Mott, F Claes, L Sovann, and **E A Karlsson***. A *Human Infection with Avian Influenza A(H9N2) Virus in Cambodia, February 2021.* Infectious Diseases. 2021. 27(10): 2742-2745. doi: 10.3201/eid2710.211039 *Corresponding Author

3. **Emerging and endemic viral discovery and surveillance in humans, domestic animals, and wild animals**. Aside from avian and human seasonal influenza, I have also contributed to a number of studies on other emerging viral diseases in Southeast Asia, including SARS-CoV-2. Selected References:

a) **Karlsson E A**, C T Small, P Freiden, M M Feeroz, F A Matsen IV , S San, M K Hasan, D Wang, G Engel, L Jones-Engel and S Schultz-Cherry. *Nonhuman primates harbor a diversity of mammalian and avian astroviruses including those associated with human infections.* PLoS Pathog. 2015 Nov 16;11(11):e1005225. doi: 10.1371/journal.ppat.1005225. PMID: 26571270. PMCID: PMC4646697
b) Delaune D*, V Hul*, **E A Karlsson***, A Hassanin, P O Tey, A Baidaliuk, F Gámbaro, Vuong T Tu, L Keatts, J Mazet, C Johnson, P Buchy, P Dussart, T Goldstein, E Simon-Lorière, Duong. *A novel SARS-CoV-2 related coronavirus in bats from Cambodia*. Nature Communications. 2021. 12; 6563. https://doi.org/10.1038/s41467-021-26809-4 *Co-first Author
c) **Karlsson E A**, V Duong. *The continuing search for the origins of SARS-CoV-2*. Cell. 2021. 184(17): 4373-4374. doi: 10.1016/j.cell.2021.07.035

d)

4. **Defining the risk of emerging viruses and the bottlenecks overcome to cross species barriers**. A major concern with zoonotic viruses, especially avian influenza virus, is the risk of zoonotic transmission. These studies seek to define factors (host-related, bacteriological, and virological) which can help to predict risk of zoonotic transmission and severity of viruses and emerging diseases. Selected References:

    a) **Karlsson, EA**, Ip HS, Hall J, Yoon S-W, Johnson J, Beck MA, Webby RJ and Schultz-Cherry S 2014. Respiratory transmission of an avian H3N8 influenza virus isolated from a harbor seal. Nat Commun 5:4791. PMID: 25183346
    b) Zaraket H, Baranovich T, Kaplan B, Carter R, Song M-S, Paulson J, Rehg F, Bahl J, Crumpton J, Seiler P, Edmonson M, Wu G, **Karlsson EA,** Fabrizio II T, Zhu H, Guan Y, Husain M, Schultz-Cherry S, Krauss S, McBride R, Webster RG, Govorkova E, Zhang J, Russell C, and Webby RJ. 2015. Mammalian adaptation of influenza A(H7N9) virus is limited by a narrow genetic bottleneck. Nat Commun 6:6553. PMID:25850788
    c) **Karlsson EA**, Meliopoulos VA, Savage C, Livingston B, Mehle A and Schultz-Cherry S**.** 2015. Visualizing Real-time influenza virus infection, transmission and protection in ferrets. Nat Commun 6:6378. PMID:25744559
    d) Rowe H M[#], **E A Karlsson**, H Echlin, T-C Chang, L Wei, T van Opijnen, S Pounds, S Schultz-Cherry and J W Rosch. *Bacterial factors required for transmission of* Streptococcus pneumoniae *in mammalian hosts.* Cell Host Microbe. 2019 Jun 12;25(6):884-891.e6. doi: 10.1016/j.chom.2019.04.012. PMID: 3112675
    e) Moncla L H, T Bedford, P Dussart, S V Horm, S Rith, P Buchy, **E A Karlsson**, L Li, Y Liu, H Zhu, Y Guan, T C Friedrich, and P F Horwood. *Quantifying within-host evolution of H5N1 influenza in humans and poultry in Cambodia.* 2020 Jan 17;16(1):e1008191. doi: 10.1371/journal.ppat.1008191 PMID: 31951644

D. Additional Information: Research Support and/or Scholastic Performance (Selected since 2019)

1. 2019 - **Grand Challenges Research Fund Networking Grant**. **GCRFNGR3\1497.** CANARIES: Consortium of Animal market Networks to Assess Risks of emerging Infectious diseases through Enhanced Surveillance (**PI**)
2. 2019 - **World Health Organization – LoA 2019/923423-0 -** Testing of ILI specimens for influenza A and B viruses; and testing of SARI specimens for influenza A/H5N1, influenza A/H7N9, other subtypes, and MERS-CoV from June – October 2019 (**PI**)
3. 2019 - **Wellcome Trust Multi-User Equipment Grant – 218310/Z/19/Z** - Advancing flow cytometry for the on-site study of tropical infectious diseases (**Co-PI**)
4. 2019-2024 - **DHHS ASIDE2 Project** - Detect threats early in Cambodia, including real-time biosurveillance, detection, characterization and reporting of emerging influenza strains and other emerging/co-emerging infectious respiratory disease pathogens. (**Co-PI**)
5. 2020 - **World Health Organization – LoA 2020/1027266-0 -** Agreement to Provide laboratory Diagnosis and Confirmation for COVID-19 Surveillance Specimens to Support the Public Health Response (**PI**)
6. 2020 - **World Health Organization – LoA 2020/1049713-0 -** Testing of ILI Specimens for Influenza A and B viruses; and Testing of SARI Specimens for Influenza A/H5N1, influenza A/H7N9, other subtypes, RSV, PIV, and human seasonal CoV from September 4th to September 29th, 2020 (**PI**)
7. 2020 - **FAO – USAID LoA** - **LOA/RAP/2020/09** – Avian and human influenza surveillance activities in Cambodia (**PI**)
8. 2021 – 2022 – **FAO – USAID LoA** - **LOA/RAP/2021/22** – Avian and human influenza surveillance activities in Cambodia and genetic analysis using novel sequencing techniques (**PI**)
9. 2021-2028 - **NIAID Centres of Excellence for Influenza Research and Response – UPenn CEIRR** - Influenza Surveillance, Risk Assessment, and Response Project 1: Swine Influenza Surveillance and Risk Assessment: Cambodia (**Country PI**)
10. 2021 – 2022 – **NIAID Centers for Research in Emerging Infectious Diseases – PICREID** – COVID Supplement: COVID-19 Sequencing Support in Cambodia (**PI**)
11. 2021 – 2022 – **DTRA STEP TD04-001** - Into the Wild: Prevalence of AIV in Wild Birds in the Kingdom of Cambodia (**PI**)

*Name of Individual: Firth, C.
Commons ID: (b) (6)

Other Support – Project/Proposal

ACTIVE

*Title: Understanding Risk of Zoonotic Virus Emergence in EID Hotspots of South East Asia

Major Goals: This project aims to identify, characterize and rank spillover risk of high zoonotic potential viruses from wildlife, identify key risk pathways for zoonotic transmission, and identify and characterize viral etiology of 'cryptic' outbreaks in clinical cohorts focusing in Thailand and Malaysia.

*Status of Support: Active

Project Number: 1U01AI15179

Name of PD/PI: Daszak, Peter

*Source of Support: NIH/NIAID

*Primary Place of Performance: EcoHealth Alliance, New York

Project/Proposal Start and End Date: (MM/YYYY) (if available): 06/2020 – 05/2025

* Total Award Amount (including Indirect Costs): $7,573,721

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	3.0 calendar
2. [2023]	3.0 calendar
3. [2024]	3.0 calendar
4. [2025]	3.0 calendar
5. [2026]	3.0 calendar

*Title: Understanding the establishment and persistence of community associated methicillin-resistant Staphylococcus aureus (CA-MRSA) in resource-limited, high-burden settings: How can we reduce the burden of disease?

Major Goals: To determine the clinical impact of MRSA-related acute hematogenous osteomyelitis (AHO) in the PNG highlands and identify interventions to reduce the burden and impact of this infection. We will evaluate the feasibility of point-of-care diagnosis of MRSA, determine the persistence and transmission pathways of CA-MRSA, and determine the diversity and phylodynamics of MRSA strains circulating in PNG highland communities and their association with AHO cases.

*Status of Support: Active

Project Number:

Name of PD/PI: Horwood, Paul

*Source of Support: National Health and Medical Research Council (Australia)

*Primary Place of Performance: James Cook University, Australia and Papua New Guinea

Project/Proposal Start and End Date: (MM/YYYY) (if available): 01/2022 – 12/2024

* Total Award Amount (including Indirect Costs): $619,987

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	0.5 calendar
2. [2023]	0.5 calendar
3. [2024]	0.5 calendar
4. [2025]	0.5 calendar
5. [2026]	0.5 calendar

PENDING

*Title: Revealing the Determinants of Virus Diversity and Cross-Species Transmission on Wildlife Farms in Southeast Asia

Major Goals: This project will characterize viral diversity, assess the rate of cross-species transmission, and evaluate zoonotic disease risk. Using innovative mathematical models, this project will estimate key epidemiological parameters for cross-species transmission within farms, simulate the impact of different farming practices, and assess the associated profit-risk trade-offs.

*Status of Support: Pending

Project Number: 2207955

Name of PD/PI: Firth, Cadhla

*Source of Support: National Science Foundation

*Primary Place of Performance: EcoHealth Alliance, New York

Project/Proposal Start and End Date: (MM/YYYY) (if available): 07/2022 – 06/2027

* Total Award Amount (including Indirect Costs): $2,999,887

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	2.0 calendar
2. [2023]	2.0 calendar
3. [2024]	2.0 calendar
4. [2025]	2.0 calendar
5. [2026]	2.0 calendar

*Title: Analyzing the Potential for Future Bat Coronavirus Emergence in Myanmar, Laos, & Vietnam

Major Goals: This project will conduct community-based surveys and biological sampling of people frequently exposed to wildlife in Myanmar, Laos, and Vietnam, to find serological evidence of spillover and assess disease spread risk; sampling and PCR screening of bats and other wildlife at community surveillance sites to identify viruses and hosts related to the human

infections; and syndromic surveillance in clinics to identify ‘cryptic’ cases or case clusters caused by bat-CoVs and assess the spread risk..

*Status of Support: Pending

Project Number:

Name of PD/PI: Daszak, Peter

*Source of Support: NIH/NIAID

*Primary Place of Performance: EcoHealth Alliance, New York

Project/Proposal Start and End Date: (MM/YYYY) (if available): 07/2022 – 04/2027

* Total Award Amount (including Indirect Costs): $3,361,851

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	3.0 calendar
2. [2023]	3.0 calendar
3. [2024]	3.0 calendar
4. [2025]	3.0 calendar
5. [2026]	3.0 calendar

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

*Signature: Firth, Cadhla

Date: January 30, 2022

*Name of Individual: Karlsson, E. A.
Commons ID: (b) (6)

Other Support – Project/Proposal

ACTIVE

*Title: Avian and human influenza surveillance activities in Cambodia and genetic analysis using novel sequencing techniques

Major Goals: The major goals of this project are continued longitudinal surveillance of avian influenza virus at the poultry-human interface in Cambodia and integration of new sequencing technologies.

*Status of Support: Active

Project Number: LOA/RAP/2021/22

Name of PD/PI: Karlsson, Erik

*Source of Support: FAO-USAID

*Primary Place of Performance: Institut Pasteur du Cambodge

Project/Proposal Start and End Date: (MM/YYYY) (if available): 06/2021 – 05/2022

* Total Award Amount (including Indirect Costs): $199,970

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	1.8 calendar

*Title: NIAID Centres of Excellence for Influenza Research and Response – UPenn CEIRR - Influenza Surveillance, Risk Assessment, and Response Project 1: Swine Influenza Surveillance and Risk Assessment: Cambodia

Major Goals: The major goals of this project are longitudinal influenza virus surveillance in swine and humans to detect, characterize, and analyze the evolution, disease dynamics, and risk profile of IAVs at the human-swine interface in Cambodia. We will monitor the evolutionary trajectories of swine IAVs currently circulating in backyard and commercial swine holdings in key border regions of Cambodia

*Status of Support: Active

Project Number:

Name of PD/PI: Karlsson, Erik (Country PI), Hensley, Scott (Center PI)

*Source of Support: NIH/NIAID

*Primary Place of Performance: Institut Pasteur du Cambodge

Project/Proposal Start and End Date: (MM/YYYY) (if available): 06/2021 – 03/2028

* Total Award Amount (including Indirect Costs): $1,503,684

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	3.6 calendar
2. [2023]	3.6 calendar
3. [2024]	3.6 calendar
4. [2025]	3.6 calendar
5. [2026]	3.6 calendar
6. [2027]	3.6 calendar
7. [2028]	1.8 calendar

*Title: Into the Wild: Prevalence of AIV in Wild Birds in the Kingdom of Cambodia

Major Goals: The major goals of this project are to obtain preliminary information on avian influenza virus prevalence in wild birds in Cambodia, develop preliminary data for building an Early Warning System (including the use of novel techniques) and to provide capacity building to Cambodian Veterinary Epidemiologists

*Status of Support: Active

Project Number: STEP TD04-001

Name of PD/PI: Karlsson, Erik

*Source of Support: DTRA

*Primary Place of Performance: Institut Pasteur du Cambodge

Project/Proposal Start and End Date: (MM/YYYY) (if available): 10/2021 – 05/2022

* Total Award Amount (including Indirect Costs): $282,154

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	1.8 calendar

*Title: CREID COVID Supplement: Support for COVID Sequencing in Cambodia. PICREID.

Major Goals: The major goals of this project are to further support sequencing and analysis of SARS-CoV-2 genomes in Cambodia as part of the national respoinse to COVID-19.

*Status of Support: Active

Project Number:

Name of PD/PI: Karlsson, Erik

*Source of Support: NIH/NIAID

*Primary Place of Performance: Institut Pasteur du Cambodge

Project/Proposal Start and End Date: (MM/YYYY) (if available): 11/2021 – 05/2022

* Total Award Amount (including Indirect Costs): $107,266

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	0.5 calendar

PENDING

*Title: Novel detection and sampling techniques for surveillance of bat-borne zoonoses in Cambodia

Major Goals: This project will utilize novel field technologies (RT-PCR, sequencing) in conjunction with novel sampling strategies (air, environment) to develop better ways to sample bats across Cambodia.

*Status of Support: Pending LoA

Project Number:

Name of PD/PI: Karlsson, Erik

*Source of Support: FAO-USAID

*Primary Place of Performance: Institut Pasteur du Cambodge

Project/Proposal Start and End Date: (MM/YYYY) (if available): 01/2022 – 11/2022

* Total Award Amount (including Indirect Costs): $77,000

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	1.8 calendar

*Title: One Health understanding of small-holder swine value chain in Cambodia

Major Goals: This study aims to enhance the health and livelihood of small-holder swine value chain stakeholders through cost-effective intervention measures following a One-Health approach. We believe that small-holder pig producers play a key role in the spread of several important zoonotic diseases, including food borne diseases. Applying cost-effective interventions and risk-based surveillance along the value chain will reduce risk and enhance the livelihood of the stakeholders. The project will tackle gender-related inequalities in the roles assigned along the pig-value chain in risk awareness, mitigation and exposure, and identify solutions to empower women in this system.

*Status of Support: Pending

Project Number:

Name of PD/PI: Karlsson, Erik

*Source of Support: ACIAR (Australia), IDRC (Canada)

*Primary Place of Performance: Institut Pasteur du Cambodge

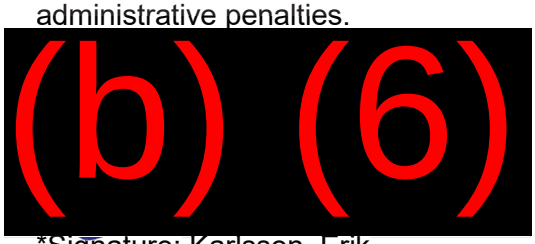
Project/Proposal Start and End Date: (MM/YYYY) (if available): 07/2022 – 07/2025

* Total Award Amount (including Indirect Costs): $698,700

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. [2022]	1.0 calendar
2. [2023]	2.0 calendar
3. [2024]	2.0 calendar
4. [2025]	1.0 calendar

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.



*Signature: Karlsson, Erik

Date: January 30, 2022

Co-PI Plan (only needed if applying as Co-PIs):

As stated in the Mentoring Plan, the co-PIs, Vireak Heang and Jurre Siegers, are at different stages of their respective careers and have discrete professional development goals. This Co-PI Plan was developed to maximize the strengths of each PI, provide opportunities to learn from each other, and create a supportive environment where they can pursue their independent professional goals while working as a team to achieve the project objectives.

Overall, the proposed research project will be jointly and equally managed by Dr. Siegers and Mr. Heang. Dr. Siegers will plan, oversee, and conduct sample management and processing. He will also lead the data analysis aspects of the work. Mr. Heang will be responsible for the technical aspects of metagenomic sequencing, including logistics and protocol development, laboratory work, and initial data curation and quality control. He will work closely with the Research Center Mentor, Dr. Cadhla Firth, to develop these aspects of the project. As the project progresses, Dr. Siegers will be exposed to the technical aspect of metagenomics, including how to prepare and load samples onto the sequencing platform, by learning from Mr. Heang. Likewise, Mr. Heang will develop skills in upstream sample preparation and handling and will have the opportunity to participate in downstream data analysis, as he learns from Dr. Siegers. This collaboration will ensure that both individuals gain technical skills in areas where they have less experience, and each will become a more well-rounded scientist as a result of their joint efforts. While Mr. Heang will be more involved with the technical aspects of sequencing and data generation, Dr. Siegers will take the lead to work with EcoHealth Alliance's modeling experts to explore the virological data and the meaning for potential surveillance efforts.

Both PIs will jointly manage data curation, analysis, and dissemination for this proposal. In addition, the Co-PIs will jointly manage the project budget with oversight from their Scientific Mentor, Dr. Erik Karlsson. The PIs will receive a budget code in the IPC system on which they can charge reagents and supplies, as well as manage spending, and purchases will be co-signed by Dr. Karlsson. In terms of specific reagents, Mr. Heang will oversee the ordering of supplies needed for sequencing, and Dr. Siegers will be responsible charges related to sample preparation. Both PIs will jointly book and conduct travel. While Mr. Heang will be more involved in generating metagenomic sequencing data in the lab, Dr. Siegers will lead the initial drafting of reports and publications that will result from this research. However, both authors will contribute to the writing and publication of any manuscripts and will jointly share authorship. The Co-PIs will work closely with both Mentors on all aspects of the project and will regularly attend IPC and EcoHealth Alliance scientific meetings to present work and progress.

References

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List of abbreviations, acronyms, symbols:

AIV: Avian Influenza Virus
BA: Bachelor of Business Administration
BI: Bioinformatics Consortiums
BS: Bachelor of Science
MBA: Master of business administration
BSL2: Biological Safety level 2
BSL3: Biological Safety level 3
CAN: Canada
CEIRR: Centers of Excellence for Influenza Research and Response
CREID: Centers for Research in Emerging Infectious Diseases
COVID-19: Coronavirus Disease 2019
Co-PI: Co-principal investigator
CVRP: Twist Comprehensive Viral Research Panel
DNA: deoxyribonucleic acid
dsDNA: double stranded deoxyribonucleic acid
ssDNA: single stranded deoxyribonucleic acid
Duke-NUS: Duke-National University of Singapore
EHA: EcoHealth Alliance
EID: emerging infectious disease
EID-SEARCH: The Emerging Infectious Disease – South East Asia Research Collaboration Hub
ES: Environmental Sample
FAO: Food and Agriculture Organization
GEIS: Emerging Infectious Surveillance and Response System
GISRS: Global Influenza Surveillance and Response System
IPC: Institut Pasteur du Cambodge
IT: information technology
LBM: live bird market
LMIC: Lover and middle income country
MoH: Ministry of Health, Cambodia
MSc: Master of Science
NAMRU-2: US Naval Medical Research Unit.2, Cambodia
NCBI: National Center for Biotechnology Information
NEB: New England Biolabs
NGS: next-generation sequencing
NIAID: National Institute of Allergy and Infectious Diseases
NIH: National Institutes of Health
NY: New York State
OFFLU: Joint OIE/FAO worldwide scientific network for the control of animal influenzas
OIE: World organization for animal health
ONT: Oxford Nanopore Technologies
PA: Pennsylvania State
cPCR: Conventional PCR
PCR: polymerase chain reaction
qRT-PCR: Quantitative Real-Time PCR
PhD: Doctor of Philosophy
PI: principal investigator

PREDICT: Pandemic Preparedness for Global Health Security
RNA: ribonucleic acid
SARS-CoV-2: Severe Acute Respiratory Coronavirus 2
ssRNA: single stranded ribonucleic acid
RT-PCR: real-time PCR
USAID: U.S. Agency for International Development
WHO: World Health Organization

Facilities and Resources

Laboratory:

The Institut Pasteur in Cambodia (IPC) is an institution of public utility engaged with the Royal Government of Cambodia by a convention and under the patronage of the Ministry of Health to meet the challenges of the future in the areas of scientific research, public health and education. It is a member of the Institut Pasteur International Network with which it develops ongoing collaborations, with 33 member institutes spread across the globe, which facilitates collaboration and scientific exchanges. Visiting scientists from Institut Pasteur Paris can provide expertise to setup specific assays and collaborations with Institut Pasteur Paris enable access state-of-the-art equipment if needed for the proposed research.

The Virology Unit at the Institut Pasteur du Cambodge (IPC) has been instrumental in public health responses in Cambodia, especially against respiratory viruses such as avian influenza. As such, IPC has served as the National Influenza Centre (NIC) for Cambodia since 2006 and was designated as a WHO H5 Reference Laboratory in October 2014 (H5RL), as part of the WHO Global Influenza Surveillance and Response System (GISRS). In addition, IPC serves as a reference laboratory for a number of other pathogens. Programs and procedures are also in place to identify, diagnose, and sequence numerous other emerging and zoonotic pathogens, such as Coronavirus (CoV). The IPC Virology Unit was designated by the Cambodian Ministry of Health as first line laboratory for diagnosis of COVID-19 and currently acts as the National Reference Laboratory for COVID-19. In April 2020, IPC Virology Unit was recently named a WHO Global Reference Laboratory for COVID-19.

The virology laboratory space covered by the Virology Unit contains all equipment needed for virology research. Biological Level 1 and 2 space (200 m^2) is dedicated for sample acquisition, processing and storage, serology, extraction, post-amplification procedures, molecular biology and PCR. Separate BSL2 spaces are also dedicated to human samples (23 m^2), animal samples (24 m^2), cell maintenance (12 m^2) and cell culture work (35 m^2). In response to A/H5N1 circulation in Cambodia the Virology Unit was upgraded in 2008 to include a BSL3 facility (142 m^2). The BSL3 facility comprises four modules dedicated to different and separate activities: arboviruses, emerging viruses (including a safety cabinet with gloves box allowing BSL3+ safety conditions) and mycobacterium (activity handled by the IPC clinical laboratory). The last module includes isolators for animal experiments.

Equipment includes: CO_2 incubators (n=3 dedicated to cell maintenance and cell culture work in a dedicated BSL2 spaces and n=2 dedicated to cell culture work in BSL3), automated extraction (n=4), real time qRT-PCR machines (n=7 BioRad CFX96), conventional PCR machines (n=8 BioRad T100), a Sanger sequencing (ABI 3500xL Genetic analyzer), 2x MinION next generation sequencer (Oxford Nanopore), 1x GridION next generation sequencer (Oxford Nanopore), 1x MiSeq next generation sequencer (Illumina), a gel imaging system (BioRad ChemiDoc WRS+), a capillary electrophoresis device (Qiagen QIAxcel advanced system), light microscopes (n=2 direct light; n=4 inverted light), a fluorescent microscope, bench top centrifuges, three ELISA washers (BioRad), two ELISA readers (BioRad and ThermoFisher Scientific) and Qubit fluorometer.

The sequencing mini-platform at IPC is a newly established platform intended to provide the sequencing services to all scientists within the institute regardless of their discipline. The sequencing mini-platform is situated in the Regional Research Platform-ASIA (PRR) building and contains all the equipment needed to perform sequencing. We have 20m2 dedicated for library preparation and another 20m2 for the instruments. Equipment includes: 1 Illumina Miseq, 1 Tapestation 4150, 1 Qubit 4.0, 1 Thermocycler.

Clinical:

The medical analysis laboratory of IPC has received ISO NF 15189 accreditation for all its applications for the analysis of clinical biology, biochemistry, hematology, and microbiology in 2018.

Animal:

A conventional mouse facility and dedicated BSL2 and BSL3 level space for animal experiments are available at IPC; however, they will not be utilized in this proposal.

Computer:

Information technology access: Since the Institute is located in a low-income country, we are eligible for the Hinari Access to Research for Health Programme of the world health organization (WHO), providing us online access to all major journals in biomedical research. Both the laboratories and PI's office are equipped with MacIntosh and PC computers and printers. Computers are protected by enterprise antivirus software. Email solution is Microsoft Exchange 2010 server. Local network is managed with Cisco catalyst switches, linked with fiberoptics or network cables, with redundant paths. VLANs are used to segregate research units, enabling more confidentiality. Two telco links provide access to the internet: primary/nominal with fiberoptics Online operator, with 8 MBbit/s bandwidth and 24/7 support. IPC has a reliable internet connection with 35Mbs download and 35Mbs upload speeds.Backup link is operated by OpenNet with 4 Mbit/s bandwidth.

The computational resources for generating, analyzing, storing and managing sequencing data are available locally. IPC acquired two Ubuntu server compute nodes (each equipped with 64 cores, 128GB of RAM, and a high-end NVIDIA GPU) connected over 10Gbs switches to 222 TB of shared networked storage.

Office:

Office space is separated from the laboratory space with dedicated offices for postdoctoral researchers, students, PI's and technicians.

Other:

Biological sample storage and access: All samples will be stored in -80°C freezers and liquid nitrogen tanks housed within the IPC biobanking system. The IPC biobank is a multisite biological resource center (BRC), which collects, transforms, analyses, stores and provides resources according to specific pre-requirements and manages associated data. All the activities of preparation, analysis and biological resource (BR) processing are realized according to stringent quality requirements. For long-term security and confidentiality, Biobank Management Software has been implemented and established and used to manage the BR and associated data from different laboratories and research units at IPC. Only the PI and relevant key personnel has access to the samples. The biobank includes human, animal and pathogenic samples.

Foreign Site Information

If the Pilot Award will have multiple foreign sites, complete this form for EACH research site

Principal Investigator Name:	Dr. Jurre SIEGERS and Mr. Vireak HEANG
Project Title:	*In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance*.
Institution:	Institut Pasteur du Cambodge
Foreign Research Site:	Phnom Penh, Cambodia
Point of Contact for Research Site:	Dr. Jurre SIEGERS Virology Unit, Institut Pasteur du Cambodge 5 Monivong Blvd, PO Box #983 Phnom Penh, Cambodia email: jsiegers@pasteur-kh.org

Introduction (3-4 sentences):
Provide a simple description of the overall goals of the project including the work that will be done at the foreign site. Indicate if any of the NIAID grant funds will be sent to the foreign site.

The goals of this project are: (1) to assess the suitability of environmental metagenomics as an early warning system for endemic and emerging infectious diseases at high-risk interfaces in Cambodia; (2) to utilize biobank environmental samples from longitudinal pathogen surveillance programs in Cambodia to build metagenomic sequencing and bioinformatics capacities and capabilities in Cambodia; and, (3) To discover new, emerging, or zoonotic pathogens of concern at high-risk interfaces in Cambodia.

To achieve these goals, investigators and primary Scientific Mentor on site will collaborate with EcoHealth Alliance and their CREID partners as part of the Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH). The EID-SEARCH team will support this project by helping the Institut Pasteur du Cambodge (IPC) team conduct and analyze metagenomic sequencing on Cambodian samples.

$147,675.96 of grant funds will be sent to IPC for these studies.

Foreign component (3-4 sentences):
Describe the specific role of the foreign site (i.e. conduct critical experiments?, conduct data analyses?, provide consultations?, provide samples?, study human and/or animal populations?, conduct observational or interventional clinical trials?, collect samples and/or data to be brought to the US?).

The site will coordinate and/or perform all laboratory work and data analyses necessary for the proposed project (including metagenomic sequencing).

Human Subjects (1 word or 1 sentence per bullet):

- **Parent Study IRB approval**
  - **IRB approval number for parent study:** Not Applicable
  - **IRB approval date:**
  - **Human Subject Assurance Number:**
- **Does this study require a modification to the IRB approval of a parent study (Yes or No)?**
  - No

- **Will existing samples from human subjects will be used: (Yes or No)?**
  - No
  - **How many subjects provided the existing samples to be used?** Not Applicable

- **Will human subjects be recruited (Yes or No)**
  - No
  - **Number of human subjects that will be recruited:** Not Applicable

- **Population parameters:** Not Applicable
  - **Gender:**
  - **Age Group:**
  - **Race/Ethnicity:**

- **Sample collection will include:** Not Applicable
  - **Blood:**
  - **Urine:**
  - **Tissues:**
  - **Other samples (describe):**

- **Sample collection will be completed in how many visits:** Not Applicable

- **Will samples be de-identified (Yes or No)? If No, describe how they will be protected.**
  - Not Applicable

- **Will local IRB approval be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals being sought or state why local IRB approval is not required.**
  - Not Applicable

- **Will informed consent be obtained prior to engaging in any research involving human subjects (Yes or No)? If No, describe alternate approvals/consents being sought or state why informed consent is not required.**
  - Not Applicable

- **Will samples be brought back to the US (Yes or No)?**
  - Not Applicable

- **Will data be brought back to the US (Yes or No)?**
  - Not Applicable

Animal Subjects (1 word or 1 sentence per bullet):

- **Parent study IACUC approval**
  - **IACUC approval number for parent study:** o Projects involving animal samples were collected (2019-2021) under a protocol approved by National Ethics Committee for Health Research under the Ministry of Health, Cambodia (NECHR143, NECHR149, and NECHR320); however, Cambodia did not have a specific animal ethics review board at the time the collections were conducted. Continuing ethics approvals (NECHR013) for projects involving further animal sample collections in 2022 are in revision and projected to be approved in February 2022.
  - **IACUC approval date:** Not Applicable
    Animal Welfare Assurance Number: Not Applicable
- **Does this study require a modification to the IACUC approval of a parent study (Yes or No)?**
  - **No**

- **Will existing samples from animal subjects will be used: (Yes or No)?**
  - **Yes**
  - **How many animal subjects provided the existing samples to be used?** 2445
- **Will vertebrate animals be collected (Yes or No)?**
  - **No**

- **Species of animals (e.g. rats, mice, rabbits, monkeys):** Not Applicable
- **Animal parameters:**
  - **Total number of animals:**
  - **Gender:**
  - **Age range:**
  - **Lab strain (e.g. Sprague-Dawley rats, Balb/C mice):**
  - **Wild animals procured in country (e.g. Rhesus monkeys from a reserve):**

- **What will be done to them or with them and how often?**
  - Not Applicable

- **What are the follow-ups?**
  - Not Applicable

- **What will be their fate at the end of the experiments – will they be euthanized?**
  - Not Applicable

- **Will in-country clearances be obtained prior to engaging in any animal research (Yes or No)? If No, describe alternate approvals or state why in-country clearances are not required.**
  - Not Applicable
- **Will samples be brought back to the US (Yes or No)?**
  - No
- **Will data be brought back to the US (Yes or No)?**
  - Yes, some of the data will be shared with EcoHealth Alliance/EID-SEARCH for relevant analysis.

ROYAUME DU CAMBODGE
NATION RELIGION ROI

Institut Pasteur du Cambodge

Le Directeur

Phnom Penh, January 31, 2022

O/Ref.: N°034/IPC/DIR/2022

Dr. Jurre Siegers & Mr. Vireak Heang
Institut Pasteur du Cambodge
Phnom Pehn, Cambodia

Object: Institutional Support for your application to the CREID Pilot Research Program, entitled "In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance."

Dear Dr. Siegers & Mr. Heang,

The Institut Pasteur du Cambodge (IPC) is pleased to support your application to the **CREID Pilot Research Program**, entitled "In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance." We are fully committed in executing the statement of work utilizing next-generation metagenomics techniques for emerging zoonoses and potentially developing novel methods of expanding our surveillance in Cambodia.

The work proposed in this project will be given the highest priority and complements nicely to all of the other endemic and emerging virus research being conducted at IPC. In addition, it serves very nicely to bring together our long-standing Virology Unit with the newly created Sequencing Mini-Platform to build capacity and capability not only in our institution, but also for the next generation of Cambodian scientists.

As such, IPC asserts that all necessary time, facilities, and resources required for the project will be made fully available to you to successfully complete the work proposed in this project.

Sincerely,

Prof. André SPIEGEL
Director

Prof André SPIEGEL
Directeur
5, Boulevard Monivong
BP 983 – Phnom Penh
Téléphone : 855 (0) 12 222 659
aspiegel@pasteur-kh.org

EID-SEARCH
Emerging Infectious Diseases
South East Asia Research Collaboration Hub

Dr. Jurre Siegers, Mr. Vireak Heang
Institut Pasteur du Cambodge
No. 5 Monivong Boulevard
P.O Box. 983
Phnom Penh, Cambodia

Dear Jurre and Vireak,

The Emerging Infectious Disease-Southeast Asia Research Collaboration Hub (EID-SEARCH) at EcoHealth Alliance is highly interested in working with you and your collaborators at the Institut Pasteur du Cambodge in Cambodia on the proposed project titled: "*In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance.*"

This project will significantly contribute to the goals of the CREID Network and EID-SEARCH to build in-country research capacity and an international research network for emerging infectious diseases in Southeast Asia. It will develop a foundation for new collaborations among EID-SEARCH, Institut Pasteur du Cambodge, and other in-country stakeholders, strengthening the multi-sectoral cooperation for emerging infectious diseases in Cambodia. Furthermore, the process to develop and implement this project will help develop your skills in project management, provide training and networking opportunities with scientists from EID-SEARCH and the CREID Network, and most importantly, expand your skills in metagenomics, to prepare you to be independent researchers leading emerging infectious disease research both within and outside of Cambodia.

Beyond capacity and network building, the proposed project strongly aligns with the EID-SEARCH's scientific objectives and research strategies by conducting surveillance among humans and animals to identify emerging pathogens with zoonotic potential at the high-risk human-animal interfaces and develop an early-warning system for zoonotic disease emergence. The research sites of this project - live animal markets, bat caves, slaughterhouses, animal farms – represent scenarios where zoonotic spillover are mostly concerned that require further in-depth investigation to reveal the spillover pathways. In addition, the innovative approach to conduct surveillance among environmental samples will not only expand our knowledge in viral discovery from the environment but also promote the One Health surveillance by establishing a validated sampling and analysis method that will significantly contribute to the disease surveillance and prevention strategies in Cambodia through collaborative and coordinative cooperation among a variety of stakeholders.

I am optimistic that this collaborative research project will bring valuable insights to advance disease surveillance and early warning systems for endemic and emerging infectious diseases in Cambodia. Members of EID-SEARCH are committed to working closely with you to develop the research project and support the efforts necessary for the success of this project. Our Senior Research Scientist, Dr. Cadhla Firth, has enthusiastically joined the project as your Mentor to advise on the study design, data collection, and analysis throughout the project. Dr. Firth will work closely with Dr. Erik Karlsson, a mentor from your IPC Virology Unit, to provide pertinent and timely career advice to assist in your professional development and brings a well-established network with leaders and experts in the ecology and evolution of infectious diseases across Southeast Asia, North America, and Australia. You will be invited to join all training conducted by EID-SEARCH and EcoHealth Alliance regarding emerging infectious disease fields, surveillance, and statistical analysis to build your skillset. In addition, Dr. Linfa

EID-SEARCH
Emerging Infectious Diseases
South East Asia Research Collaboration Hub

Wang, Co-Investigator from the EID-SEARCH, will support you on the metagenomics analysis by advising on the project and providing training or exchange opportunities at the Duke-NUS Medical School. You will be supported to present the findings of this research at international conferences, CREID Network meetings, and external partners and produce high-quality publications from this research.

I am confident that this proposed project will bring genuine opportunities for your professional development. The interdisciplinary expertise, a hallmark of "One Health", in virology, genomics, public health, extensive experience working with sequencing platforms from Dr. Siegers and Mr. Heang, and the improved environmental metagenomics methods and skills through this research will make this a successful and purposeful project to bring public health impacts in a broad region with the similar social and cultural context.

This letter conveys my strong interest and commitment to making this application a success. I look forward to collaborating with you and your team on all phases of this proposed project. I wish you success in the CREID Pilot Research Program application.

Sincerely,

Peter Daszak
Principal Investigator, EID-SEARCH
President, EcoHealth Alliance

January 25, 2022

Jurre Siegers, Vireak Heang
Institut Pasteur du Cambodge
No. 5 Monivong Boulevard
P.O Box. 983
Phnom Penh Cambodia

Dear Jurre and Vireak,

I am writing this letter in support of your CREID Network Pilot Research Program application “In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance” conducted at the Institut Pasteur du Cambodge.

I and my group at Duke-NUS Medical School have long experience working on the emerging infectious diseases associated with bats and other animals. Your current proposal complements and expands this scope to identify the at-risk groups and the risk factors of viral spillover from wildlife to human populations.

I also work with the Emerging Infectious Diseases - South East Asia Research Collaboration Hub (EID-SEARCH) to develop serological tests and conduct virus characterization. I am very much looking forward to participating in this research to contribute my expertise, advise and provide relevant laboratory training.

This letter conveys my strong interest and commitment to making this application a success. I am excited to be part of the research to build scientific evidence to better predict zoonotic disease emergence, and I look forward to working with you on this research.

Yours sincerely,

(b) (6)

Linfa (Lin-Fa) WANG, PhD FTSE
Professor in Programme in Emerging Infectious Diseases

8 College Road, Singapore 169857
T +65 6516 7666 F +65 6221 7396
www.duke-nus.edu.sg
A school of the National University of Singapore (RCB No: 200604346E)

EcoHealth Alliance

30th January, 2022

Dr. Jurre Siegers & Mr. Vireak Heang
Institut Pasteur du Cambodge
Phnom Pehn, Cambodia

Dear Dr. Siegers & Mr. Heang,

This letter is to express my support as Research Center Mentor for your application to the CREID Pilot Research Program, entitled "*In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance*."

I am extremely excited that you have chosen me to be one of your Mentors for the proposed project. The research question is timely and important, and your work has the potential to have a strong impact on our approach to pathogen surveillance at high-risk human-animal interfaces. Given what we understand to be the likely origins of SARS-CoV-2, there has never been a more pressing need to improve the efficiency, sensitivity, and cost-effectiveness of zoonotic disease surveillance in emerging infectious disease hotspots.

I would be hard-pressed to think of two more appropriate applicants to co-lead this project, given your combined expertise in pathogen surveillance, molecular diagnostics, next-generation sequencing, and emerging respiratory viruses. Although you each have unique longer-term personal and professional goals, you also have a shared vision of using your scientific knowledge and expertise to train the next generation of Cambodian scientists, while building intellectual and scientific capacity around emerging infectious diseases in the country. I believe that increased access to the CREID Network, and to the Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH), in particular, will greatly help facilitate these goals by increasing your access to other scientists working on similar research questions across Southeast Asia. Many Southeast Asian countries face similar challenges in preventing and mitigating the impacts of emerging zoonotic diseases, and stronger regional partnerships have the potential to really drive forward innovations in this area. These include the implementation of novel, cost-effective approaches to pathogen surveillance, as proposed in your pilot project application.

The well-established relationship you each have with your chosen Primary Scientific Mentor for this project, Dr. Erik Karlsson, will provide an excellent foundation for the proposed work that will be enhanced by the joint Mentorship Plan we have developed together. I believe that Dr. Karlsson and I have distinct but complementary scientific skills, expertise, and networks that will maximize your professional and scientific development throughout the duration of the project, and I look forward to working more closely with all of you. I can see multiple opportunities for this new collaboration between the four of us to develop into a fruitful, long-term scientific partnership. Towards that aim, I am committed to supporting your professional development in the following ways:

- I will actively work to maintain the healthy, open, and informal dialogue we have established while drafting the project proposal, and I will seek feedback from you on a regular basis to ensure that our communication plan continues to meet your needs as the project progresses.

EcoHealth Alliance
520 Eighth Avenue, Suite 1200
New York, NY 10018
212.380.4460
EcoHealthAlliance.org

- I will engage with your current and future in-country collaborators in a supportive and inclusive manner.
- I will create opportunities for you to interact with scientists across my network through your research project to widen your exposure to people with a range of expertise.
- I will give you timely feedback on all material in the manner that works best for you.
- I will provide you with opportunities to present your work to my colleagues at EcoHealth Alliance and across EID-SEARCH during our academic meetings and will include you in any relevant training or networking opportunities.
- I will share my scientific and technical expertise with you to help ensure the success of the project. When skills outside my area are required, I will assist you in accessing this expertise from scientists within my network.
- I will assist you in career planning, providing advice and support as required to maximize your opportunity to succeed.

Finally, I believe that the data you will collect about the potential zoonotic pathogens circulating in live bird markets, swine abattoirs, and bat roosts and guano farms in Cambodia will lead to a wealth of new research questions and hypotheses that could form the basis for additional research projects. This will present the perfect opportunity for you to continue the transition towards scientific independence, and I am committed to assisting you through the process of conceptualizing and writing your first funding applications as lead investigators.

There is significant overlap between your research interests and skills, the overall goals of the Institut Pasteur du Cambodge, and those of EcoHealth Alliance and the EID-SEARCH team. This pilot project presents a real opportunity for you to increase collaboration between these organizations, and to help drive forward future research projects on emerging zoonoses at high-risk interfaces in Cambodia. I am thrilled to be able to support you at these stages in your careers and am genuinely convinced that you both have the potential to become scientific leaders in Southeast Asia in the very near future.

Sincerely,

(b) (6)

Cadhla Firth, PhD
Senior Scientist and Program Coordinator
EcoHealth Alliance

INSTITUT PASTEUR
DU CAMBODGE
VIROLOGY UNIT

30th January, 2022

Dr. Jurre Siegers & Mr. Vireak Heang
Institut Pasteur du Cambodge
Phnom Pehn, Cambodia

Object: Support as Primary Scientific Mentor for your application to the CREID Pilot Research Program, entitled "*In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance*."

Dear Dr. Siegers & Mr. Heang,

This letter is to express my support as Primary Scientific Mentor for your application to the CREID Pilot Research Program, entitled "*In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance*."

I am proud to be able to continue to mentor both of you as one of your Mentors for the proposed project. I serve as both your mentor in the Institut Pasteur du Cambodge (IPC) Virology Unit (Dr. Siegers) and in the IPC Sequencing Mini-Platform (Mr. Heang). You are both in the top 1% of individuals I have ever had the pleasure to mentor/supervise. I have observed you both as extremely organized, productive, proactive, and responsive. You have never failed in any challenge placed in front of you and always deliver extra in your work. You are always willing to learn and adapt to new situations, and you both have extremely good leadership qualities, which will make your excellent PIs in the future.

As you well know, IPC serves as a major institution in the Institut Pasteur International Network and has been involved in research and diagnosis of human and animal infectious diseases in the Kingdom of Cambodia since 1953. IPC serves as a research institute, non-profit foundation for vaccination, diagnosis, and treatment, and is part of the Cambodian Ministry of Health (MoH). The Virology Unit at IPC was opened in 1996 and serves as the reference laboratory for Influenza, Arboviruses, and Rabies for Cambodia, as well as conducting surveillance and research on endemic, emerging, and zoonotic pathogens. The unit opened the first, and only, BSL3+ laboratory in the country in 2008. The Virology Unit works in close collaboration with the Epidemiology/Public Health, Entomology, Bacteriology, and Malaria Units at IPC as well as national and international partners to conduct One Health-focused surveillance and studies in Cambodian humans, domestic animals, and wildlife. In 2021, we opened the IPC Sequencing Mini-Platform to further increase these studies through advances in next generation sequencing equipment and support.

The research question proposed in this pilot program is both timely and important, and the work has great potential for increasing capacity and capability at IPC and in Cambodia as a whole. Indeed, the research proposed has significant potential to impact our approach to pathogen surveillance at high-risk human-animal interfaces. Indeed, the project will also serve to further link the Virology Unit and the Sequencing Mini-Platform at IPC with scientists in the CREID Network, specifically the Emerging Infectious Diseases: South East Asia Research Collaboration Hub (EID-SEARCH), which will allow future work across Southeast Asia and positively influence your networking and future careers. There is significant overlap between this proposal, the overall mission of the Institut Pasteur du Cambodge, and the goals of EcoHealth Alliance and the EID-SEARCH team. Therefore, this pilot project presents a real opportunity for you to increase

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Royaume du Cambodge
Téléphone : +855 23 426 009
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collaboration, and to spearhead future research projects on emerging zoonoses at high-risk interfaces in Cambodia. In addition, I am completely supportive of your vision to utilize the knowledge you gain from this proposal to train the next generation of Cambodian scientists, a critical part of our mission at IPC.

I fully support your choice of Research Center Mentor for this project, Dr. Cadhla Firth. Dr. Firth and I feel that our distinct but complementary scientific skills, expertise, and networks will maximize your professional and scientific development throughout the duration of the project. In the future, I see multiple opportunities for this new collaboration to develop into long-term scientific partnership. Indeed, I believe the data collected in this proposal regarding new ways to survey potential zoonotic pathogens circulating in Cambodia will lead to scientific independence and further lead investigator applications for you on novel research questions and hypotheses.

Therefore, I am committed to supporting your professional development in the following ways:

- I will continue to actively maintain the healthy, open, and informal mentoring relationship we have established as your mentor at IPC
- I will continue to meet with you on scheduled and *ad hoc* basis to support whatever needs you might have regarding this project, other ongoing projects, and future projects
- I will continue to provide (and ensure the institution provides) the time and resources needed to complete all research activities proposed in this pilot project
- I will continue to engage with our current and future in-country collaborators in a supportive and inclusive manner and promote your engagement with them
- I will continue to give you timely feedback on all material in the manner that works best for you
- I will continue to support and promote opportunities to interact with other scientists through your research project to widen your exposure to people with a range of expertise.
- I will continue to share my scientific and technical expertise with you to help ensure the success of the project. When skills outside my area are required, I will continue to assist (and encourage) you in accessing this expertise from scientists within my network.
- I continue to assist you in career planning, providing advice, and any other support as needed to maximize your opportunity to succeed.

Overall, I am thrilled you have chosen me as Primary Scientific Mentor on this project and I look forward to working with you on this project and throughout your careers.

Best Regards,

Erik A. Karlsson, Ph.D.
Deputy Head, Virology Unit
Director, National Influenza Center of Cambodia
Director, WHO H5 Regional Reference Laboratory
Coordinator, WHO Global COVID-19 Referral Laboratory
Institut Pasteur du Cambodge
5 Monivong Blvd. P.O. Box 983
Phnom Penh, Cambodia 12201
Phone: +855 (0) 70 297 804
Email: ekarlsson@pasteur-kh.org

Pilot Program Application Submission Checklist – Final Application Submission

Title of Application: In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance

Submitted by: Siegers, Jurre, Ynze and Heang, Vireak

Submission Date: 1/31/2022

Full Application Submission Requirements

- ☒ Proposal Cover Sheet
- ☒ Title and Table of Contents
- ☒ Study Personnel *(1-page limit)*
- Main Application Body Section Requirements *(7-page limit):*
  - ☒ Research Aims & Objectives
  - ☒ Study Rationale/Research Gap/Impact
  - ☒ Significance and Approach
  - ☒ Research Methods
- ☒ Project Timeline (*1-page limit*)
- ☒ Research Performance Sites *(1-page limit)*
- ☒ CREID Research Center Collaboration *(1-page limit)*
- ☒ Mentoring Plan *(2-page limit)*
- Vertebrate Animals Section Requirements *(3-page limit):*
  - ☐ Description of Procedures
  - ☐ Justifications
  - ☐ Minimization of Pain and Distress
  - ☐ Method of Euthanasia (Cover Page Supplement / PHS Fellowship Supplemental Form)
- Human Subjects Research *(3-page limit):*
  - ☐ Summary of the parent study and IRB approval information for the study
  - ☐ Risks to the subjects
  - ☐ Adequacy of protection against these risks
  - ☐ Potential benefits of the research to the subjects and others
  - ☐ Importance of the knowledge gained or to be gained
  - ☐ Country / institution-specific ethics / IRB regulations addressed
- Research, Related Project Information, and Budget/Budget Justification
  - ☒ R&R Other Project Information Form
  - ☒ Full budget, with total costs of no more than $150,000
  - ☒ Budget justification which describes the labor and other direct costs
  - ☐ If your institution does not have adequate funds for a cost-reimbursement award and requires pre-payment of funds during the award year, please note this in your budget justification and outline a payment schedule that will function for your project.
- Supporting Documentation
  - Biographical Sketch and Other Support. All applications must include:
    - ☒ Applicant PI Biographical Sketch (*4-page limit*)
    - ☒ Applicant PI Previous/Current/Pending Support (Include funding amounts, *no page limit*)
    - ☒ Mentor Biographical Sketch (*4-page limit*)
    - ☒ Mentor Current/Pending Support (*no page limit*)
    - ☐ Key Personnel Biographical Sketches (*4-page limit each*)
    - ☐ Key Personnel Current/Pending Support (*no page limit*)

Pilot Program Application Submission Checklist – Final Application Submission
Title of Application: In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance
Submitted by: Siegers, Jurre, Ynze and Heang, Vireak
Submission Date: 1/31/2022

- Co-PI Plan (only needed if applying as Co-PIs) (*1-page limit*)
  - ☒ What each Co-PI will contribute to the proposed research study
  - ☒ How the Co-PIs will jointly work with the affiliated Research Center
  - ☒ How the Co-PIs will jointly manage the proposed study

☒ References Cited (*no page limit*)
☒ List of Abbreviations, Acronyms, and Symbols
☒ Facilities, Existing Equipment, and Other Resources (*template provided*)
☒ NIH Foreign Clearance form (*template provided*)
☒ Letters of Organizational Support *(2-page limit per letter)*
☒ Letter of Collaboration from CREID Research Center PI *(2-page limit per letter)*
☒ Letter from Research Center Mentor (*2-page limit)*
☒ Letter from Primary Scientific Mentor (if different than Research Center mentor) (*2-page limit)*

From: Hongying Li on behalf of Hongying Li <li@ecohealthalliance.org>
To: Sasiprapa Ninwattana
Cc: Krongkan Srimuang; Sterling, Spencer; Supaporn Wacharapluesadee; eric.laing_usuhs
Subject: Re: Proposal submitted last year for CREID Network
Date: Wednesday, November 16, 2022 5:08:42 AM
Attachments: Final - CREIDApplication.NguyenVanCuong.Proposal.pdf
CREID_Pilot_Program_Application_SIEGERS_HEANG_FINAL_PDF_FOR_SUBMISSION.pdf

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Thank you, Bow!

Kio and Spencer,

Attached are the two successful applications for 2021 and 2022 for your reference.

And can you please quickly write up one paragraph of your proposed idea (like the abstract) **before Monday next week**? We have received eight requests to collaborate this year, so Kevin, Peter, and I need to review all of them together on Monday to decide which three to support, and it would be great if you can send something written-down.

Best,
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

On Wed, Nov 16, 2022 at 4:01 PM Sasiprapa Ninwattana <sasiprapa.n@outlook.com> wrote:

> Dear All,
>
> Attached please find the proposal that we submitted for last year's pilot research program for CREID network for your reference.
> Please note that the letter of intent needs to be submitted by December 5, 2022.
>
> Please feel free to ask me or Hongying any questions you may have.
>
> 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

CREID Annual Meeting

Welcome

Dear Meeting Attendees,

We are pleased to welcome you to the 2022 CREID Network Annual Meeting. With a focus on the science, the 2022 Annual Meeting will showcase the diverse ongoing research by members of the CREID Network.

The CREID Network was established in 2020 with overarching goals of improved rapid and coordinated outbreak research response and capacity building. CREID is a coordinated network operating in regions around the globe where emerging and re-emerging infectious disease outbreaks are likely to occur. Multidisciplinary teams of investigators are conducting pathogen/host surveillance, studying pathogen transmission, pathogenesis, and immunologic responses in the host, and developing reagents and diagnostic assays for improved detection of important emerging pathogens and their vectors.

This is the first Annual Meeting to include in-person attendees and we eagerly anticipate the collaboration and relationship building that will bring. Those of you joining virtually have an equally valuable contribution to make. We look forward to your engaged participation around this meeting and moving forward as CREID continues to develop its global network.

We look forward to a successful meeting,

Sincerely,
DMID and CREID Coordinating Center Teams

CREID
Centers for Research in Emerging Infectious Diseases

Contact

CREID Coordinating Center
info@creid-network.org

Meeting Venue

Sheraton Baltimore North

Address 903 Dulaney Valley Rd, Towson, MD 21204
Phone (410) 321-7400

On-Site Food

Coffee Corner Marketplace

Daily 6:30AM – 2:00PM

Rain 903 Restaurant

Monday - Friday 6:30AM – 10:00AM and 5:00PM – 10:00PM
Saturday—Sunday 7:00AM – 11:00AM and 5:00PM – 10:00PM

CREID
Centers for Research in Emerging Infectious Diseases

Transportation

To the Venue

BWI Airport to Sheraton Baltimore North

Driving Directions

~35 minutes via I-695

https://goo.gl/maps/ELBk7kbMfyGmHw2NA

Metro Directions

~1 hour, 30 minutes via

https://goo.gl/maps/ELBk7kbMfyGmHw2NA

Closest Metro Stop (6 minute walk)

Fairmont & Delaney Valley Rd: CityLink RED Line

Closest Bus Stop (6 minute walk)

Towson Town Center Bay 1: Bus 51

Beyond Towson and Baltimore, MD

Take a CityLink light rail from Towson Town Center Bay 2 and an Amtrak from Baltimore Penn Station into **Washington D.C.** to tour the famous National Monuments and Memorials (use Google Maps for detailed directions).

https://washington.org/visit-dc/monuments-memorials

No-Host Social Hours*

Please join CREID Network members for no-host social hours Tuesday, Wednesday, and Thursday so we can get to know each other outside of the formal meeting.

Tuesday, September 20, 6:00pm

World of Beer

125 E. Joppa Road, Towson, MD

Featuring 500+ global beers, tavern food

~15 minute walk

Wednesday, September 21, 6:00pm

The Point in Towson

523 York Road, Towson, MD

American eats, beers, & cocktails

~15 minute walk

Thursday, September 22, 6:00pm

7 West Bistro Grille

7 W. Chesapeake Avenue, Towson, MD

Mediterranean tapas & creative American dishes

~17 minute walk

* This is an optional activity and there is no central coordination of these events.

CREID
Centers for Research in Emerging Infectious Diseases

Food and Drink Nearby

Towson Town Center

0.3 miles; 7 minute walk

Shopping mall with many dining options:

- P.F. Chang's
- The Cheesecake Factory
- Stoney River Steakhouse and Grill
- Many more

Towson Tavern

0.6 miles; 14 minute walk

516 York Rd, Towson, MD 21204

Pollo Amigo

0.6 miles; 13 minute walk

714 York Road, Towson, MD

Whole Foods Market

0.8 miles; 17 minute walk

300 Towson Row, Towson, MD

FOD Poke Bar

0.7 miles; 15 minute walk

402 York Rd, Towson, MD 21204

The Fresh Market

0.3 miles; 8 minute walk

838 Dulaney Valley Road, Towson, MD

Other Nearby Restaurants

https://goo.gl/maps/f9XhD1Ypr75vNNPt8

CREID
Centers for Research in Emerging Infectious Diseases

Things to Do

Inner Harbor Baltimore

Water Taxi Ride for $20 with 13 destination points

National Aquarium

Maritime Museums Historic Ships in Baltimore, USCG Lightship Chesapeake, Seven Foot Knoll Lighthouse

Local Seafood Eat the Maryland delicacy, crab, and many other seafood delights at one of the many waterfront restaurants (may be pricier on the harbor)

baltimorewatertaxi.com

Baltimore Museums and Monuments

Fort McHenry National Monument used in the War of 1912 to defend the Baltimore Harbor, now a historic site

Baltimore Museum of Art with 19th-century, modern and contemporary art

American Visionary Art Museum with unusual and unique modern art

Reginald F. Lewis Museum Maryland African-American History and Culture

Edgar Allan Poe House and Museum

State Parks and Nature Trails

Patapsco Valley State Park – Hollofield Area; Maryland's largest state park along 32 miles of the Patapsco River south and west of the city

Patterson Park an urban park featuring 173 acres of open fields of grass, large trees, paved walkways, historic battle sites, a lake, playgrounds, athletic fields, a swimming pool, an ice skating rink and other signature attractions and buildings

Jones Falls a 17.9-mile-long stream running through the city

Lake Montebello a reservoir with a 1.4 mile biking and walking path

Sports and Other

Baltimore Orioles watch the Baltimore baseball team play a home game at Oriole Park at Camden Yards Sep. 19-25.

Maryland Zoo located in the historic Druid Hill Park in northwestern Baltimore

mlb.com/orioles/schedule/2022-09

– DETAILED SCIENTIFIC AGENDA –

FINAL AGENDA

DAY 1 | WEDNESDAY, SEPTEMBER 21

Scientific Meeting Zoom Link

Time (UMT -4)	Session	Location
13:00-14:00	**Opening, Priorities, and Vision** ▪ Welcome from DMID | Emily Erbelding (NIH/NIAID/DMID) ▪ CREID Network Priorities | Jean Patterson (NIH/NIAID/DMID) ▪ Welcome from the CREID Network | Nikos Vasilakis (CREATE-NEO), Christine Johnson (EEIDI) ▪ CREID Network Shared Vision | Njenga Kariuki (CREID-ECA), Eva Harris (A2CARES)	Fitzgerald B/C & Zoom
14:00-14:15	Break	
14:15-15:15	**Collaboration for Outbreak Research Response & Tabletop Introduction** ▪ Richard Reithinger (CREID CC) ▪ Jessica Vanhomwegen, Richard Njouom (PICREID) ▪ Scott Weaver (WAC-EID)	Fitzgerald B/C & Zoom
15:15-16:15	**CREID Network Stakeholders: Existing Collaborations** Session Chairs: Sara Woodson (DMID), Peter Daszak (EID-SEARCH) ▪ PREMISE | Peter Daszak (EID-SEARCH) ▪ WRCEVA | Scott Weaver (WAC-EID) ▪ TGHN/CSPH Virtual Biorepository | Tony Moody (CREID CC) ▪ NIBSC | Greg Sempowski (CREID CC) ▪ Abbott Pandemic Defense Coalition | Tony Moody (CREID CC), Ambroise Ahouidi (UWARN) ▪ Benefit sharing and preparing for downstream translation | Sara Woodson (DMID), Peter Daszak (EID-SEARCH)	Fitzgerald B/C & Zoom
16:15-16:30	Break	
16:30-16:45	**CREID Network Stakeholders: Potential Collaborations** Session Chairs: Tony Moody (CREID CC), Jean Patterson (DMID) ▪ CEPI, Fogarty, BV-BRC	Fitzgerald B/C & Zoom
16:45-17:00	**Day 1 Takeaways** David Wang (CREID-ESP), Sara Woodson (DMID)	Fitzgerald B/C & Zoom

DAY 2 | THURSDAY, SEPTEMBER 22

Scientific Meeting Zoom Link

Time (UMT -4)	Session (Virtual Presentation)	Location
11:00-12:00	**CREID Research Center Y2 Review Panels** Session Chair: Greg Sempowski (CREID CC) ▪ EID-SEARCH | Peter Daszak ▪ UWARN | Wes Van Voorhis ▪ CREID-ESP | David Wang ▪ WARN-ID | Kristian Andersen ▪ EEIDI | Christine Johnson	Fitzgerald B/C & Zoom
12:00-13:00	Lunch *Participants obtain on their own.*	
13:00-14:00	**CREID Research Center Y2 Review Panels (continued)** Session Chair: Tony Moody (CREID CC) ▪ PICREID | Anavaj Sakuntabhai ▪ CREATE-NEO | Nikos Vasilakis ▪ CREID-ECA | Njenga Kariuki ▪ WAC-EID | Scott Weaver ▪ A2CARES | Eva Harris	Fitzgerald B/C & Zoom
14:00-15:00	**Oral Presentations 1: Identifying and Characterizing Emerging Pathogens** Session Chair: Njenga Kariuki (CREID-ECA) Session Co-Chair: Betania Drummond (2021 Pilot Awardee, CREATE-NEO) ▪ Rift Valley Fever and Crimean-Congo Hemorrhagic Fever in Senegal: animal seroprevalence as indicator of virus circulation in nature | Déthié Ngom (PICREID) ▪ Evidence of co-circulation of multiple endemic arboviruses based on syndromic sentinel surveillance in Senegal | Gamou Fall (PICREID) ▪ Multi-RC Arbovirus Active Surveillance in Mosquito Enzootic Vectors and Potential Host in Panama | Nikos Vasilakis (CREATE-NEO) ▪ From malaria to fevers of unknown origins: genomic surveillance in Senegal | Aida Badiane (WARN-ID) ▪ Retrospective investigation of horses with encephalitis reveals unnoticed circulation of West Nile Virus in Northeastern Brazilian states | Luiz Alcantara (UWARN) ▪ Dengue-2 Cosmopolitan genotype detection and emergence in South America | Marta Giovanetti (UWARN)	Fitzgerald B/C & Zoom
15:10-15:30	Break	
15:30-16:40	**Oral Presentations 2: Ecology, Environs and Entomology** Session Chair: Mariana Leguía (EEIDI) Session Co-Chair: Janin Nouhin (2021 Pilot Awardee, PICREID) ▪ High burden of Arbovirus in Remote Rural Villages under environmental change in Ecuador | Paulina Andrade (A2CARES) ▪ Social constructs of place and their relevance in locating dengue fever outbreaks | James Trostle (A2CARES) ▪ Ecology of Aedes-transmitted arboviruses and their vectors in sylvatic and urban settings of Senegal: entomogical findings | Diawo Diallo (WAC-EID) ▪ Multi-RC Implications of land used and land coverage in the emergence of Madariaga Encephalitis in an endemic region of Venezuelan Equine Encephalitis Virus in Eastern Panama | Jean-Paul Carrera (CREATE-NEO) ▪ Multi-RC Ecological features of potential Madariaga and Venezuelan equine encephalitis virus enzootic hosts in Panama | Jean-Paul Carrera (CREATE-NEO) ▪ Larval microbiome by Aedes aegypti genotype interactions drive susceptibility to Zika virus | Laura Dickson (WAC-EID)	Fitzgerald B/C & Zoom
16:40-17:00	**Day 2 Takeaways** Njenga Kariuki (CREID-ECA), Sara Woodson (DMID)	Fitzgerald B/C & Zoom

Acronyms (p7)

DAY 3 | FRIDAY, SEPTEMBER 23

Scientific Meeting Zoom Link

Time (UMT -4)	Session (Virtual Presentation)	Location
11:00-12:00	**2021 Pilot Awardee Presentations** Session Chairs: (Tony Moody), Nikos Vasilakis (CREATE-NEO) ▪ Investigation of the spatiotemporal dynamics and ecological drivers of enzootic arbovirus circulation in non-human primates in Minas Gerais State/Southeast Brazil | Betania Drumond, 2021 Pilot Awardee (CREATE-NEO) ▪ Hantavirus detection and characterization in humans and rodents from Cambodia | Janin Nouhin, 2021 Pilot Awardee (PICREID) ▪ Defining antiviral humoral immunity against SARS-CoV-2 in Kenya | Bronwyn Gunn and Robert Langat, 2021 Pilot Awardees (CREID-ECA) ▪ Multi-species farming, viral diversity, and risks of cross-species transmission in the Central Highlands of Viet Nam | Cuong Van Nguyen, 2021 Pilot Awardee (EID-SEARCH) ▪ Revealing vector species with potential to mediate pathogen spillover from wildlife to livestock in the Pantanal | Daniel Aguiar, 2021 Pilot Awardee (CREATE-NEO) ▪ Development of a real-time pathogen surveillance system in Jordan | Issa Abu-Dayyeh, 2021 Pilot Awardee, WARN-ID	Fitzgerald B/C & Zoom
12:00-13:00	Lunch *Participants obtain on their own.*	
13:00-14:30	**Lightning Talk Concurrent Sessions** – *see pp4-6 for details* A: Ecology, Entomology and Field Methods B: One Health and Zoonotic Surveillance C: Clinical and Laboratory Science D: Epidemiology and Surveillance Across the Americas E: Epidemiology and Surveillance Across Africa and Asia	 Fitzgerald B/C & Zoom Fitzgerald A North & Zoom Fitzgerald A South & Zoom Warfields & Zoom Grason & Zoom
14:30-14:50	Break	
14:50-16:00	**Oral Presentations 3: Advanced Tools, Techniques and Late Breakers** Session Chair: Kathryn Hanley (CREATE-NEO) Session Co-Chair: Issa Abu-Dayyeh, 2021 Pilot Awardee (WARN-ID) ▪ An enrichment method for capturing SARS-CoV-2-related whole genome sequences directly from bat samples | Sininat Petcharat (EID-SEARCH) ▪ Multi-RC: Real-time RT-PCR for Venezuelan equine encephalitis complex, Madariaga and Eastern equine encephalitis viruses: application in clinical diagnostic and mosquito surveillance | Sandra Lopez-Verges (CREATE-NEO) ▪ Simple and economical extraction of viral RNA and storage at ambient temperature | Jesse Waggoner (A2CARES) ▪ Late breaker: Serological evidence of significant Middle East Respiratory Syndrome coronavirus transmission to humans among camel-owning households in Northern Kenya | Isaac Ngere (CREID-ECA) ▪ Late breaker: Concurrent non-human primate, bat, mosquito, and human One Health surveillance in the Peruvian Amazon 2021-2022 | Amy Morrison (EEIDI)	Fitzgerald B/C & Zoom
16:00-16:15	**Closing Remarks** Mark Challberg (DMID)	Fitzgerald B/C & Zoom

2022 Pilot Awardee ePosters

(*see Between Session slides*)

- Investigation of ecological drivers of sarbecoviruses spillover in Myanmar and Nepal | Ohnmar Aung (EEIDI)
- Vector surveillance in context of urban transmission and spread of Crimean-Congo hemorrhagic fever virus (CCHFV), Karachi Pakistan | Najia Ghanchi (UWARN)
- In the Air Tonight: Metagenomic Pathogen Discovery as Tools in Pathogen Surveillance | Jurre Siegers and
- Vireak Heang (EID-SEARCH)
- Pathogenic Mammarenaviruses and Orthohantaviruses in Argentina | María Martin and Carina Sen (WAC-EID)
- Surveillance for known and novel viruses with zoonotic potential at the interface between humans and livestock in Kenya | Stephanie Seifert and Isaac Ngere (CREID-ECA)
- Characterization of the mosquito microbiome and its role in arbovirus emergence and maintenance in Senegal | Laura Dickson and Alioune Gaye (WAC-EID)

Lightning Talk Concurrent Session Details

Day 3 | Friday, September 23 | 13:00-14:30

Concurrent Session A: Ecology, Entomology and Field Methods Location: Fitzgerald B/C & Zoom

Session Chair: Peter Dascak (EID-SEARCH)

Session Co-Chair: Tierra Smiley (EEIDI)

- Building capacity for ecological surveillance and molecular diagnostics of mosquito-borne viruses in Sierra Leone | Robert Cross on behalf of Aiah Lebbie (WAC-EID)
- Active surveillance to detect low-level Rift Valley Fever Virus transmission in humans in East and Central Africa | Jeanette Dawa (CREID-ECA)
- The use of satellite imagery to create robust geospatial sampling designs and examine landscape dynamics across an urban to rural gradient in Managua, Nicaragua | Kathryn Hacker (A2CARES)
- Higher infestation indices of the vector Aedes aegypti in rural areas than in urban areas in Managua, Nicaragua | Jose Victor Zambrana (A2CARES)
- Rainfall and the prevalence of Aedes aegypti in northern coastal Ecuador | Jessica Uruchima (A2CARES)
- Setting the standard for field biosafety at the human-animal interface | Marc Valitutto (EID-SEARCH)
- Effective Recruitment Strategies during an Infectious Disease Surveillance Study at Rural Health Facilities in Liberia; WARN-ID Liberia Team Experience | Bode Shobayo (WARN-ID)
- Environmental surveillance of informal sewage systems reveals community SARS-CoV-2 transmission dynamics | Joshua Levy (WARN-ID)
- Mosquito ecology and risk of arboviral infectious disease spillover in southwestern Uganda | Jalika Joyner (EEIDI)
- Report of arbovirosis surveillance prospective cohort as model of early detection of viral circulation | Mauricio Noguiera (CREATE-NEO)
- Towards the laboratory maintenance of Haemagogus janthinomys, the major neotropical vector of sylvatic yellow fever | Adam Hendy (CREATE-NEO)

Lightning Talk Concurrent Session Details, continued

Concurrent Session B: One Health and Zoonotic Surveillance Location: Fitzgerald A North & Zoom

Session Chair: Christine Johnson (EEIDI)

Session Co-Chair: Isaac Ngere (2022 Pilot Awardee, CREID-ECA)

- One Health Pathogen Surveillance in the Bwindi Impenetrable Forest Region of Uganda | John Kayiwa (EEIDI)
- Use of molecular epidemiological, serological and experimental approaches to study COVID-19 transmission in Hong Kong | Leo Poon (CREID-ESP)
- Evidence of Bourbon virus in ticks and humans in St. Louis Missouri, USA | Ishmael D. Aziati (CREID-ESP)
- Potential reservoirs hosts zoonotic pathogens in Senegal including emerging viruses such arboviruses, Lassa, Ebola, coronaviruses and henipaviruses | Mawlouth Diallo (WAC-EID)
- A One Health investigation framework for zoonotic and vector-borne disease outbreaks | Soledad Colombe (CREID-ECA)
- Coronavirus Circulation in Peridomestic Rodent Populations in Sierra Leone | Allison Smither (WAC-EID)
- One Health surveillance of Lassa fever in rodents and human close contacts in North Central Nigeria | Nathan Shehu on behalf of Pam Luka (WAC-EID)
- Plasmodium vivax infection in two non-human primates in the Amazon | Marcus Lacerda (CREATE-NEO)
- YF-neutralizing antibodies in pied tamarins (Saguinus bicolor) captured in Amazon Rainforest fragments in the urban area of Manaus, Brazil | Marcus Lacerda (CREATE-NEO)
- CREATE-NEO arboviral surveillance in mosquitoes, febrile humans and non-human primates in transition zones in Panama and Darien | Sandra Lopez-Verges (CREATE-NEO)
- Late Breaker: Predicting the zoonotic capacity of mammals to transmit SARS-CoV-2 | Barbara Han (CREATE-NEO)
- Late Breaker: Coronavirus surveillance among farmed collared peccaries (Dicotyles tajacu) and caretakers in the Peruvian Amazon | Carlos Calvo-Mac (EEIDI)
- Late Breaker: SARS-CoV-2 Genomic Variant Surveillance in Human and Non-Human Primates in Peru | Mariana Leguía (EEIDI)

Concurrent Session C: Clinical and Laboratory Science Location: Fitzgerald A South & Zoom

Session Chair: Rob Brieman (CREID-ECA)

Session Co-Chair: Bronwyn Gunn (2021 Pilot Awardee, CREID-ECA)

- Acute Neurologic Syndromes associated with Chikungunya virus infections in Salvador, Brazil | Lorena Martins on behalf of Mateus Santana do Rosário (UWARN)
- Predictors of severity in dengue-suspected pediatric patients during 2019 dengue epidemic in Brazil | Mauricio Noguiera (CREATE-NEO)
- Influence of previous Zika virus exposure on Brazilian dengue outbreak in 2019 | Cassia Fernanda Estofolete (CREATE-NEO)
- Immune escape mutations in the Spike protein of an endemic SARS-CoV-2 variant in Panama | Sandra Lopez-Verges (CREATE-NEO)
- The highly conserved stem-loop II motif is important for the lifecycle of astroviruses but dispensable for SARS-CoV-2 | David Wang (CREID-ESP)
- Antibody fucosylation predicts disease severity in secondary dengue infection | Tineke Cantaert (PICREID)
- Unexpected Acute Viral Fever Mimicking Dengue-Like Illness in Major City of Pakistan | Najeeha Talat Iqbal (UWARN)
- Comparison of the Immunogenicity of five COVID-19 vaccines in Sri Lanka | Chandima Jeewandara (A2CARES)
- SARS-CoV-2 Variant Detection and Surveillance with an Economical and Scalable Molecular Protocol | Jesse Waggoner (A2CARES)
- Multiplexed detection of respiratory viruses and SARS-CoV-2 variants with mCARMEN | Nicole Welch (WARN-ID)
- Developing Rapid Antigen Diagnostics for Emerging Viruses using Antibodies Cloned from Sorted Single Memory B-cells | Lee Gehrke (CREATE-NEO)

- A simplified Cas13-based assay for the identification of SARS-CoV-2 and its variants | Jon Artizti Sanz (WARN-ID)
- Late Breaker: Dynamics of infectious virus neutralization from convalescent and vaccine cohorts across global SARS-CoV-2 variant lineages reveals boost protection and novel monoclonal antibody efficacy against Omicron strains | Michael Gale (UWARN)

Concurrent Session D: Epidemiology and Surveillance Across the Americas Location: Warfields & Zoom

Session Chair: Wes Van Voorhis (UWARN)

Session Co-Chair: Daniel Aguiar, 2021 Pilot Awardee (CREATE-NEO)

- A Dengue outbreak in Panama, 2022 | Luis Felipe Rivera (CREATE-NEO)
- Seroprevalence of Zika, Dengue, and Chikungunya viruses in a rural area in Brazil | Marcos Vinicius Lima de Oliveira Francisco (UWARN)
- Zika virus infection surveillance in Manaus, Amazonas state | Marcus Lacerda (CREATE-NEO)
- Real-time genomic surveillance of DENV-1 and DENV-2 in Brazil: improving public health outbreaks response | Luiz Alcantara (UWARN)
- Surveillance and evolutionary analysis of Dengue viruses to understand the epidemiological dynamics of dengue outbreaks, São José do Rio Preto, São Paulo, Brazil | Lívia Sacchetto (CREATE-NEO)
- Monitoring the genetic diversity of reemerging chikungunya virus in Brazil | Luiz Alcantara (CREATE-NEO)
- Genomic and epidemiological monitoring of YFV reemergence in Brazil: unveiling the corridor of spread and the geographic hot spots for predicting and preventing other possible spillover events | Marta Giovanetti (UWARN)
- Genomic surveillance of SARS-CoV-2 in symptomatic vaccinated and unvaccinated asymptomatic patients in Brazil | Luiz Alcantara (UWARN)
- SARS-CoV-2 genomic surveillance and the impact of different lineages circulation in the epidemiological landscape of São José do Rio Preto, São Paulo, Brazil | Cecília Artico Banho (CREATE-NEO)
- Genomic epidemiology reveals the impact of national and international restrictions on the SARS-CoV-2 epidemic in Brazil | Marta Giovanetti (UWARN)
- Occurrence of SARS-CoV-2 reinfections at regular intervals in Ecuador | Paul Cardenas (A2CARES)
- Genomic characterization of SARS-CoV-2 during the COVID-19 pandemic in Nicaragua | Cristhiam Cerpas (A2CARES)

Concurrent Session E: Epidemiology and Surveillance Across Africa and Asia Location: Grason & Zoom

Session Chair: Anavaj Sakuntabhai (PICREID)

Session Co-Chair: Robert Langat, 2021 Pilot Awardee (CREID-ECA)

- Flaviviruses and Lassa fever in febrile patients in North Central Nigeria: A cross-sectional study | Nathan Shehu (WAC-EID)
- Double Stigma and discrimination: A qualitative study of Lassa fever and hearing loss in Northern Nigeria | Kachollom Best (WAC-EID)
- A brief chronicle of SARS-CoV-2 genomic surveillance in Cambodia | PICREID Representative on behalf of Erik Karlsson (PICREID)
- Identification of Genetic Variations of SARS-CoV-2 Omicron Strain and their Clinical Significance in Karachi, Pakistan | Aqsa Khalid (UWARN)
- Genetic characterization of Norovirus strains from an unusual diarrheal outbreak in the community in Thailand | Krongkan Srimuang (EID-SEARCH)
- Factors associated with changing Dengue case numbers during the COVID-19 pandemic in Sri Lanka | Dinuka Ariyarantne (A2CARES)
- SARS-CoV-2 genomic epidemiology in Sierra Leone | John Demby Sandi (WARN-ID)
- Yellow Fever outbreak in eastern Senegal, 2020–2021 | Moussa Diagne (WAC-EID)
- The evolving SARS-CoV-2 epidemic in Africa: Insights from rapidly expanding genomic surveillance | Houriiyah Tegally (UWARN)

- Detection and Characterization of Variants of Concern: Insights from the South African Epidemic | James Emmanuel San (UWARN)
- Emergence of novel combinations of SARS-CoV-2 spike receptor binding domain variants in Senegal | Amroise Ahouidi (UWARN)
- COVID-19 laboratory surveillance at IRESSEF in Senegal | Cheikh Ibrahim Lo (UWARN)
- Dynamics of Variants of Concern (VOC) during the different waves of COVID-19 in Senegal | Abdou Padane (UWARN)

Acronyms and Abbreviations

A2CARES	American and Asian Centers for Arboviral Research and Enhanced Surveillance
BV-BRC	Bacterial and Viral Bioinformatics Resource Center
CEPI	Coalition for Epidemic Preparedness Innovations
CREATE-NEO	Coordinating Research on Emerging Arboviral Threats Encompassing the Neotropics
CREID	Centers for Research in Emerging Infectious Diseases
CREID CC	CREID Coordinating Center
CREID-ECA	Center for Research in Emerging Infectious Diseases – East and Central Africa
CREID-ESP	Center for Research in Emerging Infectious Diseases – Epidemiology, Surveillance and Pathogenesis
CSPH	Colorado School of Public Health
DMID	Division of Microbiology and Infectious Diseases
EEIDI	EpiCenter for Emerging Infectious Disease Intelligence
EID-SEARCH	Emerging Infectious Diseases: South East Asia Research Collaboration Hub
NIAID	National Institute of Allergy and Infectious Diseases
NIBSC	National Institute for Biological Standards and Controls
NIH	National Institutes of Health
PICREID	Pasteur International Center for Research on Emerging Infectious Diseases
PREMISE	Pandemic Response Repository through Microbial and Immunological Surveillance and Epidemiology
RC	Research Center
TGHN	The Global Health Network
UWARN	United World Antiviral Research Network
WAC-EID	West African Center for Emerging Infectious Diseases
WARN-ID	West African Research Network for Infectious Diseases
WRCEVA	World Reference Center for Emerging Viruses and Arboviruses

– AGENDA: TABLETOP EXERCISE, APPLIED SESSIONS A and B –

DAY 1 | WEDNESDAY, SEPTEMBER 21

The ***Tabletop Exercise Situation Report*** will be introduced during the ***Collaboration for Outbreak Research Response & Tabletop Introduction*** session during the Scientific Meeting (Time: UMT -4, 14:15-15:15)

TTX Zoom Link

DAY 2 | THURSDAY, SEPTEMBER 22

Time (UMT -4)	**Session**	**Location**	**Participants**
08:30-10:40	**Tabletop Exercise: Applied Session A**		Network Members, DMID, EAC, NIAID
	Breakout Rooms		
	Combined Biorepository and Data Capture & Harmonization (hybrid session) **Facilitators:** Tony Moody (CREID CC), Nikos Vasilakis (CREATE-NEO), Nathan Vandergrift (CREID CC), Cecilia Sanchez (EID-SEARCH) **Reporters:** Hilary Bouton-Verville, Nefer Batsuli (CREID CC)	Fitzgerald A South & Zoom	
	Laboratory Assays (hybrid session) **Facilitators:** Greg Sempowski (CREID CC), Bob Garry (WARN-ID) **Reporters:** Thad Gurley, Eric Earley (CREID CC)	Fitzgerald A North & Zoom	
	Outbreak Research Response A (in-person session only) **Facilitators:** Peter Rabinowitz (UWARN), Jay Hemingway-Foday (CREID CC) **Reporter:** Megan Averill (CREID CC)	Warfields	
	Outbreak Research Response B (in-person session only) **Facilitators:** Richard Reithinger (CREID CC), Christine Johnson (EEIDI) **Reporter:** Hongying Li (EID-SEARCH)	Fitzgerald B/C	
	Outbreak Research Response C (virtual session) **Facilitators:** Rob Breiman (CREID-ECA), Souleymane Mboup (UWARN), Kathy Hanley (CREATE-NEO) **Reporters:** Danielle Wagner, Aaron Macoubray (CREID CC)	Grason & Zoom	
10:40-11:00	BREAK		
	Scientific Meeting (see detailed agenda)		
11:00-17:00			Network Members, DMID, EAC, NIAID, External and USG Stakeholders

DAY 3 | FRIDAY, SEPTEMBER 23

TTX Zoom Link

Time (UMT -4)	Session	Location	Participants
08:30-10:40	**Tabletop Exercise: Applied Session B**		Network Members, DMID, EAC, NIAID
08:30-09:40	**Breakout Rooms**		
	Combined Biorepository and Data Capture & Harmonization (hybrid session) **Facilitators:** Tony Moody (CREID CC), Nikos Vasilakis (CREATE-NEO), Nathan Vandergrift (CREID CC), Cecilia Sanchez (EID-SEARCH) **Reporters:** Hilary Bouton-Verville, Nefer Batsuli (CREID CC)	Fitzgerald A South & Zoom	
	Laboratory Assays (hybrid session) **Facilitators:** Greg Sempowski (CREID CC), Bob Garry (WARN-ID) **Reporters:** Thad Gurley, Eric Earley (CREID CC)	Fitzgerald A North & Zoom	
	Outbreak Research Response A (in-person session only) **Facilitators:** Peter Rabinowitz (UWARN), Jay Hemingway-Foday (CREID CC) **Reporter:** Megan Averill (CREID CC)	Warfields	
	Outbreak Research Response B (in-person session only) **Facilitators:** Richard Reithinger (CREID CC), Christine Johnson (EEIDI) **Reporter:** Hongying Li (EID-SEARCH)	Fitzgerald B/C	
	Outbreak Research Response C (virtual session) **Facilitators:** Rob Breiman (CREID-ECA), Souleymane Mboup (UWARN), Kathy Hanley (CREATE-NEO) **Reporters:** Danielle Wagner, Aaron Macoubray (CREID CC)	Grason & Zoom	
09:40-09:50	TRANSITION TIME		
09:50-10:40	**Tabletop General Session: Working Group Report Outs**	Fitzgerald B/C & Zoom	
10:40-11:00	BREAK		
	Scientific Meeting		
11:00-16:15	**Scientific Meeting** (see detailed agenda)		Network Members, DMID, EAC, NIAID, External and USG Stakeholders

Outbreak Research Response Tabletop Exercise Applied Sessions

Annual Meeting, Days 2 and 3

All Network Members

Research Response

Outbreak *simulation* to stress test Network capacity to launch rapid & effective research for future outbreaks.

Network-wide interactive outbreak simulation activity in support of the Network Vision to build capacity and outbreak research response readiness.

An outbreak *Situation Report* will be presented on Day 1 in the General Scientific Session.

Exercise Goals:

- Work as multidisciplinary teams to respond to a *Pathogen X* outbreak
- Build collaborative connections with Network colleagues
- Pressure test existing Network Tools/Inventories to launch coordinated research response
- Identify critical gaps/pinch points for effective research response
- Develop an After-Action Report to guide Network capacity building and outbreak research readiness

Mentimeter Tool

Using your smartphone, laptop, or other mobile device, please go to **mentimeter.com** to participate in interactive polls, quizzes, and post questions.

Applied Session Schedule: Day 2, 8:30-10:40am EDT **|** Day 3, 8:30-10:40am EDT

Additional Information:

See detailed agendas

Email: info@creid-network.org

CREID Centers for Research in Emerging Infectious Diseases

NIH National Institute of Allergy and Infectious Diseases

TTX Activities at Annual Meeting (Day 1 and 2)

15 min

Facilitator presents scripted, cross-cutting scenario (**Sit Rep 1**) and briefs participants on objectives, procedures, & expectations. Research Centers with communication/ coordination with in-country partners in preparation for applied sessions.

Presentation of Sit Rep 1 and plan for applied session

10 min

Facilitator reminds participants of outbreak scenario, reviews expectations and WG-specific objectives for session, and orients participants to the Mentimeter facilitation tool.

100 min

Facilitator uses scripted questions in the Facilitator Guide and Mentimeter facilitation tool to generate discussion about how participants would respond to the outbreak scenario presented in Sit Rep 1. Participants discuss, make decisions, and take action in real-time (e.g., test Network tools).

20 min

Facilitator (or reporter) summarizes key take-aways from Session 1 and presents Sit Rep 2, which will be used in Applied Session 2 ("Outbreak evolves")

APPLIED SESSION A: OUTBREAK IDENTIFIED

1. Presentation of WG-specific module

2. Guided Discussion with interspersed polling through menti.com

3. Wrap-up and Late Breaking news

2022 Annual Meeting Scientific Session (Day 1)

The Coordinating Center presents Situation Report (Sit Rep) #1, which describes the scenario to be discussed on Day 2 in the applied session.

2022 Annual Meeting Concurrent Working Group Sessions (Day 2 from 8:30-10:40 am ET)

Participants meet in WG-specific break-out groups to discuss response to Sit Rep 1.

1. Biorepository Collaboration & Quality/ Data Capture & Harmonization (combined group)
2. Laboratory Assay Oversight and Quality
3. Outbreak Research Response (split into 3 concurrent sessions)

Note: As a cross-cutting activity, capacity building considerations will be integrated into other WG discussions rather than convening a stand-alone session. Capacity Building WG members will have the opportunity to join the other WG session of their choice.

TTX Activities at Annual Meeting (Day 3)

10 min

Facilitator (or reporter) provides a summary of key take-aways from Session 1 and a reminder of Sit Rep 2.

50 min

Facilitator uses Facilitator's Guide and Mentimeter facilitation tool to guide participants through scripted questions and to generate discussion about how participants would respond to the new information presented in Sit Rep 2. Participants discuss, make decisions, and take action in real-time.

10 min

Facilitator (or reporter) summarizes key take-aways from Session 2.

60 min

Reporter from each WG session briefs Network on key discussion and take-aways from Applied Sessions 1 and 2.

APPLIED SESSION B: OUTBREAK EVOLVES

1. Summary of Session 1 and reminder of Sit Rep 2

2. Guided Discussion with interspersed polling using menti.com

3. Wrap-up

Report on Key Take-Aways

2022 Annual Meeting
Concurrent Working Group Sessions
(Day 3 from 8:30-9:40 am ET)

Participants re-convene in their WG-specific breakout groups from Day 2 to discuss Sit Rep #2.

1. Biorepository Collaboration & Quality/ Data Capture & Harmonization (combined group)
2. Laboratory Assay Oversight and Quality
3. Outbreak Research Response (split into 3 concurrent sessions)

2022 Annual Meeting
General Session
(Day 3 from 9:40-10:40 am ET)

Pathogen X-Like Outbreak of Suspected Animal Origin

Situation Report 01

01 September 2022 (Epi Week 36)

1. Situation at a Glance	8 Affected Countries	Cases	Deaths	CFR
		56	4	7.1%

- Since 05 August 2022, a total of 56 suspected cases have been identified in 8 countries, with geographic clusters in West Africa (Sierra Leone, Guinea) and Central Africa (DRC, Uganda). Isolated cases have also been identified in Brazil, China, France, and Senegal.
- Four deaths have been reported to date. One death has been linked to an immunocompromised patient. No further information is available about the other deaths.
- Many cases (n=31) report a history of animal exposure, including rodents, bats, monkeys (species unknown), wild pig, domesticated dogs, and livestock (poultry, pigs).
- All cases report fever and malaise. Other common symptoms include rash, swollen lymph nodes, cough, anorexia, myalgia, nausea, and skin lesions.
- Separate and distinct epidemiological linkages have been established among cases in West African cases (Sierra Leone and Guinea) and Central Africa (DRC and Uganda).
- Epidemiological investigation and laboratory analyses are ongoing.

2. Epidemiological Summary

Since 05 August 2022, WHO has received reports of a pathogen X-like illness in Brazil, China, Democratic Republic of Congo (DRC), France, Guinea, Senegal, Sierra Leone, and Uganda (Table 1). Clustering of cases has occurred in West Africa, along the Sierra Leone-Guinea border, as well as Central Africa, along the DRC-Uganda border. Among cases reported in West and Central Africa, 29 have a recent history of animal exposure, most commonly rodents. Cases also report exposure through livestock (poultry, pigs), domesticated dogs, or preparation or consumption of bushmeat (bats, monkeys, "bush rats", wild pigs).

Table 1. Suspected Cases, Deaths, and Samples for Pathogen X-like Illness (05 to 31 August 2022)

Country	Cases	Deaths	Samples Collected
Sierra Leone	21	1	18
DRC	16	2	16
Guinea	6	0	6
Uganda	7	1	7
Senegal	2	0	2
China	2	0	2
Brazil	1	0	1
France	1	0	1
Total	56	4	53

Epidemiological investigation suggests two separate and distinct transmission chains, with the index cases located in Sierra Leone and DRC. The index cases and transmission dynamics are further described in **Section 3 (Description of Cases)**. There is no known evidence of epidemiological linkages between the cases in West Africa and Central Africa.

Isolated cases have also been identified in Brazil, China, France, and Senegal; Among these cases, five have reported recent travel to West or Central Africa and two have reported animal exposure.

Clinical presentation. All cases presented with fever and malaise. Other common symptoms include rash, swollen lymph nodes, cough, anorexia, myalgia, nausea, and skin lesions. A total of four deaths have been reported (2 in DRC; 1 in Uganda; 1 in Sierra Leone). One death has been linked to an immunocompromised patient. No further information is available about the other deaths.

Diagnostic Activities. Blood samples have been collected from a total of 53 cases (see Table 1) and sent to the National Reference Laboratory in each affected country for diagnosis and viral identification by real-time PCR. Diagnostic testing results are not yet available. All samples collected in Sierra Leone, Guinea, and Senegal were sent to the Institut Pasteur de Dakar (Dakar, Senegal) for additional diagnostic and confirmatory testing.

3. Description of Cases

Suspected Clusters in Sierra Leone and Guinea. On 05 August 2022, the National IHR Focal Point in Sierra Leone notified WHO of a cluster of cases of a Pathogen X-like illness in the Kailahun District. All cases presented with fever and malaise; Other symptoms included rash, swollen lymph nodes, skin lesions, and respiratory symptoms. Epidemiological investigation indicates that the index case was a 21-year-old male with a history of animal exposure, including household exposure to rodents and domestic animals or livestock (dogs, poultry, pig) and consumption of bushmeat (bat, "bush rat"). The majority of cases (8) were identified in Yenga, a village located in a remote forest area that sits at the main international border crossing with Guinea. On 15 August 2022, health authorities in Guinea reported a household cluster of three cases presented with symptoms similar to the Sierra Leone cases. The household cluster is located in Nongoa, within the Guéckédou prefecture, and approximately 9 miles from the Sierra Leone border. Epidemiological investigations are ongoing; preliminary information indicates that one of the Guinea cases has an established travel link to affected areas in Sierra Leone and all three Guinea cases have a history of animal exposure, including rodents, bats, monkeys (unknown species), and domesticated animals or livestock. The proximity of the affected areas to international borders, cross-border movement between the Sierra Leone and Guinea, and the potential transmission of the unknown pathogen between animal vectors and humans poses an increased risk for cross-border spread. These factors also suggest a high risk at the national and regional level, given that the Kailahun District is well connected to the Guéckédou prefecture in Guinea and Lofa County in Liberia.

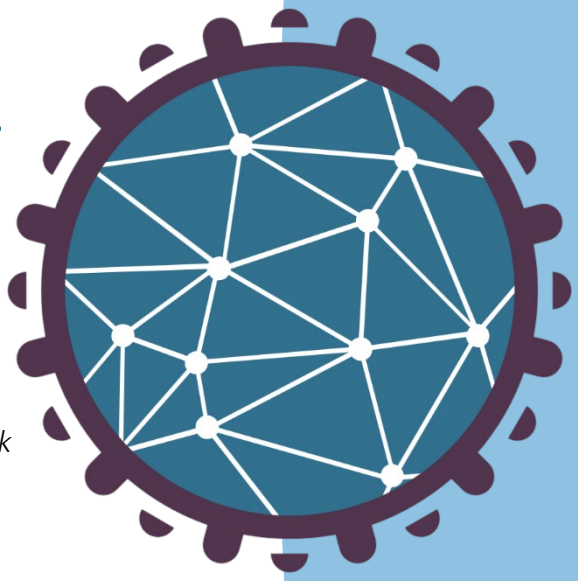
Suspected Clusters in DRC and Uganda. On 25 August 2022, the Minister of Health in DRC reported 16 cases of Pathogen X-like illness in the North Kivu Province. Epidemiological investigation indicates that the suspected index case and majority of suspected cases reside in the Beni Health zone, located on the border with Uganda. This report of the cluster in Beni directly follows the 22 August 2022 announcement by the DRC Minister of Health of a newly identified Ebola virus disease (EVD) outbreak in the same health zone. The suspected index case is a 5-year-old male who presented to a public health facility with a 10-day history of fever, malaise, swollen lymph nodes, and appearance of rashes on his skin. Three household contacts presented with similar symptoms, although the time of symptom onset was less than seven days. Before presenting to the health facility, the child and his family sought care from a traditional health practitioner who provided local herbs for the fever and rash. The suspected index case and his household contacts report a history of animal exposure, including rodents, monkeys of unknown species, and livestock (poultry, goats) within two weeks of symptom onset. The mother of the suspected index case also reports frequent trips to neighboring villages, including several villages in the Kasese District of Uganda to sell bush meat.

Suspected Cases in Senegal, China, Brazil, and France. Isolated cases of Pathogen X-like disease have also been reported in Senegal, China, Brazil, and France, five cases reported recent travel to West or Central Africa (2 from China and 1 each from Senegal, Brazil, and France). Among these five patients, only those from Senegal and France reported travel to an area with an identified cluster. Two suspected cases (1 from China and 1 from Senegal) also report a recent animal exposure.

CREID Preparedness Session 1b: Now open for YOUR input via XLEAP!

Activity Goals

- *Collectively identify evidence gaps and critical research questions that can be addressed by the Network during inter-outbreak periods*
- *Promote knowledge-sharing and relationship-building across CREID Research Centers and Sites*
- *Define mechanisms for rapid and effective research coordination, collaboration, and execution when an outbreak occurs*



Overview and Activity Methodology

Outbreak Research Preparedness Session 1b is a follow-up to the Zoom-based Session 1 on Lassa Fever (LASV) and Chikungunya Fever (CHIKV), which was held in July 2022.

For this asynchronous crowd-sourcing activity (Session 1b), the CC is employing a virtual facilitation tool called **XLeap**. Please visit the CREID Private Portal to access:

- Brief instructions on how to use and navigate within XLeap
- An informational demo of this activity in XLeap
- https://creid-network.org/login

XLeap is a secure, password protected site. Please use the login details below to access the XLeap platform and provide your responses to the four questions posed regarding CHKV and LASV within the XLeap platform **by September 26th, 2022.** Your responses will be reviewed and prioritized by a CREID Steering Committee-assigned panel of Subject Matter Experts in early October.

XLeap Login

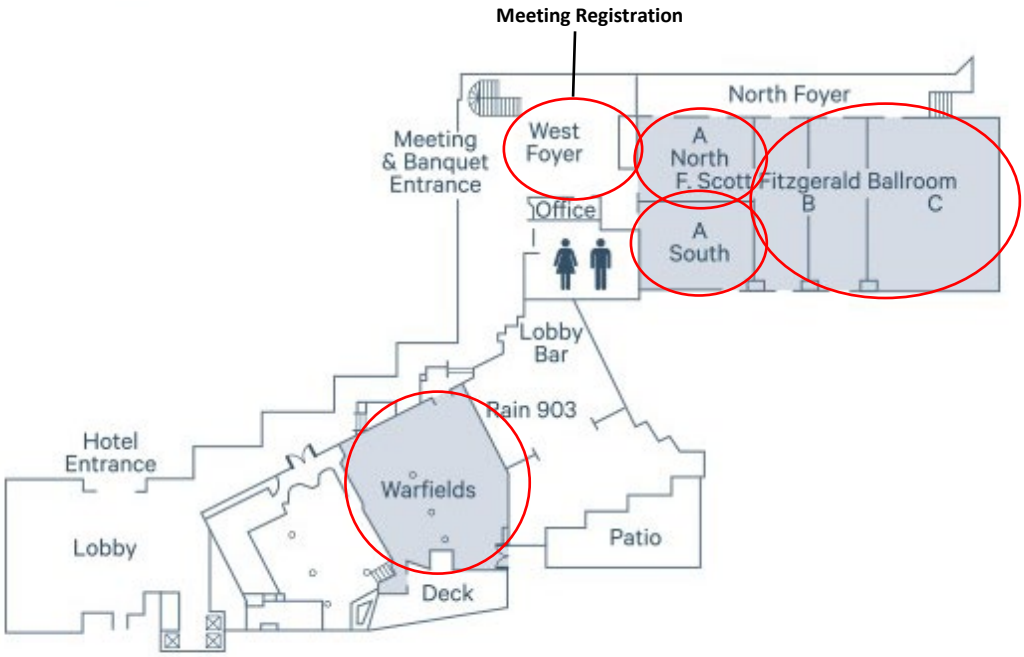
Website Link: https://rti.xleap.net/fr9o9uz47ck7/login
Your name: *Please use your full name and CREID Network affiliation* (i.e., Research Center)
Your email: *Please use your email address as your login ID*
Access Code: *CREID*

Additional Information

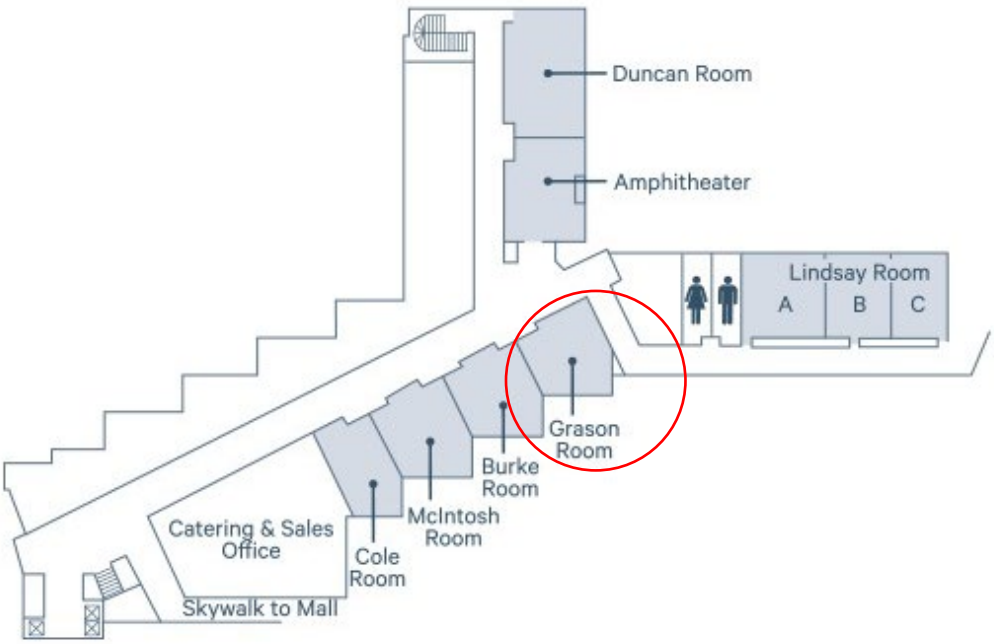
Email: info@creid-network.org

Sheraton Baltimore North Hotel: Venue Map

PLAZA LEVEL



SECOND LEVEL



2022 CREID Network Annual Meeting
COVID-19 Guidance
Updated: 09/19/2022

To In-Person CREID Annual Meeting Participants:
Included here is information about the COVID-19 Community Level in Baltimore County, Maryland, the site of the Annual Meeting, and the mitigation efforts the meeting organizers are putting in place. All information and mitigation efforts are based on guidance from the U.S. Centers for Disease Control and Prevention (CDC).

Current (as of September 19, 2022) transmission in Baltimore County: **Low**

We will update the COVID-19 information shared with meeting attendees and if the COVID-19 Community Level changes, our guidance to meeting participants will change accordingly. If you have any questions, please contact info@creid-network.org.

Annual Meeting COVID-19 logistics (for low transmission of COVID-19)

Based on updated NIH policy (as of August 24, 2022) meeting attendees are no longer required to provide vaccination attestations or a negative COVID test.

Onsite COVID-19 Mitigation

- Meeting organizers will provide masks and hand sanitizer onsite at the hotel.
- We ask all attendees to conduct a self-assessment utilizing the *CDC Coronavirus Self Checker* before attending the meeting and we ask that anyone exhibiting COVID-19 symptoms to participate in the meeting virtually: Symptoms of COVID-19: https://www.cdc.gov/coronavirus/2019-ncov/symptoms-testing/symptoms.html

Baltimore County COVID-19 Information
According to the CDC COVID-19 County Check for **Baltimore County, Maryland** on **September 19, 2022**, where the Sheraton Baltimore North Hotel is located, the COVID-19 Community Level is **Low**, which is determined by "hospital beds being used, hospital admissions, and the total number of new COVID-19 cases in an area." *Source*: https://www.cdc.gov/coronavirus/2019-ncov/your-health/covid-by-county.html

CDC Low Community Level guidance is:

- Stay up to date with COVID-19 vaccines (https://www.cdc.gov/coronavirus/2019-ncov/vaccines/stay-up-to-date.html)
- Get tested if you have symptoms (https://www.cdc.gov/coronavirus/2019-ncov/symptoms-testing/testing.html)
- People may choose to mask at any time. People with symptoms, a positive test, or exposure to someone with COVID-19 should wear a mask.

Additional information from the CDC COVID Data Tracker: County View https://covid.cdc.gov/covid-data-tracker/#county-view?list_select_state=Maryland&data-type=CommunityLevels&list_select_county=24005)

CREID Annual Meeting COVID Guidance
Updated: 09/19/2022

Health Facilities Near Meeting Location (Sheraton Baltimore North Hotel)

Health Facility	Location and Phone	Website	Hours of Operations	Distance from Meeting
Patient First Primary and Urgent Care, Towson	950 York Road, Towson, MD 21204; (410) 372-6373	https://www.patientfirst.com/locations/baltimore/towson?utm_source=local&utm_medium=organic&utm_campaign=gmb	8:00am – 10:00pm	.8 miles
Minute Clinic	1001 York Road, Towson, MD 21204; (866) 389-2727	https://www.cvs.com/minuteclinic/virtual-care/e-clinic?WT.mc_id=LS_MC_GOOGLE_2216_get_online_care_button	8:30am-7:30pm	.8 miles
University of Maryland St. Joseph Medical Center Emergency Room	7601 Osler Drive, Towson, MD 21204; (410) 337-1000	https://www.umms.org/sjmc/locations/emergency-department?utm_source=local-listing&utm_medium=organic&utm_campaign=website-link	Open 24 hours	2.5 miles
Greater Baltimore Medical Center	6701 N. Charles Street, Towson, MD 21204; (443) 849-2000	https://www.gbmc.org/	Open 24 hours	3.8 miles
University of Maryland Urgent Care, Belvedere	600 E. Belvedere Ave, Suite A, Baltimore, MD 21212; (410) 296-0018	https://www.umms.org/health-services/urgent-care/locations/belvedere-square?utm_source=local-listing&utm_medium=organic&utm_campaign=website-link	8:00am-8:00pm	5 miles
MedStar Good Samaritan Hospital	5601 Loch Raven Blvd., Baltimore, MD 21239; (443) 444-8000	https://www.medstarhealth.org/locations/medstar-good-samaritan-hospital?utm_campaign=mhs_citations&utm_medium=ad_listings&utm_source=rio_seo&utm_term=hospital	Open 24 hours	7.8 miles

From: Van Vliet, Gretchen on behalf of Van Vliet, Gretchen <gvanvliet@rti.org>

To: acmeyers@umich.edu; abalmaseda40@gmail.com; afargas@icsnicaragua.org; agajewski; Gordon, Aubree; cnarvaez@icsnicaragua.org; jeewandara@sjp.ac.lk; ccerpas@icsnicaragua.org; dinukaa@gmail.com; esanchez@icsnicaragua.org; evidea@icsnicaragua.org; emorales@icsnicaragua.org; gvasquez@icsnicaragua.org; suazolagunaharold@gmail.com; jacqueline.mdiaz.27@gmail.com; James.Trostle@trincoll.edu; jjwaggo@emory.edu; juruchi@umich.edu; jnse@umich.edu; jruiz@icsnicaragua.org; jzamb@umich.edu; kphacker@umich.edu; nerysanchez@icsnicaragua.org; mnunez@icsnicaragua.org; msalinas@icsnicaragua.org; oortega@icsnicaragua.org; paulinaandradeproano@berkeley.edu; Lakshmanane, Prem; SBennett; sarguello@icsnicaragua.org; ssanchez@icsnicaragua.org; Tulika.singh@berkeley.edu; yvillalobos@icsnicaragua.org; adhendy@utmb.edu; castellanosa@caryinstitute.org; alvillas@umtb.edu; Betania Drumond; cassiafestofolete@gmail.com; ceci.abanho@gmail.com; aguiar.daniel; edhdz@nmsu.edu; gcgray@utmb.edu; helene.cecilia3; jpcarrera@gorgas.gob.pa; liviasacchetto; lrivera@gorgas.gob.pa; marcuslacerda.br@gmail.com; mariapaula.mourao@gmail.com; Eitzen, Melissa M.; elabuen@nmsu.edu; prh2799@nmsu.edu; andrew.karani@wsu.edu; ahauner@itg.be; bbarnabas2001; Gunn, Bronwyn Mei; carolyne.nasimiyu@wsu.edu; ndumudb@gmail.com; mumvungatabitha22@gmail.com; dorbosi@gmail.com; evankleef@itg.be; ngere, isaac a.; jmasumu@hotmail.com; llepore@itg.be; nyakarahuka@gmail.com; mmeudec@itg.be; moshe.alando@wsu.edu; nicowor@gmail.com; nungari.kungu@wsu.edu; El-Duah, Philip; RLangat; skhamadi@gmail.com; s.njoga@wsu.edu; shemakiala@yahoo.fr; silvia.situma@wsu.edu; scolombe@itg.be; stephanie.seifert@wsu.edu; wvanbortel@itg.be; Annie Elong Ngono; awalbal@gmail.com; bschalise@gmail.com; feyesag@yahoo.com; getas73@yahoo.com; getachewtollera@gmail.com; idaziati@wustl.edu; krishna.manandhar@gmail.com; Leo Poon; rawaldrmanisha1976@gmail.com; runa75jha@gmail.com; shravan.nepal@gmail.com; ajmoo@ucdavis.edu; isaacjamesahwera@gmail.com; garcia.alejandra@pucp.edu.pe; akawiecki@ucdavis.edu; bssebide@gorilladoctors.org; brenov.munozs@pucp.edu.pe; calo.25388@gmail.com; diana.juarez@pucp.edu.pe; dibesh@cmdn.org; edward.gaitan@pucp.edu.pe; gsalmonm@pucp.edu.pe; jjjoyner@ucdavis.edu; jkayiwa; jjlutwama03@yahoo.com; katherine.jamanca@pucp.edu.pe; keliee.vilcahuaman@pucp.edu.pe; kkvanrompay@ucdavis.edu; lcwells@ucdavis.edu; Marcela Uhart; mugisacharles4@gmail.com; hahotice@googlemail.com; felistanannono@gmail.com; ohnmara@gmail.com; oisaacmagezi@gmail.com; pbarrerab@pucp.edu.pe; Pranav Sudhir Pandit; Kading,Rebekah; armero; ananporn.su@gmail.com; Cadhla Firth; caitedw@unc.edu; Cecilia Sanchez; cuongnv; Eric Laing; keusch@bu.edu; jsiegers; khwankamon.r; krongkan srimuang; lgralins@email.unc.edu; Marc Valitutto; rbcorley@bu.edu; sasiprapa.n; sini.petcharat@hotmail.com; Su Yadana; hvireak@paster-kh.org; lorenacmartins@hotmail.com; ahmed.abd_el_wahed@uni-leipzig.de; djuicy@pasteur-yaounde.org; dethie.ngom@pasteur.sn; diawo.diallo@pasteur.sn; ekarlsson@pasteur-kh.org; gamou.fall; Janin NOUHIN; jvhomweg@pasteur.fr; abarry@pasteur.sn; Paul Alain Tagnouokam; Richard Njouom; sboyer@pasteur-kh.org; souand.mohamed-ali@pasteur.fr; Tineke CANTAERT; Veasna DUONG; abdou.padane@iressef.org; Ambroise Ahouidi; aqsa.khalid@aku.edu; Badara Cisse; Davide F. Robbiani; Geoffrey S. Gottlieb; houriiyah.tegally@gmail.com; Isadora Cristina de Siqueira; sanemmanueljames@gmail.com; Elizabeth.dykstra@doh.wa.gov; alcantaraluiz42; marcosv19@live.com; Marta Giovanetti; msrosario@uneb.br; Najeeha Iqbal; Najia Ghanchi; Noelle Benzekri; Edlefsen PhD, Paul T; Renee Ireton; Souleymane Mboup; alioune.gaye@pasteur.sn; arsmithe@utmb.edu; amehigoche@gmail.com; usfilmalgwi@yahoo.com; carinanoesen@gmail.com; yilgwanc@unijos.edu.ng; geothankgod2020@gmail.com; dungd.pam@gmail.com; otakpaeche@gmail.com; emanuelsaidu@gmail.com; damif@unijos.edu.ng; ganijoel65@gmail.com; grivik2000@yahoo.co.uk; jesse4xt@gmail.com; helenluka28@gmail.com; ularamuhussaini@yahoo.co.uk; leokonti@yahoo.com; judithbakam@gmail.com; kachbest@gmail.com; ldickson; imohc@unijos.edu.ng; mvbecker@utmb.edu; malalamar@gmail.com; mmyke842@gmail.com; MoussaMoise.DIAGNE@pasteur.sn; olaoluolushola@gmail.com; Pam Luka; beckybitiyong@gmail.com; bekkyweka@gmail.com; amosgamborimfa@gmail.com; Cross, Robert W.; ezekielstv@gmail.com; taiyeadeyanju@gmail.com; temidayoadeyanju@gmail.com; aida.badiane@cigass.org; alhajiboumaloljalloh@gmail.com; amy.gaye@cigass.org; deme.awa; bodeishobayo@gmail.com; nosamiefani@run.edu.ng; fjaryan68@gmail.com; hlutz; uniqueissa; johnatsandi@gmail.com; jon96as@mit.edu; jolevy@scripps.edu; Jyothi Purushotham; Katie Siddle; mambumomoh@gmail.com; zellerm@scripps.edu; Michelle Platero; mouhamad.sy@cigass.org; nbond@tulane.edu; nicole hoff; nwelch@broadinstitute.org; amoo.oluwaseun1@gmail.com; Shirlee Wohl; thelma.nelson; tkallon03@gmail.com; tolla.ndiaye@cigass.org; zlevine@broadinstitute.org

Cc: Macoubray, Aaron; Batsuli, Nefer; Beaubien, Candice (NIH/NIAID) [E]; Megan Averill

Subject: CREID Network Annual Meeting: Final Agenda and Materials

Date: Monday, September 19, 2022 2:22:33 PM

Attachments: CREID_welcomepacket.pdf
CREIDAgenda_Detailed_Scientific.pdf
TTX Materials_v3.pdf
Preparedness_Session1b.pdf
Venue Map_v3.pdf
COVIDguidance_09192022.pdf

Dear CREID Network Members,

We are looking forward to seeing you – in person or virtually – on Wednesday as our CREID Network Annual Meeting gets underway.

Please find final materials attached for your reference:

1. Welcome Packet
2. Final Scientific Agenda (Zoom links included)
3. Tabletop Agenda and Materials (Zoom links included)
4. Preparedness Session 1b flyer
5. Venue Map
6. COVID guidance

For those attending in person:

- A registration table will be set up (see venue map) for you to pick up your badges and a hard copy of meeting materials starting Tuesday afternoon (for about an hour at 4pm EDT) or in the mornings before the sessions start.
- Good news! COVID transmission in Baltimore County is now low; updated COVID information is attached.
- We hope you take advantage of the no-host social hours – Tuesday, Wednesday, and Thursday evenings – to get to know one another better. See the Welcome Packet for location details.
- Look for the CREID CC team members with the yellow ribbons on their name badges if you have questions or concerns during your stay.

If you have any questions, please email info@creid-network.org.

For those who are traveling, safe travels!

Gretchen

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

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Commented [NR1]: Please check that your affiliation is as it should be!

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].

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.

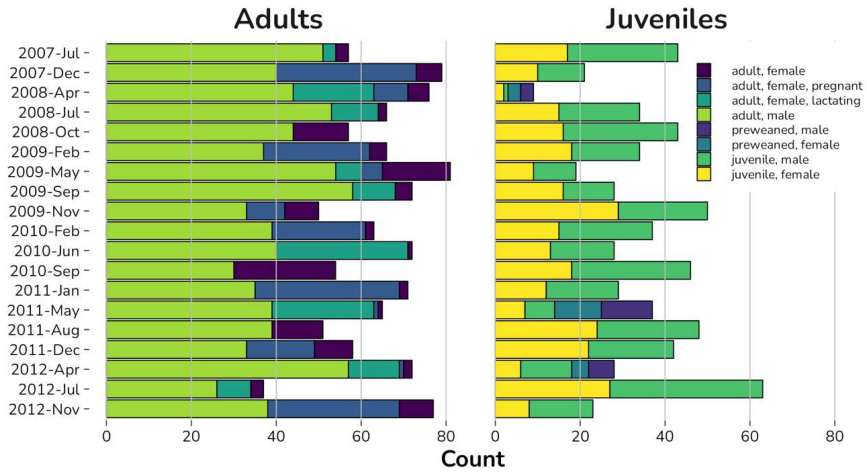


Figure 1. Demographics of bats captured in longitudinal sampling. Pregnant females were captured in months from November to June, lactating females were captured in months from April to July. Females with pre-weaning juveniles attached were found from April to May.

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).

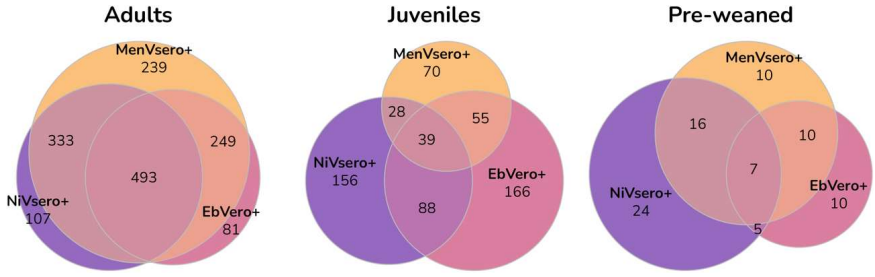


Figure 2. Detection of IgG antibodies against multiple viruses in individual *Pteropus medius*: Venn diagrams for adult, juvenile, and pre-weaned bats testing positive for antibodies against Nipah virus, filovirus, and Rubulavirus. Numbers under labels are counts of bats with only those viruses, numbers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

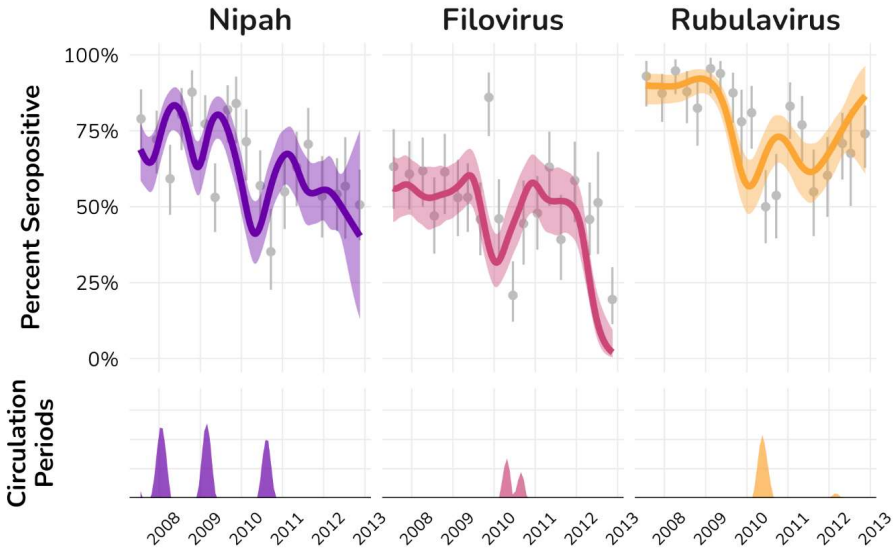


Figure 3. Inter-annual serodynamics in adult bats. Top: Measured and modeled population seroprevalence in adults over the term of the study. X-axis ticks mark the first day of the year. Grey points and bars represent measured population seroprevalence from individual sampling events on and 95% exact 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.

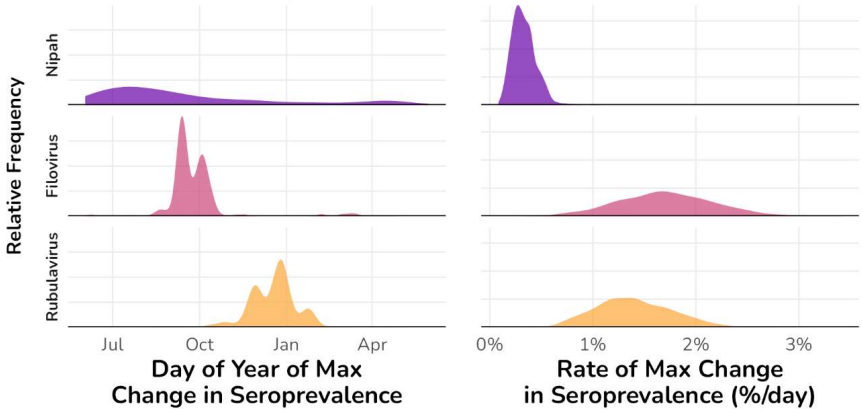


Figure 4. Timing and strength of seasonality of viral circulation in adults. Plots show posterior densities of the date for which increase in seroprevalence is greatest (left), and the rate of increase (right), for adult bats, drawn from GAMM models of seasonality. Densities are of these quantities derived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

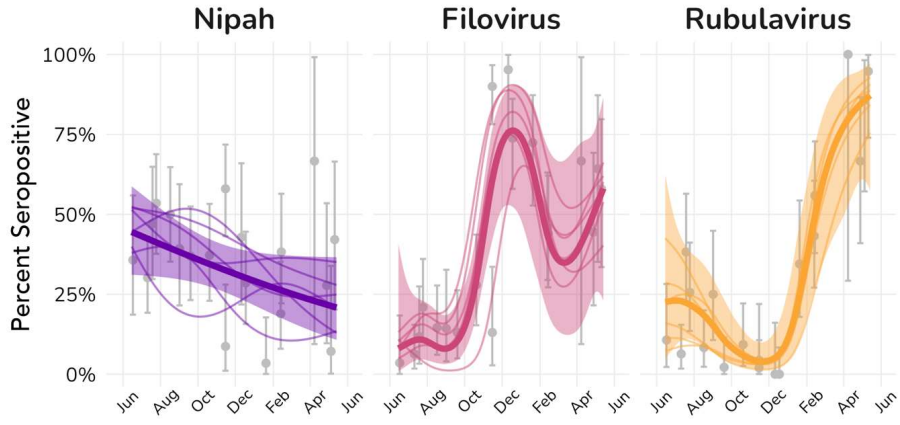
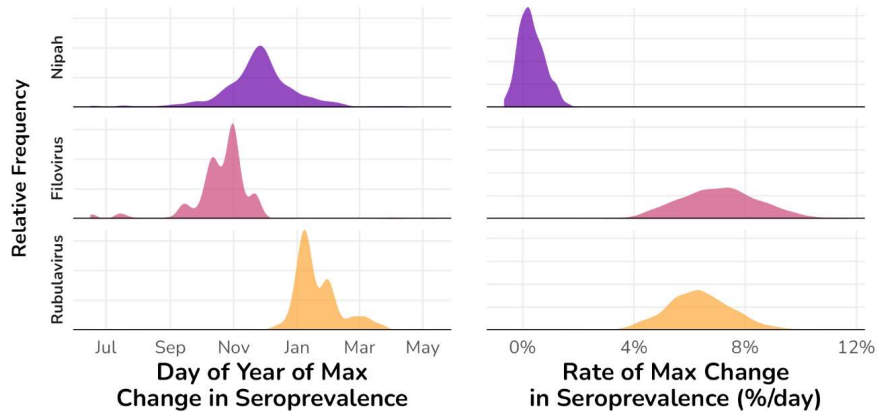


Figure 5. Serodynamics in juvenile bats in five-year study. Measured and modeled population seroprevalence in juvenile bats caught over their first year of life. Sampling events from multiple years are transposed onto a single year starting June (the earliest month free-flying young-of-the-year juveniles 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.



158

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

163 Filovirus seroprevalence exhibited different patterns. Among adults, seroprevalence against
164 filovirus was lower than for Nipah virus or Rubulavirus. As with Nipah virus, filovirus
165 seroprevalence decreased over the study period, from 64% (50%-76%) to 20% (12%-30%), and had
166 periods of distinct increases in spring/summer 2010 (concurrently with a circulation period for Nipah
167 virus) (Fig. 3). filovirus seroprevalence did not exhibit cyclic annual patterns in adults (Fig. 4).
168 Among juveniles within the average year, filovirus seroprevalence started very low in June at
169 8% (2%-40%), but increased to a peak of 44% (32%-58%) in juveniles approximately six months of age
170 before declining. (Fig 5). This pattern was consistent across years; though the peak varied in size, it
171 consistently occurred in December or January. The average date with the greatest rate of
172 seroprevalence increase was Oct 31 (Jul 14-Nov 25), and the maximum rate of increase was 7.1%/day
173 (4.5%/day-9.9%/day).
174 Rubulavirus seroprevalence had a distinct dynamic pattern. Among adults, rubulavirus
175 seroprevalence was high at 92% (82%-98%), and remained high through the end of the period at 74%
176 (62%-84%), though there were some temporary periods of decline. There were distinct periods of
177 increasing circulation in early 2010 and early 2012. Only for the rubulavirus did adult seroprevalence
178 exhibit patterns of seasonality. Periods of increasing seroprevalence in adults exhibited strong
179 seasonality of 1.6%/day (1.0%/day-2.4%/day) increase on peak days, with the mean peak date
180 occurring on Dec 15 (Oct 15-Feb 1)
181 In juveniles, seroprevalence against the rubulavirus in the average year was 22% (4%-62%) in
182 the youngest bats in June, though in individual years this could range from 7%-43%. In all years
183 juvenile seroprevalence declined to a low of 1.7% (0.6%, 8.3%) that occurred between Oct 9 and Dec
184 11, then increased starting in winter, reaching high values in yearling juveniles of 88% (56%-99%) The
185 peak rate of increase for juvenile rubulavirus seroprevalence was 6.4%/day (4.2%-8.8%) and occurred
186 Jan 9 (Dec 21-Mar 16).

187 *Spatial Comparisons*

188 Seroprevalence trends in both adult and juvenile bats within the one-year studies in Chakhoria
189 and Ramnagar broadly mirrored those in the five-year study in Faridpur (Supplementary Figure 2).
190 In adults, Nipah virus seroprevalence increased in both Ramnagar and Chakhoria over the course of
191 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.

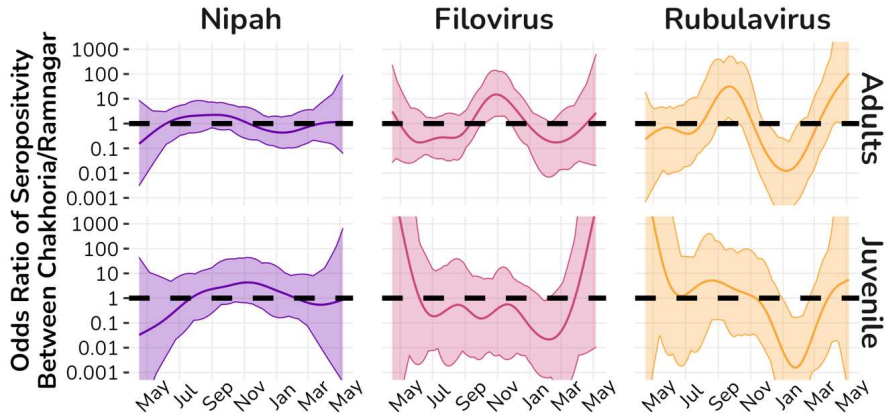


Figure 7. Comparison of splines for seroprevalence trends between locations in adults and juveniles over one-year study. Thick lines and shaded errors represent the estimated difference in trends and their associated 95% confidence intervals. The horizontal dashed line is set at zero. Where the confidence 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.

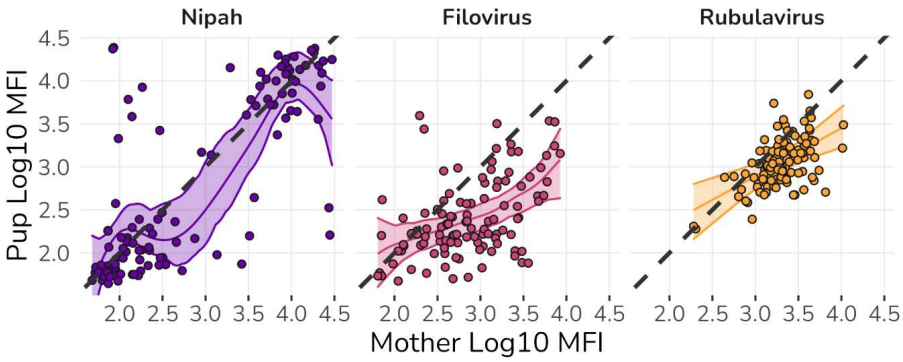


Figure 8. Maternal inheritance of antibodies. In each plot, points are measured log-MFI values for adults and juveniles in mother-pup pairs for each of the three antibody tests. Lines represent the predicted mean relationship between the two and their associated 95% confidence intervals.

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

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From:	Noam Ross on behalf of Noam Ross <ross@ecohealthalliance.org>
To:	Laing, Eric
Cc:	Jon Epstein
Subject:	Fwd: Draft manuscript: Co-circulation dynamics of viruses in a bat population
Date:	Wednesday, September 7, 2022 2:57:50 PM
Attachments:	Ross-et-al_bangladesh-bats-cocirculation-serology_2022-08-11.docx

Chris let us know that you should be on the attached paper as you developed some of the proteins used in the panel run in our previous Bangladesh study. Glad to have you on, as this way we'll have another joint reference for the grant.

We're doing final revisions on it now before sending to preprint before the end of the month. If you have any feedback, maybe we touch on it when we meet next week?

Best,
Noam

---------- Forwarded message ---------
From: **Jon Epstein** <epstein@ecohealthalliance.org>
Date: Fri, Aug 19, 2022 at 11:22 AM
Subject: Draft manuscript: Co-circulation dynamics of viruses in a bat population
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>

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

Vice President for Science and Outreach

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

SPECIFIC AIMS

We propose to develop robust analytical approaches for using quantitative serological data to better target novel disease discovery and surveillance in both human and wildlife populations. Multiplex and quantitative serological assays are increasingly used in disease surveillance to identify spillover, but are underutilized because they produce large datasets without a standardized analytical framework. The confounding effects of cross-reactivity, and a lack of true controls in wildlife species or for yet-uncharacterized viruses stymies use of rich quantitative data that they produce. Using PCR and other direct viral detection methods is costly because of the rarity of positive detection. **Our approach will allow more cost effective and statistically powerful serological assays to be used for identifying high risk populations for pathogen spillover**.

We will test and validate our methods using paired serological and viral detection data from extensive surveillance studies of wildlife and human populations collected and curated by our team. Our team has unique experience applying multiplex serological assays in regions of pathogen emergence in parallel with PCR-based viral detection, allowing us to use paired samples to validate our approach. This includes work on SARS-related bat coronavirus spillover in Southeast and East Asia, and filovirus and henipavirus exposure in Africa and Asia. This strategy leverages access to extensive, comparable, field data and also prior investments by NIH and other federal agencies (DTRA, USAID) in zoonotic disease emergence.

Our statistical approaches are applicable to a broad range of serological technologies that can quantify immune response including Luminex, epitope serochips, and quantitative LFIA and ELISA. **They will be published as open-source software packages to enable their rapid adoption and validation.**

Our specific aims are to:

1. **Develop an approach for identifying and characterize novel viruses from multiplex panels of quantitative serological assays.** We will develop detailed reaction/cross reaction profiles of a broad panel of Luminex serological tests and incorporate these into statistical models with explicit representations of sampling and measurement processes. With these, we will determine whether serological "profiles" are more likely to represent antibodies of known viruses, mixtures of multiple known antiviruses, or previously uncharacterized viruses. We extend techniques of antigenic mapping, 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
2. **Create a robust bioinformatics pipeline for multiplex detection platforms**. "Serochips" consist of hundreds of thousands of synthetic epitopes drawn from libraries of viral genetic sequences. While promising platforms for broad surveillance, they suffer from multiple testing dilemmas combined with problems cross-reaction and within-sequence correlation. We will build mechanistic, hierarchical Bayesian models to translate the large data generated by these platforms into usable measures of immune response. 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.
3. **Use spatio-temporal quantitative serological data to identify populations with distinct profiles indicative of novel pathogens.** We will develop spatio-temporal models that capture population-level immunological patterns from spatially extensive quantitative multiplex serological assays. Using ordination techniques drawn from community ecology, we will scale up the approach to individual-scale serological profiles to populations. We will work directly with an active NIAID-funded CREID research center in Southeast Asia (EID-SEARCH), along with a global consortium of research groups using similar datasets to develop our models. These analyses will allow us to characterize groups and regions with distinct exposure profiles, characterize spatial variation, and identify populations that have unique mixtures of immune assay responses that indicate exposures to novel pathogens or distinct combinations of pathogens – targets for intensive surveillance, viral detection and characterization.

From:	Noam Ross on behalf of Noam Ross <ross@ecohealthalliance.org>
To:	Laing, Eric
Cc:	Sterling, Spencer
Subject:	Fwd: NIH R01 on serology bioinformatics
Date:	Wednesday, August 24, 2022 4:58:43 PM
Attachments:	EcoHealth_SerologicalStats_conceptnote-Aug2022.pdf

---

Hi Eric,

Hi! I hope you are well. I had a call with Spencer yesterday about this grant as I know you've been away for family reasons. (CC'ing him to chat with you about this when he has the chance). We discussed things that we could write into the grant on your side, notably validation/calibration testing using the Filovirus standard coming out of Oxford, and also running tests on the sera from experimental infections that are at BSL-4 labs at RMN, UTMB, and maybe South Africa. These could be used to model how we expect real responses to look in related virus and host species.

Here are my follow-up items:

- I'd like to have a conversation with you about nuts and bolts and budgeting. I'm headed out for vacation Friday afternoon through Labor day. ***If you want to chat tomorrow or Friday pick a time*** at this link, if not I will write when I return: https://calendly.com/noamross/60-minute-chat-extended-hours

- ***I'll get you a draft*** with a bulleted section on this stuff that you and Spencer can make sound like a virologist who knows what they are talking about. I expect this ***around September 12.***

- I think it would be good for me to ***come down to USU for a day or two in early-mid September*** for us to work through some of this stuff if you agree and have the time. If so, let's schedule that.

Best,
Noam
--

Dr. Noam Ross
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On Mon, Aug 8, 2022 at 8:51 AM Noam Ross <ross@ecohealthalliance.org> wrote:

> Hi Eric,
>
> After many false starts, I really am planning to submit an R01 on bioinformatics and statistical methods for Luminex serology in the next cycle (due Oct 6). Everyone came back from the BatID conference really jazzed about this. I still think it works best with you and I as co-PIs. If you are still game, want to have a call about it? I'd still like to come down to USU for a day in the next month, too, if you'd like to spend a day working on it and other related issues.
>
> Schedule a call on my calendar here: https://calendly.com/noamross/60-min-chat
>
> Last draft of specific aims attached.
>
> Best,
>
> Noam

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

EID-SEARCH
Emerging Infectious Diseases
South East Asia Research Collaboration Hub

CREID
Centers for Research in Emerging Infectious Diseases

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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 (CoVs), paramyxoviruses (PMVs), filoviruses (FVs), 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 and Kevin…

- Developed and optimized multiplex serological assays including pan-sarbecovirus and Henipavirus multiplex surrogate virus neutralization test (sVNT); and transferred these technologies to labs in Thailand and Malaysia.
- Conducted extensive sampling of bat, rodent and non-human primate populations; discovered 8 putative novel CoVs and 8 novel PMVs – including full genome of a bat SARS2-related CoV.
- Began human community surveillance in Thailand at sites with high human-wildlife contact.
- Obtained preliminary evidence of filovirus and henipavirus zoonotic spillover in humans.
- Supported viral diarrhea outbreak investigation in Chanthaburi province, Thailand.
- Strengthened outbreak preparedness for Monkeypox virus with Ministry of Public Health and helped detect first cases in Thailand (July 2022).
- Conducted experimental research on Swine Acute Diarrhea Syndrome coronavirus (SADS-CoV) and pangolin coronavirus to identify essential factor for infection, potential for spillover, and efficacy of existing CoV therapeutics and vaccines.
- Published 9 peer-reviewed papers; 4 others in revision, and 3 more in prep.
- Drafted extensive Field Biosafety Manual (presented at the CREID Scientific Meeting), to be peer-reviewed, revised and shared broadly with CREID Network in Fall 2022.
- Strengthened cross-Research Center communication via information sharing for outbreak research response and sharing protocol and diagnostic assays.

Commented [KO1]: Agree w Eric, great if we could reference some pre-prints or in review titles of papers. Also prob could cut this section a little more (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 of research partners across almost all Southeast Asian countries. By enhancing and focusing research within the three hub countries, and coordinating communication among the network, EID-SEARCH is 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 has facilitated our 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 (in 2021) and Cambodia (in 2022) through the CREID Pilot Research Program*. Our geography covers 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. Our field sites in mainland Southeast Asia allows us to sample representative wildlife species that occur across the greater biogeographic region, including India, Bangladesh, Myanmar, Southwest China, Laos, Cambodia, Philippines, and Indonesia, and thus characterize zoonotic disease risk more broadly outside our study area.

Commented [LH2]: This is from the original proposal. Tom suggested removing this because we don't actually work in Sarawak.

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.** To date, we have collected 18,033 specimens from 2,660 animals of 31 bat species, one non-human primate (NHP) species, and five rodent species from 9 sites at high-risk human-wildlife interfaces in Malaysia and Thailand (Table 1).

Commented [KO3]: Should we put in a short update about Cecilia's paper published last week? Or was this fully covered last time? I guess the revisions proposed by reviewers and slightly new estimated numbers would be new? OR THE VIRAL CURVE stuff?

Commented [LH4R3]: We have highlighted the paper last year

EID-SEARCH
Emerging Infectious Diseases
South East Asia Research Collaboration Hub

CREID
Centers for Research in Emerging Infectious Diseases

Table 1. EID-SEARCH summary of sampling and virus surveillance in wildlife in Thailand and Malaysia.

	Thailand	Malaysia
No. of collected specimens[1]	3,862	14,171
No. of individuals	454 bats, 106 rodents, 100 NHPs	2,000 bats
No. of species	6 (bats), 5 (rodents) 1 (NHP)	24 (bats)
No. of sites	5	4
No. of tested individuals	506	1,332
No. of PCR tests for CoVs, PMVs, FLVs, influenza viruses	5,063	9,228
Detected divergent viral PCR sequences (RdRp)[2]	3 novel CoVs from 11 bats[3] (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)
No. of specimens for multiplex immunoassay for FLVs, CoV and Henipavirus	100 (macaques) 92 (rodents)	720 (bats)

**Abbreviations: CoV – Coronavirus; PMV – Paramyxovirus; FLV – Filovirus*

- **Discovery of novel bat SARS-related coronavirus in Thailand**. Whole genomes of novel sarbecoviruses from *Rhinolophus pusillus* 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 receptor binding domain (RBD) is most closely related to bat-CoV RaTG13. Experimental assays suggest that this new virus binds to human ACE2 receptors and is able to mediate cell entry. Whole-genome sequencing of additional novel 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 Swine Acute Diarrhea Syndrome coronavirus (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

[1] Collected specimens include oral swabs, rectal swabs, urogenital swabs, and whole blood, types of specimens may vary among individual animals.
[2] Further studies are needed to understand the characteristics and classification of these divergent viral sequences, as partial-gene phylogenetic trees suggest that these are potentially "novel viruses".
[3] Complete whole genome sequences were obtained from 3 samples and partial whole genome sequences obtained from (8 samples), further viral characterization studies are undergoing.

Commented [MOU5]: 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…

Commented [LH6R5]: Added footnote

Commented [SN9]: Based on complete whole genome sequences (3 samples) and partial whole genome sequences (8 samples)

Commented [LH10R9]: Added to the footnote

Commented [MOU11]: Same species? Details.

Commented [LH12R11]: Added no. of species, not sure if we need to detail the species, no much space

Commented [MOU7]: 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.

Commented [LH8R7]: Added footnote

Commented [KO13]: Agree w Eric, great if we could reference some pre-prints or in review titles of papers. Also prob could cut this section a little more (Peter).

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 6highlights 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 demonstrate the efficacy of FDA-approved therapeutic antibodies against a range of CoVs with emergence potential, allowing for the creation on-the-shelf therapeutics to target future emerging coronaviruses. As all FDA approved or emergency authorized vaccines express the prefusion stabilized spike this work further 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 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 human surveillance work in Thailand, with 56 participants enrolled at one community surveillance site with high levels of bat-human contact, and 2 individuals from a clinic site, with a total of 58 participants enrolled. Serology testing using multi-family MMIA showed evidence of previous infection by Asiatic FLVs, PMVs, and CoV (Fig. 1). Preliminary data suggest several interesting findings. First, a single human serum sample 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 due to exposure to a phylogenetically-related, likely novel, Asiatic ebolavirus (Figure 1A). 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 would imply. Second, Henipavirus serology showed one individual reactive with Hendra and Cedar virus, and several individuals with evidence of exposure to Mojang or Mojang-related viruses. Lastly, CoV serology was complicated by prior COVID-19 vaccination of participants. At the low testing titer, cross-reactions with phylogenetically related bat sarbecoviruses were expectedly observed (Figure 1C) – patterns we are currently exploring in more detail. 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 (A), HNVs (B), and CoVs (C) among community participants in (n=56).

Commented [MOU14]: ? 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?

Commented [GLE15R14]: The transmission studies showed occasional, but less, transmission to exposed hamsters than was seen with SARS2 infected animals. No preprint at this time.

Commented [MOU16]: List these?

Commented [GLE17R16]: B38, REGN10933, REGN10987, LY-COV016

Commented [MOU18]: 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?

Commented [GLE19R18]: All of the early SARS2 mAbs targeted RBD so those are the most broadly available for testing. We tested 3 SARS2 NTD binding antibodies and none neutralized pangolin. 13 of 15 RBD targeting antibodies had efficient neutralization of pangolin.

Commented [MOU20]: Is this an FDA EUA or authorized treatment?

Commented [GLE21R20]: In trials, published in Science as having pan-coronavirus efficacy

Commented [MOU22]: This is something not FDA EUA? Will stakeholders understand what S-2P etc means? This is what NovaVax is making.

Commented [GLE23R22]: Removing S2P to be less jargony makes sense here.

Commented [MOU24]: Replication, not infection or disease model? What did these mAb and vaccine prot… [1]

Commented [GLE25R24]: Mice show no weight loss after pangolin infection but have high virus titer at day… [2]

Commented [MOU26]: What does this mean, sera from humans with memory to seasonal beta-human … [3]

Commented [GLE27R26]: We are not sure what Ralph means here.

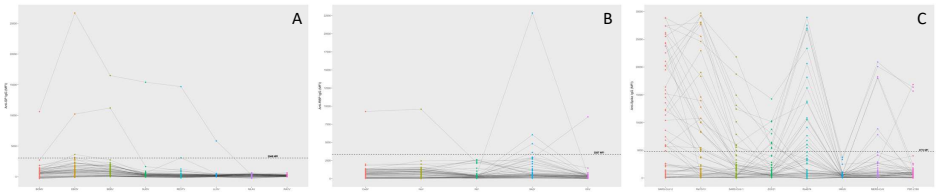
Commented [MOU28]: I didn't see any mention of FDA-approved antibody therapies or vaccine countermeasu… [4]

Commented [KO29]: Think it's okay to make a little longer than one sentence as this is interesting stuff. I … [5]

Commented [LH30]: Peter and Kevin - please shorten this if possible, we don't have space, and may not wan… [6]

Commented [MOU31]: Needs a proper legend.

Commented [KO32]: Unless we get a much simpler figure, I would suggest we ditch the figures and just de… [7]



- **Design and application of pan-sarbecovirus multiplex surrogate virus neutralization test (sVNT)**
  The assay covers SARS-CoV-2 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 and in identifying the progenitor of SARS-CoV-2 in animals.

  We have also optimization and established a pan-henipavirus multiplex sVNT at Chulalongkorn University to screen human and animal samples for neutralizing antibodies via a virus-free/cell-culture free, bead-based serology assay.

Outbreak research response 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, which appears to be the likely etiological agent.
- 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 PCR 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 papers have been published or posted as preprints from EID-SEARCH resesarch, four (4) 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,

Commented [SN33]: 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.

Commented [KO34R33]: I edited it to be part of the assay estabilishment and optimization, no results.

Commented [KO35]: Hongying, are the preprints the same ones that are in revision? Can we add refs anywhere and list all these, then just give reference numbers here?

Commented [SN36]: 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

Commented [LH37R36]: Counted!

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

EID-SEARCH is committed to in-country capacity building to strengthen outbreak response and research and develop next-generation EID researchers in Southeast Asia. Within our network, nine (9) training sessions were conducted by EID-SEARCH partners in Thailand, Malaysia, and Viet Nam for project members, local government partners, universities, research institutes, and NGOs within the reporting period, 89 participants were trained in lab biosafety (2 training 32 participants), research biosafety (1 training 3 participants in Malaysia), lab monitoring (1 training 3 participants), Bio-plex and multiplex immunoassay introduction (1 training, 2 participants), applying data sharing tools (1 training 10 participants), serology data analysis (1 training, 14 participants), field biosafety and animal sampling SOPs (2 training sessions with 25 participants, virtual and in-person), to strengthen in-country research capacity and improve information sharing. Regular virtual meetings were held for results analysis, hands-on lab and field training were conducted in Thailand and Viet Nam, all 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.

Commented [LH38]: These training were organized by Conservation Medicine. From the feedbacks on annual report, Tom was very upset that we lumped all these as overall EID-SEARCH efforts, just FYI, if you want to edit

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 research responses, capacity building, and cross-center collaboration by sharing expertise, leveraging existing local partnerships, and sharing protocols and relevant resources.

- In outbreak response and research to Monkeypox, EID-SEARCH partner Uniformed Services University (Dr. Eric Laing) works with PICREID to co-lead the efforts in sharing protocols or approaches and planning for the development and optimization of a multiplex, species independent, Luminex-bead based serology assay for MPXV, collaborating with DMID. Meanwhile, 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 (Dr. Eric Laing) is working with WAC-EID PIs, Dr. Robert Cross and Dr. Scott Weaver, to establish an 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 have been communicating with multiple Research Centers regarding the Field Biosafety Manual presented at the CREID Scientific Meeting by EID-SEARCH senior field veterinarian (Dr. Marc Valitutto). The Manual and corresponding training resources will be made available to all CREID members once the peer review process is completed in Fall 2022.
- EID-SEARCH has provided scientific expertise on CCHF, Henipavirus, RVF related research by connecting CREID members with our research partners via the Outbreak Research Response working group or other *ad hoc* requests.

Commented [KO39]: Correct date?

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 continue to follow the research plan laid out in our original proposal which aligns with the CREID network overall research priorities, as follows:

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 plans to increase sampling of rodents and non-human primates and potential longitudinal sampling at 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. Refine models to analyze cost-efficacy of viral discovery.
- Conduct viral family-level molecular screening of collected wildlife samples for coronaviruses, filoviruses, and paramyxoviruses.
- Perform serology testing on the wildlife samples for coronaviruses, filoviruses, and henipaviruses.

- 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 in-country whole-genome and spike glycoprotein sequencing for 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 Y1-3.

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. 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. Test pre-COVID-19 archived human sera using sarbecovirus multiplex serology panel.
- Conduct 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.

Commented [KO40]: We didn't mention the CDC collaboration in Thailand, but probably as the details are not yet worked out – so maybe best.

Commented [SN41]: Would it be possible to propose training on RBD and/or spike glycoprotein design and coupling?

- 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 workshop 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 as requested.

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

Page 6: [1] Commented [MOU24] **Microsoft Office User** **8/2/2022 8:39:00 AM**

Replication, not infection or disease model? What did these mAb and vaccine protect from, infection?

Page 6: [2] Commented [GLE25R24] **Gralinski, Lisa E** **8/16/2022 2:11:00 PM**

Mice show no weight loss after pangolin infection but have high virus titer at days 2 and 4 post-infection so mice are a good virus replication model. We can block virus replication in mice with ADG2 antibody treatment or vaccination for SARS2.

Page 6: [3] Commented [MOU26] **Microsoft Office User** **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?

Page 6: [4] Commented [MOU28] **Microsoft Office User** **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.

Page 6: [5] Commented [KO29] **Kevin Olival** **8/15/2022 8:29:00 PM**

Think it's okay to make a little longer than one sentence as this is interesting stuff. I shortened a bit tho.

Page 6: [6] Commented [LH30] **Li, Hongying** **8/15/2022 12:02:00 PM**

Peter and Kevin - please shorten this if possible, we don't have space, and may not want to include these details.

Page 6: [7] Commented [KO32] **Kevin Olival** **8/15/2022 8:36:00 PM**

Unless we get a much simpler figure, I would suggest we ditch the figures and just describe the results as we do. OR, maybe we just include the FV and Henipa figures and not the CoVs as it's complicated.

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

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 (CoVs), paramyxoviruses (PMVs), filoviruses (FVs), 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:

- Developed and optimized multiplex serological assays including pan-sarbecovirus and Henipavirus multiplex surrogate virus neutralization test (sVNT); transferred these technologies to labs in Thailand and Malaysia; shared protocols with other CREID Network centers.
- Conducted extensive sampling of bat, rodent and non-human primate populations; discovered 8 putative novel CoVs and 8 novel paramyxoviruses
- Sequenced full genome of a SARS-CoV-2-related CoV from bat samples in Thailand associated with bat guano collectors. New CoV has higher RBD sequence identity to SARS-CoV-2 than BANAL group, is most closely related to RaTG13 (RBD), but binds to human ACE2.
- Began human community surveillance in Thailand at sites with high human-wildlife contact.
- Obtained serological evidence of filovirus and paramyxovirus (Mojiang-related) spillover in humans.
- Supported viral diarrhea outbreak investigation in Chanthaburi province, Thailand.
- Strengthened outbreak preparedness for Monkeypox virus with Ministry of Public Health and detected first cases in Thailand (July 2022).
- Tested whether Swine Acute Diarrhea Syndrome coronavirus (SADS-CoV) and pangolin CoV can infect human cells, and efficacy of existing CoV therapeutics and vaccines against them.
- Published 9 peer-reviewed papers; 4 others in revision, and 3 more in prep.
- Drafted first ever comprehensive Field Biosafety Manual. Currently being peer-reviewed, revised and will be shared broadly with CREID Network in Fall 2022, then publicly.
- Strengthened cross-Research Center communication via information sharing for outbreak research response and sharing protocol and diagnostic assays.

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:

1. Identify, characterize, and rank spillover risk of high zoonotic potential viruses from wildlife.
2. Identify evidence and analyze risk factors for viral spillover in high-risk communities using novel serological assays.
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 of research partners across almost all Southeast Asian countries. By enhancing and focusing research within the three hub countries, and coordinating communication among the network, EID-SEARCH acts 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 has facilitated our 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 include Viet Nam (in 2021) and Cambodia (in 2022) through the CREID Pilot Research Program*. Our geography covers one of the most significant foci of EID risk globally. We have also assembled an informal 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. These provide updates on outbreaks and research in their countries. Our work includes sampling wildlife and people in Thailand, Peninsular Malaysia, Sabah, Viet Nam, and Cambodia. Our field sites in mainland Southeast Asia allows us to sample representative wildlife species that occur across the greater biogeographic region, including India, Bangladesh, Myanmar, Southwest China, Laos, Cambodia, Philippines, and Indonesia, and thus characterize zoonotic disease risk more broadly outside our study area.

Section 3: Research Center Progress and Accomplishments

A. Projects and Studies

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.** To date, we have collected 18,033 specimens from 2,660 animals of 31 bat species, one non-human primate (NHP) species, and five rodent species from 9 sites at high-risk human-wildlife interfaces (Table 1).

Table 1. EID-SEARCH summary of sampling and virus surveillance in wildlife in Thailand and Malaysia.

	Thailand	**Malaysia**
# samples[1]	3,862	14,171
# individuals	454 bats, 106 rodents, 100 NHPs	2,000 bats
# species	6 (bats), 5 (rodents) 1 (NHP)	24 (bats)
# sites	5	4
# individuals tested	506	1,332
# PCR tests for CoVs, PMVs, FLVs, influenza viruses	5,063	9,228
Divergent viral PCR sequences (RdRp) detected[2]	3 novel CoVs from 11 bats[3] (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 multiplex immunoassay for FLVs, CoV, Henipaviruses	100 (macaques) 92 (rodents)	720 (bats)

[1] Specimens include oral swabs, rectal swabs, urogenital swabs, and whole blood, types of specimens may vary among individual animals.
[2] Further studies are underway to classify and characterize these divergent viral sequences. Partial-gene phylogenetic trees suggest that these are potentially "novel viruses".
[3] Complete whole genome sequences were obtained from 3 samples and partial whole genome sequences obtained from (8 samples), further viral characterization studies underway.

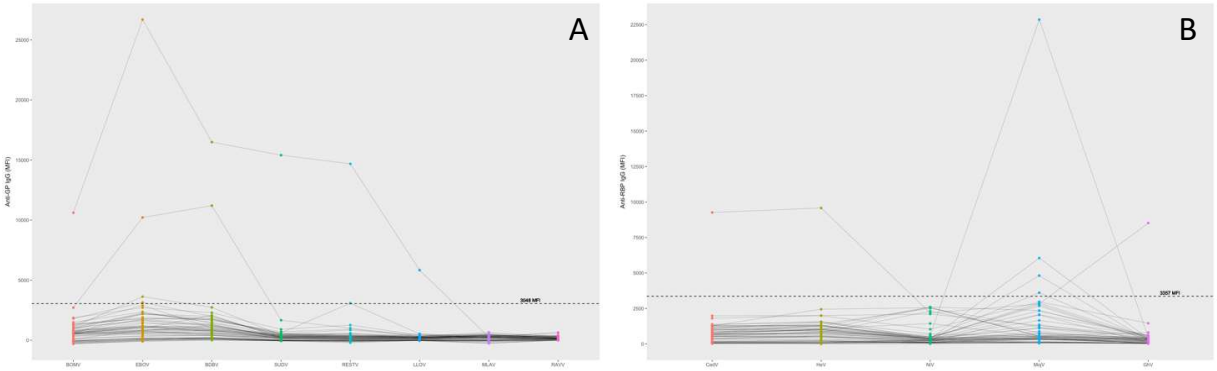
- **Discovery of novel bat SARS-related coronavirus in Thailand**. Whole genomes of novel sarbecoviruses from *Rhinolophus pusillus* revealed a 91.72% nucleotide similarity to SARS-CoV-2. Phylogenetic analysis suggested that this virus is closer to pangolin CoV-GD-1 than to SARS-CoV-2; however, the receptor binding domain (RBD) is most closely related to bat-CoV RaTG13. Experimental assays suggest that this new virus binds to human ACE2 receptors and is able to mediate cell entry. Whole-genome sequencing of additional novel CoVs from Thailand and Malaysia is ongoing, plans to look for furin cleavage sites using NGS are underway.
- **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 Swine Acute Diarrhea Syndrome coronavirus (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 Guangdong 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 highlights 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. A pan-sarbecovirus antibody, ADG-2, and a SARS-CoV-2 prefusion stabilized spike protein recombinant protein vaccine protected mice in a PgCoV-GD replication model. 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 demonstrate the efficacy of FDA-approved therapeutic antibodies against a range of CoVs with emergence potential, allowing for the creation on-the-shelf therapeutics to target future emerging coronaviruses. As all FDA approved or emergency authorized vaccines express the prefusion stabilized spike this work further 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 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 human surveillance work in Thailand, with 56 participants enrolled at one community surveillance site with high levels of bat-human contact, and 2 individuals from a clinic site, with a total of 58 participants enrolled. Serology testing using multi-family MMIA showed evidence of previous infection by Asiatic FLVs, PMVs, and CoV (Fig. 1). Preliminary data suggest several interesting findings: **First,** a single human serum sample 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 due to exposure to a phylogenetically-related, likely novel, Asiatic ebolavirus. 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 would imply. **Second,** Henipavirus serology showed one individual reactive with Hendra and Cedar virus, and several individuals with evidence of exposure to Mòjiāng virus or Mòjiāng virus-related viruses. **Third,** CoV serology was complicated by prior COVID-19 vaccination of participants. At the low testing titer, cross-reactions with phylogenetically related bat sarbecoviruses were expectedly observed – patterns we are currently exploring in more detail. 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 Mulitiplex serology testing results for filoviruses (A), paramyxoviruses (B) among high-wildlife contact community participants (n=56). Note the presence of antibodies against an unidentified filovirus in one participant, and a cluster of people seropositive for Mòjiāng-related virus.



- **Design and application of pan-sarbecovirus multiplex surrogate virus neutralization test (sVNT)** The assay covers SARS-CoV-2 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 in people and progenitors of SARS-CoV-2 in animals.
  We also have optimization and established a pan-henipavirus multiplex sVNT at Chulalongkorn University to screen human and animal samples for neutralizing antibodies via a virus-free/cell-culture free, bead-based serology assay.

Outbreak research response 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, which appears to be the likely etiological agent.

- 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 PCR testing platform in April 2022 and detected the first Monkeypox case in Thailand in July 2022.

B. Dissemination of Results

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 papers have been published or posted as preprints from EID-SEARCH research, four (4) manuscripts are currently under revision, and three (3) manuscripts are in preparation for submission, including a risk assessment of HKU-2 viruses in the region, details of a novel SARS-CoV-2-like virus in Thailand, and serological results from high wildlife-exposure cohorts. 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

EID-SEARCH is committed to in-country capacity building to strengthen outbreak response and research and develop next-generation EID researchers in Southeast Asia. Within our network, nine (9) training sessions were conducted by EID-SEARCH partners in Thailand, Malaysia, and Viet Nam for project members, local government partners, universities, research institutes, and NGOs within the reporting period, 89 participants were trained in lab biosafety (2 training 32 participants), research biosafety (1 training 3 participants in Malaysia), lab monitoring (1 training 3 participants), Bio-plex and multiplex immunoassay introduction (1 training, 2 participants), applying data sharing tools (1 training 10 participants), serology data analysis (1 training, 14 participants), field biosafety and animal sampling SOPs (2 training sessions with 25 participants, virtual and in-person), to strengthen in-country research capacity and improve information sharing. Regular virtual meetings were held for results analysis, and hands-on lab and field training was conducted in Thailand and Viet Nam, all 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.

D. Challenges and Proposed Solutions

The only challenges we encountered during the reporting period were travel restrictions in our field partner countries, as well as a large number of hospitalized patients in local hospitals in Thailand and Malaysia due to COVID-19. The patient influx 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

Through the participation of different CREID Working Groups, EID-SEARCH members participated in the network's outbreak research responses, capacity building, and cross-center collaboration by sharing expertise, leveraging existing local partnerships, and sharing protocols and relevant resources.

- In outbreak response and research to Monkeypox, EID-SEARCH partner Uniformed Services University (Dr. Eric Laing) works with PICREID to co-lead the efforts in sharing protocols or approaches and planning for the development and optimization of a multiplex, species independent, Luminex-bead based serology assay for MPXV, collaborating with DMID. Meanwhile, 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 (Dr. Eric Laing) is working with WAC-EID PIs, Dr. Robert Cross and Dr. Scott Weaver, to establish an 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 have been communicating with multiple Research Centers regarding the Field Biosafety Manual presented at the CREID Scientific Meeting by EID-SEARCH senior field veterinarian (Dr. Marc Valitutto). The Manual and corresponding training resources will be made available to all CREID members once the peer review process is completed in Fall 2022.
- EID-SEARCH has provided scientific expertise on CCHF, Henipavirus, RVF related research by connecting CREID members with our research partners via the Outbreak Research Response working group or other *ad hoc* requests.

Section 4: Future Directions

In the next reporting period, we will continue to follow the research plan laid out in our original proposal which aligns with the CREID network's overall research priorities, engage multiple disciplines of social science, anthropology, health policy, and behavioral science toward EID risk mitigation strategies on key human-animal interfaces, and share relevant research methods (e.g., behavioral questionnaires) and best practices with the CREID Network members:

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 plans to increase sampling of rodents and non-human primates and potential longitudinal sampling at 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. Refine models to analyze cost-efficacy of viral discovery.
- Conduct viral family-level molecular screening of collected wildlife samples for coronaviruses, filoviruses, and paramyxoviruses.
- Perform serology testing on the wildlife samples for coronaviruses, filoviruses, and henipaviruses.
- 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 in-country whole-genome and spike glycoprotein sequencing for 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 Y1-3.

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. 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. Test pre-COVID-19 archived human sera using sarbecovirus multiplex serology panel.
- Conduct epidemiological analyses of biological and behavioral data
- Work with US CDC and Thailand MoH to test human samples for range of antibodies to high-risk viral agents

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.

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 workshop 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 as requested.

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

EID-SEARCH
Emerging Infectious Diseases
South East Asia Research Collaboration Hub

CREID
Centers for Research in Emerging Infectious Diseases

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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	5 new CoVs from 112 bats
	3 known PMVs from 4 rodents 4 new PMVs from 11 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

Commented [MOU1]: 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…

Commented [MOU2]: 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.

Commented [MOU3]: Same species? Details.

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Commented [MOU5]: The fusion data Chee Wah presented indicated that it does bind to and mediate cellular entry/fusion via the human ACE-2 orthologue.

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, aA pan-sSarbecovirus 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, ~~however, efficient PgCoV replication in primary nasal airway epithelial cells and transmission in the hamster highlights the high emergence potential of this virus.~~ wWe 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 ~~Designed and secured the expression of~~ bat-CoV spike proteins into the multi-family~~for incorporation into our~~ multiplex-based immunoassay (MMIA) ~~(multi-family) serological assay~~**. Specific bat~~t borne~~ -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~~to include priority coronaviruses such as -SARS-CoV-2 and bat SARSr-CoVs/bat MERSr-CoV ~~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.

- **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,~~ henipaviruses (HNVs) (family *Paramyxoviridae*)~~Henipavirus~~, filoviruses

Commented [MOU6]: ? 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?

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Commented [MOU7]: List these?

Commented [MOU8]: 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?

Commented [MOU9]: Is this an FDA EUA or authorized treatment?

Commented [MOU10]: This is something not FDA EUA? Will stakeholders understand what S-2P etc means? This is what NovaVax is making.

Commented [MOU11]: Replication, not infection or disease model? What did these mAb and vaccine protect from, infection?

Commented [MOU12]: 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?

Commented [MOU13]: 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.

Commented [MOU14]: What does this mean?

(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 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 eptiopes 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 recptor for cellular entry, and it quite distinct from the sarbecoviruses.

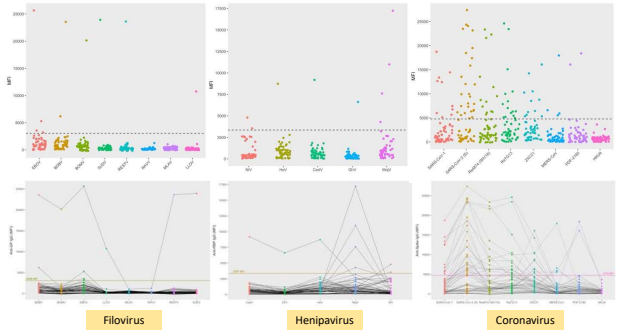

Fig. 1 Serology testing results for FLVs, HNVs~~Henipavirus~~, and CoVs among community participants in Thailand (n=56).

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- **Design and application of pan-sarbecovirus 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 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.

- **Optimization and establishment of pan-henipavirus multiplex sVNT at Chulalongkorn University.** 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 assa.

- ~~**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-~~

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~~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.

Commented [MOU17]: If not including the PMID, I'd at least add the *Surrname, First Initial, et al. J. Infect Dis. 2022.*

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 UWARE~~E~~N 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-~~to share the multiplex microsphere immunoassay (MMIA) with the regional partners for~~ EBOV serology testing. Enquiries ~~,~~has been made with ~~and plan to work~~Dr. Mark Page and ~~with~~ the National Institute for Biological Standards and Control (NIBSC) regarding additional MARV and NiV serology standards. ~~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

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)

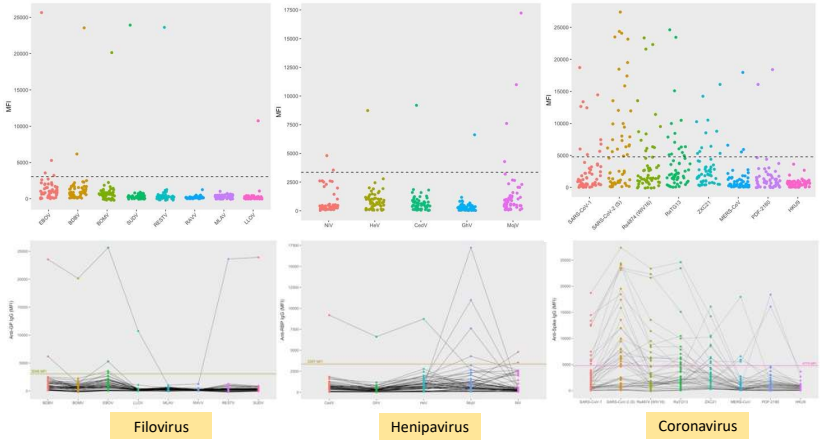

Fig. 1 Serology testing results for FLVs, Henipavirus, and CoVs among community participants in Thailand (n=56)

- **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: Hongying Li on behalf of Hongying Li <li@ecohealthalliance.org>
To: Laing, Eric
Subject: Re: For you to edit and review by August 15 Monday_CREID EAC report
Date: Wednesday, August 17, 2022 2:11:42 PM
Attachments: 2022 EAC Report_EID-SEARCH_draft v03.docx
2022 EAC Report EID-SEARCH FINAL_formatted.docx

Hi Eric,

Just sharing an updated version of draft v03 where Lisa and Cait from UNC addressed some of your comments on "Host range, transmissibility, and antigenicity of a pangolin coronavirus", in case you want to know (the paper is under revision, and no preprint). Also attached the final version from Peter for your information.

Cheers,
Hongying

On Mon, Aug 8, 2022 at 2:37 PM Laing, Eric <eric.laing@usuhs.edu> wrote:

> Hey Hongying,
>
> One edit. The cross RC collaboration is with Bob Cross and Scott Weaver, WAC-EID. I incorrectly wrote "UWARN."
>
> 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 Mon, Aug 8, 2022 at 2:21 PM 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

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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
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>
> 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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SPECIFIC AIMS

We propose to develop robust analytical approaches for using quantitative serological data to better target novel disease discovery and surveillance in both human and wildlife populations. Multiplex and quantitative serological assays are increasingly used in disease surveillance to identify spillover, but are underutilized because they produce large datasets without a standardized analytical framework. The confounding effects of cross-reactivity, and a lack of true controls in wildlife species or for yet-uncharacterized viruses stymies use of rich quantitative data that they produce. Using PCR and other direct viral detection methods is costly because of the rarity of positive detection. **Our approach will allow more cost effective and statistically powerful serological assays to be used for identifying high risk populations for pathogen spillover**.

We will test and validate our methods using paired serological and viral detection data from extensive surveillance studies of wildlife and human populations collected and curated by our team. Our team has unique experience applying multiplex serological assays in regions of pathogen emergence in parallel with PCR-based viral detection, allowing us to use paired samples to validate our approach. This includes work on SARS-related bat coronavirus spillover in Southeast and East Asia, and filovirus and henipavirus exposure in Africa and Asia. This strategy leverages access to extensive, comparable, field data and also prior investments by NIH and other federal agencies (DTRA, USAID) in zoonotic disease emergence.

Our statistical approaches are applicable to a broad range of serological technologies that can quantify immune response including Luminex, epitope serochips, and quantitative LFIA and ELISA. **They will be published as open-source software packages to enable their rapid adoption and validation.**

Our specific aims are to:

1. **Develop an approach for identifying and characterize novel viruses from multiplex panels of quantitative serological assays.** We will develop detailed reaction/cross reaction profiles of a broad panel of Luminex serological tests and incorporate these into statistical models with explicit representations of sampling and measurement processes. With these, we will determine whether serological "profiles" are more likely to represent antibodies of known viruses, mixtures of multiple known antiviruses, or previously uncharacterized viruses. We extend techniques of antigenic mapping, 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
2. **Create a robust bioinformatics pipeline for multiplex detection platforms**. "Serochips" consist of hundreds of thousands of synthetic epitopes drawn from libraries of viral genetic sequences. While promising platforms for broad surveillance, they suffer from multiple testing dilemmas combined with problems cross-reaction and within-sequence correlation. We will build mechanistic, hierarchical Bayesian models to translate the large data generated by these platforms into usable measures of immune response. 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.
3. **Use spatio-temporal quantitative serological data to identify populations with distinct profiles indicative of novel pathogens.** We will develop spatio-temporal models that capture population-level immunological patterns from spatially extensive quantitative multiplex serological assays. Using ordination techniques drawn from community ecology, we will scale up the approach to individual-scale serological profiles to populations. We will work directly with an active NIAID-funded CREID research center in Southeast Asia (EID-SEARCH), along with a global consortium of research groups using similar datasets to develop our models. These analyses will allow us to characterize groups and regions with distinct exposure profiles, characterize spatial variation, and identify populations that have unique mixtures of immune assay responses that indicate exposures to novel pathogens or distinct combinations of pathogens – targets for intensive surveillance, viral detection and characterization.

From: Noam Ross on behalf of Noam Ross <ross@ecohealthalliance.org>
To: Laing, Eric
Subject: NIH R01 on serology bioinformatics
Date: Monday, August 8, 2022 8:52:38 AM
Attachments: EcoHealth_SerologicalStats_conceptnote-Aug2022.pdf

---

Hi Eric,

After many false starts, I really am planning to submit an R01 on bioinformatics and statistical methods for Luminex serology in the next cycle (due Oct 6). Everyone came back from the BatID conference really jazzed about this. I still think it works best with you and I as co-PIs. If you are still game, want to have a call about it? I'd still like to come down to USU for a day in the next month, too, if you'd like to spend a day working on it and other related issues.

Schedule a call on my calendar here: https://calendly.com/noamross/60-min-chat

Last draft of specific aims attached.

Best,

Noam

Mitra, Marcia, Admela (CS) – NSF
Peter, Ehsan, KJO
17 June 2022

Introductions.

Mitra: They needed to consider things other than review scores, had different panels from 4 disciplines. Had to balance across panels and optics (or topics?). Geographic diversity, and various other factors.

Panel summary. Scoring can be high, but in some disciplines like CS, scores are usually low.

Will be funding around 25. They haven't announced them all yet. Can't tell statistics at this point (including how many proposals they received), probably won't be published.
Score not only factor.

NSF had 125 internal meetings to develop this project.

Not stopping here, going on to center stage. We can still apply for center stage. Can think about our team as already well formed, maybe we're beyond the planning stage. Can't say how many proposals they got – but had several panels.

Prob not another round of planning grants. Full center RFP expected in 2023. Guessing it will be 5-10 center grants max.

PD: Political issues influence decision at all?
Marcia – no mention of that anywhere in the panel.

KJO was concerned that negative panel summary misread our proposal – e.g. reviewers pick up on China "samples" that we never said existited when we referenced the Sanchez et al. *modeling* study. They noted how are we going to get data was a concern.

Ehsan – EAGR grant to keep momentum up?
Mitra – PIPP is a new program, not like old program where they have extra money around. Maybe can break it down in to smaller pieces and approach other program directors, to submit as separate proposal.

Admela: If you find some aspects of our program that we want to deepen, to submit to keep momentum going. Would be happy to help us navigate that. Many programs in CS, engineering, etc. Try to reshape some to make it more.

EAGER grants are dependent on program director, and you can't just submit for a previously unfunded proposal. A full proposal would be another 1-year timeline. NSF is memory-less,

needs to go from scratch every time. Can't submit EAGER from something that has been turned down.

Mitra does DNA computing, etc. it's a little different than our proposal, so she may not be best person in CS to talk to for EAGER, etc. Approach someone in RIS? Division.

KJO mentioned how the panel summary was based on a mis-read of several aspects of our proposal. Recourse to complain? – there is a formal process but doesn't look at the reviews, just process, i.e. if process was biased.

Mitra: CS perspective that image recognition part wasn't new.
PD: need to be clearer in our language about everything the reviewers pointed out.

EHA being a sub on BU proposal did "not at all" affected our proposal. Did not come in to discussion. Problem would have only been if we proposed to do the same work, which we did not.

Admela: For center scale, need to really strengthen intellectual merit. "Upscaling" (i.e. spillover surveillance) is not an intellectual endeavor, so need to strengthen IM more and discuss in terms of difficulties of scaling up. Make it more intellectually appealing.
For CS – look at data, code, etc as a sharable good. Also highlight what is there for the rest of the community, what will be open and who will use the data.
If we're already part of a team, look for synergistic work – i.e. maybe we team up more w BU.

Will be fewer but bigger Center grants – so building synergistic activities. Could build new activities around that.

Debrief (PD, EH, KO):

Need to keep momentum
Think of specific focus and work on it. Use facebook data and keep in touch w them.
REU – only $16k if you have an existing grant, so this may not work for support
PD: "Future of epidemiology needs to have computer science in it." So let's work to get ahead of this for our work.
Clue as to why we didn't get it: Geography. They seem to have tried to balance all the top projects in terms of geography or disciplinary focus.
Some real weaknesses mentioned: image recognition wasn't novel enough, and challenge of finding rare events.
BU proposal – Peter will follow up, but probably won't be able to do more work under their proposal. Will need to see if it makes sense to team up on Full Center. Need to review what they proposed to do and revisit.
Ehsan – mystery how 4 reviewers read panel summary and signed off on something that was incorrect.
KJO to reach out to Katarina Ditmar to see if there's anything fundable on the BIO side.

NSF PIPP Panel Summary and reviews
3 June 2022

"PIPP Phase I: Multidisciplinary advances to scale up zoonotic spillover intelligence". PI Olival
Tracking number: **2201117**

Panel Summary

Panel Summary

1. PROPOSAL OVERVIEW

The grand challenge of this proposal is to capture spillover data quickly enough for it to be actionable. It aims to capture data at a sufficiently wide geographic & temporal scale to measure spillover.
The researchers have an existing model based on the flying fox bat – pig interaction, which can serve as a model for other spillovers. They will develop advances in rapid serological testing with lateral flow assays (LFAs) deployed to the field, to build a zoonotic virus surveillance early warning system. The team was deeply involved in SARS-related viruses, well before COVID-19.
The surveillance data will be merged with social media survey data, human mobility patterns and visual data to map potential spillover sites for further investigation.

2. REVIEW CRITERION I: INTELLECTUAL MERIT

Strengths:

A major intellectual strength is that they have distilled the fundamental problems of pandemic, spillover.
It is a strength that they have already developed a working collaboration and data network with colleagues in the field. In some sense, they are the tip of the spear, working in the field to detect spill over. They have been in field for quite some time, developing expertise.

Expanding their expertise to social media delivered surveys is much faster and broader than in-person surveys. Not particularly innovative, but fast and flexible. This project will use innovative combinations of big data, merging social media survey data, visual data and geographic base layers. Expanding their expertise to social media provides a means to gather data much faster and broader than in person surveys. While not particularly innovative, this approach is fast and flexible.

A major component element of actionable data is the proposed mapping of human/animal interaction. Spatial analysis and mapping will enable targeting and interventions in potential spillover locations before the virus becomes more widespread.

Another major strength is the development of in-field lateral flow assays (LFAs) tests to advance rapid serological testing. They plan to pilot this for Nipah virus antibody detection. This could be a major engineering and biological advancement, in that LFAs have not yet been used in the field for zoonotic virus surveillance. This field based LFA could serve as a critical tool in a virus early warning system.

The focus on viral chatter or sporadic cases in early phases, which leads to pandemics, is especially forward thinking.

Weaknesses:

The proposal makes mention of 50,000 seropositive human cases that have already collected. The proposal could have elaborated on this early finding, citing the MedArchive 2021 preprint.

There would appear to be a weakness in the origin-destination and how is this used to measure exposure. This is not a new methodology. The researchers plan to use Facebook's Space Maps and Co-location Maps. First, question is the issue of ready access to data in China. The second question is, what is the temporal and spatial scale of these tools?

How will interactions be visualized and captured when much exposure is at night. How will they capture that interaction? That was not sufficiently explained in the text.

3. REVIEW CRITERION II: BROADER IMPACTS

Strengths:
This is a strong team, integrated across the disciplines. This is the potential for novel applications such as the merging of computer science, AI, image data and mapping to generate spillover zones of interest.
Another major strength is that they are growing an on-the-ground training and experience network. Individual tasks are embedded with education and training activities. The resulting technology will have benefits far beyond individual viruses or geographic locations.

Weaknesses:
The review team was unsure if there would be barriers to using data from Facebook's Data for Good platform, given the existence of the Chinese Great Firewall. The proposal did not go into sufficient detail on whether or not this is an issue that could affect access to survey results. The same for the Internet-based image data.

4. SOLICITATION-SPECIFIC CRITERIA

Potential for interdisciplinary innovation:
The team has already conducted interdisciplinary work, and that work has advanced science in several innovative ways. The potential for location specific spillover targeting, informed by field testing, visual data, social media surveys and the transformation of those data, is a novel advancement of the science.

Qualification of investigators and team synergy:
The team has already demonstrated their expertise, as well as solid field operations, as a product of their fieldwork. It is a strength that they are already working with Southeast Asian colleagues who provide cultural and scientific expertise necessary for successful fieldwork.

Approach to center scale vision and plan:
This is an excellent "planning proposal" which aims to develop a center for spillover intelligence.
It engages a wide range of individuals and institutions.
The proposal clearly lays out the vision for scaling up from a planning and research activity project to a functional Center.

Project management quality:

Their plan calls for an Advisory Board, a Steering Committee and the three focus area research groups will be led by a multidisciplinary team of Co-PIs. Notably, they have designated a Project Manager to administer operations.

5. SUMMARY STATEMENT AND RECOMMENDATION

This is a well-developed proposal that builds on excellent research and results to date. They have already accomplished the formation of a team of collaborators and piloted a data collection system. They are on the cusp of making major advance in basic science (computer and information science, engineering, biological sciences and social and behavioral science), pandemic intelligence, collaboration and education. The rapid serological testing, supported by computer science expertise, merged with a unique combination of social media, will be a major advance for monitoring and alerts. The end product of this planning grant would be the formation of a transdisciplinary center that is devoted to early warnings of zoonotic spillovers that threaten mankind.

The panel recommendation is: Highly Competitive [HC]

This summary was read by the assigned panelists, and they concurred that the summary accurately reflects the panel discussion.
PANEL RECOMMENDATION: Highly Competitive

Proposal Review 1 : 2201117

Agency Name:

National Science Foundation

Agency Tracking Number:

2201117

Organization:

NSF Program:

PIPP-Pandemic Prevention

PI/PD:

Olival, Kevin

Application Title:

PIPP Phase I: Multidisciplinary advances to scale up zoonotic spillover intelligence

Rating:

Good

Review

Summary
In the context of the five review elements, please
evaluate the strengths and weaknesses of the proposal with respect to intellectual merit.

1. Synopsis
This application from Dr. Kevin Olival and colleagues at EcoHealth Alliance, University of Rochester, and Georgetown University aims to develop a Center for Spillover Intelligence (CSI), an intellectual and operational construct engaging a range of individuals and institutions that will endeavor to develop systems of surveillance and prediction that capture at a sufficiently wide geographic and temporal scales when and where viral spillovers from wildlife or domesticated animals will occur to humans. The concept focuses on the problem of pre-viral emergence and viral chatter, that "gray zone" of spillover transmission when human cases of infection occur sporadically but prior to the generation of human to human transmission and expansion of the spillover phase to outbreak or pandemic conditions. The Grand Challenge statement is a distillation of public health and scientific concerns brought into sharp focus by the COVID-19 pandemic episode, namely, how can actionable data (about the risk of zoonotic spillover and spread at the scale and speed required to prevent outbreaks) be captured? This, of course, is not a new question and is at the heart of any proactive disease surveillance system. The aim for this center is to satisfy the demands of this question through research and training activities of a collaborative nature.

2. Intellectual Merit
Strengths:

1. Somewhat counterintuitively, an intellectual strength of this application is in the way it distills the fundamental problems of pandemic prediction to clear and basic, simple problems (surveillance at adequate scale) and from this rather frank admission proposes specific pathways of case studies to advance knowledge in this research space.
2. The rapid application of technology to measure prevalence of seropositivity as a measure of spillover, while conceptually not knew, is an example of the distillation to simple problems as addressed above. The intellectual merit is in re-visiting this old idea not merely with more rapid technology but with the analysis of the information into the decision making framework that is proposed, bridging from Aim 2 to Aim 3.
3. This is an exceptionally qualified team to address the problem of spillover dynamics; it is the science that this group has been doing for some time operationally in real life contexts.

Weaknesses:
1. Conceptually, no one knows if examination of anyone emergence scenario (such as Nipah virus as proposed here) will allow generalizations of the principles under investigation and elucidation for all such scenarios; thus the transformative nature of it (that is, to truly change the way the scientific community and public community can predict spillover leading to pandemic system behavior) is simply uncertain.
2. The argument is well made but generally understood that more intensive, up-scaled surveillance is more likely to capture spillover events than are others. This is not an intellectual bridge to new ways to investigate the problem, but a sort of admission that it is was is needed. The problem is that it has serious limitations: costs, infrastructure, capacity, public support (or lack thereof), and the kind of invasiveness into life that not all people or societies find acceptable or implementable.
3. The case is made that the EHA had evidence of covid bridging from serology data of some 50,000 positive samples in the China study setting, pre-pandemic. It is hard to know from this observation if this empirical finding represents virus bridging dead ends, stuttering, or viral chatter;

indeed these descriptive categories are not entirely separated or epidemiologically recognizable. If pandemic breakouts occur in tandem with these events, and they are results of spillovers or bridge transmission from enzootic reservoirs, what is the frequency of them when pandemic breakouts don't happen? The latter must be a rare event given what appears to be evidence for frequent spillover. That appears to be the intellectual challenge to this application, and is a comment regarding assessment of success: what exactly is an anomalous event?

In the context of the five review elements, please
evaluate the strengths and weaknesses of the proposal with respect to broader impacts.

3. Broader Impact
Strengths:

The lateral flow technology with multiplex serologic analysis has the potential to allow rapid and broad assessment of changes in seropositivity to suites of pathogens, offering a means of measuring spillover events with large data sets.

Weaknesses:
It is hard to envision the broad impact of the image analysis approach to human / mammal contact as a means to assess the potential for spillover. What are the limitations, such as when contact occurs when it is dark and images cannot be derived, or when the contact is not direct but indirect through excreta or contaminated surfaces? As a generalized methodology with broad impact, this concept has uncertainties. It is also weakened by some individual and societal resistance to the invasiveness required to implement it. One would have to justify the invasion of the privacy of people in public spaces (market settings, for example).

The origin/destination analysis may provide data on human movement and inform pathogen movement through movement networks and pathways, but this is a rather well studied area that has been addressed through such means as cell phone network and use analysis. For example, it has been used to explain the excess of human malaria cases in cities of SubSaharan Africa where the origin of those cases is rural exposure but infection and disease manifests in cities.

Please evaluate the strengths and
weaknesses of the proposal with respect to any additional solicitation-specific review criteria, if applicable

1. An ambitious grand challenge. The grand challenge statement regards scientific activities that address the problem of capturing adequate, robust data at scale to inform spillover events that may lead to pandemic outbreaks. This is a fundamental problem that nearly everyone will agree with. How to go to adequate scale (sample size to measure meaningful and real changes confidently) is a true limitation to the science of prediction of outbreaks of disease.
2. Going beyond current science. The utilization of recognition software and imaging systems as means of measuring nonhuman animal hosts of pathogens and human interfaces with them was not convincing.
3. Sub-project innovation and synergy. The three research areas were well defined and synergistic. There is an especially good bridge from Aim 2 to Aim 3.
4. Disciplinary component and context. Cross disciplinary elements are emphasized throughout; the anthropological dimension including development of gamed scenarios was difficult to envision in the context of the other proposed components of the research plan.
5. Center-scale vision and plan. The concept of a Center for Spillover Intelligence is well

articulated and designed. The notion of a Spillover Situation Room has appeal.
6. Project management. A Project Management Plan is evident.

Summary Statement

The application from Dr. Kevin Olival and colleagues at EcoHealth Alliance, University of Rochester, and Georgetown University takes advantage of robust, on going programs of emerging infections to quantify extent of exposure to zoonotic pathogens and predict anomalous spillover events. The basic concept of scaled up surveillance is facilitated by rapid-processing recognition systems and multiplex assessment of seropositivity rates in human populations which information can be integrated quickly into models towards decision making regarding the nature of the spillover. The grand challenge statement, while compelling, admits to the common problem that scale up of surveillance is a limitation that faces nearly public health and disease surveillance, including research, activities and fundamentally becomes a problem of sample size to capture rare events. How to accomplish this in the face of logistic, funding, and societal limitations places constraints on the broader impact of the proposed effort.

Proposal Review 2 : 2201117

Agency Name:

National Science Foundation

Agency Tracking Number:

2201117

Organization:

NSF Program:

PIPP-Pandemic Prevention

PI/PD:

Olival, Kevin

Application Title:

PIPP Phase I: Multidisciplinary advances to scale up zoonotic spillover intelligence

Rating:

Excellent

Review

Summary

In the context of the five review elements, please
evaluate the strengths and weaknesses of the proposal with respect to intellectual merit.

This proposal tackles a crucial challenge of pandemic prediction and prevention: acquiring actionable intelligence about what happens at the zoonotic spillover boundary fast enough to make a difference. There are two components here: describing conditions where a zoonosis is likely to emerge and become established in a human population, and actually detecting such an event when it occurs. By integrating big-data analytics and social media engagement with established medical anthropology approaches, the investigators aim to identify and map behaviors likely to bring people into contact with potential zoonotic reservoir animals, much faster than was possible with traditional surveys. The investigators have also developed ambitious and potentially transformative approaches to detecting zoonosis emergence through advanced rapid serological testing and looking for behavior anomalies that could signal events of high spillover likelihood or actual disease taking hold.
These approaches represent ambitious but feasible advances in their respective fields and represent synergistic collaborations across disciplines. EcoHealth Alliance has extensive experience uncovering behavioral and ecological conditions that enhance spillover, and the team brings in expertise and massive datasets to develop methods to identify potential spillover events, as well as medical anthropology, network modeling and analysis, and disease assays and surveillance. Resource to begin this work identified for phase I appear to be available at the participating institutions, and the partnership with Facebook is key.

In the context of the five review elements, please
evaluate the strengths and weaknesses of the proposal with respect to broader impacts.

This work has strong potential to generate positive broader impacts. The grand challenge of this project has strong potential to provide societal benefit through mediation of spillover prone conditions and early detection of zoonoses that become established and begin to spread among people. The investigators show a thorough grasp of the ethical and privacy considerations of the data they aim to use, and the work would provide a vital opportunity for training and education in multidisciplinary epidemiological and ecological research. They also indicate products will be made publicly available. Finally, the investigators aim to enhance recruitment and participation of underrepresented groups although the specific mechanisms are vague.

Please evaluate the strengths and
weaknesses of the proposal with respect to any additional solicitation-specific review criteria, if applicable

This proposal identifies a very specific yet very important and ambitious grand challenge, and the proposal describes a thorough and sophisticated approach to tackling it. The assembled team definitely provides complementary expertise and are connected to broader research and public policy contexts, and the collaborations are vital to success of the effort. The Project Management Plan does a good job of sketching the investigators' roles and the overall management structure. Coordination mechanisms are well described and appropriately ambitious. Metrics for success provided are clear and there is a concrete vision for scaling up to phase II. The budget reflects the activities described.

Summary Statement

This proposal tackles the crucial challenge of providing information at the site and time of zoonotic spillover, potentially allowing pandemics to be prevented or stopped at very early stages. The work described is ambitious yet feasible, and strongly multidisciplinary with strong potential to advance

beyond the existing strengths of the team. Effective prediction and prevention of pandemics would have enormous societal benefits, and the proposed work would enhance knowledge dissemination, generate new research infrastructure, and provide valuable training to students and early researchers.

Proposal Review 3 : 2201117

Agency Name:

National Science Foundation

Agency Tracking Number:

2201117

Organization:

NSF Program:

PIPP-Pandemic Prevention

PI/PD:

Olival, Kevin

Application Title:

PIPP Phase I: Multidisciplinary advances to scale up zoonotic spillover intelligence

Rating:

Excellent

Review

Summary
In the context of the five review elements, please
evaluate the strengths and weaknesses of the proposal with respect to intellectual merit.

This is an excellent “planning proposal” to build a collaborative research and training network with ambitious to grown and scaled up to a future Center for Spillover Intelligence (CSI). Its vision and rationale for “piloting innovative approaches to large-scale spillover intelligence” are clearly articulated and easy to appreciate. The grand challenge is important and has clear societal impact and benefit: How can we capture actionable data about the risk of zoonotic spillover (and early spread) at the scale and speed required to prevent outbreaks? The proposal outlines a truly multidisciplinary team approach and recognizes the need for working beyond “previously siloed scientific domains”. The proposal, pilot projects, network (cross-)membership and project management mechanisms are structured by three clearly articulated aims: 1) Measuring human-animal contact at scale; 2) Detecting zoonotic disease exposure at scale; 3) Rapidly Identifying Anomalous Health Events. Not only are network members linked to specifically to these aims

through designated working groups, but specific education and training events and activities are embedded within them.

2. Intellectual Merit
Strengths: There is considerable potential to advance knowledge of poorly understood zoonotic spillover effects through the network operating in a truly multidisciplinary way beyond siloes. It clearly articulates the need and ambition to undertake disease surveillance and research “across a wide enough geography [and at an appropriate] temporal or spatial scale needed for disease mitigation”. They propose data collection at a global scale using a variety data types.

The proposal contains considerable creativity, originality, and is potentially transformative both in its overall objective of both establishing and providing “spillover intelligence” and also in different data collection and harvesting activities associated with different research aims and associated pilot projects (e.g. quantitative/qualitative behavioral data collection through partnership with Facebook; image recognition and AI to quantify and map human-wildlife contact; first field-forward lateral-flow assay for Nipah virus antibody detection; using machine learning to detect changes in human mobility patterns that may giving warning of potential spillover).

The research plan is particularly well-reasoned, well-organized, and based on a sound rationale, and has appropriate mechanism to assess success including proposed metrics. There is very clear vision to grow the a collaborative research and training network with ambitious to grown and scaled up to a future Center for Spillover Intelligence (CSI) and do this using pilot projects associated with the research aims. Education and training are clearly embedded and communicated with these aims/project and will deliver capacity building.

The multidisciplinary team is extremely well qualified and experienced. It has an outstanding track record in undertaking research, NSF support, publication, and translating research into practice with impressive stakeholder impact.

Weaknesses: I have not identified anything specific at this stage but anticipate others might have (and look forward hearing about these from other panelists).

In the context of the five review elements, please
evaluate the strengths and weaknesses of the proposal with respect to broader impacts.

3. Broader Impact

Strengths: There is considerable potential to benefit society through facilitating zoonotic spillover intelligence at global and local scales.

There is considerable creativity originality, and novelty in the proposed plan to implement broader impact. A very strong feature is the plan for capacity building which will to “initiate 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. Other training and research exchanges related to pilot research projects are also detailed. A wide range of stakeholders are also alluded to (e.g. EcoHealth Alliance).

Is the plan to achieve the BIs is well-reasoned, well-organized, and based on a sound rationale and embedded with the proposed networks activities (i.e. 3 project aims) and project management plan.

Weaknesses: Might have said a little bit more about potential for broader impact activities with non-academic public sector stakeholders

Please evaluate the strengths and
weaknesses of the proposal with respect to any additional solicitation-specific review criteria, if applicable

An ambitious grand challenge is presented that is forward-looking, and if successful to provide scientific breakthroughs in large-scale spillover intelligence (see synopsis).
Going beyond current science. The proposal include an innovative vision with potential to be transformative in undertaking truly multidisciplinary research by building a research network for spillover intelligence and developing and proof of concept pilot projects (see intellectual merit)
Sub-project innovation and synergy are a particular strength of what is proposed and draws upon the comprehensive multi-disciplinary research team/network. The targeted pilot projects and outputs are well-integrated have focused synergetic goals (c.f. Fig.2). They appear to provide considerable potential to advance the involved disciplines
Disciplinary component and context. This is well articulated as are the inter-disciplinary linkages.
Center-scale vision and plan is clearly articulated with vision to grow from a research network to a fully-fledged Centre.
Project management and data management plans are exemplary, clearly articulated and fully

Summary Statement

This is a very thoughtful and realistic truly multi-disciplinary “planning proposal” to build a collaborative research and training network with ambitious to grown and scaled up to a future Center for Spillover Intelligence (CSI). It clearly articulates and timely and important grand challenge with clear societal impact and benefit: How can we capture actionable data about the risk of zoonotic spillover (and early spread) at the scale and speed required to prevent outbreaks? It is an excellent fit with the PIPP Phase 1 solicitation with 3 excellent aims that structure the network, it aims, outputs, membership, and project management mechanisms proposals. It details very suitable and appropriate pilot projects, education and capacity building which is at the Centre of its vision and demonstrate clear impact benefits and activities. In summary compelling case is made for supporting the establishment of research and training network.

Proposal Review 4 : 2201117

Agency Name:

National Science Foundation

Agency Tracking Number:

2201117

Organization:

NSF Program:

PIPP-Pandemic Prevention

PI/PD:

Olival, Kevin

Application Title:

PIPP Phase I: Multidisciplinary advances to scale up zoonotic spillover intelligence

Rating:

Multiple Rating: (Excellent/Very Good)

Review

Summary
In the context of the five review elements, please
evaluate the strengths and weaknesses of the proposal with respect to intellectual merit.

1. Synopsis
The research team plans to collaborate among the disciplines of computer science, engineering, data science and Facebook, a.k.a. Meta, to develop an Internet-sourced early warning system using AI, anomaly detection and machine learning. The team will also develop a unique lateral-flow assay field detection device, to be deployed in the field. The plan is premised on global scale data collection. Detection of pathogens is primarily based on the assumption of anomalies in human behavior that would be detected via social media.

2. Intellectual Merit
Strengths:

The background and statement of the problem is strong and compelling.
The research team plans to leverage existing funded research from NIH and DTRA to develop and test novel rapid serology tests and lateral flow assay tests. This increases the likelihood of major advances in the development of rapid bioinformatics and in-field testing, which is critical to a functional early warning system.
The reliance on social media image recognition, while not novel, does access a potentially useful data source.
The overall plan, as well as the initial aims, are well defined, developed and reasonable. Varying degrees of success in realizing the goals will help lay the foundation for a Center moving forward.
The research team is well qualified in terms of experience, past and current projects, collaborations, and resources. As outlined, the team is fully capable of undertaking Phase 1 research.

Weaknesses:
The researchers mention the “SARS-related coronaviruses in China” as a specific example of spill-over threat to humans. The team will partner with Facebooks’ Data for Good. How will the team pierce the Great Firewall that prevent access to China’s Internet, given that pathogens in China are a specific concern?

Regarding the surveys of specific populations (p. 11 of 167), how will they target geographic areas using Internet-based social media. The researchers will be using the Facebook platform. How ubiquitous is the Internet in edge locations? I would think not very. What alternatives do the research have to conducting surveys where the Internet is not present, or not in high use?

The researchers mention their extensive experience and infrastructure in Southeast Asia. Why not

pilot pre-emergent flu detection, since most flu originates in that region, early detection would facilitate the creation of that season's vaccine and would be beneficial world-wide.

In the context of the five review elements, please
evaluate the strengths and weaknesses of the proposal with respect to broader impacts.

3. Broader Impact
Strengths:
The team's aims, if successful, would have broader societal impacts both in terms of advancing scientific knowledge and also, given the collaborations with industry, the development of early warning and surveillance tools for multiple threats.
The team plans to develop lateral flow assays (LFAs), tests that can be conducted in the field. The researchers note that LFAs have not yet been used in field for zoonotic virus surveillance. Such an advance would have societal benefits since such as test would be a critical tool in a virus early warning system, regardless of the particular virus.
Overall, the approach is well-ground, internally consistent and builds on the team's current experience and expertise.

Weaknesses:

The social media component's reliance on samples of convenience and self-reporting is weak. Any intelligence gleamed from this data would have to be ground truthed which, given the number of "hits" on any day, could mushroom to unsustainable scales.

Please evaluate the strengths and
weaknesses of the proposal with respect to any additional solicitation-specific review criteria, if applicable

In addition to the two National Science Board-approved merit review criteria of IM and BI, the PIPP Phase I solicitation has the following program-specific criteria, which need to be addressed:

1. An ambitious grand challenge.

The proposal is ambitious, but not beyond the realm of achievability. It is a logical extension, and in the case of field testing, a technological leap, in developing the tools and methodologies for an early warning system.

5. Going beyond current science.

The greatest innovations appear to be in the field testing and deployment. I leave it to my other reviewers to assess the potential.

6. Sub-project innovation and synergy.

The individual aims, while separate disciplines and domains, are necessary for the development of the final early warning system. The barrier to success will be not successfully integrating the two domains into a cohesive system.

7. Disciplinary component and context
Aim 3 seeks to identify signs of health-seeking behaviors using social media, communication patterns and mobility (cell phone?) patterns. I would caution the team on three points. First, Google's

Flu Tends was eventually abandoned after overestimating flu cases. The primary reason was that on-line health seeking behavior was not as correlated with actual cases as expected, and thus gave rise to inflated estimations. Second, individuals are not trained to recognize symptoms nor ask the right Google questions. Thus flu, colds, seasonal allergies, and hangovers comingled in the Google space. Third, what we are interested in are very, very rare events, assuming that we can identify it before it becomes an epidemic in a region. Thus, the challenge is not only to analyze massive data streams, but also to be able to correctly identify the rare event, without making a false positive or false negative mistake. To put this in perspective, worldwide there are 5.4 BILLION Google searches per day.

The use of Instagram image-recognition should be more fully developed and exploited. Posts and image contain both a time and location stamp, which makes them a valuable source of image remote sensing. Further, I get the sense that the demographics of social media use are becoming skewed away from Facebook. Thus, it become even more important to scrap other social medial platforms in order to capture the younger and higher-use social media posters.

I agree that origin-destination matrices do not typically operationalize a temporal component and are thus less useful for tracking the length of time (spreading infection) at a location. The researchers plan to use Facebook's Activity Space Maps and Colocation Maps. What is the temporal scale and what is the spatial scale of these tools?

The researchers plan to "target areas known to include health care facilities in pursuit of abnormal trends." (p. 16 of 167). Given that these facilities are (relatively) near edge land use, what is the likelihood that the facility can report anomalies quickly (likely lacking electronic medical records) and what is the likelihood that the facility will recognize a novel spill-over quickly?

8. Center-scale vision and plan.

The proposal clearly lays out the vision for scaling from a planning and research activity project to a functional Center.

9. Project management.
Project management will consist of an Advisory Board, a Steering Committee, and the three focus area research groups will be led by a multidisciplinary team of Co-PIs. The management plan calls for meetings every month. I would feel more comfortable with the commitment to meet at least twice monthly, and more like weekly for the initial phase. True collaboration comes from deeply understanding other disciplines, the lens through which they view the work, and the nomenclature that they use. Notably, they do designate a (part-time) Project Manager to administer operations.

Summary Statement

10. Summary Statement:
This is a well-developed proposal, with clearly defined short term objective with an eye toward fleshing out a Center. The research objectives are important extensions to established science, extensions necessary to developing an anomaly and early warning zoonotic intelligence system. The individual aims are reasonable and achievable. The overall research objective appears to be scalable. The research team has the necessary experience in the topical areas and, in some cases, are already doing preliminary research in those areas. As the researchers note, these are fresh collaborations. As such, I would revisit and strengthen the formal and informal channels of communication. The institutional resources and support are appropriate.

From:	Kevin Olival on behalf of Kevin Olival <olival@ecohealthalliance.org>
To:	Peter Daszak; M. Ehsan Hoque; Mateos Buckstein, Gonzalo; Noam Ross; Emma Mendelsohn; Emily Mendenhall; alexpompe@fb.com; Eric Laing
Cc:	Aleksei Avery Chmura; Luke Hamel
Subject:	Summary of discussion w NSF PIPP program officers
Date:	Sunday, June 19, 2022 7:31:02 PM
Attachments:	Discussion_NSFprogramofficers_17June2022.docx ATT00002.bin Olival_NSF PIPP Panel Summary and reviews_3June2022.docx ATT00004.bin

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Dear all,

We (me, Ehsan, Peter) had a 1 hour call w NSF on Friday afternoon to discuss our unfunded NSF PIPP proposal. Here are my notes summarizing the call.

In short, we ranked very high but will not get funded. They encouraged us to try and fund parts of the proposal via other mechanisms, but these could involve putting whole proposals together (~1 year time frame before funding), but we will pursue other mechanisms that could be quicker like EAGER grants. I will also follow up w Katharina Ditmar (the BIO NSF PIPP program officer) to get her thoughts.

In any case, this is very frustrating as we were "highly competitive" and clearly came so close to getting funded. I still believe (as I summarized in my previous email to the NSF program officers) that the reviewers just flat out got some things wrong that we laid out quite clearly, esp. 2 of the main negative aspects in the panel summary - but seems we have no real recourse here but to try other funding mechanisms, and/or apply for a Center level grant in ~1 year. We also agreed it would be great to not lose momentum before the RFP for Center level grants, and maybe find ways to do some of what we proposed (albeit without funding!). Chime in if you have some specific ideas.

Appreciate everyone's hard work on this. Upward and onward.

Cheers,
Kevin

P.S. Sending the panel summary and reviews again too, as I didn't send them to everyone here before.

Notes - PIPP planning call - 2 September 2021

Participants:

- *EHA*: Kevin Olival, Emma Mendelsohn, Noam Ross, Luke Hamel
    - Unable to attend: Peter Daszak
- *Univ. of Rochester*: Gonzalo Buckstein
    - Unable to attend: Ehsan Hoque
- *USU*: Eric Laing
- *Georgetown Univ*ersity:
    - Unable to attend: Emily Mendenhall

Action Items:

- Kevin to send around 1-page 'Project Summary' by early next week
- Luke to request biosketches and other required documents from senior personnel by early next week
- Noam to share his method for anomaly detection of serological results, with Gonzalo
- Kevin to follow up with Eric to discuss novel serological ideas that could be included on the proposal
- PIPP team (especially Kevin, Noam, Emma, Gonzalo and Ehsan) to review meeting notes from the call with Facebook. Explore various links to preprints/datasets/online tools
    - Gonzalo to start a notes document and write down ideas about how to leverage the datasets referenced in the notes for our proposal
- All to continue thinking about 'broader impacts' and draft any text related to training or outreach at your institution

Agenda/Notes:

- *Introduce Dr. Eric Laing - serology and biosurveillance*
    - (Kevin) Eric works at Uniformed Services University (USU). Extensive experience with serological surveillance
    - (Eric) I'm a molecular virologist by training, but I work on biosurveillance. Doing a lot of COVID-19 work recently

- *Brainstorm intellectual and engineering innovations on "Detecting zoonotic disease exposure at scale" (Serology)*
    - (Kevin) We have a current NIH project sampling people at high risk communities in SE Asia (e.g. bat guano harvesters, people presenting symptoms at health facilities). Using serological platforms to find evidence of spillover (perhaps of novel bat viruses)
    - (Kevin) First question for Eric: Are you working with any engineers/product designers to think about scaling up technologies (cheaper, smaller, easier to use)?
        - (Eric) No we aren't. We haven't looked at anything that's point of care focused. No handheld devices. No antigens on lateral flow.

- This is possible to do, but you wouldn't be able to do multiplex
- You could develop a small test for a specific virus (e.g. Nipah) but nobody wants to develop it. No money in that venture.

- (Noam)
- I spoke with Linfa Wang (of Duke-NUS) who works on serology. He's less focused on point of care, more focused on working at a larger scale (e.g. working with blood banks to access lots of samples at a larger scale/larger fractions of the population)
  - The main goal of this Phase 1 proposal is to plan for the Phase 2 proposal. Who do we want to bring together to brainstorm big ideas for Phase 2? Question is about piloting different approaches. Show what could be possible in a 5-year project.
  - (Kevin) Also thinking about our working group structure. Eric, given your familiarity with the serological workshop space, **who else would you bring to the table, re next-generation serological multiplexing for zoonoses?** These are the things you (Eric) could help us think about
    - (Eric) Serology is challenging. Always limited by what it is you're looking for. CoVs, paramyxos, filos potentially have the greatest risk for pandemic potential. So do you make rapid tests for those viral families? We have everything we need to develop test for Nipah, but no monetary interest there. What would be needed for ebolaviruses? **Also, I don't know how you'd make a 'disease x' assay**
      - (Kevin) NSF is thinking big picture. So one idea is a 'serochip' assay where you test for 1000s of viruses at once. Maybe this doesn't work that well compared to standard methods, but **this planning grant is focused on thinking about big picture ideas.**
  - (Eric) You can look at this pandemic as a blueprint for moving forward. The research diagnostic field moved very quickly (ELISAs were rapidly approved, etc.)
    - **Re the serochip idea, the big question is more about: Do we have the analytics in place to understand what the data means?**
      - (Noam) Probably not. Even for multiplex of 15 tests, we barely have the bioinformatics set up to run those and have rapid interpretation. **So the rapid analytics side of things needs to be further explored.**
      - (Kevin) Gonzalo, would you and Ehsan be able to assist with anomaly detection of antibody positives?
      - (Noam) I have a method that I can share with you Gonzalo.
        - (Gonzalo) That's maybe more in Ehsan's field. I'm more about scaling up methods. I'm happy to take a look though.

(Noam) There are several ideas for scaling serological surveillance. 1. Strategy to test more people, would involve having a more centralized high throughput lab. 2. Another strategy would involve moving more towards point of care. We could talk to Bill Busa? Developed lateral flow assay that is optical reader so that lateral flow assay can be calibrated to give quantitative output at point of care scan. It measures light absorption that otherwise would just read positive or negative. 3. **Think about which of these methods would require having a rapid analytical pipeline in place.**

- (Kevin) **How do you detect exposure for a virus you're not looking for (e.g. one that's truly novel, i.e. the 'Disease X' problem?) Problem that I don't know the answer to**
    - With PCR, you can apply a viral family wide primer set, but you don't have that with serology
        - (Eric) Ideally, you make serological tests as specific as possible
- (Noam) In 1st aim (scaling up the measurement of human-animal contact), the idea is to identify (with more granularity and more geographic specificity), populations that are likely encountering probable viral hosts, and to identify the types of contact that are likely to lead to exposure. I think of serology in the same way. We can think about the degree of regional specificity. Looking at particular classes of viruses in certain areas, etc. Or some other stratified, scaling approach where one has higher cost multiplex approaches covering many different viruses, then using that to do more intensive/geographically extensive sampling for particular viruses. **This would be a surveillance design question.**
    - (Kevin) **The problem is that we don't know the geographic extent of pathogen groups. So we can't necessarily target serological testing of particular viruses in particular geographies.**
    - (Eric) So the knowledge gap is that viruses emerge where testing isn't deployed and that viruses diverge enough to avoid seroglocial testing.
        - So if you implemented more serological platforms at more interfaces (e.g. markets), would this be helpful or should we focus on hospitals/clinics?
        - (Noam) There's a low level virus exposure that may not be human to human transmitted that we want to detect.
            - But logical framework is that with many viruses emerging, one of them will go pandemic.

        - (Noam) Given the widespread nature of SARS-CoV-2, identifying people who have been exposed to CoVs won't be very helpful (unless we can discriminate between SARS-CoV-2 and other related viruses)
        - (Noam) PCR - too much of a "needle in haystack" problem, could be overcome by novel, scaled-up serology approaches.
        - (Kevin) Eric, we should circle back with you on some novel seroglocial ideas.
            - (Eric) There's a nearby company that does 3D printing. Could 3D print the lateral flow plastic component. Issue would be access to positive controls. If we had outbreak, we could run it and look at acute periods vs. convalescent periods of exposure. Need to think about where we'd get validating serum samples
                - (Kevin) Re: positive control to EHA could potentially help procure samples (perhaps from EHA project w EIDRC on Nipah). We'll follow up on this

- *Debrief from Facebook (FB) <-> Rochester discussion earlier today*
    - (Kevin) FB would primarily support Aim 1 re human-animal contact (image recognition) as well as Aim 3 (post-exposure human mobility data)
        - Next steps will be to have discussions with the FB team about exploring the available data and discuss methodology used to weight the co-location dataset. Alex (form FB) will set up discussions with his colleagues.
        - **Think this would be a nice private-public partnership to highlight for NSF**
    - (Gonzalo) I like the fact that FB is thinking seriously about privacy concerns, and that they're transparent about data sharing. Also data is available on a global scale.
    - (Kevin) I agree that it's important we highlight the importance of data privacy
    - PIPP team will review the notes from the discussion with Facebook
        - Gonzalo will start notes document as he works through datasets mentioned during Facebook call
    - (Kevin) Co-location dataset is available and updated weekly. Activity space dataset should be made available within a month or so. Might be available to us now, just not made public yet?
    - (Emma) **For image recognition, do we want to hone in on a specific geography?**
        - (Kevin) **SE Asia is obviously of interest. Probably pretty good coverage from Facebook too. Central Africa not so much.**
        - (Emma) For surveys, we'll have to refine our geographic scope.

- (Kevin) **We should include a schematic in the proposal for how we hone down our strategy** (e.g. conduct global image recognition of human bat contacts, and where we identify high rates of contact we push out surveys, etc)
- (Kevin) **It'd be helpful if we could map out all of the wildlife markets in a particular geography (e.g. Thailand), then use the activity map space to look at who's coming in and out of the areas where those markets exist**. Could be an interesting way to look at human flow in and out of markets. Perhaps we could implement a pilot study of this where markets have already been mapped out (Sulawesi bat markets that Alice Latine helped to identify). We could take the GPS coordinates of those markets and look at how human movement in and out of there changes over time.

- *Brainstorm "Broader Impacts" of our proposed 18 months*
  - (Kevin) **Curious what we want our broader impacts to be?**
  - (Gonzalo) Perhaps mention how methodologies could be used for societal good? (e.g. widespread implementation of FB surveys).
  - (Noam) In the past, NSF's broader impacts have been very specific. Typically, they're heavily aimed at bringing underrepresented groups into the research field, with a big focus on the training and education sides of things. **On that front, the best thing to do was to already have a program at your institution that you could partner with. e.g. Are there REU programs that target recruitment of underrepresented groups?** Seems NSF has relaxed a bit about how narrow broader impacts are
  - (Gonzalo) **I think it's important to be creative about your broader impacts.** Rochester has a lot of programs focused on increasing diversity that track success of participants over time.
    - https://www.rochester.edu/college/kearnscenter/
    - At the same time, I don't think this piece of the proposal is quite as important as intellectual merit side of the proposal
  - (Noam) We could highlight the EcoHealthNet (EHN) program. Mention that although the project is wrapping up in 2022, that we can extend the EHN model through to this PIPP project. We need to make sure the proposal includes funding for this though (and that we draw from financial numbers from EHN to accurately budget for this)
  - (Eric) I had a friend that was part of an IB program in Maryland. They introduced me to a program that connected high school students to various research opportunities. I'll try to remember what this was.
  - (Kevin) We should think about this more. Need to be cognizant of diversity on our proposal as well.

    - **(Gonzalo) Other things that I've seen that have been well received include: making lectures public (through Zoom/Zoom recordings) and making datasets open access**

- *AOB*

From:	Kevin Olival on behalf of Kevin Olival <olival@ecohealthalliance.org>
To:	Luke Hamel; Peter Daszak; Noam Ross; Emma Mendelsohn; Emily Mendenhall; M. Ehsan Hoque; gmateosb@ur.rochester.edu; Eric Laing
Cc:	Aleksei Avery Chmura; Alison Andre
Subject:	Re: [Rescheduled] PIPP call this week, Thursday (9/2) from 3-4 PM EDT
Date:	Tuesday, September 7, 2021 11:38:03 AM
Attachments:	Notes - PIPP planning call - 2 September 2021.docx ATT00002.bin

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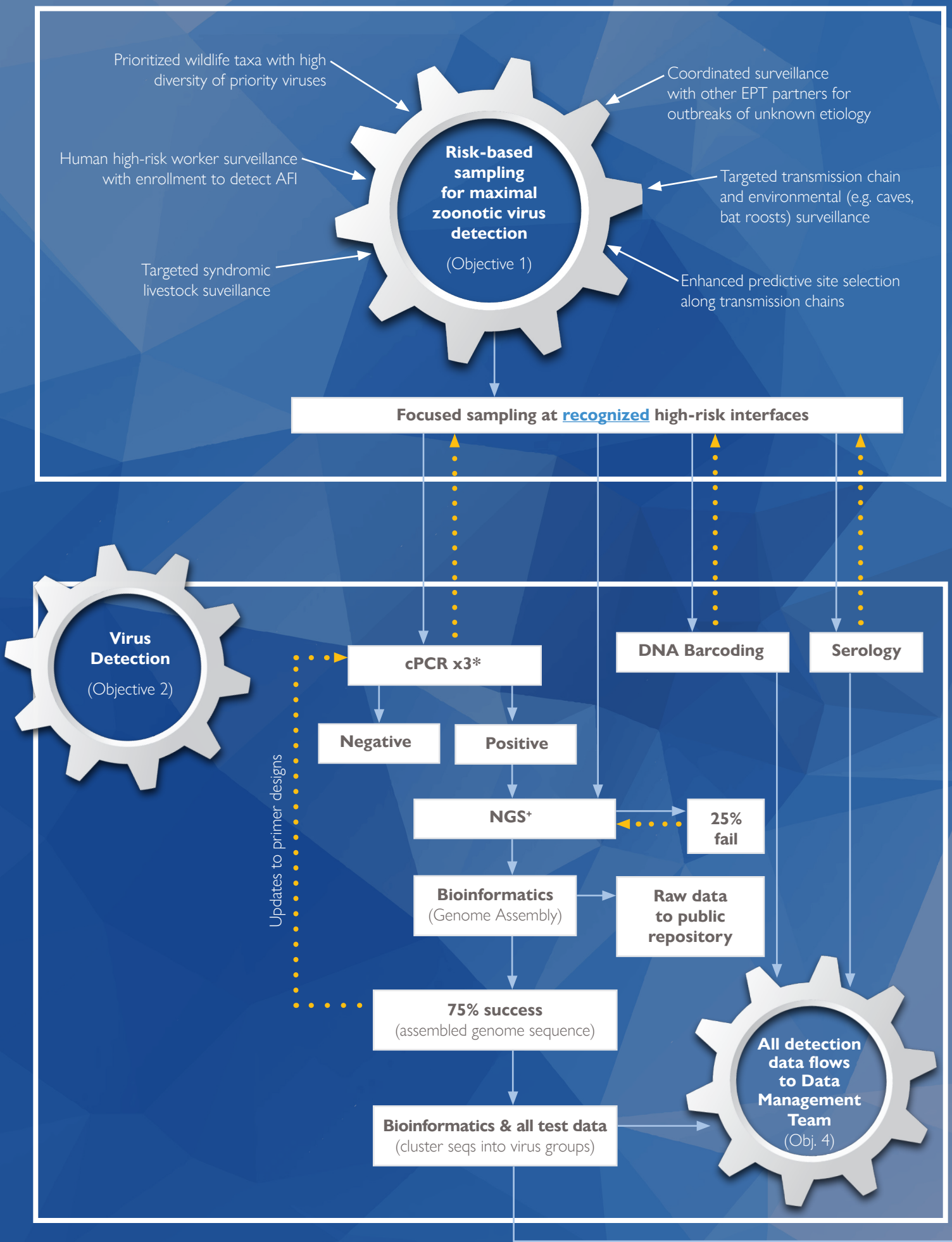
Dear all,

Attached are detailed notes from our NSF PIPP proposal planning call last week Thursday. Hope you all had a good Labor Day weekend. Will be following up throughout this week to start getting our proposal text together, NSF biosketches and Conflict of Interest lists from you, etc.

Cheers,
Kevin


Prioritized wildlife taxa with high diversity of priority viruses
Coordinated surveillance with other EPT partners for outbreaks of unknown etiology
Human high-risk worker surveillance with enrollment to detect AFI
Targeted transmission chain and environmental (e.g. caves, bat roosts) surveillance
Targeted syndromic livestock suveillance
Enhanced predictive site selection along transmission chains
Risk-based sampling for maximal zoonotic virus detection
(Objective 1)
Focused sampling at recognized high-risk interfaces
Virus Detection
(Objective 2)
cPCR x3*
DNA Barcoding
Serology
Negative
Positive
NGS+
25% fail
Updates to primer designs
Bioinformatics
(Genome Assembly)
Raw data to public repository
75% success
(assembled genome sequence)
Bioinformatics & all test data
(cluster seqs into virus groups)
All detection data flows to Data Management Team
(Obj. 4)

DEEP VZN LABORATORY TOOL BOX

†Detection Assays:

- Consensus PCR: coronaviruses; filoviruses; paramyxoviruses
- NGS: Illumina; Oxford Nanopore
- Serology: Luminex multiplex; ELISA

***Cell Entry:**

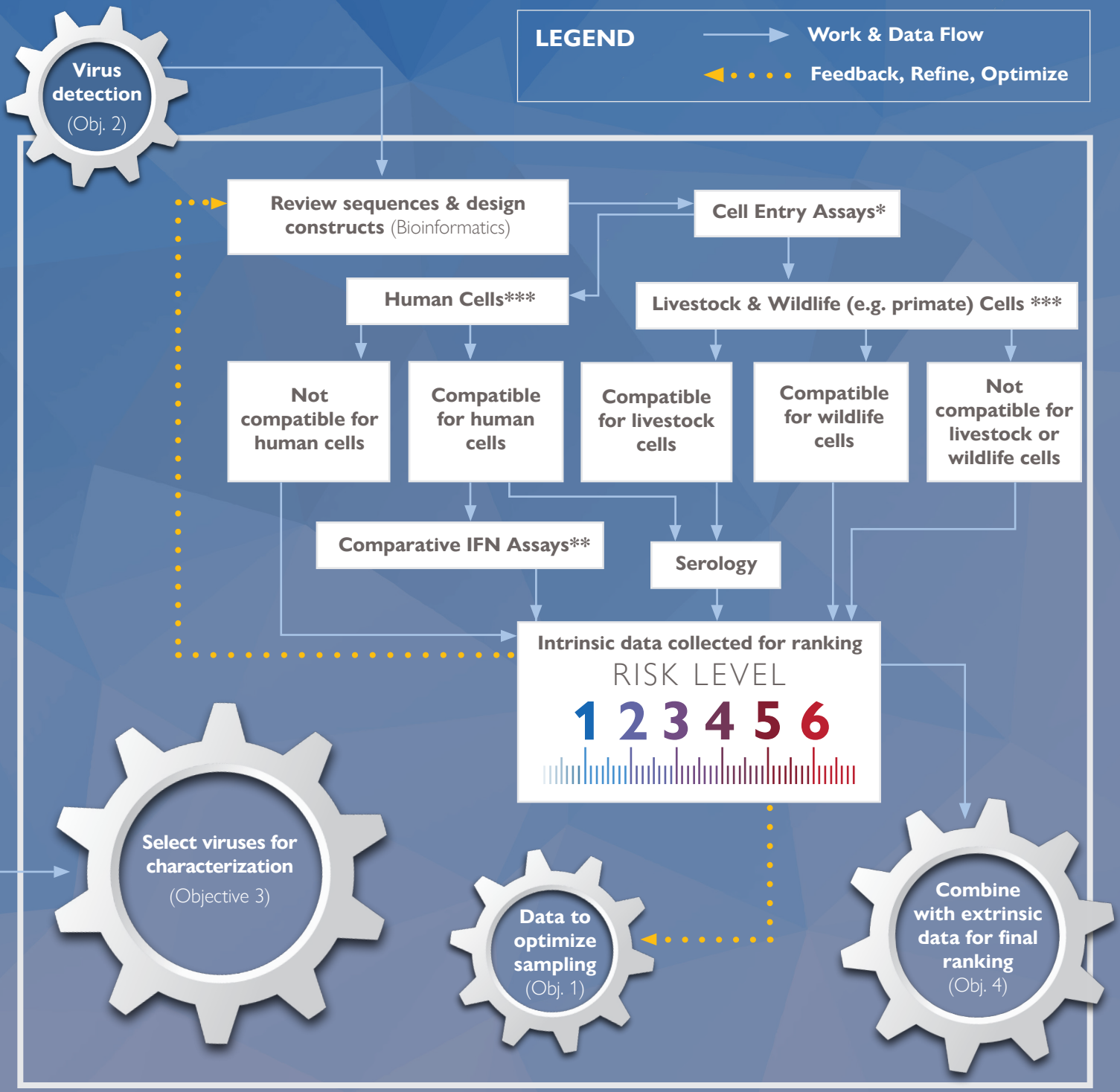
- Recombinant VSV-GPs
- Pseudotyped VSV-GPs
- Binding Assays

****IFN Antagonism**

- Luciferase Assays
- IFN ELISAs
- IFN qPCRs

*****Cell Types:**

TBD, but relevant for each virus being evaluated (e.g. for filoviruses start with Huh7 then move to primary Macrophages)



BACKGROUND AND JUSTIFICATION

The increasingly frequent and deadly consequences of emerging virus outbreaks on animal and human health is a global clarion call to action. Even before COVID-19 emerged as the first globally disruptive pandemic in over 100 years, diseases like Ebola, Nipah, MERS, SARS, and others signaled the need for dramatic and transformative approaches to global disease surveillance and zoonotic pathogen characterization. The DEEP VZN project will be a leading light in this transformation. For the first time in history, we have the tools required to forge direct actionable links between field surveillance, virus discovery, pathogen characterization, and countermeasure development. We are poised to move beyond the past centuries of reactionary health response towards comprehensive and prospective global health security paradigms. Our Consortium is the most ambitious formal collaboration of its kind, established to support focus countries in building the capacity to detect, characterize, and analyze novel virus threats and rapidly share data, results, and findings for maximum equitable public health benefit worldwide.

TECHNICAL APPROACH

Our goal is to develop locally-based leaders across a global network capable of working together, and with USAID and other EPT partners, to build a sustainable network of institutions, laboratories, and host-country agencies that can **rapidly assess ongoing and novel threats across the spectrum of wildlife, livestock, and human health, and conduct data analyses that will be of direct relevance to local and global policy makers, health authorities and translational partners** for vaccines, therapeutics, and diagnostics development (Figure 1). Working hand-in-hand with our trusted partners across the globe, we can begin the work described here as our vision for a DEEP VZN that will transform global health security.

Figure 1: The DEEP VZN technical framework integrating sampling, detection, characterization, and reporting to inform risk mitigation through local leadership.

Objective 1. Conduct Sampling in Focus Countries for Unknown Viruses from the Priority Viral Families

We will leverage decades of success empowering local stakeholders to implement early detection and pre-emergence virus surveillance programs and introduce technological innovations, sampling prioritization models, and new partnerships to cost-effectively scale up sampling and reporting in close to real-time. Our sampling strategy is informed by the many lessons learned

from the ongoing COVID-19 pandemic and our substantial experience in animal and human biosurveillance, recognizing that true resilience from future pandemic threats requires more coordinated local and global viral surveillance. We will use next-generation and cross-cutting analytical approaches combined with scalable surveillance tools to strategically target sites, species, and high-risk human-livestock-wildlife interfaces to begin sampling rapidly as well as iteratively refine sampling to improve rates of novel virus discovery.

DEEP VZN's Data-Driven Sampling Strategy

Biosafety and security. We consider biosafety and biosecurity practices to be the central pillar of our entire approach. To best address this challenge, our Biosafety Officer will oversee all aspects of biosafety from the field to the lab. In consultation with USAID, we also will conduct independent periodic reviews with outside consultants to ensure that we adhere to all best practices, regulations, and procedures across the global network. Our plan to scale up biosecure sampling involves collecting specimens directly into virus inactivating lysis reagents. This will dramatically reduce the biosafety and biosecurity concerns, risks, and costs across the project's global collection of biological specimens.

Innovative sample-multiplier approaches and cost-effective gold-standards. We will implement innovative approaches to scale, automate, and improve the cost efficiency of virus discovery. Approaches we have validated previously include non-invasive sampling of wildlife (e.g., plastic sheets for pooled urine and feces collection; baited ropes or stations for primate oral swabs). We are also collaborating with engineers on development of autonomous air sampling at sites with high animal density (e.g., caves, indoor markets), and water-outflow sampling (e.g., market drainage areas; rivers in bat caves). Teams will leverage our dispersed network of trained professionals for opportunistic samples and utilize new virus inactivation lysis reagents that greatly reduce cold-chain requirements and increase biosafety. Efforts will complement gold standard methods of sampling wildlife using non-lethal, high-quality blood, fecal, urine, and saliva samples conducted by our teams previously.

High priority interfaces. Previous analyses to investigate high priority viruses identified wildlife-human interfaces that yielded 2-5x the number of RNA virus detections over baseline sites. Working with local partners, DEEP VZN will prioritize wildlife, livestock, and human populations at these high-risk transmission pathways, including a) along the wildlife supply chain (e.g., hunting sites, wildlife farms, confiscated animals, large and small live-animal markets), b) cave and roost sites with high bat diversity and human disturbance, and c) areas with ongoing resource extraction (e.g., deforestation, mining). We will collect standardized data on individual, demographic, occupational, and environmental factors that influence spillover and could serve as critical risk mitigation points for disease control.

Key wildlife species. We propose to sample a diversity of bats that serve as reservoirs for the priority virus families (e.g., bats for betacoronaviruses, filoviruses, and henipaviruses), rodents, and primates that can serve as spillover hosts, as well as species from other mammal families found at high-risk interfaces (i.e., traded or synanthropic), that have been inadequately sampled and are predicted to harbor viruses.

High-risk human sampling targets. Molecular detection of novel viruses in healthy asymptomatic people, even those living or working in high-risk zoonotic transmission settings can be difficult. To overcome this challenge, we will conduct focused enrollment of high-risk communities and occupations (e.g., wildlife-associated work) where the risk of spillover is maximized. Surveillance will be supported by community health workers to identify and sample acute febrile illness cases **at the earliest opportunity**. Our consortium's existing network of

clinics and hospitals will be expanded with new partnerships, by establishing formal linkages with national event-based surveillance systems to focus on clusters of disease with unknown origin. In collaboration with government programs, we will be on standby to detect novel viruses in any specimens that meet criteria for non-routine diagnostics.

High-risk livestock sampling targets. At selected sites, we will support syndromic and risk-based surveillance in livestock that meet criteria for non-routine testing. This will be implemented in coordination with international and in-country livestock surveillance systems, to 1) enhance training for the local network of veterinarians and para-veterinary staff to identify and report unusual livestock illnesses, and 2) facilitate earlier detection and characterization of novel viruses from wildlife that could cause devastating diseases in livestock.

Cost-effective sampling. Viral diversity scales with host diversity, thus it is critical to select sites with a maximum diversity of mammalian hosts to maximize efficiencies. We will apply tools developed for species conservation to identify the most cost-effective sites for virus sampling in each country and account for land-use designations (e.g., future logging concessions, development, conversion to cropland) that could project risk.

Scale and scope. Based on our collective experience in wildlife health, we anticipate sampling approximately 10,000 unique individuals or discrete environmental units (e.g., wet-markets, caves, bat roosts, etc.) per country. We hope to expand our sample size estimates several fold over the course of the project if anticipated innovations in technologies being developed by our consortium are validated to provide the level of accuracy needed for this project. Using a data-driven approach across all objectives, teams will sample key wildlife taxa from high-risk interface sites until the local viral discovery curves indicate saturation of predicted novel priority viruses present (beyond which rarer virus discoveries are not cost effective). Based on data from previous projects, we anticipate intensive sampling at 4 sites per country per year, and moving on to new sites once discovery curves are saturated or viruses are being found repeatedly in the same species. This strategy should enable overlapping longitudinal sampling (2-3 years) for 5-10 sites per country over the project period.

Ongoing strategy refinement. We will iteratively refine sampling targets locally using models and data visualization techniques over the course of DEEP VZN with real-time assessments of sampling sites and species to maximize efficiency of novel virus discovery. Local data scientists will be supported in risk modeling to steward their data, guide their sampling teams, transform findings into actionable surveillance strategies, collaborate regionally for trans-boundary inferences, and lead publications.

New Partnerships to Scale-up 'Trans-sectoral' One Health Surveillance

Robust One Health surveillance for novel zoonoses is currently hampered by a limited number of trained personnel. Working synergistically with other capacity development projects like OHW-NG and STOP Spillover, we will implement multi-partner stakeholder workshops, dedicated in-service trainings, and specific instructor training to strategically engage new institutional partners, stakeholders, and government sectors typically left out of disease surveillance activities to more efficiently operationalize a scalable virus discovery sampling strategy in the target countries. New partnerships to ensure long-term sustainability of surveillance include academic institutions and natural history and zoology centers personnel, wildlife market vendors and workers, and authorities involved in wildlife management (e.g., customs officials, border patrol, wildlife inspectors, protected area staff, conservation IGOs including IUCN and CITES, and wildlife rehabilitation center staff) to develop communities of practice built around training opportunities. Open-source monitoring and reporting tools will facilitate real-time surveillance at

the wildlife-human interface. We will also engage paravets, paramedics, and community health workers to foster the One Health community approach and make gains in local health security and zoonotic disease awareness as a critical step towards global health security.

Objective 2. Strengthen Detection in Focus Countries for Novel Viruses from the Priority Viral Families

Rapid detection of novel viruses requires a safety-first multi-layered strategy and a "best-fit" approach in alignment with regional and host-country needs and priorities. We will optimize direct peer-to-peer and south-south collaborations to drive the development, optimization, and scale-up of detection assays and analytical approaches to strengthen capabilities and directly synergize with host-country government partners. Innovative technology, training, and detection strategies will be leveraged to augment and reinforce national priorities and virus detection capabilities through the dissemination of knowledge, technical approaches, and training to promote country-level engagement, sustainability, and progression in external frameworks (e.g., IHR, JEE). This will maximize gains for in-country partner laboratories to advance scientific excellence and sustainability and transition laboratories towards regional and global leadership. All laboratories will implement updated and refined standardized protocols along with state-of-the-art platforms for virus detection within the first year of project implementation. Our DEEP-VZN laboratory network spans a wide range of technical capacities, bridging across low to high-resource host-country partners and includes global subject matter experts that together foster a global community of laboratory practice.

DEEP VZN's Innovative Detection Strategy

Laboratory biosafety/biosecurity and quality-management systems. Coordinated by our dedicated project Biosafety officer with technical input from delegates at each partner laboratory and in consultation with USAID and consortium partner CDC, we will begin with rigorous evaluation of existing practices and identify gaps in BS&S and QMS knowledge and practices at each partner laboratory. We will conduct our initial and ongoing in-service training and education to ensure compliance with all national, regional, and international requirements (e.g., BMBL, WHO Stepwise QMS, etc.). A secure electronic inventory and laboratory data tracking system will be utilized in each partner laboratory for precise and real-time inventory management and tracking of specimen, results, and data.

Rapid training implementation using bio-archived and other surveillance project specimens. Where available, archived and well-curated bio-banked specimens (from the USAID-PREDICT, other consortium surveillance projects, and other USAID programs (e.g., STOP Spillover when synergistic and novel etiologic agents are suspected) will be provided so that each partner laboratory can rapidly begin their training, internal quality assessments, pilot testing, optimization of results, and data handling pipelines even before the first DEEP-VZN prospective specimens are collected. This rapid roll-out and implementation of the testing strategy will allow for optimization to local conditions and further in-service identification of training gaps and infrastructure/equipment needs, as well as lead to enhanced national level technical capacities early-on in the DEEP-VZN project to empower much needed laboratory surge capacity for diagnostic needs during outbreaks of unknown etiology.

Optimized, more thermostable specimen handling and extraction for surge capacity. Semi-automated to automated nucleic acid extraction using newly developed and more thermostable virus inactivation and extraction buffers will be utilized to ensure that medium to high-throughput surge capacity exists in each partner laboratory. These platforms have been utilized and validated by our global consortium in the 2014-2016 West Africa Ebola outbreak and

COVID-19 pandemic and will ensure that both the technical and technological capacity exists in each partner laboratory to conduct virus detection at the robust throughput required.

Prioritized specimen selection and testing. We propose to use a data-driven prioritization scheme developed from more than a decade of experience in novel virus detection and other biosurveillance and virus discovery projects. Thus, time, human and financial resources, and effort will be maximized for efficient novel virus detection across a variety of species and sample types as described in Objective 1 and detailed in the "Decision Tree" (Annex 4).

The DEEP VZN Virus Detection Toolbox

Over the past 10 years, members of our consortium have implemented molecular tools that led to the detection of over 1,000 viruses, including 177 coronaviruses, 230 paramyxoviruses, and 3 filoviruses. With our targeted field surveillance plan (Objective 1) and cutting-edge detection platforms (Objective 2) we expect to greatly surpass these previous discovery efforts, truly transforming the capacity for viral discovery. Our virus detection toolbox addresses **two critical needs** 1) to identify and sequence unknown viruses, and 2) to use laboratory data to refine and optimize sampling activities in Objective 1.

Identify and sequence novel viruses: We will deploy robust NGS protocols and provide low-cost equipment for in-house sequencing of prioritized specimens in near real-time. To target the priority virus families, we will use a **capture-based NGS sequencing approach** that uses a set of nucleic acid probes to enrich samples for coronavirus, paramyxovirus, and filovirus targets. Working with world-leading NGS consortium partner, Illumina, we will update existing capture-based sequencing protocols using data generated from the USAID PREDICT project and will conduct robust training on data processing to build critical bioinformatic capacity to assemble virus genomes from NGS datasets. To validate our NGS protocols, we will routinely sequence a subset of specimens from high-risk species and interfaces using **unbiased NGS approaches** to ensure we can detect highly divergent viruses. Recognizing the likely spectrum of laboratory capacity, we also plan to implement alternative NGS strategies such as **Amplicon based NGS by MinIon (Oxford Nanopore)**. By expanding our NGS toolkit, we maximize our ability to tailor NGS needs to each laboratory or situation (e.g., sequencing in field settings). Amplicon-based sequencing was used successfully to sequence Ebola virus genomes during the 2014-2016 epidemic and offers greater flexibility in less-resourced laboratories. In addition to our highly innovative NGS platforms, we will continue to build on the incredible success of consensus PCR, which was implemented in more than 60 labs and 30 countries during PREDICT and led to the detection of >1000 viruses from >20 viral families. We will update **priority virus family-level consensus RT-PCR detection assays** to pre-screen samples for NGS. This combined strategy will capitalize on the cPCR capacity that was built under PREDICT and maximize cost-effectiveness, sample throughput, and flexibility in the range of laboratory tools available (i.e., ranging from simple methods like cPCR to more complex methods like NGS).

Laboratory assays to refine Objective 1 surveillance: A critical part of our strategy involves optimizing field-based surveillance using laboratory-generated data. To help target species, locations, and populations with signatures of priority viral families, we will use two approaches. First, we will use ***serologic assays*** to identify species, locations, and populations with evidence of high-priority viruses. Multiple assays, including two advanced multiplexed bead-based assays designed by consortium partners USU and Duke-NUS, will be utilized for the detection of antibodies in humans and animals (including select wildlife species) across the priority virus families. Using tiny volumes of serum (~2μL, feasible for even the smallest insectivorous bat

and rodent species), these quantitative assays rely on expected serologic cross-reactivity across the diversity of each virus family in animals and humans against these target viruses, including those which are unknown but antigenically related. Beyond broad serosurveillance, we will also employ a highly specific and sensitive assay designed to detect neutralizing antibodies that target the specific receptor binding domain (RBD) on the spike protein of each of the known sarbecoviruses (SARS-related viruses, including SARS-CoV-2) to screen both animal and human sera for coronaviruses with zoonotic potential and identify possible reservoirs and exposed human populations. Critically, this newly developed assay will allow us to differentiate antibodies against SARS-CoV-2 from antibodies against other bat-associated CoVs in human sera from populations with frequent exposure to bats or bat excreta. Both of these panels are easily updated when new viral sequences are discovered, making them flexible and powerful screening tools. When coupled with the ecology and exposure history of animals or humans tested, they can provide evidence of an as yet undetected virus or variant of concern from all three high priority viral groups, in the sampled population.

The second approach will be to develop **rapid point-of-collection detection assays** that can provide in-situ and real-time evidence of virus circulation, allowing us to rapidly expand the number of sites that can be evaluated for evidence of priority viruses and transforming the surveillance landscape at scale. In order to assess the ability of current state-of-the-art rapid-diagnostic devices to be brought to scale, we will test, validate, and verify select novel technologies (e.g., Conservation X Labs, and others) in target countries. These approaches will be cross validated with our other detection platforms to determine real-world utility under field conditions. Broad-based rapid detection assays in each priority virus family will be designed to provide an alert-signature indicating the probable existence of high-consequence virus threats. We will evaluate the power of these new technologies to vastly expand the scope of novel pathogen detection across a much broader suite of locations. All of our **laboratory detection assays will be reviewed and updated routinely,** iteratively incorporating new data as it is generated to ensure that the toolbox is robust and remains state-of-the-art. Continuing education and re-training for lab teams will be also conducted across all years of the project implementation.

Objective 3. Strengthening Characterization in Focus Countries of Novel Viruses from the Priority Viral Families

Describing the ‘pre-emergent’ diversity of viruses circulating in bats, rodents, and other high-risk wildlife taxa is a critical component of pandemic prevention. However, finding an unknown viral sequence in wildlife does not reveal whether it has zoonotic or pandemic potential. Using experimental and computational approaches we will explore the compatibility of wildlife viruses for human infection. Our consortium includes some of world’s foremost subject matter experts in coronaviruses, paramyxoviruses, and filoviruses, and together we will build the technical capacity in-country to perform a variety of cutting-edge, biosecure, and informative assays that evaluate the risk of novel viruses and variants of concern.

The DEEP VZN Characterization Toolbox

To evaluate novel viruses detected in Objective 2, we will build a risk characterization toolbox to identify potentially hazardous viruses before they emerge in humans. We define ‘potentially hazardous’ as any virus with the ability to enter a human cell, replicate efficiently, and antagonize host immune responses. Recognizing that there is no singular or definitive method to evaluate these characteristics, we will build a range of tools to identify viruses with the capacity

to infect humans. **These tools will be scalable, versatile, and built specifically for use in standard laboratories without the need to work directly with live viruses that pose an unknown threat to humans.** Our goal is to rank the hazard potential of unknown viruses so that they can be prioritized for the development of downstream countermeasures.

Targeting high-risk viral traits. The genetic factors that determine zoonotic potential are complex and are the result of hundreds of molecular interactions between viral and host-cell proteins. However, viruses with the ability to 1) enter human cells, 2) utilize cellular resources (i.e., replicate efficiently), and 3) avoid or suppress host responses like interferon (IFN) are most likely to establish productive infections in humans. We will characterize these high-risk traits to 'rank' viruses according to their zoonotic potential. *Low containment BSL2 tools:* Virus characterization can be a time consuming and costly process that traditionally requires live virus, high-containment facilities (e.g., BSL3 or BSL4), and highly-trained personnel. It is neither feasible nor safe to implement this work where biosecurity is in question. To build much-needed technical capacity for virus characterization, we will use surrogate systems that examine the function of individual viral proteins rather than entire infectious viruses. Surrogate tools targeting proteins involved in cell entry and suppression of host immunity can effectively identify viruses with key zoonotic traits. *Cell entry*: The ability to enter a human cell is the first critical step in infection. Thus, the identification of viruses that can circumvent this primary barrier is a critical component of spillover risk assessment. We will use pseudotyped or recombinant Vesicular Stomatitis virus particles (VSV) as a technique to incorporate coronavirus, paramyxovirus, or filovirus surface glycoproteins (VSV-GPs), and evaluate their ability to enter a range of relevant human cells. As broad host range is an additional risk factor for zoonotic emergence, we will also investigate the ability of these non-hazardous VSV-GPs to enter the cells of animal species relevant to each country and region, in particular the cells of livestock or other wildlife that could serve as intermediate hosts. *Innate Immunity (Interferon; IFN)*: The ability to antagonize IFN is a major determinant of pathogenicity, as all viruses express proteins capable of avoiding or suppressing the IFN response. For filoviruses, these proteins include VP35, VP24, and VP40, while for coronaviruses, NSP1, NSP12, and ORF6 contribute to IFN antagonism. The degree to which different viral proteins suppress the human IFN response will have important implications for their pathogenic potential. For instance, Ebola virus (species: *Zaire ebolavirus*) is a potent antagonist of human IFN and is highly pathogenic, whereas the related Reston virus (species: *Reston ebolavirus*) is a less efficient antagonist and is non-pathogenic. Thus, evaluating the ability of novel viruses to suppress IFN will indicate their potential to cause disease. We will use dual luciferase reporter assays and standard ELISAs to measure how effectively different viral proteins antagonize human IFN induction and signaling in vitro. *Serology:* One of the most direct approaches to identifying future viral threats is to look for evidence that spillover has already occurred. Thus, for novel viruses with hazard potential, we will develop specific serologic tools to survey humans, including high-risk workers along key transmission chains and look for evidence of spillover and community transmission. We will utilize the VSV-GPs built for evaluating cell entry and re-purpose them effectively for specific serology, maximizing cost effectiveness, maintaining stringent biosafety conditions, and increasing sustainability. A second strategy will build on our extensive experience with multiplex serology on the Luminex platform, which we have successfully implemented in multiple low resource settings. We will expand our existing multiplex assays to include surface glycoproteins for potentially hazardous viruses, as well as closely related viruses to help evaluate potential cross-reactivity.

High-Containment (BSL3, BSL4) validation of risk assessments. Surrogate tools provide critical insights into the zoonotic potential of viruses. However, it is important to acknowledge that they are reductionist and may not recapitulate the course of natural infection where a multitude of traits are expressed simultaneously. In consultation with and with approval from USAID, in-country governments, and a third-party independent auditor, select viruses will be considered for advanced virus characterization at BSL3 or BSL4 facilities where we can safely recover virus by reverse genetics and verify our surrogate-based assessments of cell entry and IFN antagonism in vitro. As we are not proposing to collect samples into viral preservative for biosafety and security reasons, we will not have the option to isolate viruses directly from samples. Thus, using the recovered viruses under highly secure and permitted conditions will allow us to evaluate other key viral traits such as efficiency of replication in different cell types, including those associated with transmission. This high containment work will only be initiated following a consultative auditing process to assess feasibility and ensure biosafety measures are in place that meet or exceed national and institutional biosafety regulations. In countries where BSL3 or BSL4 containment facilities are not available, we will utilize our DEEP VZN network to perform this work at another facility in the region or the United States as needed. Our consortium partners include institutions with BSL3 facilities (e.g., Thailand, Viet Nam, Singapore, US), and BSL4 facilities (e.g., US CDC, NIH RML, and CSIRO-Australia).

Selecting viruses for characterization. By utilizing surrogate and scalable tools that can be implemented in standard BSL2 laboratories, we will be able to characterize and perform risk assessments for a large number of viruses. We plan to characterize any virus that has not previously been evaluated and for which data on hazard potential is lacking. Based on our previous work, we anticipate fully characterizing approximately 672 distinct novel viruses and 120 variants of concern across the proposed 12 country network.

Ranking criteria for viruses with zoonotic potential. Empirical data generated from the characterization toolbox will be used to rank the hazard potential of viruses using a computational approach that includes phylogenetics, protein modeling, and machine learning. An example of proposed risk levels based on criteria generated by our staged testing strategy is presented in Figure 2. Our goal is to help country-level stakeholders make decisions about which viruses to target for further characterization work, ecological studies, or interventions. Our ranking strategy will be refined throughout the duration of the project with in-country partners.

Examples from Our Previous Work: Successes in Novel Virus Risk Assessment and Characterization

A key focus of our work in DEEP VZN will be to ensure that we provide the knowledge depth required to ensure the long-term sustainability and growth of the characterization toolbox in each country. Below we have highlighted three examples that collectively demonstrate our experience characterizing viruses from all priority regions and virus families. *Africa Region (Filoviruses):* A novel ebolavirus, Bombali virus (BOMV), was discovered in insectivorous bats in Sierra Leone. The bats were found roosting in human houses, which was concerning because ebolaviruses can be highly pathogenic. To assess the zoonotic risk of this virus, we generated recombinant VSVs expressing the GP and found that BOMV is able to infect human cells. We then partnered with the US CDC to rescue infectious BOMV under BSL4 conditions and confirmed this finding with authentic virus. Next, we built a serologic assay targeting the BOMV GP to survey humans across multiple countries and found evidence of exposure to BOMV in a single person in the DRC. This validated our in vitro risk assessments and indicated a wider geographic range for the

virus. Indeed, following this discovery other research groups found evidence of the virus also circulating in bats in Kenya confirming a wide distribution across Africa. Finally, we used dual luciferase reporter assays to show that BOMV is a poor antagonist of human IFN, relative to highly pathogenic ebolaviruses, raising questions about its pathogenic potential. Altogether, the data generated to date support the following risk assessment: BOMV can infect humans but there is limited evidence of human-to-human transmission (at this time) and no strong evidence that it has the capacity to cause severe disease. Thus, in its current form BOMV does have zoonotic potential, but limited pandemic potential.

Figure 2: Risk levels ranking the hazard potential of viruses based on a staged testing for virus characterization.

Latin America Region (Paramyxoviruses): We identified a novel bat paramyxovirus from Brazil - Myotis Bat Morbillivirus (MBaMV) - which is related to several viruses of human (e.g., measles (MeV)) and veterinary (e.g., canine distemper) concern. As with BOMV, we generated VSV-GPs with MBaMV surface proteins using a pseudotyping approach and showed that the receptor binding proteins of MBaMV are able to mediate entry into human cells. We rescued infectious virus from the genome sequence and confirmed this ability using human myeloid cells. We also demonstrated that MBaMV replicates in human epithelial cells and uses human NECTIN4 almost as well as MeV. These data demonstrated the unusual ability of MBaMV to infect and replicate in human cells that are critical for MeV pathogenesis and transmission. Critically, MBaMV was not able to replicate efficiently in human lymphoid cells that are required for amplification during the early stages of infection. Thus, based on available data our current risk assessment is that MBaMV has not yet adapted sufficiently to replicate well in critical early cell types, limiting its pandemic potential. If the virus evolves this ability, however,

it would be considered a high-risk virus. *Asia Region (Coronaviruses):* A bat coronavirus related to SARS-CoV-2 was detected in horseshoe bats in Thailand. This virus, RacCS203, was evaluated for its ability to infect human cells at the sequence, structure, and function levels. We found that while RacCS203 was 91% similar to SARS-CoV-2 across the whole genome, it shared only 71% identity across the spike gene and 61% in the critical receptor-binding domain. Structural modeling predicted that the RacCS203 receptor-binding domain was unlikely to bind with the human ACE2 receptor, which was subsequently confirmed using functional binding assays. We therefore assessed the zoonotic risk of RacCS203 as low, given its inability to use ACE2 to enter human cells. However, we acknowledge the potential for this virus to enter human cells via a different receptor or acquire an ACE2-competent receptor binding domain in the future via subsequent evolution.

Objective 4. Strengthen Host Country Capacities for Data Management and the Viral Characterization Process

Our vision is to enhance capacity for detection and characterization of unknown viruses through data management and ensure that data availability to inform preparedness and response spans the full scope of DEEP VZN. Our efforts are directed at improving the speed and efficiency of generating actionable scientific evidence on virus detections, virus spillover, outbreaks, and novel virus discoveries. Our approach is based on a collaborative peer-to-peer mentorship program, continuous iterative training and technology transfer, tool-building, and networking. Key characteristics of the proposed Data Management approach include:

- DEEP VZN "Data Management Help Desk" to support Objective Teams with data science expertise in engineering, computer science, and computationally complex bioinformatics, as well as technical experts and host country data staff.
- Peer-to-peer community of practice for assistance in data quality assurance, system management and customization, data integration and processing, data analyses, data visualization, and publishing, via in-person and remote trainings.
- Common schema for essential metadata that addresses privacy and security, established with input from Regional Councils, to facilitate querying and augment existing data systems.
- Data transfer software tools to simplify workflows for data packaging, extrinsic dataset integration, data visualization, and deposition of data to local and international platforms.
- Decentralized data management systems to meet local stakeholder needs and priorities with customizable data integration and visualization to promote local ownership of data and support local leadership in data management, data sharing, and publication.

Data Collection. We will facilitate electronic collection tools for field and laboratory metadata standards that builds on data collection platforms optimized over decades of field data collection in a wide range of resource settings. In countries lacking data collection systems, we will use open-source online platforms, designed for low-resource settings to support online, offline, and paper-based data entry. All information required to identify nodes and chains of transmission will be captured, as well as provide epidemiological context for novel viruses to inform risk mitigation strategies. We will work with local and national government partners, international stakeholders, One Health Workforce, One Health Platforms, and national and international data reporting standards and requirements to ensure compatibility and long-term use of a common data schema and data dictionary linked field and lab data.

Data and Metadata Management. Local data managers and bioinformaticians will coordinate data transfer and synthesis between field, lab, analytics, and local and stakeholder outputs. The

Data Management Team will support these day-to-day operations and workflows, organizing data and software distribution. We will ensure access to diverse data streams and the flexibility required to capture local and varied programs in a common data model. This common model will enable more rapid reporting and robust data-sharing by 1) facilitating data exchange in national and regional networks, 2) enabling greater automation of dataset integration, risk modeling, visualization, and reporting, and 3) linking in platforms without loss of information, especially for national and international data-sharing platforms designed for very narrow data types. We will develop data checking and validation tools to test data quality locally and across the entire project.

Data Storage. Where possible, we will build on local experience with data platforms, and use existing project data storage and management systems within local partner laboratories and institutions. This will facilitate integration into relevant national platforms. Systems can be hosted on DEEP VZN servers for rapid start-up and transferred to in-country over the course of the project. In-country data management systems will communicate and share data routinely with a centralized data system for project tracking and management. The project's internal data-deposition system will also serve as a "staging" system to support next steps in data use and management, and peer-to-peer training in data management.

Further Characterization with Extrinsic Datasets for Risk Analyses. Large scale datasets on recognized zoonotic viruses, hosts, host traits, host genomes, ecological niches, and geography will be integrated for local, national and international stakeholder-led analyses. Similarly, extrinsic data sources on future land use, climate projections, and other environmental drivers will also be integrated into local data systems for predictive models estimating future risk. We will support in-country bioinformatics in near-real-time with flexible and automated workflows packaged as open-source software (R, Python, and Jupyter Notebooks). This will enable users to run pre-defined analyses, risk characterization summaries, and interactive visualizations, linking in local data to global open-source extrinsic data that can provide situational context for virus characterization data. These resources will be modular, allowing in-country bioinformaticians to customize analyses for their needs. We will favor low/no-cost open-source software to maximize accessibility.

Rapid Information Sharing. The Data Management Team will support analytical pipelines to provide epidemiological context for highly ranked viruses to share with partners and stakeholders in rapidly produced situation reports. Situation rapid reports will combine intrinsic virus traits described in Objectives 2 and 3 with extrinsic data identified in Objectives 1 and 4 to provide relevant epidemiological context for highly ranked virus findings. In addition, a web-based data visualization system will be optimized to streamline semi-automated reporting of results and sharing situation reports with local, national, and international stakeholders, including USAID. Data visualization will be designed with stakeholders to 1) meet local capacity and need for outputs, 2) provide in-country teams capabilities to customize outputs, and 3) be automatable to allow for quick dissemination of preliminary results with appropriate context for interpretation. The Help Desk will coordinate data validation tests, synthesize datasets, integrate risk characterization tools, and ensure models are tailored into actionable outputs. Along with dissemination of results, these tools will support in-country teams in generating data summaries that can be readily used for data publication.

Results Reporting and Depositions. Data approval procedures and priority contact lists will be established in collaboration with USAID and national government authorities to ensure an informed, transparent, and timely approach to reporting results, securing approvals for broader dissemination, and meeting local, national and international (e.g., IHR and OIE) reporting requirements. At project start in each host country, **data sharing protocols and mechanisms for rapid results communications will be developed and agreed upon in collaboration with local implementing partners and national authorities** spanning Ministries of Health, Agriculture, and Environment, and national One Health platforms (or equivalent) to ensure timely approval and robust public data sharing procedures. Host country data leads in coordination with local partners will jointly identify national, regional, and international scientific data platforms for routine and regular publication of updated DEEP VZN situation reports and accompanying data, while customizing data workflows to target these platforms as needed. Preliminary updates will be shared routinely on biweekly Senior Management Team discussions and more immediately as warranted by project findings. Summaries of sampling and testing data will be shared with USAID quarterly or as requested.

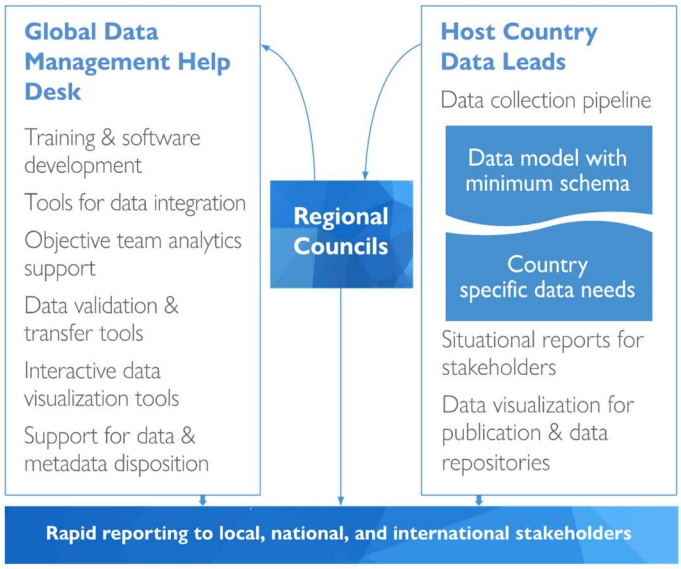


Figure 3: Data management team core activities.

Data depository targets will follow best practices in international data disposition, and further promote enhanced metadata inclusion at global data depositories, such as the NIH National Center for Biotechnology Information (NCBI) and the Development Data Library. Workflows, software, and training will ensure quality assurance during conversion of project data to formats appropriate for publication on each platform.

Strengthening Long-Term Sustainability and Country Ownership

Key to pandemic preparedness and our future legacy is the achievement of independent, sustained, local capabilities in One Health surveillance at high-risk animal interfaces, virus discovery, virus characterization, and data management. Therefore, we must not only ensure specialized training for individuals in these activities, but we must also create an environment in which some of those who are trained become experts who then later train others. Universities are well suited to this challenge, as the nature of academia is such that any given current academic workforce instructs most students broadly, while training some students with interest and aptitude to become the next instructors. Many of our implementing partners will have strong connections to universities that serve as research capacity for their nations, especially in outbreak investigations. Our consortium is therefore poised to not only strengthen capabilities in safe and secure virus surveillance for emerging threats, but to engage local trainees in academic pursuits of research, scholarship, and publication, thus simultaneously establish the next generation of scientific leaders in all aspects of this work in partnership with other USAID projects.

We fully recognize the benefits of training a One Health professional workforce by leveraging existing regional training platforms. Only through experiential training and multidisciplinary mentorship can young faculty and students be empowered to truly take on the pressing global health challenges ahead of us. To meet this demand, we propose that a DEEP-VZN Training Academy be coordinated through AFROHUN and SEAOHUN for their respective regions, with a similar model applied for the Latin America region. Over the life of project, highly talented university undergraduate and graduate students as well as in-service professionals, balanced across genders, will be provided training opportunities in our DEEP-VZN network laboratories, to receive in-depth, state-of-the-art training and mentorship in virology, disease ecology, virus diagnostics, scientific writing and communications. A requirement of participation will be for trainees to put their training towards publication(s) and future work, whether that be through their current jobs or through engaging with academic or government led institutions to invigorate the host-country's virology and disease detection workforce.

MONITORING, EVALUATION, AND LEARNING (MEL)

For over a decade, we have worked with USAID, USG interagency partners, and host country stakeholders to develop collaborative and adaptive approaches to performance monitoring, learning, and capacity strengthening. Our consortium has led efforts with in-country implementing partners to design performance monitoring plans and adapt to challenging and sometimes rapidly evolving international and national priorities such as the GHSA Action Packages and indicators, the WHO's Joint External Evaluations (JEE) and Benchmarks for IHR capacity, and USAID's own Self Reliance Metrics and country road maps. Through EPT-1 and -2 and the current USAID One Health Workforce-Next Generation M&E design and implementation process, we have learned how a more robust and design-centered plan, which strengthens capacity for performance monitoring at the country level, is best grounded in a theory of change, and includes a learning agenda to inform decision making for improved management, collaboration and return on investment.

MEL plan. Based on our theory of change, this Monitoring, Evaluation, and Learning (MEL) plan provides the road map for overall project design and performance. Performance-based MEL elements are built into the core of each objective's activities (along with crosscutting objectives such as capacity strengthening, biosafety and biosecurity, and gender equity). This results and change-focused MEL plan is incorporated into the organizational culture of our consortium and our management approach through a learning agenda that facilitates critical examination of the project for strategic and collaborative decision making.

At project inception, we will engage in-country implementing partners to establish a MEL team, develop our collaborative learning agenda, and design systems for performance tracking, data collection, and information and knowledge management. At this initial stage, we will establish MEL as a foundational element of project design and implementation that is compatible and synergistic with international and country-level monitoring systems and tools. Building on innovations established by the OHW-NG MEL team, we propose to work in close coordination with our Objective 4 Data Management team to create electronic data capture tools for streamlined and effective data collection, reporting, and summaries/visualizations that serve to inform country, regional, and senior management teams of project performance, impact, and opportunities for corrective action and adaptation.

Process and timeline. The MEL team, chaired by the project's Operations Lead, will be led by specialists with experience developing and implementing performance monitoring plans for

USAID and the GHSA communities and will include membership from our Objective Teams, Regional Councils, country-based Implementation Teams, and Biosafety/Biosecurity and Gender consultants. The MEL team will work closely with consortium partners and USAID to create the project's learning agenda, developing core questions that address the project's goals over time and are flexible and responsive in implementation. Our plan is designed around an iterative process that will include feedback and entry points for host country stakeholders, public/private, and government partners (as appropriate) in both development and implementation stages with defined approaches to monitoring and evaluations. The MEL team will meet at least monthly with each objective team and will hold quarterly project-level reviews to track performance progress toward targets and deliverables.

MEL data will be collected in real-time at the activity level and routinely analyzed and prominently featured in project meetings for discussion, enabling routine performance monitoring and opportunities to collaboratively adapt the MEL plan for improved monitoring and enhanced project performance. Data validation will occur bi-annually during the reporting process, enabling a feedback loop with objective and country implementation teams. Performance monitoring will be built into project meetings as they are planned (semi-annual and annual meetings, etc.) with virtual MEL team meetings held routinely throughout project implementation for decision making, adaptation, and improvements in performance, as needed. Our consortium is committed to open data systems, and MEL data collected through this project will be accessible to USAID and other international and GHSA partners as appropriate to support evaluation and inform policy and strategic direction in global health and health security.

External evaluations. Through the leadership of our external advisory committee, we will support and conduct project-level external evaluations at key points in the project lifecycle. Because a core element of DEEP VZN is safe and effective implementation, biosafety and biosecurity assessments will play a major role in monitoring and evaluation, conducted by an external consultant group (to be determined in consultation with USAID). In addition to regular and ongoing evaluation and assessments, additional major overall project and biosecurity evaluations will be planned midway in Years 2 and 4 of the project at critical phases when our activities will be mature for rigorous evaluation and yielding results that will inform project management and strategic direction.

Indicators and targets. We have crafted output, outcome, and long-term impact indicators and will refine these indicators and the MEL framework in collaboration with USAID and implementing partners to ensure buy-in and support of the MEL process. Each indicator will be accompanied by Performance Indicator Reference Sheets that include: rationale or justification for indicator, expected/desired change, calculation and unit of measure, disaggregation, frontline data source, means of verification/data collection methods, frequenting of reporting, baseline, targets, and limitations/considerations. We will develop both life of project targets and short-term achievable ones as part of each year's workplan process to enable more real-time performance monitoring. By design, our outcome-level indicators will be continually evolving and informed by project implementation, project data and findings, and through collaborative engagement with key stakeholders. A selection from our proposed MEL framework with illustrative indicators (qualitative and quantitative), targets, country stage gates representing key capacity and sustainability gains, and anticipated long-term impact is provided in Table 1.

Strengthening national systems. Our overarching goal is to strengthen national capacity to detect and characterize unknown viruses with zoonotic and epidemic potential, and our partners include national health authorities and close partners of national programs. To support national

systems, our proposed activities are designed to achieve outcomes in alignment with national WHO Benchmark Technical Areas for enhancing country-level IHR capacities. Indicators, outputs, outcomes, and targets will complement national goals, and we anticipate that our project's investments in capacity strengthening will support national progress towards GHSA and WHO action plans. Through a network of host country and regional partners, virus detection and characterization platforms will integrate with and strengthen national laboratory systems and our data and findings will feed into multisectoral coordination bodies (e.g., national task forces, EOCs, One Health platforms, interagency coordination desks, etc.) to directly inform decision making, policy, and interventions. Within the proposed MEL framework, we highlight key areas of overlap and synergy between the project and WHO Benchmark Technical Areas.

Table 1: Snapshot from proposed MEL framework to highlight objective technical areas, overlap with WHO Benchmarks for IHR capacities, sample output and outcome indicators with associated targets and anticipated long-term impacts.

Objective(s) ***WHO Benchmark Technical Areas***	Illustrative Output Indicators and Sample Targets		Expected Outcome Indicators and Sample Targets		Expected Long-term Impact
Crosscutting Virus Detection (Obj. 2) & Characterization (Obj. 3) - ***Biosafety and Security, Zoonotic Disease, and National Lab Systems***	**Outputs** 1. # specimens safely collected and secured 2. # ,% labs implementing detection & characterization tools 3. % selected viruses fully characterized	**Targets** 1. Up to 10,000 specimens per country by Y5 2. At least 1 lab/country in Y1; 100% of engaged labs by Y3 3. 100% by the end of the project	**Outcomes** 1. # ,% labs safely performing <u>all</u> project detection and characterization protocols 2. # ,% labs sharing data and findings to inform policy, intervention strategies, and potential countermeasures	**Targets** 1. 100% of engaged labs by Y3 2. 100% of engaged labs by Y5	10-fold increase in detection and characterization rates over current rate, led by in-country lab networks
Crosscutting (Obj. 4) - Capacity & Data Management - Zoonotic Disease, National Lab System, Biosafety and Biosecurity, and Risk Communication	**Outputs** 1. # individuals trained in full suite of skills and protocols required for safe sample handling, virus detection, characterization, data management, and communications 2. # publications and products led by country teams	**Targets** 1. ~100 individuals per country 2. At least 2 publications and/or products per country team	**Outcomes** 1. Improved time from sampling to reporting of sequence data 2. Approx. 675 virus sequences per country published on national and/or international platforms	**Targets** 1. TBD with in-country partners 2. 100% of all genomes by Y5	Improved national capacity and stronger, more cost-effective systems for safe sampling, virus detection and characterization, risk modeling and analytics, and communication

Learning agenda. Our learning agenda is based on the following proposed questions (to be confirmed post-award in consultation with USAID):

1. Is the viral surveillance, detection, and characterization strategy working (e.g., cost-effective sampling resulting in enhanced detection and characterization of novel priority family viruses and variants of concern)? *To be addressed semi-annually each year.*
2. Are we improving the efficiency and scale of virus discovery in-country each year?
3. Is the strategy safe, secure, and adhering to international and country-related BS&S regulations and best practices? *Addressed on an ongoing basis and systematically reviewed quarterly in close consultation with Objective Teams, Country Implementation Teams, and Biosafety/Biosecurity compliance officers.*
4. Is the strategy adoptable and transitioning well to country partners, institutions, and labs (e.g., is the strategy/technology/approach/platform(s) appropriate for capacity levels in host countries and with host country partners)? *To be addressed semi-annually each year.*
5. Are we expanding human resources and core technical capacities for virus surveillance, detection, characterization, data management, analyses, and reporting? *To be addressed semi-annually each year.*
6. Is our investment coordinated with national health and laboratory systems, and are we contributing data to inform policy (why or why not)? *Addressed quarterly in close consultation with Objective Teams and Country Implementation Teams.*
7. Are our data and findings contributing to policy reform and development of new diagnostics and countermeasures? *Longer-term question to be addressed Years 3-5.*

Learning agenda questions will serve to orient project direction and will be routinely reviewed and updated throughout the project lifecycle. The MEL team will incorporate the learning agenda into quarterly MEL meetings, and performance monitoring data and stakeholder input and feedback (via interviews and surveys) collected through the bi-annual reporting process will inform the agenda. Findings and insights will be summarized and used for decision making and adjustments to the project strategy and activities in collaboration with USAID and key partners.

From:	Brian Bird on behalf of Brian Bird <bhbird@ucdavis.edu>
To:	Christine Kreuder Johnson; Simon J Anthony; S Wacharapluesadee; ksaylors; Murray, Suzan; Walzer, Christian; Kevin Olival; William Karesh; Towner, Jonathan (Jon) (CDC/DDID/NCEZID/DHCPP); Peter Daszak; Elizabeth Leasure; Kirsten Gilardi; kartik.chandran; Fine, Amanda; Lee, Benhur; mariana.leguia; ztq9@cdc.gov; Moeder, Vanessa; Marcela Uhart; Zimmerman, Dawn; Alex; gschroth; Ava Sullivan; Latinne, Alice; Christian Lange; Olson, Sarah; Paul Bunje; hkretser; ccs8@cdc.gov; Jon Epstein; Keatts, Lucy; Tierra Smiley Evans; Pranav Sudhir Pandit; Susie.Welty; Aleksei Chmura; Alberga, Jeremy J; Eric Laing (Uniformed Services University); Mike Whelan; hlw2124; LintonY; Kathryn Vosburg; Mary Guttieri; Kaczmerek@ecohealthalliance.org; Cadhla Firth; Maryska Kaczmarek; Sarah Munro; Churchill, Carolina; Matthew Blake; Nicole R Gardner
Cc:	David John Wolking; Michael Ziccardi
Subject:	Re: DEEP VZN proposal submitted!
Date:	Thursday, June 17, 2021 5:21:09 PM
Attachments:	DEEP VZN Decision Tree_v12.pdf DEEP VZN Consortium Technical Application[37].docx

Hi everyone!

It has indeed been a great experience for almost 6 months working on this since we first started brainstorming on DEEP Vision. I want to thank everyone for the great insights and world-leading inputs along the way!

I've attached here the main technical section of the proposal we submitted last night, plus the optional decision tree we submitted as an annex that shows the flow of the major testing platforms/concepts and then the feedback loops to refine our various strategies along the way. For best effect, open the decision tree in Acrobat and look at it in the two-page (side-by-side) view. A huge shout-out to Eunah Cho at OHI for taking our initial caveman like etch-a-sketch drawings and turning them into a really neat and unified graphics package!

We of course ask that you keep these documents close hold/confidential within our group since we don't want this floating around somewhere and then somehow interfering with the review process.

I hope everyone has as great weekend, and we'll keep you in the loop as we hear back from USAID… most likely not until August if we're lucky!

Thanks again and all the best,

-Brian

From: Christine Kreuder Johnson <ckjohnson@UCDAVIS.EDU>
Date: Thursday, June 17, 2021 at 10:59 AM
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<benhur.lee@mssm.edu>, mariana.leguia <mariana.leguia@pucp.edu.pe>, ztq9@cdc.gov <ztq9@cdc.gov>, Moeder, Vanessa <vmoeder@illumina.com>, Marcela Uhart <muhart@ucdavis.edu>, Zimmerman, Dawn <ZimmermanD@si.edu>, Alex <Alex@conservationxlabs.com>, gschroth <gschroth@illumina.com>, Ava Sullivan <sullivan@ecohealthalliance.org>, Latinne, Alice <alatinne@wcs.org>, Christian Lange <clange@labyrinthgh.com>, Olson, Sarah <solson@wcs.org>, Paul Bunje <Paul@conservationxlabs.com>, hkretser <hkretser@wcs.org>, ccs8@cdc.gov <ccs8@cdc.gov>, Jon Epstein <epstein@ecohealthalliance.org>, Keatts, Lucy <lkeatts@wcs.org>, Tierra Smiley Evans <tsmevans@ucdavis.edu>, Pranav Sudhir Pandit <pspandit@ucdavis.edu>, Susie.Welty <Susie.Welty@ucsf.edu>, Aleksei Chmura <chmura@ecohealthalliance.org>, Alberga, Jeremy J <Jeremy.Alberga@ucsf.edu>, Eric Laing (Uniformed Services University) <eric.laing@usuhs.edu>, Mike Whelan <mike.whelan@cepi.net>, hlw2124 <hlw2124@columbia.edu>, LintonY <LintonY@si.edu>, Kathryn Vosburg <kathryn.vosburg@ucsf.edu>, Mary Guttieri <mguttieri@labyrinthgh.com>, Kaczmerek@ecohealthalliance.org <Kaczmerek@ecohealthalliance.org>, Cadhla Firth <firth@ecohealthalliance.org>, Aleksei Chmura <chmura@ecohealthalliance.org>, Maryska Kaczmarek <kaczmarek@ecohealthalliance.org>, Sarah Munro <munro@ecohealthalliance.org>, Churchill, Carolina <cchurchill@wcs.org>, Matthew Blake <mblake@ucdavis.edu>, Nicole R Gardner <nrgardner@ucdavis.edu>
Cc: Brian H Bird <bhbird@ucdavis.edu>, David John Wolking <djwolking@ucdavis.edu>, Michael Ziccardi <mhziccardi@ucdavis.edu>
Subject: DEEP VZN proposal submitted!

Colleagues,
Our proposal was submitted late last evening and we received confirmation of receipt by USAID this morning.
Brian will follow up shortly with the final version of the technical application so you can see how this all came together. We hope you will be pleased with this collaborative vision for the next steps in combatting emerging pandemic threats. We greatly appreciate your dedication, talents, and generosity in giving your time to pull this together over the last many months (including thousands of pages for the cost application) - we could not have done this without each of you.
Your collective expertise and experience is humbling. We think we have developed an exceptional proposal that reflects our strengths and a strong vision for the future of this work.
We are very grateful to have you all as partners in this endeavor, wherever this leads. We will be in touch as soon as we hear any news.
In the meantime, enjoy the summer!
/Chris

On 6/17/21, 4:40 AM, "Discovery & Exploration of Emerging Pathogens – Viral Zoonoses" <deepvzn@usaid.gov> wrote:

Confirming receipt of 8 emails.

Thank you.

On Thursday, June 17, 2021 at 1:20:07 AM UTC-4 Christine Kreuder Johnson wrote:

Dear Ms. Bradley,
It is with great pleasure that I submit, on behalf of UC Davis and our consortium partners, the attached application to implement the Discovery & Exploration of Emerging Pathogens - Viral Zoonoses (DEEP VZN) project. For your reference, a list of the documents comprising our full application is included below; these documents will be submitted in multiple emails due to the file size limitations noted in the NOFO. Thank you for your consideration.

1. Technical Application Part 1 of 3
2. Technical Application Part 2 of 3
3. Technical Application Part 3 of 3
4. Business (Cost) Application (Excel)
5. Business (Cost) Application (Narrative) Part 1 of 5
6. Business (Cost) Application (Narrative) Part 2 of 5
7. Business (Cost) Application (Narrative) Part 3 of 5
8. Business (Cost) Application (Narrative) Part 4 of 5
9. **Business (Cost) Application (Narrative) Part 5 of 5**

Sincerely,
Christine K Johnson
One Health Institute
School of Veterinary Medicine
University of California
1089 Veterinary Medicine Drive
Davis, California 95616
+1.530.752.1238

Laboratory Protocols for PREDICT II Surveillance

Priority Viral Family Protocols

Version 2: 2017-01

Dr T. Goldstein[2], Dr D. Rejmanek[2], Dr A. Tremeau-Bravard[2], E. Liang[1,4], I. Navarrete[1,4], Dr I. Lipkin[1], Dr S.J Anthony[1,4]

[1]=Columbia University, [2]=UC Davis, [3]=Global Viral Forecasting, [4]=EcoHealth Alliance, [5]=Wildlife Conservation Society

* with contributions from Dr T. Seimon[1,5], Dr J. Fair[3], Dr B. Schneider[3], Dr J. Epstein[4], K.A. Jackson[2], Dr E. Mertens[2], B. Smith

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I. Introduction

Predict Diagnostics

The diagnostic scope of PREDICT is uniquely ambitious, and its success clearly dependent on the availability of good molecular assays. The purpose of this document is therefore to provide a list of recommended molecular assays for PREDICT collaborating laboratories.

The approach taken for molecular testing of PREDICT samples will vary depending on sample, location and laboratory. Our main approach is broad testing for indiscriminate detection of all viruses within a particular family or genus by consensus PCR – and in so doing potentially identify novel agents belonging to that particular taxonomic group. In some instances testing will focus on the detection of specific pathogens of interest, in order to gain information on prevalence and pathogenesis (including host and tissue tropisms) of a known virus. Pathogens and sample types for testing will be prioritized with the lab and field teams based on the interfaces, host species sampled and potential routes of transmission of viruses between the wildlife sampled and humans.

Unfortunately, many of the published protocols for this latter (consensus-PCR) approach are not well validated, and their efficacy on diverse clinical and field samples, such as we have with PREDICT, is currently quite undetermined. Thus, these assays are not considered to be validated diagnostic tests, and are designed more for pathogen discovery. These tests often simply represent primer pairs that have been designed from available sequences for a specific study or purpose. On occasion their use may have been demonstrated on diverse laboratory virus strains within a family or genus, or (infrequently) even on clinical samples. However for the most part, the literature is not rich with protocols that might be fit for our purposes. That is, protocols that will detect divergent members of different viral families and/or genera in clinical and field samples. Thus we have modified and designed a number of these assays in an attempt to develop protocols that are more broadly reactive.

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Use in Clinical Settings

It is important to recognize that the assays present in these protocols, are not validated diagnostic assays. Their sensitivity and specificity for known pathogens has not been assessed in the majority of cases. There is an important distinction between diagnostics for known pathogens (where sensitivity and specificity are known) and discovery (where they are not). These assays are not intended for diagnostics.

Diagnostic assays are often designed against specific agents and are therefore more sensitive for detecting those specific agents. Consensus PCR is broadly reactive thus their strength is for viral discovery, however, they are not as sensitive and have the potential to result in false negatives. For this reason, they are inappropriate for clinical diagnostics when specific assays exist.

We are not recommending that diagnostic labs or facilities replace specific assays with these consensus PCR assays when screening for known pathogens, but rather start incorporating them into diagnostic investigations when specific assays are unavailable or have failed to produce a positive result. At this point, this would be considered discovery and not diagnostics.

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Reagents

We realize that each laboratory will no doubt be familiar with, or have preference for, certain reagents and PCR kits. All the protocols in this manual have been tested using one set of reagents used routinely in our laboratories and all assays performed well using these reagents. Thus, we suggest PREDICT collaborating laboratories use the same reagents described in our document or in the original publication of a particular assay. The details provided here refer to kits used by scientists at Columbia University's Center for Infection and Immunity (CII), University of California Davis Wildlife Health Center and Global Viral Forecasting Inc.

- RNA extraction is performed with using the Zymo Research Direct-zol RNA MiniPrep Kit . Catalogue number: Zymo Research Cat **# R2050 (kit of 50), R2052 (kit of 200)**; http://www.zymoresearch.com/rna/total-rna-purification/samples-in-tri-reagent-trizol-etc/direct-zol-rna-miniprep

- Reverse-transcription is performed using Invitrogen's SuperScript III First-strand cDNA synthesis kit, according to the manufacturer's instructions.
  Catalogue Number: **18080-400 (50 reactions)**

- PCR is performed using Invitrogen Platinum TAQ DNA polymerase kit, according to the manufacturer's instructions.
  Catalogue Number: **10966-026 (250 x 25 µl reactions)**

II. Pan (Consensus) RNA Virus Assays

Reverse-Complementation

Samples to be tested for RNA viruses must first be reverse-transcribed (RT) in order to provide a suitable cDNA template for PCR. **We recommend that cDNA is generated prior to PCR in a separate reaction, and primed by random hexamers (ie. perform two-step PCR)** using Invitrogen's SuperScript III First-strand cDNA synthesis kit**.** This approach has been taken as sample quantity is often limited and budgetary restrictions and/or the availability of reagents also influence the preference to target cDNA (RT performed prior to PCR in a separate reaction).

Coronaviruses

NOTE: Please use both Coronavirus protocols for screening all samples. These two assays target non-overlapping regions of the RNA-Dependent RNA Polymerase in ORF 1b and it is useful to have both regions for phylogenetic discrimination.

PROTOCOL P-001

REFERENCE: Quan, PL et al (2010). MBio 1:1-9 (e00208-10)

NOTES: Reverse-transcription performed separately using Superscript III, followed by nested PCR. On the Human Coronavirus genome (strain 229E) it roughly amplifies the region 17,480-17,820.

TARGET: RNA-Dependent RNA Polymerase (RdRp)

PRIMERS:

Round 1:

CoV-FWD1:	CGTTGGIACWAAYBTVCCWYTICARBTRGG
CoV-RVS1:	GGTCATKATAGCRTCAVMASWWGCNACATG

Round 2:

CoV-FWD2:	GGCWCCWCCHGGNGARCAATT
CoV-RVS2:	GGWAWCCCCAYTGYTGWAYRTC

PROTOCOL: 95°C 5 min, then 15 cycles of 95°C for 30 seconds, 65°C for 30 seconds and 72°C for 45 seconds, then 40 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 45 seconds. Finish with 72°C for 7 minutes.

Same protocol for round 2 but for 35 cycles.

AMPLICON: First Round: 520 bp
Second Round**:** 328 bp

CONTROL: Universal Control 1, or appropriate Coronavirus cDNA

PROTOCOL P-002

REFERENCE: Modified from Watanabe, S *et al* (2010). Emerging Infectious Diseases 16:1217-1223

NOTE: Like the Quan protocol, this assay also targets the polymerase. However, it targets a different region slightly more upstream. On the Human Coronavirus genome (Strain 229E) it targets roughly nucleotides 14,370-14,750. If you are looking for coronaviruses in bats, this may be a good protocol to use because many of the partial sequences that are published on CoV (in bats) have been generated using these primers.
Please note that this assay has been modified from the original publication: Initial primer sequences have been modified to increase the ability of the assay to detect widely variant coronaviruses. A second, heminested step has also been added to increase sensitivity. This step can be performed using a forward primer that is optimized for bat viruses, or other coronaviruses, depending on the sample being investigated.

TARGET: RNA-Dependent RNA Polymerase (RdRp)

PRIMERS: Round 1:
CoV-FWD3: GGTTGGGAYTAYCCHAARTGTGA
CoV-RVS3: CCATCATCASWYRAATCATCATA

Round 2:
CoV-FWD4/Bat: GAYTAYCCHAARTGTGAYAGAGC
(or CoV-FWD4/Other: GAYTAYCCHAARTGTGAUMGWGC)
CoV-RVS3: Same reverse primer as round 1

PROTOCOL: 94°C 2 min, then 35 cycles of 94°C for 20 seconds, 50°C for 30 seconds and 72°C for 30 seconds. Finish with 72°C for 7 mins.

Same protocol for both round 1 and round 2.

AMPLICON: First Round: 440 bp
Second Round: 434 bp

CONTROL: Universal Control 1, or appropriate Coronavirus cDNA

Filoviruses

PROTOCOL P-003

REFERENCE: Unpublished, Designed at UCD, modified from Zhai, J *et al* (2007). Journal of Clinical Microbiology, 45:224-226

NOTES: The previous primers used in PREDICT were well validated for known filoviruses, but we were concerned their lack of degeneracy might inhibit their ability to detect novel viruses of this group and that the one round of PCR might not be sensitive enough for our needs with field samples.

Thus we redesigned the set of modified primers to introduce more degeneracy into the sequence to improve their use for pathogen discovery and also to add a nested PCR step ton increase sensitivity. These primers are able to detect known Ebola and Marburg viruses, as well as the divergent bat filovirus, Lloviu virus, and we hope will also improving broad reactivity.

Please note that these primers may produce non-specific products of the incorrect size in clinical samples, so labs should not follow up on bands are clearly the wrong size, and focus only on bands that are approximately 630 bp. All positives must be confirmed by sequencing.

TARGET: L-Gene

PRIMERS: Round 1:

Filo-MOD-FWD:	TITTYTCHVTICAAAAICAYTGGG
FiloL.conR:	ACCATCATRTTRCTIGGRAAKGCTTT

Round 2:

Filo-MOD-FWD:	TITTYTCHVTICAAAAICAYTGGG
Filo-MOD-RVS:	GCYTCISMIAIIGTTTGIACATT

PROTOCOL: 94°C for 5 min, followed by 40 cycles of 94°C for 1min, 52°C for 1min and 72°C for 1min. Finish with a final extension of 72°C for 7min.

Same protocol for rounds 1 and 2.

AMPLICON: First Round: 680 bp (round 1 band may not always be visible)
Second Round: 630 bp
Please note: In the nested round 2 assay a larger second band can sometimes appear at >750 bp, please do not consider this band as positive, the lower 630bp band is the correct size in a positive sample

CONTROLS: Appropriate filovirus control (provided)

Flaviviruses

PROTOCOL P-004

REFERENCE: Moureau, G *et al,* (2007). Vector-borne and zoonotic diseases 7:467-477

NOTES: We perform this assay using conventional PCR on cDNA following reverse-transcription, and recommend this method. This is a good screening assay. Positive samples should be sequenced to confirm the presence of a flavivirus, and then followed with the Unpublished UCD Designed Flavi assay below to try to obtain additional sequence for further identification.

Please note: While this test works very well overall, some species (particularly insects) are known to contain integrated flaviviral sequences that can, on occasion, be detected using this assay. The confirmation of all PCR products by sequencing is therefore extremely important to confirm a positive result.

TARGET: NS5 gene

PRIMERS: Flavi-FWD: TGYRTBTAYAACATGATGGG
Flavi-RVS: GTGTCCCAICCNGCNGTRTC

PROTOCOL: 95°C 5 min, then 45 cycles of 94°C for 15 sec, 50°C for 30 sec and 72°C for 45 sec. Finish with 72°C for 10 minutes for final elongation.

AMPLICON: ~270 bp

CONTROL: Universal Control 1, or appropriate flavivirus control.

Paramyxoviruses

PROTOCOL P-005

REFERENCE: Tong, S *et al*, (2008). Journal of Clinical Microbiology, 46:2652-2658

NOTES: This is a conventional hemi-nested PCR, with the same reverse primer used for both rounds. The paper provides good validation of the primers against various paramyxoviruses, but when applied to clinical samples this assay can cross-react with host sequences quite readily. We have seen that this particular problem with bat samples.

Note: When a paramyxovirus *is* present in a sample, the assay does seem to work well, and (mostly) amplifies a single and specific product, with very little non-specific amplification. Investigators should therefore

proceed cautiously with any sample that looks to have products that are 'almost' the right size, but which also have a high background of non-specific amplification. It is probable that such samples are in fact negative.

TARGET: Polymerase (*pol*) gene

PRIMERS: Round 1
PAR-F1: GAAGGITATTGTCAIAARNTNTGGAC
PAR-R: GCTGAAGTTACIGGITCICCDATRTTNC

Round 2
PAR-F2: GTTGCTTCAATGGTTCARGGNGAYAA
PAR-R: Same Reverse primer as round 1

PROTOCOL: Round 1:
94°C for 5 min, followed by 40 cycles of 94°C for 1min, 48°C for 1min and 72°C for 1min. Finish with a final extension of 72°C for 7min.

Round 2:
94°C 2 min, then 40 cycles of 94°C for 30 seconds, 48°C for 30 seconds, 72°C for 30 seconds. Finish with 72°C for 7 minutes.

AMPLICON: Round 1**: ~**639 bp (*Note: Band many not be visible on a gel after Round 1*)
Round 2: ~561 bp

CONTROL: Universal Control 1, or appropriate paramyxovirus RNA

Influenzas

PROTOCOL P-006

REFERENCE: Anthony *et al.* (2012). MBio 3: e00166-12.

NOTE: Two-step PCR is performed using Superscript to make cDNA. This assay has been used successfully identify a variety of 'classic' influenza A viruses in different species. It will not however detect the newly identified divergent bat 'influenza A-like' viruses. A more divergent assay below is provided that is capable of detecting these. Please also note that subtype cannot be inferred from the sequence of the matrix. While sequence(s) may match certain subtypes on blast, this must not be considered diagnostic for that (or any other) subtype. Only amplification of the H and N genes can confirm subtype.

TARGET: M gene

PRIMERS: FLUAV-M-U44: GTCTTCTAACCGAGGTCGAAACG
FLUAV-M-L287: GCATTTTGGACAAAGCGTCTACG

PROTOCOL: 94°C 2 min, then 45 cycles of 94°C for 30 seconds, 52°C for 30 seconds and 72 °C for 30 seconds. Finish with 72°C for 7 minutes.

AMPLICON: 243 bp

CONTROLS: Universal Control 2, or appropriate influenza A RNA

PROTOCOL P-007

REFERENCE: Liang, E. Unpublished. Developed at CII.

NOTES: Consensus based on 2009-2013 published influenza A sequences. This assay was designed to amplify both 'classic' influenza A viruses and the newly identified divergent bat 'influenza A-like' viruses.

This assay uses a touch-down protocol. While a nested assay is presented here, we have (to date) not seen any increased sensitivity from the nested round. For now, we recommend running both rounds in order to assess performance – however be sure to run both rounds out on gels.

Please also note that subtype cannot be inferred from the sequence of the matrix. While sequence(s) may match certain subtypes on blast, this must not be considered diagnostic for that (or any other) subtype. Only amplification of the H and N genes can confirm subtype.

TARGET: PB1

PRIMERS: Round 1
FLUAPB1-F: ATGATGATGGGNATGTTYAAYATG
FLUAPB1-R: GCNGGNCCNAKDTCRYTRTTDATCAT

Round 2
FLUAPB1-NF: GATGGGNATGTTYAAYATGYTDAGYAC
FLUAPB1-R: Same reverse primer as Round 1

PROTOCOL: *Note this is a touchdown PCR:*
95°C for 5 min, then 14 cycles of 95°C for 30 seconds, 65°C for 35 seconds (-1°C /cycle) and 72°C for 50 seconds. Then perform 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 50 seconds. Finish with 72°C for 7 minutes.

Same protocol for both round 1 and 2.

AMPLICON: Round 1: 407 bp
Round 2: 402 bp

CONTROL: Appropriate influenza A control (provided)

IV. Controls

Given the diversity of viral pathogens targeted in PREDICT surveillance strategies, the availability of appropriate control materials is of obvious concern. The ability to distinguish a true positive result from contamination (from control material, or any other source) is also particularly important.

We have produced synthetic DNA constructs for use as 'universal controls'. These DNA plasmids contain short regions of overlapping viral sequence that act as primer binding sites for different PCR assays (Figures 1-3). They have also been synthesized under the control of a T7-promoter sequence, and can therefore be transcribed into RNA for reverse-transcriptase based assays on RNA pathogens. It is important to note that these controls can *only* be used with the specific assays for which they were designed. Please refer to sections II and III for details of which control to use in each assay.

These constructs offer significant advantage to large-scale laboratory surveillance efforts, such as we have with PREDICT. The sequence spanning any two primer binding regions is unique (it contains the primer binding sites for other assays and will not be found anywhere in nature), allowing for contamination to be readily identified. They can also be produced in large quantities, safely and quickly, and distributed to PREDICT regional partners very easily. *However, there can also be one significant drawback to using these controls!* Given that the controls are in a plasmid construct, they are extremely small particles that are highly stable at room temperature and can be easily transferred onto gloves, tubes, pipetters and other equipment, if not handled carefully and with dedicated pipetters and filter tips. Additionally, since many of the protocols included here are nested PCR assays, the additional handling of PCR products and controls when transferring test samples and controls between round 1 and round 2 PCR reactions, allows ample opportunity for contamination with the controls. The primers and protocols are provided below and should be used to rule out the possibility of contamination in PCR samples prior to shipment of PCR products to other laboratories for sequencing.

Figure 1: Schematic of Universal Control 1

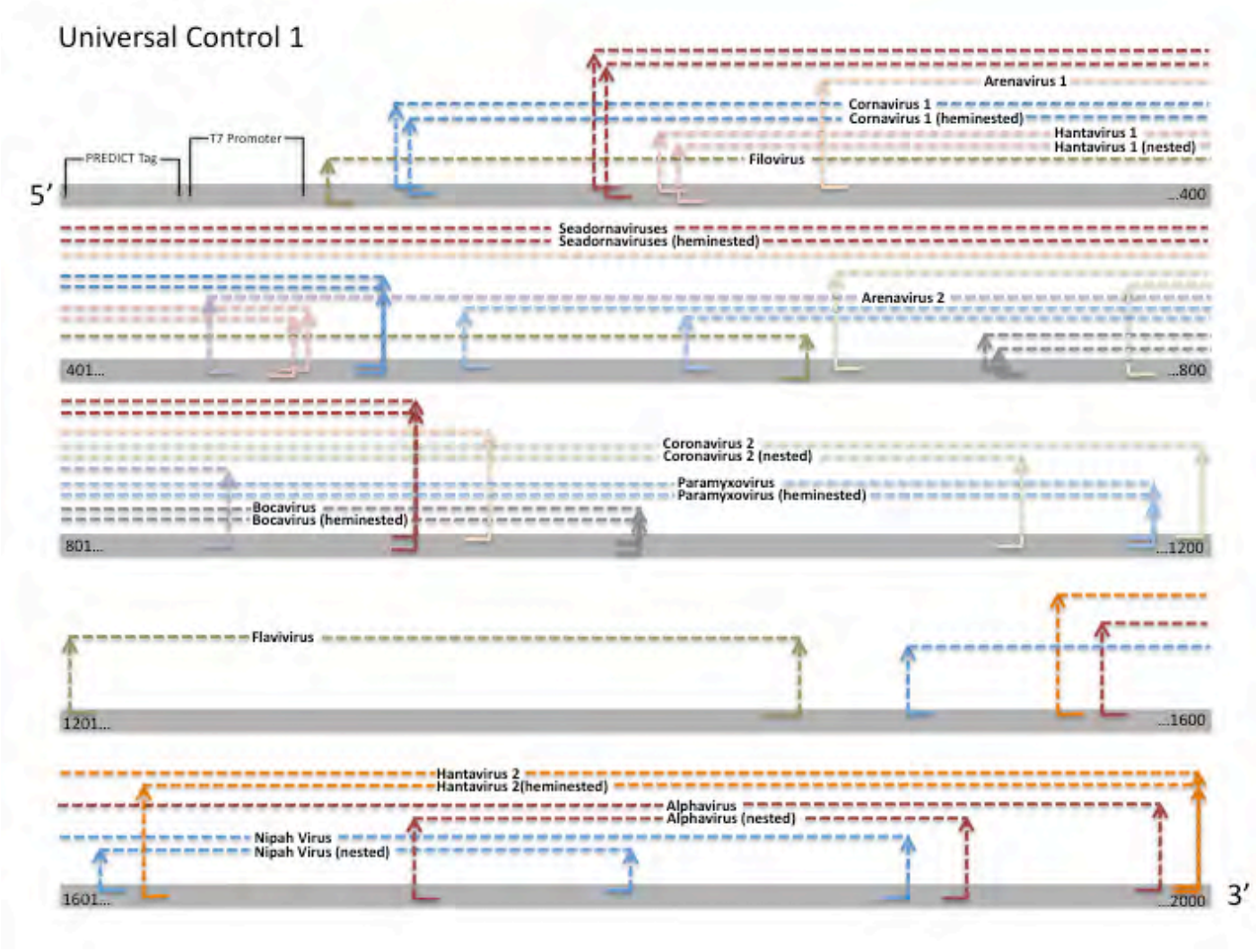

Figure 2: Schematic of Universal Control 2

Contamination Control PCR for Universal Control 1

The primers below should be used to rule out the possibility of contamination in a sample with the Universal Control 1 when a positive PCR result is obtained. This PCR should be run each time a positive result is obtained. The amplified product targets a PREDICT tag of nucleotides that corresponds to amino acid sequence P-R-E-D-I-C-T after sequencing and translation.

PRIMERS: PREDICT-Fwd: GGGCCTAGAGAAGATATTTGTACT
PREDICT-Rvs: CGCCATTGACATCCTCGAAG

PROTOCOL: 94°C for 2 min, then 40 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 minute. Finish with 72°C for 2 minutes

AMPLICON: 412 bp

Contamination Control PCR for Universal Control 2

The primers below should be used to rule out the possibility of contamination in a sample with the Universal Control 2 when a positive PCR result is obtained. This PCR should be run each time a positive result is obtained. The amplified product targets a group of nucleotides that correspond to the amino acid sequence D-A-V-I-S after sequencing and translation.

PRIMERS: DAVIS-Fwd: 5'- CGACTCACTATAGGGAGAGACTTCG -3´
DAVIS-Rvs: 5'- CCGAGTTACATAACGCTTTGATTGCC -3´

PROTOCOL: 94°C for 2 min, then 40 cycles of 94°C for 30 sec, 54°C for 30 sec, 72°C for 1 minute. Finish with 72°C for 2 minutes

AMPLICON: 318 bp

VI. Support

The purpose of this manual is to guide assay selection and control. It is not designed to present comprehensive methodologies or discuss troubleshooting, as we are not seeking to dictate the molecular diagnostics of each laboratory. That said, investigators should feel reassured that additional custom support will be available to provide further guidance beyond this manual, should it be required. Please contact Dr Simon Anthony (anthony@ecohealthalliance.org or sja2127@columbia.edu) and Dr Tracey Goldstein (tgoldstein@ucdavis.edu).

VII Appendices

All of the published papers presented in this document are included here as appendices for reference.

Laboratory Protocols for PREDICT II Surveillance - Priority Viral Families

Version 2: 2016-05

Dr T. Goldstein[2], Dr D. Rejmanek[2], Dr A. Tremeau-Bravard[2]; E. Liang[1,4] , Dr I. Lipkin[1], Dr S.J Anthony[1,4],

[1]=Columbia University, [2]=UC Davis, [3]=Global Viral Forecasting,
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  Catalogue Number: **10966-026 (250 x 25 µl reactions)**

II. Pan (Consensus) RNA Virus Assays

Reverse-Complementation

Samples to be tested for RNA viruses must first be reverse-transcribed (RT) in order to provide a suitable cDNA template for PCR. **We recommend that cDNA is generated prior to PCR in a separate reaction, and primed by random hexamers (ie. perform two-step PCR)** using Invitrogen's SuperScript III First-strand cDNA synthesis kit**.** This approach has been taken as sample quantity is often limited and budgetary restrictions and/or the availability of reagents also influence the preference to target cDNA (RT performed prior to PCR in a separate reaction).

Coronaviruses

NOTE: Please use both Coronavirus protocols for screening all samples. These two assays target non-overlapping regions of the RNA-Dependent RNA Polymerase in ORF 1b and it is useful to have both regions for phylogenetic discrimination.

PROTOCOL P-001

REFERENCE: Quan, PL et al (2010). MBio 1:1-9 (e00208-10)

NOTES: Reverse-transcription performed separately using Superscript III, followed by nested PCR. On the Human Coronavirus genome (strain 229E) it roughly amplifies the region 17,480-17,820.

TARGET: RNA-Dependent RNA Polymerase (RdRp)

PRIMERS:

Round 1:

CoV-FWD1:	CGTTGGIACWAAYBTVCCWYTICARBTRGG
CoV-RVS1:	GGTCATKATAGCRTCAVMASWWGCNACATG

Round 2:

CoV-FWD2:	GGCWCCWCCHGGNGARCAATT
CoV-RVS2:	GGWAWCCCCAYTGYTGWAYRTC

PROTOCOL: 95°C 5 min, then 15 cycles of 95°C for 30 seconds, 65°C for 30 seconds and 72°C for 45 seconds, then 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 45 seconds. Finish with 72°C for 7 minutes.

Same protocol for rounds 1 and 2.

AMPLICON: First Round: 520 bp
Second Round**:** 328 bp

CONTROL: Universal Control 1, or appropriate Coronavirus cDNA

PROTOCOL P-002

REFERENCE: Modified from Watanabe, S *et al* (2010). Emerging Infectious Diseases 16:1217-1223

NOTE: Like the Quan protocol, this assay also targets the polymerase. However, it targets a different region slightly more upstream. On the Human Coronavirus genome (Strain 229E) it targets roughly nucleotides 14,370-14,750. If you are looking for coronaviruses in bats, this may be a good protocol to use because many of the partial sequences that are published on CoV (in bats) have been generated using these primers.
Please note that this assay has been modified from the original publication: Initial primer sequences have been modified to increase the ability of the assay to detect widely variant coronaviruses. A second, heminested step has also been added to increase sensitivity. This step can be performed using a forward primer that is optimized for bat viruses, or other coronaviruses, depending on the sample being investigated.

TARGET: RNA-Dependent RNA Polymerase (RdRp)

PRIMERS: Round 1:
CoV-FWD3: GGTTGGGAYTAYCCHAARTGTGA
CoV-RVS3: CCATCATCASWYRAATCATCATA

Round 2:
CoV-FWD4/Bat: GAYTAYCCHAARTGTGAYAGAGC
(or CoV-FWD4/Other: GAYTAYCCHAARTGTGAUMGWGC)
CoV-RVS3: Same reverse primer as round 1

PROTOCOL: 94°C 2 min, then 35 cycles of 94°C for 20 seconds, 50°C for 30 seconds and 72°C for 30 seconds. Finish with 72°C for 7 mins.

Same protocol for rounds 1 and 2.

AMPLICON: First Round: 440 bp
Second Round: 434 bp

CONTROL: Universal Control 1 or (when available), or appropriate Coronavirus cDNA

Filoviruses

PROTOCOL P-003

REFERENCE: Modified from Zhai, J *et al* (2007). Journal of Clinical Microbiology, 45:224-226

NOTES: The previous primers used in PREDICT were well validated for known filoviruses, but we were concerned their lack of degeneracy might inhibit their ability to detect novel viruses of this group and that the one round of PCR might not be sensitive enough for our needs with field samples.

Thus we redesigned the set of modified primers to introduce more degeneracy into the sequence to improve their use for pathogen discovery and also to add a nested PCR step ton increase sensitivity. These primers are able to detect known Ebola and Marburg viruses, as well as the divergent bat filovirus, Lloviu virus, and we hope will also improving broad reactivity.

Please note that these primers may produce non-specific products of the incorrect size in clinical samples, so labs should not follow up on bands are clearly the wrong size, and focus only on bands that are approximately 630 bp. All positives must be confirmed by sequencing.

TARGET: L-Gene

PRIMERS: Round 1:

Filo-MOD-FWD:	TITTYTCHVTICAAAAICAYTGGG
FiloL.conR:	ACCATCATRTTRCTIGGRAAKGCTTT

Round 2:

Filo-MOD-FWD:	TITTYTCHVTICAAAAICAYTGGG
Filo-MOD-RVS:	GCYTCISMIAIIGTTTGIACATT

PROTOCOL: 94°C for 5 min, followed by 40 cycles of 94°C for 1min, 52°C for 1min and 72°C for 1min. Finish with a final extension of 72°C for 7min.

Same protocol for rounds 1 and 2.

AMPLICON: First Round: 680 bp (round 1 band may not always be visible)
Second Round: 630 bp
Please note: In the nested round 2 assay a larger second band can sometimes appear at >750 bp, please do not consider this band as positive, the lower 630bp band is the correct size in a positive sample

CONTROLS: Appropriate filovirus cDNA (provided)

Flaviviruses

PROTOCOL P-004

REFERENCE: Moureau, G *et al,* (2007). Vector-borne and zoonotic diseases 7:467-477

NOTES: We perform this assay using conventional PCR on cDNA following reverse-transcription, and recommend this method. This is a good screening assay. Positive samples should be sequenced to confirm the presence of a flavivirus, and then followed with the Unpublished UCD Designed Flavi assay below to try to obtain additional sequence for further identification.

Please note: While this test works very well overall, some species (particularly insects) are known to contain integrated flaviviral sequences that can, on occasion, be detected using this assay. The confirmation of all PCR products by sequencing is therefore extremely important to confirm a positive result.

TARGET: NS5 gene

PRIMERS: Flavi-FWD: TGYRBTTAYAACATGATGGG
Flavi-RVS: GTGTCCCAICCNGCNGTRTC

PROTOCOL: 95°C 5 min, then 45 cycles of 94°C for 15 sec, 50°C for 30 sec, 72°C for 45 sec, and 77°C for 15 sec. Finish with 72°C for 10 minutes for final elongation.

AMPLICON: ~270 bp

CONTROL: Universal Control 1, or appropriate flavivirus control.

Paramyxoviruses

PROTOCOL P-005

REFERENCE: Tong, S *et al*, (2008). Journal of Clinical Microbiology, 46:2652-2658

NOTES: This is a conventional hemi-nested PCR, with the same reverse primer used for both rounds. The paper provides good validation of the primers against various paramyxoviruses, but when applied to clinical samples this assay can cross-react with host sequences quite readily. We have seen that this particular problem with bat samples.

Note: When a paramyxovirus *is* present in a sample, the assay does seem to work well, and (mostly) amplifies a single and specific product, with very little non-specific amplification. Investigators should therefore proceed cautiously with any sample that looks to have products that are 'almost' the right size, but which also have a high background of non-specific amplification. It is probable that such samples are in fact negative.

TARGET: Polymerase (*pol*) gene

PRIMERS: Round 1
PAR-F1: GAAGGITATTGTCAIAARNTNTGGAC
PAR-R: GCTGAAGTTACIGGITCICCDATRTTNC

Round 2
PAR-F2: GTTGCTTCAATGGTTCARGGNGAYAA
PAR-R: Same Reverse primer as round 1

PROTOCOL: Round 1:
94°C for 5 min, followed by 40 cycles of 94°C for 1min, 48°C for 1min and 72°C for 1min. Finish with a final extension of 72°C for 7min.

Round 2:
94°C 2 min, then 40 cycles of 94°C for 30 seconds, 48°C for 30 seconds, 72°C for 30 seconds. Finish with 72°C for 7 minutes.

AMPLICON: Round 1**: ~**639 bp (*Note: Band many not be visible on a gel after Round 1*)
Round 2: ~561 bp

CONTROL: Universal Control 1, or appropriate paramyxovirus RNA

Influenzas

PROTOCOL P-06

REFERENCE: Anthony *et al.* (2012). MBio 3: e00166-12.

NOTE: Two-step PCR is performed using Superscript to make cDNA. This assay has been used successfully identify a variety of 'classic' influenza A viruses in different species. It will not however detect the newly identified divergent bat 'influenza A-like' viruses. A more divergent assay below is provided that is capable of detecting these. Please also note that subtype cannot be inferred from the sequence of the matrix. While sequence(s) may match certain subtypes on blast, this must not be

considered diagnostic for that (or any other) subtype. Only amplification of the H and N genes can confirm subtype.

TARGET: M gene

PRIMERS: FLUAV-M-U44: GTCTTCTAACCGAGGTCGAAACG
FLUAV-M-L287: GCATTTTGGACAAAGCGTCTACG

PROTOCOL: 94°C 2 min, then 45 cycles of 94°C for 30 seconds, 52°C for 30 seconds and 72 °C for 30 seconds. Finish with 72°C for 7 minutes.

AMPLICON: 243 bp

CONTROLS: Universal Control 2, or appropriate influenza A RNA

PROTOCOL P-07

REFERENCE: Liang, E. Unpublished. Developed at CII.

NOTES: Consensus based on 2009-2013 published influenza A sequences. This assay was designed to amplify both 'classic' influenza A viruses and the newly identified divergent bat 'influenza A-like' viruses.

This assay uses a touch-down protocol. While a nested assay is presented here, we have (to date) not seen any increased sensitivity from the nested round. For now, we recommend running both rounds in order to assess performance – however be sure to run both rounds out on gels.

Please also note that subtype cannot be inferred from the sequence of the matrix. While sequence(s) may match certain subtypes on blast, this must not be considered diagnostic for that (or any other) subtype. Only amplification of the H and N genes can confirm subtype.

TARGET: PB1

PRIMERS: Round 1
FLUAPB1-F: ATGATGATGGGNATGTTYAAYATG
FLUAPB1-R: GCNGGNCCNAKDTCRYTRTTDATCAT

Round 2
FLUAPB1-NF: GATGGGNATGTTYAAYATGYTDAGYAC
FLUAPB1-R: Same reverse primer as Round 1

PROTOCOL: *Note this is a touchdown PCR:*

95°C for 5 min, then 14 cycles of 95°C for 30 seconds, 65°C for 35 seconds (-1°C /cycle) and 72°C for 50 seconds. Then perform 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 50 seconds. Finish with 72°C for 7 minutes.

Same protocol for both round 1 and 2.

AMPLICON: Round 1: 407 bp
Round 2: 402 bp

CONTROL: Appropriate influenza A cDNA (provided)

IV. Controls

Given the diversity of viral pathogens targeted in PREDICT surveillance strategies, the availability of appropriate control materials is of obvious concern. The ability to distinguish a true positive result from contamination (from control material, or any other source) is also particularly important.

We have produced synthetic DNA constructs for use as 'universal controls'. These DNA plasmids contain short regions of overlapping viral sequence that act as primer binding sites for different PCR assays (Figures 1-3). They have also been synthesized under the control of a T7-promoter sequence, and can therefore be transcribed into RNA for reverse-transcriptase based assays on RNA pathogens. It is important to note that these controls can *only* be used with the specific assays for which they were designed. Please refer to sections II and III for details of which control to use in each assay.

These constructs offer significant advantage to large-scale laboratory surveillance efforts, such as we have with PREDICT. The sequence spanning any two primer binding regions is unique (it contains the primer binding sites for other assays and will not be found anywhere in nature), allowing for contamination to be readily identified. They can also be produced in large quantities, safely and quickly, and distributed to PREDICT regional partners very easily. *However, there can also be one significant drawback to using these controls!* Given that the controls are in a plasmid construct, they are extremely small particles that are highly stable at room temperature and can be easily transferred onto gloves, tubes, pipetters and other equipment, if not handled carefully and with dedicated pipetters and filter tips. Additionally, since many of the protocols included here are nested PCR assays, the additional handling of PCR products and controls when transferring test samples and controls between round 1 and round 2 PCR reactions, allows ample opportunity for contamination with the controls. The primers and protocols are provided below and should be used to rule out the possibility of contamination in PCR samples prior to shipment of PCR products to other laboratories for sequencing.

Figure 1: Schematic of Universal Control 1

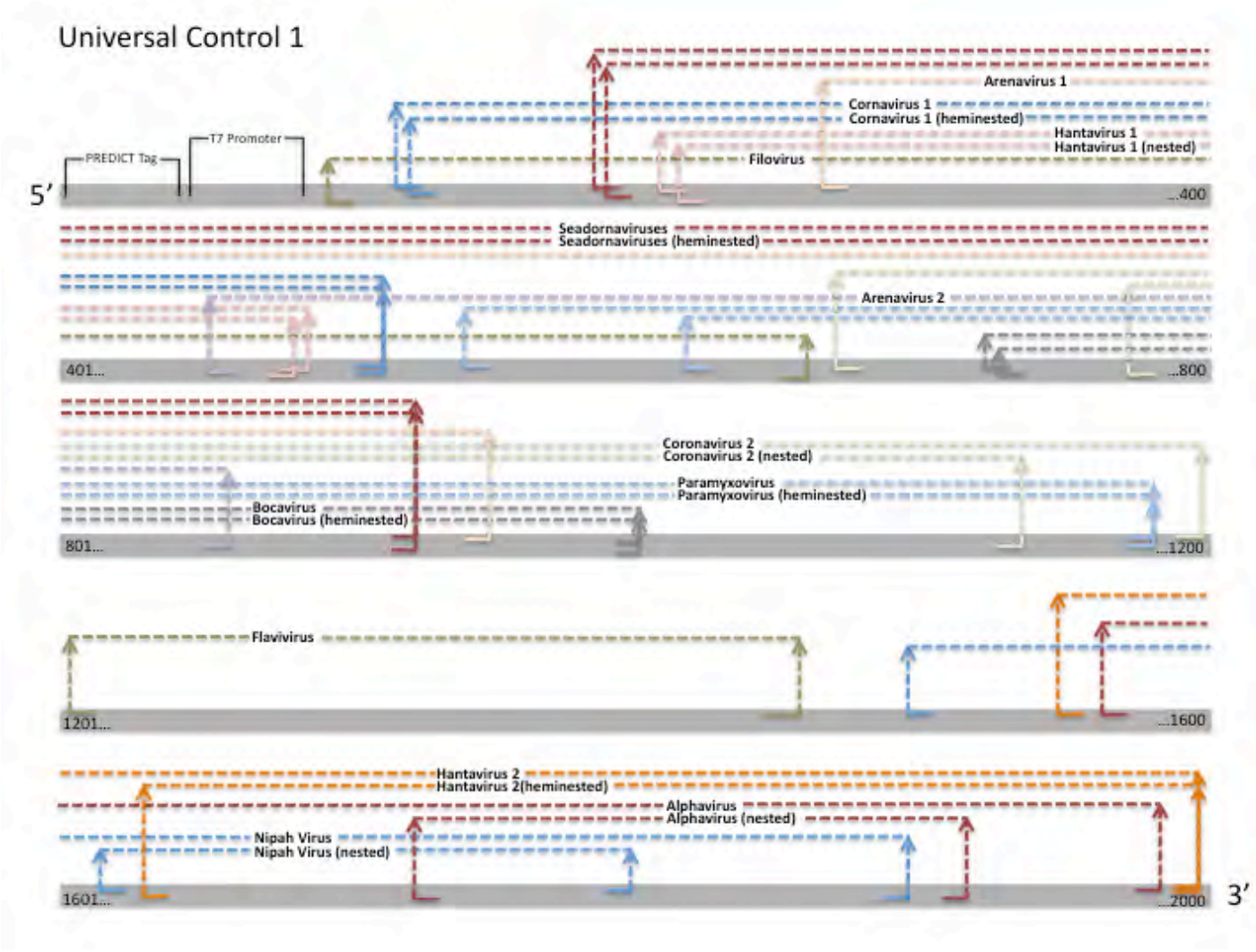

Figure 2: Schematic of Universal Control 2

Contamination Control PCR for Universal Control 1

The primers below should be used to rule out the possibility of contamination in a sample with the Universal Control 1 when a positive PCR result is obtained. This PCR should be run each time a positive result is obtained. The amplified product targets a PREDICT tag of nucleotides that corresponds to amino acid sequence P-R-E-D-I-C-T after sequencing and translation.

PRIMERS: PREDICT-Fwd: GGGCCTAGAGAAGATATTTGTACT
PREDICT-Rvs: CGCCATTGACATCCTCGAAG

PROTOCOL: 94°C for 2 min, then 40 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 minute. Finish with 72°C for 2 minutes

AMPLICON: 412 bp

Contamination Control PCR for Universal Control 2

The primers below should be used to rule out the possibility of contamination in a sample with the Universal Control 2 when a positive PCR result is obtained. This PCR should be run each time a positive result is obtained. The amplified product targets a group of nucleotides that correspond to the amino acid sequence D-A-V-I-S after sequencing and translation.

PRIMERS: DAVIS-Fwd: 5'- CGACTCACTATAGGGAGAGACTTCG -3´
DAVIS-Rvs: 5'- CCGAGTTACATAACGCTTTGATTGCC -3´

PROTOCOL: 94°C for 2 min, then 40 cycles of 94°C for 30 sec, 54°C for 30 sec, 72°C for 1 minute. Finish with 72°C for 2 minutes

AMPLICON: 318 bp

VI. Support

The purpose of this manual is to guide assay selection and control. It is not designed to present comprehensive methodologies or discuss troubleshooting, as we are not seeking to dictate the molecular diagnostics of each laboratory. That said, investigators should feel reassured that additional custom support will be available to provide further guidance beyond this manual, should it be required. Please contact Dr Simon Anthony (anthony@ecohealthalliance.org or sja2127@columbia.edu) and Dr Tracey Goldstein (tgoldstein@ucdavis.edu).

VII Appendices

All of the published papers presented in this document are included here as appendices for reference.

Identification of a Severe Acute Respiratory Syndrome Coronavirus-Like Virus in a Leaf-Nosed Bat in Nigeria

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ABSTRACT **Bats are reservoirs for emerging zoonotic viruses that can have a profound impact on human and animal health, including lyssaviruses, filoviruses, paramyxoviruses, and severe acute respiratory syndrome coronaviruses (SARS-CoVs). In the course of a project focused on pathogen discovery in contexts where human-bat contact might facilitate more efficient interspecies transmission of viruses, we surveyed gastrointestinal tissue obtained from bats collected in caves in Nigeria that are frequented by humans. Coronavirus consensus PCR and unbiased high-throughput pyrosequencing revealed the presence of coronavirus sequences related to those of SARS-CoV in a Commerson's leaf-nosed bat (*Hipposideros commersoni*). Additional genomic sequencing indicated that this virus, unlike subgroup 2b CoVs, which includes SARS-CoV, is unique, comprising three overlapping open reading frames between the M and N genes and two conserved stem-loop II motifs. Phylogenetic analyses in conjunction with these features suggest that this virus represents a new subgroup within group 2 CoVs.**

IMPORTANCE **Bats (order Chiroptera, suborders Megachiroptera and Microchiroptera) are reservoirs for a wide range of viruses that cause diseases in humans and livestock, including the severe acute respiratory syndrome coronavirus (SARS-CoV), responsible for the global SARS outbreak in 2003. The diversity of viruses harbored by bats is only just beginning to be understood because of expanded wildlife surveillance and the development and application of new tools for pathogen discovery. This paper describes a new coronavirus, one with a distinctive genomic organization that may provide insights into coronavirus evolution and biology.**

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Citation Quan, P.-L., C. Firth, C. Street, J. A. Henriquez, A. Petrosov, et al. 2010. Identification of a severe acute respiratory syndrome coronavirus-like virus in a leaf-nosed bat in Nigeria. mBio 1(4):e00208-10. doi:10.1128/mBio.00208-10.

Editor Anne Moscona, Weill Cornell Medical College

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Coronaviruses (order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae*) infect a wide range of vertebrates and cause respiratory, enteric, or less frequently, neurological diseases (1, 2). Coronaviruses were originally divided into three groups based on their antigenic cross-reactivities and nucleotide sequences (3). They have been recently reclassified by the International Committee on Taxonomy of Viruses into 3 genera, designated *Alphacoronavirus* (former group 1), *Betacoronavirus* (former group 2), and *Gammacoronavirus* (former group 3) (4). Whereas the alphacoronaviruses and betacoronaviruses are associated with diseases of mammals, including humans, the gammacoronaviruses are implicated chiefly in diseases of birds. Interest in coronaviruses was largely focused on their impact on domestic porcine and avian husbandry and their utility in animal models of virus-induced demyelination (5) until the emergence of severe acute respiratory syndrome (SARS) in 2003 (6). Thereafter, with recognition of the causative agent SARS coronavirus (SARS-CoV) (7–10) and of the presence of SARS-CoV-like viruses in Chinese horseshoe bats (*Rhinolophus* spp.) (11), efforts to explore the genetic diversity of coronaviruses and their host range intensified (12).

Bats are suggested to be important reservoir hosts of many zoonotic viruses with significant impact on human and animal health, including lyssaviruses, henipaviruses, filoviruses, and coronaviruses (13–17). Viruses of bats may be transmitted to humans directly through bites or via exposure to saliva, fecal aerosols, or infected tissues as well as indirectly through contact with infected intermediate hosts, such as swine (18). In the course of a project focused on pathogen discovery in situations where human-bat contact might facilitate more efficient interspecies transmission of emerging viruses, we surveyed bats in Nigeria. Through consensus PCR (cPCR) and unbiased high-throughput pyrosequencing (UHTS) of bat tissue samples, we identified a coronavirus that is most closely related to the genus *Betacoronavirus* (subgroup 2b), which includes SARS-CoV and SARS-CoV-like viruses. However, the genomic organization of this coronavirus, obtained from a Commerson's leaf-nosed bat (*Hipposideros commersoni*), is unique in that it is comprised of three overlapping open reading frames (ORFs) between the M and N genes and two conserved stem-loop II motifs (s2m). Based on these observations and phylogenetic analyses, we propose that this new member of

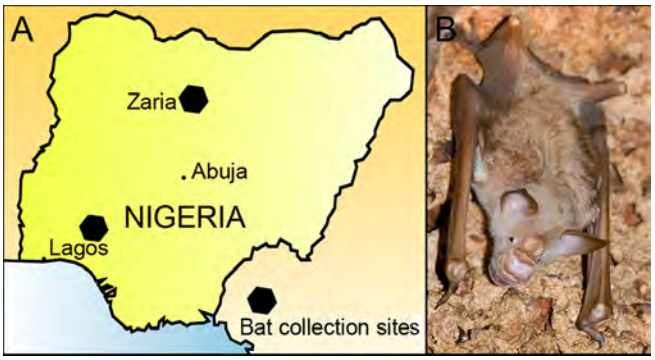

FIG 1 (A) Map of Nigeria showing the locations of bat collection sites. (B) Photograph of a male Commerson's leaf-nosed bat (*Hipposideros commersoni*), courtesy of Ivan V. Kuzmin, reproduced with permission.

the family *Coronaviridae*, tentatively named Zaria bat coronavirus (ZBCoV) after the city near to where the bat was captured, represents a new subgroup of group 2 CoVs.

RESULTS

Identification of a coronavirus in intestinal tissue of a Commerson's leaf-nosed bat (*Hipposideros commersoni*). Total RNA extracts from gastrointestinal tract (GIT) specimens obtained from 33 bats of 6 different species (*Eidolon helvum, Hipposideros commersoni, Pipistrellus* sp., *Rousettus aegyptiacus, Scotophilus nigrita*, and *Scotophilus leucogaster*) captured at 2 different sites from a roost inside a cave in Nigeria (Fig. 1A) were screened for the presence of coronaviruses by consensus PCRs of a 400-nucleotide (nt) fragment of the RNA-dependent RNA polymerase (RdRp) gene. One specimen obtained from a Commerson's leaf-nosed bat (Fig. 1B) yielded products that shared no more than 70% nt identity to any known coronavirus. RNA from ZBCoV was submitted for UHTS, resulting in a library comprising 74,133 sequence reads. Alignment of unique singleton and assembled contiguous sequences to the GenBank database (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (Blastn and Blastx) (19) indicated coverage of approximately 6,500 nt of sequence distributed along coronavirus genome scaffolds and homology to regions of replicase, spike (S), and nucleocapsid (N) sequences.

Genome organization and coding potential of ZBCoV. The additional genomic sequence of ZBCoV was determined by filling in gaps between UTHS reads, applying consensus PCRs, and 3′ and 5′ rapid amplification of cDNA ends (RACE). Overlapping primer sets based on the draft genome were synthesized to facilitate sequence validation by conventional dideoxy sequencing. Due to exhaustion of the sample, we were unable to completely sequence the open reading frame 1ab (ORF 1ab) region (Fig. 2A).

ZBCoV has a genome organization similar to that of other coronaviruses, with the following characteristic gene order:

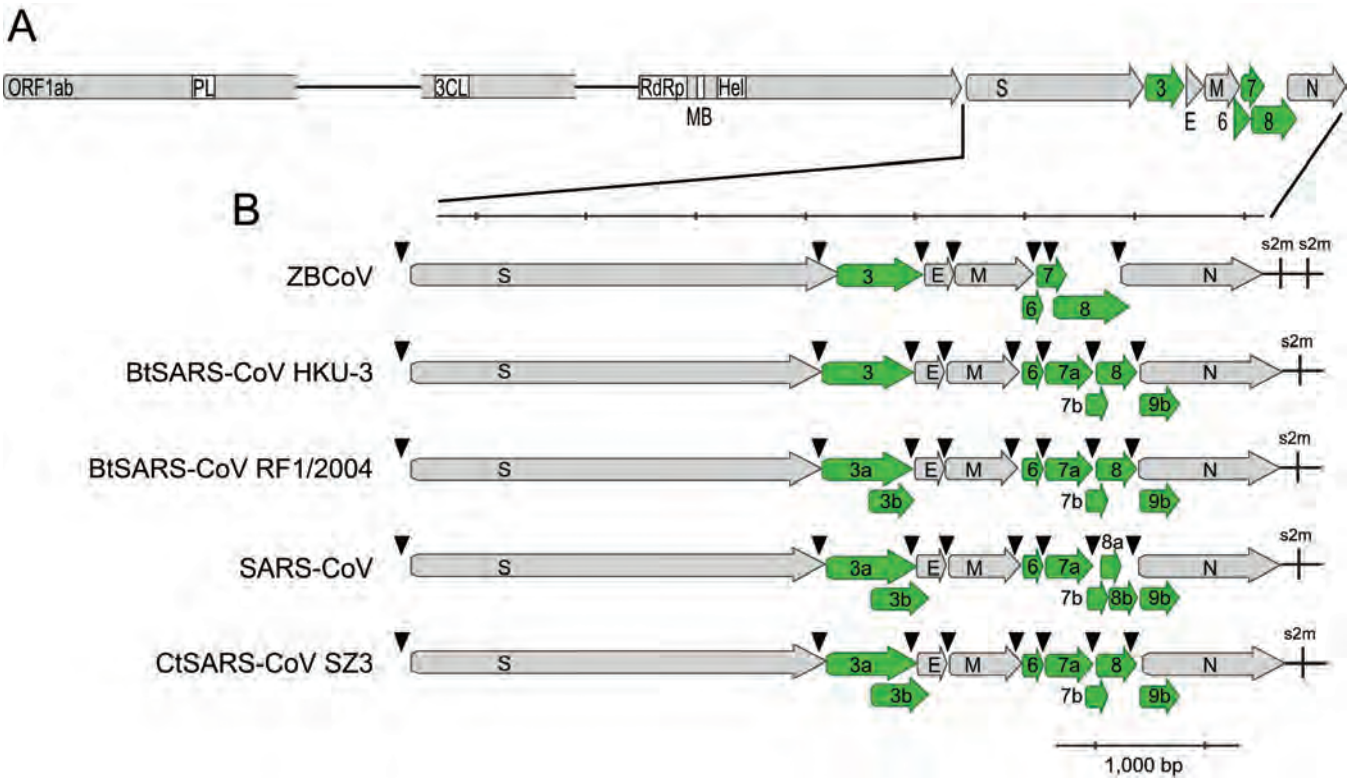

FIG 2 Genome organization of ZBCoV in comparison to that of representative coronaviruses from subgroup 2b. (A) Overall genome organization of ZBCoV. The ORF 1ab, spike (S), envelope (E), membrane (M), and nucleocapsid (N) genes are shown in gray arrows, whereas putative accessory genes ORF 3, ORF 6, ORF 7, and ORF 8 are indicated as 3, 6, 7, and 8 and illustrated by green arrows. The following conserved functional domains in ORF 1ab are represented in boxes: papain-like protease (PL), 3C-like protease (3CL), RNA-dependent RNA polymerase (RdRp), metal ion-binding domain (MB), and helicase (Hel). The two regions in ORF 1ab where sequences are incomplete are indicated by black lines. (B) Expanded diagram of the 3′ region of the ZBCoV genome in comparison to representative CoVs from subgroup 2b. TRS motifs and s2m are represented by black arrowheads and vertical lines, respectively.

TABLE 1 ORFs and putative TRS motifs

ORF	Length in:		TRS
	nt	aa	
1ab	NC[a]	NC	$ACGAAC_{221}AUG$
Spike	3,897	1,299	ACGAACAUG
3	750	250	$ACGAAC_{28}AUG$
Envelope	237	79	$ACGAAC_{23}AUG$
Membrane	729	243	$ACGAAC_{30}AUG$
6	147	49	NA[b]
7	237	79	$ACGAAC_{3}AUG$
8	654	218	$ACGAAC_{10}AUG$
Nucleocapsid	1,260	420	$ACGAAC_{12}AUG$

[a] NC, not complete.
[b] NA, not applicable.

5′-replicase ORF 1ab-spike (S)-envelope (E)-membrane (M)-nucleocapsid (N)-3′. Both the 5′ and 3′ ends contain short untranslated regions of 297 nt and 363 nt, respectively. The conserved putative transcription regulatory sequence (TRS) motif 5′-ACGAAC-3′ identified in subgroup 2b, 2c, and 2d viruses (2) is present in ZBCoV at the 3′ end of the leader sequence and upstream of potential initiating methionine residues of each ORF except ORF 6 (Table 1).

All domains within replicase polyproteins of coronaviruses that are implicated in viral replication are found in ZBCoV, including the papain-like protease (PL^{pro}), 3C-like protease ($3CL^{pro}$), RNA-dependent RNA polymerase (RdRp), and helicase (Hel) domains (Fig. 2A). ORFs consistent with the S, E, M, and N proteins present in all other coronaviruses are also present in ZBCoV (Table 1; Fig. 2). Pairwise identity (I) and similarity (S) comparisons of a deduced amino acid sequence of ZBCoV to that of representative coronaviruses in other groups showed that the predicted proteins of ZBCoV are more similar to those of subgroup 2b CoVs than to those of other subgroups, with Hel and RdRp having the highest homologies (Hel: I, 80%; S, 90%; RdRp: I, 74%; S, 85%) and the S protein having the lowest (I, 36 to 38%; S, 50 to 53%) (http://cait.cumc.columbia.edu:88/dept/greeneidlab/IdentificationofaSARS-Coronavirus-likevirusinaleaf-nosedbatinNigeria.html).

The putative spike (S) protein of ZBCoV, comprising 1,299 amino acids (aa) in length, is slightly larger than those of other subgroup 2b CoVs (see Table S8 in the supplemental material). ZBCoV showed the highest amino acid conservation to human and civet SARS-CoV (I, 38%; S, 53%) (http://cait.cumc.columbia.edu:88/dept/greeneidlab/IdentificationofaSARS-Coronavirus-likevirusinaleaf-nosedbatinNigeria.html). Pfam (20) analysis identified a spike receptor binding domain (PF09408) that corresponds to the immunogenic receptor binding domain that binds to angiotensin-converting enzyme 2 (ACE2) and the coronavirus S1 (PF01600) and S2 (PF01601) spike glycoprotein domains. Transmembrane region prediction (TMHMM 2.0) (21) revealed a long ectodomain (aa 1 to 1240), a transmembrane domain near the C-terminal end (aa 1241 to 1263), and a short cytoplasmic tail (aa 1264 to 1298). A predicted signal peptide (SignalP 3.0) ($P = 1$) (22) was identified with a cleavage site ($P = 0.768$) between residues A_{16} and A_{17}. NetNGlyc 1.0 identified 25 putative N-linked glycosylation sites. The S protein of ZBCoV displays major sequence differences compared to that of subgroup 2b CoVs, especially in the S1 domain involved in receptor binding. The critical residues suggested to be important for the cleavage of the SARS-CoV S protein are present in the S protein of ZBCoV (23–25) (see Fig. S1A in the supplemental material). Motifs at the carboxyl terminus of the S protein that are conserved among coronaviruses are also found in the ZBCoV S protein, including the conserved motif Y(X)KWPW(Y/W)(V/I)WL present as Y_{1237}EKWPWYIWL and the cysteine-rich cytoplasmic tail (10) (see Fig. S1B in the supplemental material).

In addition to the five genes present in all genomes, coronaviruses also have several group-specific genes between the S gene and the 3′ end of the genome that encode accessory proteins (Fig. 2) (26, 27).

An ORF (ORF 3) encoding a putative 250-aa protein was observed between the S and E proteins of ZBCoV (Table 1). ORF 3 corresponds to the genomic position of ORF 3a in subgroup 2b CoVs. Similar to subgroup 2b CoVs, ORF 3 is the largest accessory gene of ZBCoV and is 75 nt shorter than ORF 3a of subgroup 2b CoVs (see Table S8 in the supplemental material). ORF 3 shows 21 to 23% aa identity and 31 to 35% aa similarity to the ORF 3a protein of subgroup 2b CoVs (see Table S9 in the supplemental material). Pfam analysis showed a relationship with PF11289, a viral family protein of an unknown function; TMHMM analysis predicts the presence of 4 transmembrane regions, spanning residues P_{43} to L_{65}, A_{72} to E_{94}, V_{99} to L_{121}, and Y_{196} to V_{218}. NetOGlyc 3.1 predicted two potential O glycosylation sites in ZBCoV. ORF 3 contains only a portion of the cysteine-rich domain identified in the ORF 3a protein of SARS-CoV; however, the cysteine potentially involved in ORF 3a protein polymerization (28) is present in ORF 3. No signal peptide, YXXΦ, or diacidic motifs were identified in ORF 3 of ZBCoV (29).

ZBCoV has a set of ORFs located between the M and N genes that are not shared by any of the known coronaviruses. These ORFs, ORF 6, ORF 7, and ORF 8, encode predicted proteins of 49, 79, and 218 aa, respectively (Table 1). A TRS was identified upstream of ORF 7 and ORF 8 but not ORF 6. ORF 6 overlaps with the M gene at the 3′ end by 101 nt, ORF 7 overlaps with ORF 6 by 31 nt, and ORF 8 overlaps with ORF 7 and the N gene by 83 and 35 nt, respectively. Blastx and Pfam analyses of ORF 6, ORF 7, and ORF 8 revealed no significant similarities or functional domains. Pfam analysis of ORF 7 indicated nonsignificant associations to the PRA1 (prenylated Rab acceptor 1) proteins (PF03208) (E value = 0.02) and the 7 transmembrane G-protein-coupled-receptor protein families (PF10323) (E value = 0.025). TMHMM analysis of ORF 7 suggested the presence of a transmembrane region between residues L_{10} and I_{32}. No signal peptide was predicted.

TMHMM and SignalP analyses of ORF 6 indicated no transmembrane region or signal peptide. TMHMM analysis of ORF 8 predicted 2 transmembrane regions, and a third transmembrane region located downstream was predicted by TMpred (30). SignalP revealed a signal peptide ($P = 0.988$) with a putative cleaved signal sequence ($P = 0.804$) between residues G_{29} and A_{30}.

At only 788 nt, the region in ZBCoV between the M and N genes is significantly shorter than those observed for subgroup 2b CoVs (see Table S8 in the supplemental material). Alignment of the region between the M and N genes of ZBCoV with those of subgroup 2b CoVs indicated large deletions in ZBCoV (see Fig. S2 in the supplemental material).

Another distinctive genomic feature of ZBCoV is the presence downstream from the N gene of two conserved motifs corresponding to the conserved stem-loop II motif (s2m) (31). A unique s2m is observed in coronaviruses from subgroups 2b, 3a, and 3c and in astroviruses and in the picornavirus equine rhinitis

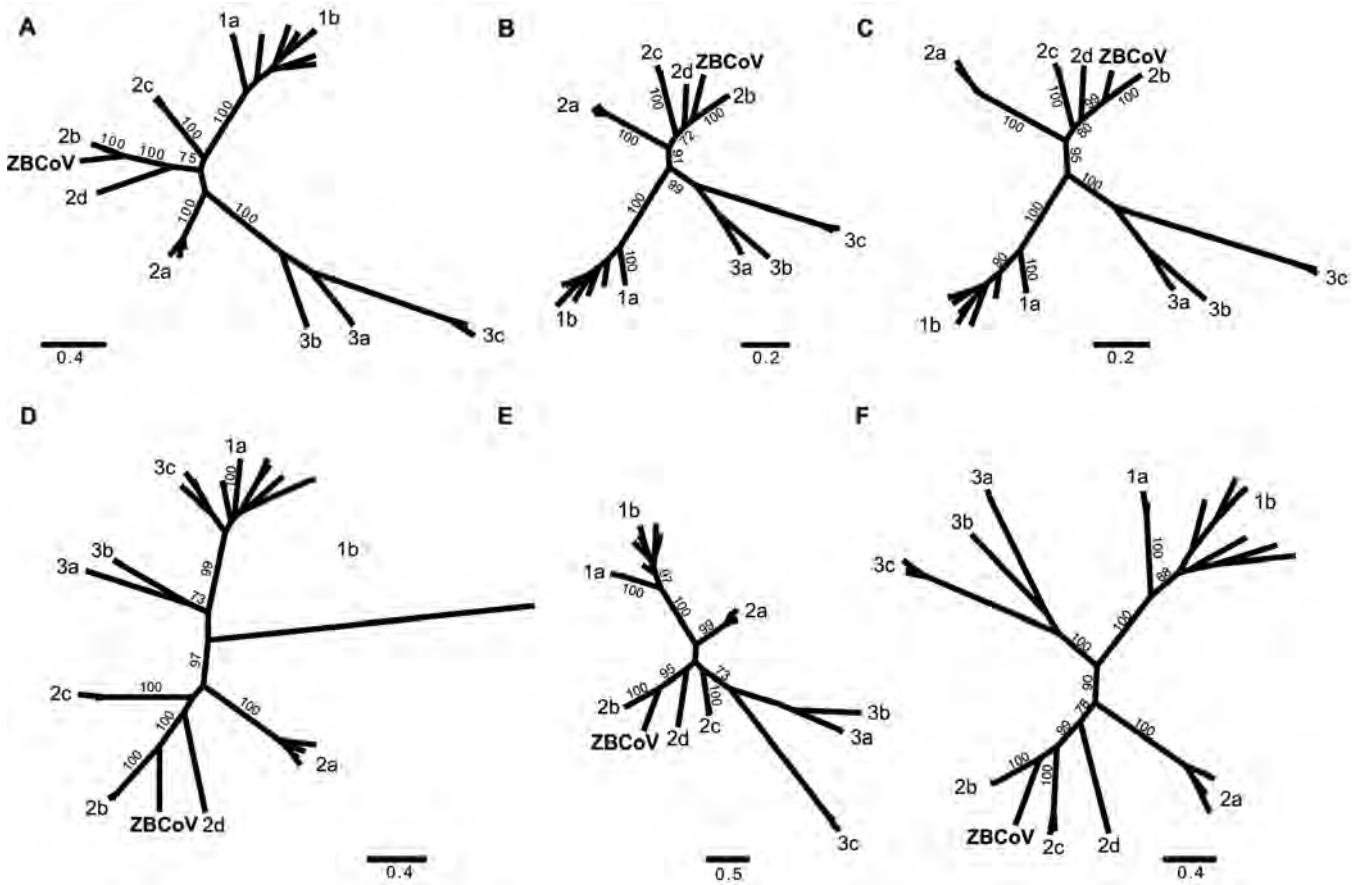

FIG 3 Phylogenetic analysis of the 3CL^{pro}, RdRp, Hel, S, M, and N proteins of ZBCoV. Unrooted maximum likelihood phylogenies of the 3CL^{pro} (A), RNA-dependent RNA polymerase (B), helicase (C), spike (D), membrane (E), and nucleocapsid (F) proteins. All phylogenies were constructed using the complete amino acid alignments of each protein, with the exception of RdRp (partial region available) and spike (only an 884-aa region could be reliably aligned). The scale bar indicates the number of substitutions per amino acid site. The numbers at each branch node represent the maximum likelihood bootstrap support; only major nodes where values exceed 70% are shown. The CoV subgroups are indicated as 1a and b, 2a to d, and 3a to c, and the following sequences obtained from GenBank were included, with the GenBank accession numbers given in parentheses: PRCV, porcine respiratory coronavirus (DQ811787); FIPV, feline infectious peritonitis virus (AY994055); HCoV-229E, human coronavirus 229E (NC_002645); HCoV-NL63, human coronavirus NL63 (NC_005831); BtCoV-512/2005, bat coronavirus 512/2005 (NC_009657); BtCoV-HKU2, bat coronavirus HKU2 (NC_009988); BtCoV-1B, bat coronavirus 1 B (NC_010436); BtCoV-1A, bat coronavirus 1A (NC_010437); BtCoV-HKU8, bat coronavirus HKU8 (NC_010438); BCoV, bovine coronavirus (NC_003045); HCoV-OC43, human coronavirus OC43 (NC_005147); HCoV-HKU1, human coronavirus HKU1 (NC_006577); MHV, mouse hepatitis virus (NC_006577); PHEV, porcine hemagglutinating encephalomyelitis virus (NC_007732); ECoV, equine coronavirus (NC_010327); BtSARS-CoV HKU3, bat SARS coronavirus HKU3 (NC_009694); CtSARS-CoV SZ3, civet SARS coronavirus SZ3 (AY304486); SARS-CoV, SARS coronavirus (NC_004718); BtSARS-CoV Rp3, bat coronavirus Rp3 (NC_009693); BtSARS-CoV Rf1/2004, bat coronavirus Rf1/2004 (NC_009695); BtSARS-CoV RM1, bat coronavirus RM1 (NC_009696); BtCoV-HKU4, bat coronavirus HKU4 (NC_009019); BtCoV HKU5, bat coronavirus HKU5 (NC_009020); BtCoV HKU9, bat coronavirus HKU9 (NC_009021); IBV, infectious bronchitis virus (NC_001451); TCoV, turkey coronavirus (NC_010800); SW1, beluga whale coronavirus (NC_010646); BuCoV HKU11, Bulbul coronavirus HKU11 (NC_011548); ThCoV HKU12, thrush coronavirus HKU12 (NC_011549); and MuCoV HKU13, Munia coronavirus HKU13 (NC_011550).

B virus (ERBV) (31–33) (see Fig. S3A in the supplemental material). Alignment of the 3′ end of ZBCoV with subgroup 2b CoVs showed deletions in the genome of subgroup 2b CoVs where the second s2m of ZBCoV is identified (see Fig. S3B). The s2m of ZBCoV are almost identical in sequence and are separated by 19 nt (see Fig. S3B). mfold prediction (34) of RNA secondary structure indicated that both s2m fold into RNA stem-loop motifs (see Fig. S3C).

Phylogenetic analyses. Phylogenetic trees constructed from 3CL^{pro}, RdRp, Hel, S, M and N amino acid sequences of ZBCoV and representative coronaviruses show that ZBCoV is most closely related to but distinct from the subgroup 2b CoVs, which include SARS-CoV and SARS-CoV-like viruses (Fig. 3). This finding is in accord with results obtained from pairwise amino acid comparisons of ZBCoV and other coronaviruses (http://cait.cumc.columbia.edu:88/dept/greeneidlab/IdentificationofaSARS-Coronavirus-likevirusinaleaf-nosedbatinNigeria.html). To further define the phylogenetic position of ZBCoV, an additional phylogeny was constructed using a conserved 659-nt sequence of RdRp, and the time to the most recent common ancestor (TMRCA) between ZBCoV and related coronaviruses was estimated. Based on the best-fit model (SRD06 with informative rate prior), the results of this analysis indicated that ZBCoV is most closely related to GhanaBt-CoV, a recently identified coronavirus found in bats in Ghana (35) (Fig. 4). Furthermore, ZBCoV and GhanaBt-CoV together form a well-supported clade distinct from that of the subgroup 2b CoVs. The

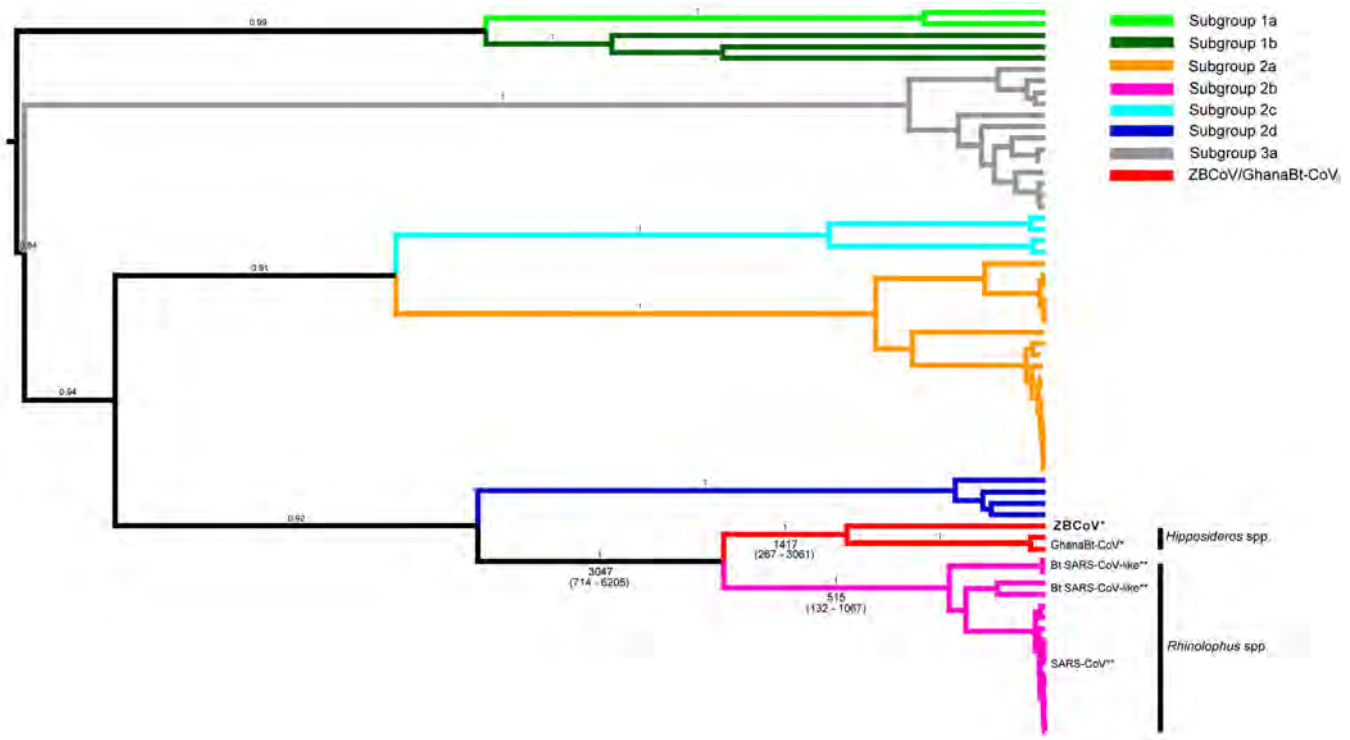

FIG 4 Estimation of the time of divergence between ZBCoV and representative coronaviruses. Bayesian MCMC phylogeny of a 659-nt region of the RNA-dependent RNA polymerase gene of ZBCoV and representative members of group 1, 2, and 3 coronaviruses. The host bat species and their geographic origins (*, Africa; **, Asia) are indicated for ZBCoV, GhanaBtCoV, and subgroup 2b CoVs. The times given at branch tips represent the dates of viral sampling, and the tree is rooted through the use of a relaxed molecular clock. Bayesian posterior probability values greater than 0.8 are shown above the branches leading to each major node. The mean TMRCAs for the taxa in subgroup 2b CoVs and ZBCoV are given below each branch, with the 95% highest probability densities indicated in parentheses. The following sequences from GenBank were included, with the GenBank accession numbers given in parentheses: for subgroup 1a CoVs, feline coronavirus (FJ938055) and canine coronavirus (GQ477367); for subgroup 1b CoVs, bat coronavirus HKU2 (DQ249213), bat coronavirus BtCoV/512/2005 (DQ648858), and human coronavirus NL63 (DQ445911); for subgroup 2a CoVs, murine hepatitis virus (AB551247), human coronavirus HKU1 (AY597011, DQ422731, DQ422728, DQ422732, DQ422737, and DQ422733), bovine respiratory coronavirus (AF220295, AF391541, AF391542, EF424615, EF424620, FJ938066, and U00735), equine coronavirus (EF446615), human enteric coronavirus 4408 (FJ415324), human coronavirus OC43 (AY391777 and AY903460), and waterbuck coronavirus (FJ425184); for subgroup 2b CoVs, bat SARS coronavirus Rf1 (DQ412042 and DQ648856), SARS coronavirus (AY313906, AY545914, AY559085, AY559097, AY595412, DQ071615, FJ882929, FJ882931, FJ882941, FJ882944, FJ882959, and FJ88686), bat SARS coronavirus HKU3 (DQ084199), and bat SARS coronavirus RM1 (DQ412043); for subgroup 2c CoVs, bat coronavirus HKU5 (DQ249217 and DQ249218), bat coronavirus HKU4 (DQ074652), and bat coronavirus BtCov/133/2005 (DQ648794); for subgroup 2d CoVs, bat coronavirus HKU9-1 (EF065513), bat coronavirus HKU9-2 (EF065514), bat coronavirus HKU9-3 (EF065515), and bat coronavirus HKU9-4 (EF065516); and for subgroup 3a CoVs, avian infectious bronchitis virus (AY514485, AY641576, AY646283, DQ001339, DQ646405, EU714029, FJ888351, FN430414, FN430415, HM245923, and HM245924) and turkey coronavirus (GQ427174, GQ427175, and GQ427176).

TMRCA between ZBCoV and GhanaBt-CoV was estimated at 1,417 years before present (ybp) (95% highest population density [HPD] = 267 to 3,061 ybp). The TMRCA between the ZBCoV/GhanaBt-CoV clade and subgroup 2b CoVs was estimated at 3,047 ybp (95% HPD = 714 to 6,205 ybp), whereas the TMRCA between SARS-CoVs and SARS-CoV-like viruses was only 515 ybp (95% HPD = 132 to 1,067 ybp). Estimates of the TMRCAs between subgroup 2b CoVs and the rest of the coronavirus groups are not provided due to the potential for nucleotide site saturation at deeper phylogenetic levels to artificially create too recent TMRCA estimates.

Whereas the mean pairwise nucleotide similarity of the partial RdRp gene region was 85% (standard deviation [SD] = 9.75) within coronavirus subgroups (excluding ZBCoV/GhanaBt-CoV), the mean pairwise similarity between coronavirus subgroups was 66% (SD = 5.14) (see Fig. S4 in the supplemental material). Based on the results of the Mann-Whitney U test, these distributions are statistically different ($P < 0.0001$). Additionally, whereas the mean pairwise similarity within the clade ZBCoV/GhanaBt-CoV was 85% (SD = 9.01), the pairwise similarity between the clade ZBCoV/GhanaBt-CoV and subgroup 2b CoVs was only 73% (SD = 0.84). Based on the results of the Mann-Whitney U test, these distributions are statistically different ($P = 0.0092$). Together, these findings indicate that the clade containing ZBCoV and GhanaBt-CoV should be considered a separate subgroup within group 2 CoVs, distinct from subgroup 2b CoVs (see Fig. S4 in the supplemental material).

DISCUSSION

Differences in phylogenetic relationships and genomic organization and the low amino acid similarities of ORF 3 and the S protein of ZBCoV compared to the ORF 3a and S proteins of subgroup 2b CoVs suggest that ZBCoV represents a new subgroup of coronaviruses within the group 2 CoVs. Although ZBCoV has features found in subgroup 2b CoVs, including the TRS, a unique PL^{Pro}, ORFs between the M and N genes, and the presence of the s2m, ZBCoV forms a unique branch distinct from subgroup 2b CoVs in

all phylogenetic trees analyzed. Furthermore, it differs from subgroup 2b CoVs in that ZBCoV contains three (versus four to five) ORFs between the M and N genes and has two (versus one) s2m.

Whereas the S proteins of subgroup 2b CoVs share 78 to 98% aa sequence identity, the S protein of ZBCoV has only 36 to 38% identity in the deduced amino acid sequence with those of subgroup 2b CoVs. Despite limited primary sequence conservation of the spike protein among ZBCoV and subgroup 2b CoVs, particularly in the S1 domain, Pfam analyses indicated the presence of a receptor domain that binds to the receptor ACE2, the cellular receptor for SARS-CoV (36). However, the residues in SARS-CoV that interact with the human ACE2 molecule are not conserved in ZBCoV, suggesting that human ACE2 is not a *bona fide* receptor for ZBCoV (37).

ORF 3, located between the S and E proteins of ZBCoV, is slightly shorter than the 3a proteins of subgroup 2b CoVs and has at most only 22% aa identity to the 3a proteins of subgroup 2b CoVs. In contrast, the 3a proteins of subgroup 2b CoVs share 81 to 98% aa identity. ORF 3 is predicted to contain four transmembrane domains with extracellular N and C termini. In contrast, ORF 3a of SARS-CoV is predicted to contain three transmembrane domains with extracellular N termini and intracellular C termini (28, 29). Whereas four O glycosylation sites are predicted in the ORF 3a protein of SARS-CoV (38), only two putative O glycosylation sites were identified in the ORF 3 of ZBCoV. The 3a protein of SARS-CoV has a cysteine-rich region important for polymerization and ion channel activity (28), as well as YXXΦ and diacidic motifs suggested to be involved in the intracellular trafficking (29). These domains were recently suggested to be important for the proapoptotic function of ORF 3a of SARS-CoV (39). However, ORF 3 of ZBCoV contains only a portion of the cysteine-rich domain and has no YXXΦ diacidic motifs. In contrast to human and civet SARS-CoV and bat RF1/2004, there is no ORF 3b in ZBCoV. The 3b protein may function as an interferon antagonist (40).

ZBCoV contains a unique set of ORFs located between the M and N genes. In subgroup 2b CoVs, ORF 6, ORF 7, and ORF 8 between the M and N genes do not overlap. In contrast, the three ORFs between the M and N genes overlap in ZBCoV. Alignment with subgroup 2b CoVs indicated deletions in ZBCoV, and as a result, one continuous ORF, ORF 8, is present in ZBCoV in place of ORFs 7a, 7b, 8, 8a, and 8b of subgroup 2b CoVs.

Similar to SARS-CoV, the putative products of ORF 6, ORF 7, and ORF 8 of ZBCoV show no sequence homology to other viral proteins. No TRS upstream of ORF 6 is found, suggesting that if ORF 6 encodes a *bona fide* protein, that protein is likely expressed by the subgenomic RNA M. There is precedent in SARS-CoV for functional bicistronic RNAs in the expression of ORF 3b, ORF 7b, ORF 8b, and ORF 9b (26, 41). Coronaviruses possess accessory genes, the size and location of which are group specific (2). By analogy to SARS-CoV, ORF 6, ORF 7, and ORF 8 of ZBCoV may encode accessory proteins important for virus-host interactions that may contribute to virulence and pathogenesis (26). Recent studies suggest that the SARS-CoV accessory proteins 6 and 7b are incorporated into virus particles and that 3a, 7a, and 9b are structural components of the virion (26, 41, 42). The SARS-CoV accessory proteins are suggested to have biological functions that include virus release, interferon antagonism, apoptosis induction, and inhibition of cellular protein synthesis (26, 41).

Another unique feature of ZBCoV is the presence of two highly conserved RNA sequences (s2m) downstream of the N gene. A single s2m is identified at the 3′ end of the genomes of members of several RNA virus families, including the *Coronaviridae* and *Astroviridae*, as well as the picornavirus ERBV (31–33). Recent data suggest that the SARS-CoV s2m RNA is a functional molecular mimic of the 530 stem-loop region in small-subunit ribosomal RNA, which could facilitate viral hijacking of the host's protein synthesis machinery (43). The presence of a second s2m in ZBCoV may further increase the efficiency of this process. Interestingly, secondary structures downstream of the N gene, including bulged stem-loop and pseudoknot structures, are also identified in the genomes of subgroup 2a and 2c CoVs (44, 45).

Lagos bat virus (family *Rhabdoviridae*, genus *Lyssavirus*) was initially identified in Nigeria in the 1950s. The discovery of ZBCoV in a bat of the genus *Hipposideros* (family Hipposideridae), is the first identification of a coronavirus in wildlife from Nigeria. Recently, bat coronaviruses closely related to ZBCoV were isolated from roundleaf bats (*Hipposideros caffer* and *Hipposideros ruber*) in Ghana, a country that is close to Nigeria (35). Phylogenetic analysis indicates that ZBCoV and GhanaBt-CoV form a unique clade that is distinct from those in subgroup 2b CoVs. However, as the only sequence available for GhanaBt-CoV is a fragment of the RdRp gene, a comparison of the genome organization between ZBCoV and GhanaBt-CoV is not possible. Our findings and recent published data, wherein a SARS-CoV-like virus was found to lack ORF 8, suggest that there is considerable diversity in the genome organization of SARS-CoV-like viruses (46).

SARS-CoV-like viruses have been isolated from various rhinolophid bats (family Rhinolophidae, genus *Rhinolophus*), common insectivorous bats found in Africa and Eurasia. However, despite extensive studies, no SARS-CoV-like viruses have been reported in *Hipposideros* sp. bats in China (32). The *Rhinolophus* species suggested as reservoirs of SARS-CoV-like viruses are not present in Africa. A sequence fragment of a SARS-CoV-like virus was identified in Kenya in bats of the *Chaerephon* genus (family Molossidae) (47), and antibodies reactive with SARS-CoV antigen have also been detected in the sera of seven different genera of insectivorous and fruit bats sampled in central and southern Africa (48). In concert, these findings suggest that there may be no strict species-specific host restriction of SARS-CoV-like viruses in African bats.

Our phylogenetic analysis indicates that the clade containing ZBCoV and GhanaBt-CoV occupies an ancestral position to the group 2b CoVs, which include SARS-CoV and SARS-CoV-like viruses. Similar to previous estimates, the TMRCA of these two clades was estimated at ~3,047 ybp (although with large 95% HPDs). Although SARS-CoV-like viruses have been identified exclusively in bats in China, a recent sequence fragment (~120 bp) recovered from a Kenyan bat was found to occupy a position just outside subgroup 2b and may represent the ancestral African lineage of all subgroup 2b CoVs (47). Together with the position of the African clade of ZBCoV/GhanaBt-CoV relative to subgroup 2b CoVs, this finding suggests that a migration event from Africa to China within the last 100 to 1,000 years may have resulted in the subgroup 2b lineage of CoVs. Indeed, the geographic distribution and the phylogenetic relationships of bat coronaviruses seen both here (Fig. 4) and in previous work (35) suggest the presence of multiple independent migration events between Africa and Asia

throughout the history of bat coronaviruses. Additional sequence data for the bat coronaviruses identified in Kenya along with increased sampling for coronaviruses in Africa as well as central and eastern Asia will likely be necessary to unveil the timing and origin of this diverse group of coronaviruses.

Bats are important reservoir hosts of zoonotic viruses with significant impact on human health, including rabies, Nipah virus, Hendra virus, Zaire Ebola virus, Marburg virus, and SARS-CoV. The wide genetic diversity that exists among zoonotic viruses in bats may allow an increased emergent potential of interspecies variants that may cause outbreaks of disease in humans and domestic animals. The giant leaf-nosed bat, *Hipposideros commersoni*, is widespread in sub-Saharan Africa, from Gambia to Ethiopia, Mozambique, and Madagascar, but little is known concerning its ecology, population biology, or vector competence. Clearly, in order to enhance our knowledge of the diversity and cooccurrence of potential reservoir hosts, it is essential to better understand emerging pathogen dynamics and public health relevance as a means to prevent and control future disease outbreaks.

MATERIALS AND METHODS

Bat sample collection. During June 2008, bats were collected with mist netting in caves and around human dwellings or manually from roost locations near Idanre and Zaria, Nigeria. All bats appeared clinically normal. Captured bats were anesthetized by intramuscular inoculation with ketamine hydrochloride (0.05 to 0.1 mg/g of body weight) and euthanized under sedation by intracardiac exsanguination and cervical dislocation. The species of each captured bat was recorded, as well as the sex, forearm and body lengths (in cm), and weight. All samples were initially stored, transported on ice packs, and stored thereafter at −20°C, until shipment on dry ice and final storage at −80°C. No lyssavirus-specific antigens were identified in bat brains by use of direct fluorescent antibody testing.

Coronavirus consensus PCRs. Coronavirus screening was performed by nested PCR, amplifying a 400-nt fragment of the RdRp genes of coronaviruses using consensus primer sequences 5′-CGTTGGIACW AAYBTVCCWYTICARBTRGG-3′ and 5′-GGTCATKATAGCRTCA VMASWWGCNACNACATG-3′ for the first PCR and consensus primer sequences 5′-GGCWCCWCCHGGNGARCAATT-3′ and 5′-GGWAWCCCCAYTGYTGWAYRTC-3′ for the second PCR. Primers were designed by multiple alignments of the nucleotide sequences of available RdRp genes of known coronaviruses. Reverse transcription was performed using the SuperScript III kit (Invitrogen, San Diego, CA). PCR primers were applied at 0.2-μM concentrations with 1 μl cDNA and HotStar polymerase (Qiagen, Valencia, CA). Cycle conditions used were as follows: 1 cycle at 95°C for 15 min; 15 cycles at 95°C for 30 s, 65°C for 30 s (−1°C/cycle), and 72°C for 45 s; 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s; and 1 cycle at 72°C for 5 min.

UHTS. Total RNA obtained from the gastrointestinal tract specimen positive for coronavirus was extracted for UHTS. Purified RNA (0.5 μg) was DNase I digested (DNA-free; Ambion, Austin, TX) and reverse transcribed using a Superscript II kit (Invitrogen) with random octamer primers linked to an arbitrary, defined 17-mer primer sequence (MWG, Huntsville, AL). cDNA was RNase H treated prior to random amplification by PCR, applying a 9:1 dilution mixture of a primer corresponding to the defined 17-mer sequence and the octamer-linked 17-mer sequence primer, respectively. Products of >70 bp were purified (MinElute; Qiagen) and ligated to linkers for sequencing on a GS FLX sequencer (454 Life Sciences, Branford, CT).

Genome sequencing. PCR primers for amplification across sequence gaps were designed (available upon request) based on the UTHS data, and the draft genome was sequenced by overlapping PCR products. Products were purified (QIAquick PCR purification kit; Qiagen) and directly dideoxy sequenced in both directions with ABI Prism BigDye Terminator 1.1 cycle sequencing kits (PerkinElmer Applied Biosystems, Foster City, CA). Additional methods applied to obtain the genome sequence included additional consensus PCR and 3′ and 5′ RACE (Invitrogen).

Phylogenetic and sequence analyses. Alignments were constructed using MUSCLE 3.7 (49) and adjusted manually using Se-Al (50). Maximum likelihood (ML) phylogenetic trees containing representative taxa from each coronavirus genus ($n = 31$) (Fig. 3, legend) were constructed using the subtree pruning and regrafting (SPR) method of branch swapping in PhyML (51). Phylogenies were constructed using amino acid alignments for the complete proteins of 3CL, Hel, M, and N and partial protein alignments for the available RdRp protein sequence and for the S protein after regions with low alignment confidence were removed. In all cases, the Whelan and Goldman model of amino acid replacement was used (52), with a gamma distribution of rate heterogeneity. The value of the shape parameter for gamma (α) was estimated from the data and approximated by six rate categories. The reliability of each branch in all phylogenies was estimated using a bootstrap resampling procedure, with 100 ML replications.

To estimate the time to the most recent common ancestor (TMRCA) for the taxa contained within subgroup 2b CoVs and including ZBCoV, an additional 659-nt alignment of the RdRp gene was constructed and chosen for homology to the gene region sequenced for the coronaviruses most closely related to ZBCoV (GhanaBt-CoV). All sequences for which time-of-sampling information was available were included ($n = 64$). TMRCAs were estimated using the Bayesian Markov chain Monte Carlo (MCMC) method with the BEAST package, version 1.5.2 (53), and both the general time-reversible (GTR) model plus Γ distribution and the SRD06 model of nucleotide substitution. A relaxed uncorrelated lognormal molecular clock was used, calibrated by the time-stamped sequences, both with and without informative rates prior on the molecular clock of $2.0 \times 10^{-4} \pm 0.0009$ nt substitutions/site/year (35). This analysis was run until all parameters converged, with 10% of the MCMC chains discarded as burn-in. Statistical confidence in the TMRCA estimates is given by the 95% highest probability density (HPD) interval around the marginal posterior parameter mean.

The classification of ZBCoV and GhanaBt-CoV as a putative new subgroup within group 2 CoVs was determined by first calculating the percent pairwise nucleotide similarity of the same 659-nt region of RdRp genes between and within the existing subgroups of coronaviruses and then extending this comparison to include the clade ZBCoV/GhanaBt-CoV. To verify this approach, a nonparametric Mann-Whitney U test was used to assess if the pairwise nucleotide similarity within the currently accepted subgroups is different from that between subgroups. This test was then used to determine if the percent pairwise similarity within the clade ZBCoV/GhanaBt-CoV is statistically different from that of the most closely related subgroup 2b CoVs.

Protein family analysis was performed using Pfam (http://pfam.sanger.ac.uk/). Predictions of signal peptide cleavage sites, glycosylation sites, and transmembrane domains were performed using respective prediction servers available at the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/ and http://www.ch.embnet.org/software/TMPRED_form.html). The percent amino acid sequence identity and similarity were calculated using the Needleman algorithm with an EBLOSUM62 substitution matrix (gap open/extension penalties of 10/0.1 for nucleotide and amino acid alignments; EMBOSS [54]), using a Perl script to iterate the process for all versus all comparisons. Prediction of RNA secondary structures was performed with the mfold program (http://mfold.bioinfo.rpi.edu/).

Nucleotide sequence accession number. The GenBank accession number for the ZBCoV sequence is HQ166910.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00208-10/-/DCSupplemental.

Table S8, PDF file, 0.036 MB.
Table S9, PDF file, 0.027 MB.
Figure S1, PDF file, 0.034 MB.
Figure S2, PDF file, 0.020 MB.
Figure S3, PDF file, 0.140 MB.
Figure S4, PDF file, 0.297 MB.

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Bat Coronaviruses and Experimental Infection of Bats, the Philippines

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Fifty-two bats captured during July 2008 in the Philippines were tested by reverse transcription–PCR to detect bat coronavirus (CoV) RNA. The overall prevalence of virus RNA was 55.8%. We found 2 groups of sequences that belonged to group 1 (genus *Alphacoronavirus*) and group 2 (genus *Betacoronavirus*) CoVs. Phylogenetic analysis of the RNA-dependent RNA polymerase gene showed that groups 1 and 2 CoVs were similar to Bat-CoV/China/A515/2005 (95% nt sequence identity) and Bat-CoV/HKU9–1/China/2007 (83% identity), respectively. To propagate group 2 CoVs obtained from a lesser dog-faced fruit bat (*Cynopterus brachyotis*), we administered intestine samples orally to Leschenault rousette bats (*Rousettus leschenaulti*) maintained in our laboratory. After virus replication in the bats was confirmed, an additional passage of the virus was made in Leschenault rousette bats, and bat pathogenesis was investigated. Fruit bats infected with virus did not show clinical signs of infection.

Severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) is a newly emerged zoonotic CoV that caused an international epidemic in 2003. Epidemiologic studies have demonstrated that the first human cases of SARS were caused by CoVs closely related to those found in Himalayan palm civets and raccoon dogs in wildlife markets (*1*). This finding accelerated surveys of CoVs specific for various animals in Southeast Asia to identify reservoirs for SARS-CoV. These survey findings suggested that palm civets and raccoon dogs are an intermediate host of, but not a primary reservoir for, SARS-CoV because of the low prevalence of SARS-like CoVs in these animals (*2*). Moreover, a large variety of novel CoVs in these surveys, including bat SARS–like CoVs, were detected in many bat species in the People's Republic of China and Hong Kong Special Administrative Region (*3–6*).

Phylogenetic analysis of bat CoVs and other known CoVs suggested that the progenitor of SARS-CoV and all other CoVs in other animal hosts originated in bats (*5*,*7*). Recently, bat CoVs in North and South America, Europe, and Africa were also reported (*8–12*). Although extensive bat surveys have been conducted, no infectious bat CoVs have been isolated from cell cultures, which hinders characterization of bat CoVs and evaluation of the risks posed by these viruses to public health.

In this study, we detected bat CoVs in the Philippines. We attempted to isolate bat CoVs and virus RNA from cell cultures and from Leschenault rousette bats (*Rousettus leschenaulti*) orally infected with intestinal tissues and contents from a lesser dog-faced fruit bat (*Cynopterus brachyotis*). After infection, clinical signs of infected bats were examined, and pathogenesis in bats was investigated.

Materials and Methods

Bat Collection

We obtained 52 bats of 6 species during July 2008 from Diliman and Los Baños, the Philippines, after receiving permission from the government. All captured bats were anesthetized with an intraperitoneal injection (15 mg/kg) of tiletamine and zolazepam (Virbac, Carros, France) and killed by cardiac exsanguination. The experiment was conducted in accordance with the Guidelines for the Care

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Extraction of RNA and Reverse Transcription

Virus RNA was extracted from samples obtained from field bats and from experimentally infected bats by using an SV Total RNA Isolation System Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Extracted RNA was eluted in 50 μL of RNase-free water. For cDNA synthesis, RNA (5 μL), a random hexamer, and a SuperScript III Kit (Invitrogen, Carlsbad, CA, USA) were used.

PCR and DNA Sequencing

All cDNA samples obtained from field bats and experimentally infected bats were tested by using conventional and nested PCR. On the basis of previous reports, we used a PCR and a pair of consensus primers specific for a highly conserved region of the RNA-dependent RNA polymerase (RdRp) gene (*13*).

Two microliters of cDNA was added to a 25-μL reaction mixture containing 2× GoTaq PCR Master Mix (Promega) and 0.2 μM of 5′-GGT TGGGACTATCCTAAGTGTGA-3′ (primer 1) and 5′-CCATCATCAGATAGAATCATCATA-3′ (primer 2). The PCR conditions were 2 min at 94°C; 35 cycles for 20 s at 94°C, 30 s at 50°C, and 30 s at 72°C; and 1 min at 72°C. PCR amplicons were gel purified by using NucleoSpin Extract II (Machrey-Nagel, Düren, Germany) and cloned by using a TOPO-TA pCR2.1 Cloning Kit (Invitrogen). Sequencing was performed in an ABI 3130 XL DNA analyzer (Applied Biosystems, Foster City, CA, USA).

On the basis of the sequences obtained, we designed new specific primer pairs for the Bat-CoV/Philippines/Diliman1552G1/2008 sequence (5′-TGATTT CTGCAATGATACTTGGTTC-3′ and 5′-ACTTGATGAT CTGTAACAACAATCG-3′) and for the Bat-CoV/Philippines/Diliman1525G2/2008 sequence (5′-TACAAC CTACGCTGCAACTC-3′ and 5′-ATGAGTGTGCACAA GTGCTTAG-3′). These primers were used as the inner primer set for the nested PCR after the first PCR was performed with primers 1 and 2. Aliquots (2 μL) of cDNA for primary amplification were added to 2× GoTaq Master Mix (Promega) and primers 1 and 2. Amplification was performed by using 15 cycles at conditions described above. Aliquots (2 μL) of primary amplification products were used for the second PCR with GoTaq Master Mix and the inner primers. The second PCR was performed by using 35 cycles at the conditions described above. PCR products were extracted from gels by using NucleoSpin Extract II and subjected to direct sequencing or TA cloning.

Bat Samples

Leschenault rousette bats were obtained from zoos in Japan. Seven bats were randomly selected for the experiments. In each experiment, 2 bats were placed in a negative-pressure isolator. One additional bat was kept in a separate isolator as a control. A sample of large intestine from a lesser dog-faced fruit bat (*C. brachyotis*) was homogenized in a sterile mortar. After low-speed centrifugation (2,000 × *g* for 10 min), the supernatant was used for oral infection. Experimentally infected bats were examined daily for clinical signs of infection. Fecal specimens were obtained from a clean translucent plastic sheet spread along the bottom of the cage. All bats were killed after being anesthetized with diethyl ether, and organs (liver, kidney, spleen, lung, brain, and intestine) and serum samples were obtained.

Detection of Virus mRNA in Bats

To determine membrane, nucleocapsid, nonstructural (Ns)7a, Ns7b, and Ns7c protein nucleotide sequences, we conducted PCR and DNA sequencing in the same manner as for determination of partial RdRp nucleotide sequence described above by using the HKU9-Leader42–64 primer (5′-CCGTTTCGTCTTGTACGAATCAC-3′) and the 3siteAd20T primer (5′-CTGATCTAGAGGTACCGGA TCCTTTTTTTTTTTTTTTTTTTT-3′). To detect virus mRNA, we conducted reverse transcription–PCR (RT-PCR) by using 2 primer sets: HKU9-Leader42–64 and N468–448r (5′-GTTACGTGTGCCCATGTCACC-3′) and HKU9-Leader42–64 and Ns7a440–420r (5′-CAAGCCA CAACAACATTAGG-3′).

Quantitative Real-Time RT-PCR

cDNA synthesis was performed by using 0.5 μL total RNA and the PrimeScript RT Reagent Kit (TaKaRa, Shiga, Japan) according to the manufacturer's protocol. Virus RNA was quantified by using Power SYBR Green PCR Master Mix (Applied Biosystems) with 2 μL of reverse-transcribed cDNA. Quantitative real-time PCR was performed by using the Thermal Cycler Dice System (TaKaRa). The temperature program consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The primer pair for the real-time PCR was designed on the basis of the partial RdRp sequences of Bat-CoV/Philippines/Diliman1525G2/2008; primers used were 5′-TCCTAAGTGTGATAGAGCTATGCC-3′ and 5′-GTGCACACTCATTTGCTAACCG-3′. In each experiment, 10-fold serial dilutions of plasmid DNA containing the partial RdRp gene were tested in duplicate to establish a standard curve for calculating the relative amount of RNA in each sample. All samples were analyzed at least 3 times. To confirm the specificity of each PCR product, we conducted a melting curve analysis immediately after

the amplification phase of each PCR. The amount of RNA in each sample was expressed as the average value (copy number per weight [milligrams] of sample).

Results

Virus Detected

During July 2008, a total of 52 bats were obtained at 3 locations in Diliman and 1 location in Los Baños, the Philippines (Table 1). RT-PCRs for a 440-bp fragment in the RdRp gene of CoVs were performed for large intestine samples, including intestinal contents; 9 (17.3%) of 52 bats were positive. Differences in the 440-nt sequence in the RdRp region were determined after TA cloning of the 9 positive samples. Sequences indicated that the 2 groups of sequences obtained belonged to group 1 CoV (genus *Alphacoronavirus*) (n = 4) and group 2 CoV (genus *Betacoronavirus*) (n = 5).

A 440-bp consensus nt sequence of the group 1 CoV was obtained on the basis of alignment of 4 group 1 CoV sequences detected (>98% nt identity with each other) and deposited in GenBank as Bat-CoV/Philippines/Diliman1552G1/2008 (DNA Database of Japan [DDBJ] accession no. AB539080). BLAST (www.ncbi.nlm.nih.gov/BLAST) search findings of GenBank indicated that the partial RdRp sequence was most similar to that of Bat-CoV/China/A515/2005 (95% nt identity).

A 440-bp consensus nt sequence of group 2 CoVs was also obtained (>98% nt identity with each other) and deposited in GenBank as Bat-CoV/Philippines/Diliman1525G2/2008 (DDBJ accession no. AB539081). A BLAST search suggested that the partial RdRp sequence was novel but most similar to that of Bat-CoV/HKU9–1/China/2007 (83% nt identity). A phylogenetic tree was constructed with the partial RdRp-deduced amino acid sequence (120 aa) and available sequences of known CoVs (Figure 1). Data in the tree suggested that Bat-CoV/Philippines/Diliman1552G1/2008 belonged to group 1b CoVs and Bat-CoV/Philippines/Diliman1525G2/2008 belonged to group 2d to CoVs.

Specific and nested primer pairs for group 1b bat CoV and group 2d bat CoV sequences were designed, and nested PCR was performed by using cDNAs of all samples. Twenty additional amplicons (≈200-bp sequences) were obtained by using primers specific for group 2d bat CoVs. After direct sequencing or TA cloning, partial sequences of all amplicons obtained were found to be nearly identical to group 2d bat CoVs (>98% nt identity) and resulted in a total CoV prevalence of 55.8% (Table 1). All sequences of group 1b bat CoVs were obtained from insectivorous bats (4/7, 57.1%), and all sequences of group 2d bat CoVs were obtained from frugivorous bats (25/45, 55.6%).

Virus in Cell Cultures

Cytopathic effects were not observed in any of the cells (Vero E6, Vero, Hrt18, A549, fcwf-4, BKT-1, Tb-1 Lu, or primary kidney cells derived from Leschenault rousette bats) tested with bat intestinal specimens positive for both detected viruses by RT-PCR. Results of RT-PCR for cell lysates to detect viral replication also were negative.

Virus Propagation in Fruit Bats

To obtain bat CoVs from field samples, we administered virus orally to 2 Leschenault rousette bats maintained in the Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, University of Tokyo. The volume of intestine samples collected from insectivorous bats was less than that from fruit bats because of their body size, and all positive samples for the group 1b bat CoV genome were derived from small insectivorous bats. Oral infection was conducted only with samples positive for the group 2d bat CoV genome. A homogenized large intestine sample (60 mg) derived from a lesser dog-faced fruit bat, which contained 7.8×10^6 copies of viral genome, was given orally to 2 fruit bats (bats A and B). After confirmation that these bats showed

Table 1. Prevalence of coronavirus in bats, the Philippines

Sampling site	Common name (species)	No. intestine samples tested	No. positive (group 1)	No. positive (group 2)
Los Baños	Lesser dog-faced fruit bat (*Cynopterus brachyotis*)	4	0	2
	Cave nectar bat (*Eonycteris spelaea*)	3	0	2
	Greater musky fruit bat (*Ptenochirus jagori*)	14	0	11
Diliman (site A)	Lesser dog-faced fruit bat (*C. brachyotis*)	1	0	1
	Cave nectar bat (*E. spelaea*)	1	0	1
	Greater musky fruit bat (*P. jagori*)	1	0	0
Diliman (site B)	Cave nectar bat (*E. spelaea)*	1	0	1
	Java pipistrelle bat (*Pipistrellus javanicas*)	3	0	0
	Lesser Asiatic yellow bat (*Scotphilus kuhlii*)	4	4	0
Diliman (site C)	Lesser dog-faced fruit bat (*C. brachyotis*)	18	0	6
	Greater musky fruit bat (*P. jagori*)	1	0	0
	Geoffroy rousette bat (*Rousettus amplexicaudatus*)	1	0	1
Total		52	4	25

no clinical signs of infection, they were killed 6 days after infection.

Virus genome was detected only in the small and large intestines of both bats by RT-PCR (Table 2). Virus was not detected in these intestine samples by cell cultures. Virus genome was detected by RT-PCR in fecal samples obtained during daily observations for clinical signs, and viral genome copy number was determined by real-time RT-PCR (Table 3). Virus copy number peaked on day 3. On day 4, a fecal sample was not collected because feces were not found on the bottom of the isolator.

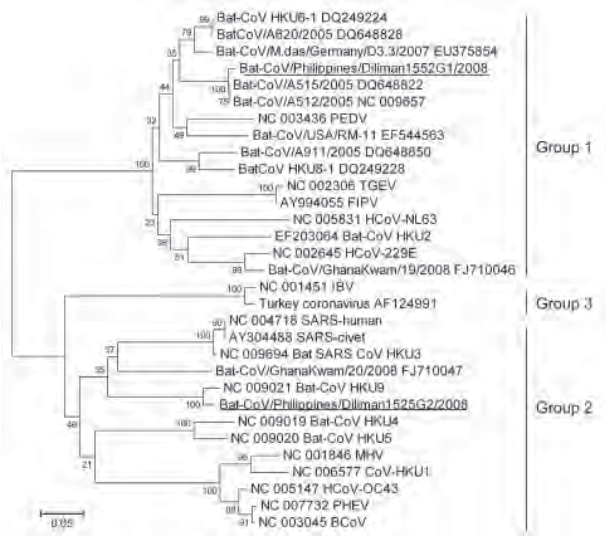

Figure 1. Phylogenetic tree based on deduced amino acid sequences of partial RNA-dependent RNA polymerase of coronaviruses (CoVs), the Philippines. The 2 new viruses detected in this study are underlined. Percentage of replicate trees in which the associated taxa clustered in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed by using the Poisson correction method and are shown as number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. The final dataset included 120 positions. Phylogenetic analyses were conducted in MEGA4 (*14*). Coronaviruses used for comparisons and their GenBank accession numbers are human coronavirus (HCoV) 229E (HCoV-229E) (NC_002645), porcine epidemic diarrhea virus (PEDV) (NC_003436), transmissible gastroenteritis virus (TGEV) (NC_002306), feline infectious peritonitis virus (FIPV) (AY994055), human coronavirus NL63 (HCoV-NL63) (NC_005831), bat-CoV/A512/2005 (NC_009657), bat-CoV/A515/2005 (DQ648822), bat-CoV/A620/2005 (DQ648828), bat-CoV/A911/2005 (DQ648850), bat-CoV/GhanaKwan/19/2008 (FJ710046), bat-CoV/GhanaKwan/20/2008 (FJ710047), bat-CoV/M.das/Germany/D3.3/2007 (EU375854), bat-CoV/USA/RM-11 (EF544563), bat-CoV HKU2 (EF203064), HKU4 (NC_009019), HKU5 (NC_009020), HKU6 (DQ249224), HKU8 (DQ249228), HKU9 (NC_009021), CoV-HKU1 (NC_006577), human coronavirus (HCoV-OC43) (NC_005147), murine hepatitis virus (MHV) (NC_001846), bovine coronavirus (BCoV) (NC_003045), porcine hemagglutinating encephalomyelitis virus (PHEV) (NC_007732), human severe acute respiratory syndrome coronavirus (SARS) (SARS-human) (NC_004718), civet SARS-like coronavirus (SARS-civet) (AY304488), bat-SARS-like coronavirus HKU3 (bat-SARS-CoV HKU3) (NC_009694), infectious bronchitis virus (IBV) (NC_001451), and turkey coronavirus (AF124991).

Virus mRNA in Experimentally Infected Bats

For murine hepatitis virus and several CoVs, an ≈70-bp leader sequence is added to the 5′ end of the transcription regulatory sequence of each nested mRNA during mRNA processing (*15*,*16*). For bat CoVHKU9–1, which was most similar to group 2d bat CoV, a complete genome sequence and putative transcription regulatory sequence of Bat-CoV HKU9 were predicted (*17*). On the basis of that report, primer HKU9-Leader42–64, including a leader sequence, was designed (Figure 2). The HKU9-Leader42–64 primer and 3siteAd20T primer, which included the oligo dT sequence, were used for PCR with RNA extracted from intestines of bats A and B. Amplicons were cloned, and partial genomic sequences of group 2d bat CoV membrane, nucleocapsid, Ns7a, Ns7b, and Ns7c genes were determined. These sequences were deposited in GenBank (DDBJ accession no. AB543561). A phylogenetic tree was also constructed with the deduced amino acid sequence (463 aa) of the complete N gene of group 2d bat CoV and available sequences of known CoVs. The tree showed the same topology as that constructed with deduced amino acid sequence of the partial RdRP gene. The N gene nucleotide sequence was most similar to that of Bat-CoV/HKU9–1/China/2007 (72% identity).

To confirm presence of transcribed virus mRNA in bats A and B (Table 2), RT-PCR specific for mRNA of the group 2d bat CoVs was conducted with HKU9-Leader42–64, N468–448r, and Ns7a440–420r primers. Virus mRNAs were detected in RNA extracted from the small intestines of bats A and B (Figure 3). All amplicons were sequenced and included the nucleotide sequence of the HKU9-Leader42–64 primer sequence at the 5′ end of the sequences obtained (Figure 2). These results suggest that virus mRNAs were transcribed in bats A and B.

Experimental Infection of Bats

To determine whether this 2d bat CoV was pathogenic, we experimentally infected 5 *R. leschenaulti* fruit bats. A 60-mg sample of the small intestine from bat A was given orally to 2 bats (bats C and D), and 500 μL of phosphate-buffered saline was given orally to 1 bat (bat E) as a control. These bats were killed 6 days after infection. Clinical signs were not observed in the experimentally infected bats. Virus genome amplification was not detected by RT-PCR in any samples (serum, brain, kidney, liver, lung, spleen, and feces). However, virus RNA was detected in the small intestine (Table 4). No pathologic changes were observed in the intestines or other organs.

Table 2. PCR results for bat coronavirus in fruit bats infected by using bat intestinal samples, the Philippines*

Bat	Assay	Liver	Kidney	Lung	Spleen	Brain	Small intestine	Large intestine	Serum
A	RT-PCR	–	–	–	–	–	–	+	–
	qRT-PCR	ND	ND	ND	ND	ND	1.25×10^6	3.53×10^6	ND
B	RT-PCR	–	–	–	–	–	+	+	–
	qRT-PCR	ND	ND	ND	ND	ND	1.47×10^6	1.50×10^6	–

*Bat intestinal samples containing 7.8×10^6 copies of coronavirus genome. Values are copies per milligram. RT-PCR, reverse transcription–PCR; –, virus RNA not detected; +, virus RNA detected; qRT-PCR, quantitative RT-PCR; ND, not done.

Table 3. Time course of detection of coronavirus viral genome by PCR in feces from 2 fruit bats, the Philippines*

	Days after infection					
Test	0	1	2	3	4	5
RT-PCR	–	–	–	+	ND	+
Quantitative RT-PCR	–	–	5.31×10^4	1.74×10^7	ND	1.5×10^6

*Values are copies per milligram. RT-PCR, reverse transcription–PCR; –, virus RNA not detected; +, virus RNA detected; ND, not done.

Because virus growth in fruit bats was weaker than virus growth in bats after primary infection with field samples, experimental conditions were changed. Samples (300 mg) from the small intestine of 2 bats (A and B) were given orally to 2 other bats (F and G), which were killed 3 days after infection. Six intestinal samples were obtained from each bat to determine site specificity of virus growth. The entire intestine (duodenum to the large intestine) was divided into 6 equal parts (1–6). Virus RNA was detected only by RT-nested PCR in the small and large intestines (Table 4), and no pathologic effect was detected in these bats. Virus RNA was detected in the lower region (parts 3–4 from bat F and parts 3–6 from bat G). However, we could not determine the specific site of virus replication.

Discussion

After the SARS epidemic in 2003, bats were identified as carriers of CoVs in China. Recently, bat CoVs have also been detected in several other regions, including Germany, North and South America, and Africa. In the current study, we confirmed the presence of 2 CoVs in bats in the Philippines. Our findings suggest that CoV circulation in bats is worldwide. Although only 52 bats were tested, CoV RNA was present in 55.8% of large intestine samples from these bats. Moreover, all bats tested seemed to be healthy. Thus, bats may be persistently infected carriers of CoVs. These data are consistent with results of previous reports of CoV detection in bats (*3*–*6*).

RNA of group 1b bat CoV was detected in 4 (57.1%) of 7 insectivorous bats. All bats positive for group 1b bat CoV RNA belonged to the same species, the Lesser Asiatic yellow bat (*Scotophilus kuhlii*). However, the partial RdRp sequence of the virus was most similar to that of Bat-CoV/People's Republic of China/A515/2005 (95% nt sequence identity), which was also detected in bats of the same species in the southern China on Hainan Island (*5*). The Lesser Asiatic yellow bat is distributed widely in eastern Asia in Philippines, Pakistan, Hainan Island, Taiwan, and Borneo (*18*). High similarities of sequences between group 1b bat CoV and Bat-CoV/China/A515/2005 suggest that these viruses are distributed widely in bats enzootic to eastern Asia.

Although group 1 bat CoV was detected in 1 species of insectivorous bats, group 2d bat CoV was detected in 4 species of frugivorous bats. Five of the 45 frugivorous bats were positive by RT-PCR, and an additional 20 were positive by RT-nested PCR (prevalence 55.6%). This finding suggests that replication of group 2d bat CoV in the intestine is low.

The complete N sequence of group 2d bat CoV suggests that it is a novel virus and most similar to that of Bat-CoV/

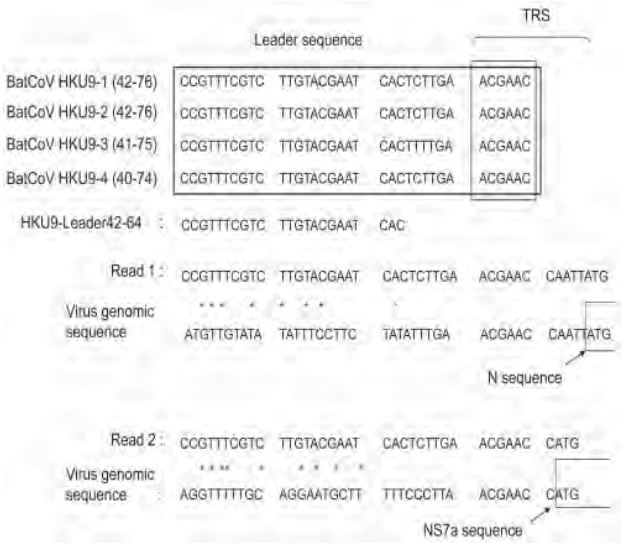

Figure 2. Comparison of mRNA sequences of bat coronavirus (BatCoV) with viral genomic sequences. Read 1 was obtained by using reverse transcription–PCR and HKU9-Leader42–64 and N468–448r primers. Read 2 was obtained by using HKU9-Leader42–64 and Ns7a440–420r primers. Asterisks indicate sequence identity for read and virus genome sequences. TRS, transcription regulatory sequence; N, nucleocapsid; NS, nonstructural.

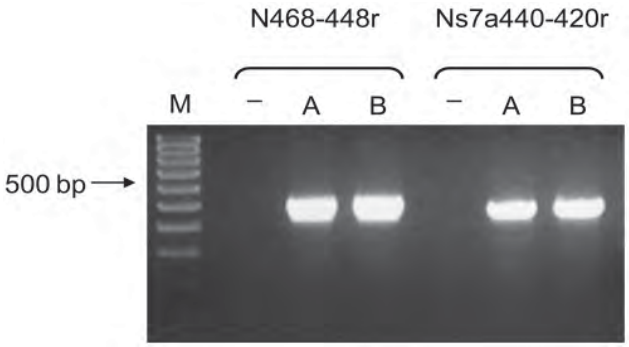

Figure 3. Bat coronavirus/Philippines/Dilliman1525G2/2008 mRNA in experimentally infected fruit bats, the Philippines. Reverse transcription–PCR results for small intestines of bats A and B. Lane M, 100-bp DNA ladder; lane –, nontemplate control.

HKU9–1/China/2007 (77% aa sequence identity). Woo et al. (*17*) detected Bat-CoV/HKU9–1/China/2007 and classified the viral nucleotide sequence as that of group 2d CoV. Our phylogenetic data (Figure 1) suggest that group 2d bat CoV and Bat-CoV/HKU9–1/China/2007 belong to the same group.

We attempted to isolate bat CoVs from several cell lines and primary cultured cells. However, virus replication was not observed, which is consistent with results of a previous report (*17*). No infectious bat CoV has been isolated from cell culture. In the current study, the amount of large intestine obtained per bat was ≤100 mg. Therefore, most samples were inadequate for virus isolation, especially virus-positive samples for group 1 bat CoVs from insectivorous bats.

To obtain sufficient tissue to isolate virus RNA, we attempted to infect fruit bats with bat CoV. Although we could not obtain bats of the species from which group 2d bat CoV was detected in the field survey, we obtained Leschenault rousette bats from zoos in Japan. In addition, Bat-CoV/HKU9–1/China/2007, which was most similar to group 2d bat CoVs by phylogenetic analysis, was identified in this species in Hong Kong (*17*). This finding indicates that fruit bats can be infected with this virus. No signs of clinical disease were observed after oral infection with an intestine sample derived from a lesser dog-faced fruit bat. However, virus RNA was detected in the small and large intestines (Table 2), and these intestinal samples contained more genome copies than input copies. Furthermore, virus RNA was amplified in fecal samples by real-time PCR, and viral mRNAs were detected in bats A and B (Figure 3). These findings indicate that group 2d bat CoVs can be orally transmitted to fruit bats and replicate in them.

Experimental infection was conducted in fruit bats by using tissues from virus-infected bats to determine virus pathogenicity. However, infected bats showed no signs of a pathologic effect, although low levels of virus RNAs were detected in the small and large intestines of these bats. These findings suggest that fruit bats can be infected with bat CoV without showing any signs of infection. However, compared with primary infection by field samples obtained from *C. brachyotis*, the level of viral genome amplification was low in experimental infection. This finding may have been caused by a difference in viral replication in bats of different species. In the field survey, partial nucleotide sequences of group 2d bat CoVs, were detected in 4 bat species. A high prevalence of virus RNA was observed in each bat species (Table 1). These findings suggest that the group 2d bat CoVs may infect fruit bats of many species. The oral infection study showed that CoV is easily transmitted across species. These results, and the fact that most reported bat CoV sequences have been detected in several bat species (*12*,*17*), imply that interspecies transmission in bats may be common.

Further investigation of bat CoV ecology is needed to better understand the risk for infection with this virus. Knowing this risk could help elucidate emergence of SARS. Although we demonstrated in vivo propagation of a bat CoV, a bat CoV culture system is needed to obtain additional information about this virus.

Table 4. Results of nested and quantitative RT-PCRs of cDNA from bat samples, the Philippines*

Bat	Liver	Kidney	Lung	Spleen	Brain	Small intestine				Large intestine		Serum
C	–	–	–	–	–	+ (ND)				–		–
D	–	+ (ND)	+ (ND)	+ (ND)	–	+ (6.57×10^4)†				–		+ (ND)
E	–	–	–	–	–	–				–		–
						Intestine section						
						1	2	3	4	5	6	
F	–	–	–	–	–	–	–	+ (ND)	+ (ND)	–	–	–
G	–	–	–	–	–	–	–	+ (ND)	+ (ND)	+ (ND)	+ (5.91×10^4)	–

*cDNA was synthesized from bat samples obtained after experimental infection (second passage of the group 2d coronavirus in Leschenault rousette bats). Values are copies per milligram. Virus load was quantified by real-time PCR. RT-PCR,reverse transcription–PCR; –, virus RNA not detected; +, virus RNA detected; ND, not detected by real-time PCR in RT-PCR–positive samples.
†Result of nested PCR.

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A Real-Time RT-PCR Method for the Universal Detection and Identification of Flaviviruses

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ABSTRACT

Here we describe an optimized molecular protocol for the universal detection and identification of flaviviruses. It combines the convenient real-time polymerase chain reaction (PCR) format with a broad spectrum of flavivirus detection. This assay, based on the amplification of a 269–272 nt (depending on the flavivirus tested) region at the N terminal end of the NS5 gene, enabled the amplification of 51 flavivirus species and 3 tentative species. Sequencing of the amplicons produced by reverse transcriptase (RT)-PCR permitted the reliable taxonomic identification of flavivirus species by comparison with reference sequences available in databases, using either the BLASTN algorithm or a simple phylogenetic reconstruction. The limit of detection of the assay (2–20,500 copies/reaction depending on the virus tested) allowed the detection of different flaviviruses from a series of human sera or veterinary samples. Altogether, the characteristics of this technique make it a good candidate for the identification of previously identified flaviviruses in cell culture and the investigation of field samples, and also a promising tool for the discovery and identification of new species, including viruses distantly related to "classical" arthropod-borne flaviviruses.

INTRODUCTION

THE GENUS *Flavivirus* COMPRISES more than 50 recognized species (of which approximately one half are human pathogens responsible for biphasic fever, encephalitis, or hemorrhagic fever) and a number of tentative species, according to the VIIIth report of the ICTV (Thiel et al. 2005). It has been suggested that the genus may include a large number of additional virus species yet to be identified (Pybus et al. 2002). The recent discovery of new arboviruses transmitted by *Culex mosquitoes* (Bakonyi et al. 2005, Kono et al. 2000, Nisbet et al. 2005) supports this hypothesis. However, the characterization of Tamana bat virus (de Lamballerie et al. 2002) and new strains of Cell fusing agent in the New World (Cook et al. 2006), together with the discovery of Kamiti River virus (Crabtree et al. 2003, Sang et al. 2003), Cell Silent agents 1 and 2 (Crochu et al. 2004), Ngoye virus (Grard et al. 2006), and "Culex flavivirus " (Hoshino et al. 2007), suggest that a large number of viruses distantly related to the group of classical arthropod-borne flaviviruses also remains to be discovered. Crucial in the identification of new flaviviruses has been the use of molecular amplification systems employing highly conserved enzymatic motifs of the NS3 and NS5 genes, and it is evident that they will continue to constitute valuable tools for the discovery of classical and atypical flaviviruses.

In the past, such systems have been described (Ayers et al. 2006, Chao et al. 2007, Chow et al. 1993, Dyer et al. 2007, Eldadah et

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al. 1991, Figueiredo et al. 1998, Fulop et al. 1993, Gaunt et al. 2005, Kuno 1998a, Kuno et al. 1998b, Pierre et al. 1994, Puri et al. 1994, Sanchez-Seco et al. 2005, Scaramozzino et al. 2001, Tanaka 1993). However, their ability to detect a large number of species was, in most cases, based only on sequence analysis and the virus identification has been experimentally confirmed only for a few species. Moreover, their use in large screening analyses was hampered by the traditional reverse transcriptase polymerase chain reaction (RT- PCR) format and the frequent use of nested PCR, a common cause of false-positive results arising from PCR contamination.

The detection system presented in this study makes available a modern, high-performance molecular tool for the study of flaviviruses. It has proved its capability to detect 54 different recognized or tentative flavivirus species using a convenient one-step real -time RT- PCR amplification protocol. In addition, phylogenetic clustering of the derived amplicons with the previously published reference sequences (Kuno et al. 1998b), allowed the valid identification of the viruses detected. In combination with sequencing, our protocol represents a simple tool for the identification of flaviviruses and the discovery of new flavivirus species.

MATERIALS AND METHODS

Viruses and cell culture

All flaviviruses were handled and propagated according to their biosafety level requirement to comply with safety regulation in use at the Université de la Méditerranée. *Aedes* spp and *Culex* spp–associated viruses were propagated in *Aedes albopictus C6/36* cells grown at 28°C in Leibovitz' 15 medium supplemented with 3% decomplemented calf serum, 5% tryptose phosphate broth and penicillin G, streptomycin, at 100 IU/mL and 100 μg/mL, respectively. Tick-borne viruses or viruses with no known vector were propagated in human (SW13), Green monkey (Vero), or hamster (BHK 21) cells cultured as monolayers at 37°C under 5% CO_2 in Eagle's minimum essential medium with 3% calf serum and penicillin G, streptomycin, at 100 IU/mL and 100 μg/mL, respectively (Table 1). Bukalasa bat virus was tested from an infected mouse brain suspension, kindly provided by Dr. E.A. Gould.

RNA isolation

In the case of cell cultures prepared at the Unité des Virus Emergents, v iral RNAs were extracted from supernatant medium onto a Biorobot EZ1 automat (Qiagen) using the EZ1 Virus mini kit v2.0 in the presence of RNA Carrier (Qiagen, Poly-A, 30 μg/mL) according to the manufacturer's recommendations. Human and veterinary samples received at the Unité des Virus Emergents were preserved and inactivated using phenol- guanidinium thiocyanate solution (RNA Now TM TC-Kit, Biogentex Inc.). Viral RNA extraction was performed according to the manufacturer 's recommendation.

Oligonucleotide primers

A first pair of primers (Cook et al. 2006, Crochu et al. 2004, Grard et al. 2006), [PF1S: 5′-TGY-RTB-TA Y -AA C- ATG-ATG-GG, positions 8869–8888 of yellow fever virus strain 17D open reading frame (ORF) (GenBank accession number NC_002031) and PF2R: (5′-GT-GTC -CCA-DCC-DGC-DGT-RTC, positions 9, 121-9, 140], was designed to amplify a 269–272 nucleotides sequence (according to the flavivirus tested). The region amplified is located in the N-terminal end of the RNA-dependent RNA polymerase domain, between Motif 2 of the methyltransferase and Motif A of the polymerase (the reverse primer is located within Motif A) . The existence of these highly conserved patterns was previously identified by Kuno and collaborators and used to design a first generation of consensus primers (FU1/MA -cFD2 primer set) (Kuno et al. 1998a). The PF1S-PF 2R primers were originally degenerated to take into account the variability of the sequence among a large number of flaviviruses and to allow a strong hybridization at the 3′ termini (the 3′ terminal nucleotide is either at the first or second position of the corresponding codon).

Several modifications of this original set of

Table 1. Viruses Reference List

Species	*Virus*	*Abbreviation*	*Strain*	*Melting temperature Tm*	*GenBank Ac number*	*Sample*
Tick-borne viruses						
Gadgets Gully	Gadgets Gully	GGYV	CSIRO 122	82.1	EU073988	SW13 cells
Kadam	Kadam	KADV	AMP 6640	82.4	EU073994	BHK21 cells
Langat	Langat	LGTV	TP 64	84	EU073999	SW13 cells
Omsk haemorrhagic fever	Omsk haemorrhagic fever	OHFV	Bogoluvovska	82.5	EU074005	Vero cells
Powassan	Powassan	POWV	L. B.	82.2	EU074007	SW13 cells
Powassan	Deer Tick	DTV	CT-390	83.3–83.6	EU074035	Vero cells
Royal farm	Royal farm	RFV	Eg Art 371	83	EU074008	SW13 cells
*Karshi**	Karshi	KSIV	30517	84	EU073997	SW13 cells
Tick-borne encephalitis	Eastern subtype*	TBEV	Japan Os hima 5-10	83.1	EU074029	SW13 cells
Tick-borne encephalitis	Western subtype	TBEV	Hypr	82.8	EU073989	SW13 cells
Tick-borne encephalitis	Eastern subtype*	TBEV	Bochkova	82.5–82.8	EU074022	SW13 cells
*Tick-borne encephalitis**	Looping ill Negishi subtype*	LIV	Not Cent	83.3–83.8	EU074002	SW13 cells
*Tick-borne encephalitis**	Looping ill British subtype*	LIV	369T	82.8	EU074000	SW13 cells
*Tick-borne encephalitis**	Looping ill Spanish subtype*	SSEV	87/2617	83.8	EU074016	SW13 cells
*Tick-borne encephalitis**	Turkish sheep encephalitis*	TSEV	TTE80	84.1	EU074018	SW13 cells
*Tick-borne encephalitis**	Turkish sheep encephalitis Greek subtype*	GGEV	Vergina	84	EU073987	SW13 cells
Meaban	Meaban	MEAV	BREST AR T70	81.8–82	EU074001	SW13 cells
Saumarez reef	Saumarez reef	SREV	CSIRO 4	82.8	EU074015	SW13 cells
Tyuleniy	Tyuleniy	TYUV	6017	81	EU074019	SW13 cells
Tyuleniy	Tyuleniy	TYUV	3 ARCH#37	81.2	EU073973	SW13 cells
Kyasanur Forest disease	Alkhurma	ALKV	Isolates 5975, 7586, 7471, 9518, 1209, 7466, 7344, 87, 1176, MOS, 228	82.7	Published in §	Human sera
Kyasanur Forest disease	Alkhurma	ALKV	JE-7	82.1–82.5	Published in ○	Crushed tick
Kyasanur Forest disease	Kyasanur Forest disease	KFDV	It P 9065	82.7	EU074030	Vero cells
Mosquito-borne viruses						
Dengue	Dengue serotype 1	DENV-1	CY 436 D1 606	79.4	EU073979	C6/36 cells
Dengue	Dengue serotype 1	DENV-1	Indonesia 1186 TVP 949	79.5–80.1	EU074031	C6/36 cells
Dengue	Dengue serotype 2	DENV-2	Central Java JKT 85-544	80.8–81.1	EU074032	C6/36 cells
Dengue	Dengue serotype 2	DENV-2	H/IMTSSA-MART/98-703	81.4	EU073980	C6/36 cells
Dengue	Dengue serotype 2	DENV-2	Papete 341.175 17/12/96	80.1–80.5	EU074033	C6/36 cells
Dengue	Dengue serotype 2	DENV-2	Trinidad 1751	80.8–81.1	EU073981	C6/36 cells
Dengue	Dengue serotype 3	DENV-3	H87 prototype	81.4–81.7	EU073982	C6/36 cells
Dengue	Dengue serotype 4	DENV-4	Dak HD 34 460	81.4–81.7	EU073983	C6/36 cells
Dengue	Dengue serotype 4	DENV-4	BeH403714	81.5–81.7	EU073984	C6/36 cells
Kedougou	Kedougou	KEDV	Dak Ar D1470	82.8	EU073995	C6/36 cells

(*continued*)

Table 1. Viruses Reference List (Cont'd)

Species	*Virus*	*Abbreviation*	*Strain*	*Melting temperature Tm*	*GenBank Ac number*	*Sample*
Zika	Zika	ZIKV	MR 766	81.3	EU074027	C6/36 cells
Zika	Spondweni	SPOV	SA Ar 94	83.3–83.6	EU074014	C6/36 cells
Banzi	Banzi	BANV	SA H 336	82.2	EU073975	C6/36 cells
Bouboui	Bouboui	BOUV	Dak Ar B 490	82.5	EU073976	C6/36 cells
Edge Hill	Edge Hill	EHV	C-281	80.2	EU073985	C6/36 cells
Jugra	Jugra	JUGV	P-9-314	82.7	EU073993	C6/36 cells
Saboya	Saboya	SABV	Dak Ar D 4600	82.5	EU074010	C6/36 cells
Saboya	Potiskum	POTV	IBAN 10069	81.3	EU074006	C6/36 cells
Sepik	Sepik	SEPV	MK-7148	82	EU074011	C6/36 cells
Uganda S	Uganda S	UGSV	Original	80.7–81	EU074020	C6/36 cells
Wesselsbron	Wesselsbron	WESSV	SAH-177 99871-2	81	EU074039	C6/36 cells
Yellow fever	Yellow fever	YFV	17D vaccinal	81.8	EU074025	C6/36 cells
Aroa	Aroa	AROAV	Maracay 01809	81.8–82	EU074041	C6/36 cells
Aroa	Bussuquara	BSQV	Be An 4073	81.7	EU073977	C6/36 cells
Aroa	Iguape	IGUV	SP An 71686	83.5	EU074054	C6/36 cells
Aroa	Naranjal	NJLV	25008	81.5	EU074003	C6/36 cells
Cacipacore	Cacipacore	CPCV	Be An 327600	82.2	EU073978	C6/36 cells
Koutango	Koutango	KOUV	Dak Ar D 5443	81	EU073996	C6/36 cells
Japanese encephalitis	Japanese enc ephalitis	JEV	SA-14	81.7	EU073992	C6/36 cells
St Louis encephalitis	St Louis encephalitis	SLEV	MSI-7	81.5	EU074012	C6/36 cells
Usutu	Usutu	USUV	SA-AR-1776	82.2–82.5	EU074021	C6/36 cells
West Nile	West Nile	WNV	NY 385-99	82.8–83.1	EU074024	C6/36 cells
West Nile	West Nile	WNV	Ar B 3573/82	82.4–82.8	EU074034	C6/36 cells
West Nile	West Nile	WNV	PaAn001	82.5	EU074023	C6/36 cells
West Nile	Kunjin	KUNV	MRM 16	82.8–83.1	EU073998	C6/36 cells
Kokobera	Kokobera	KOKV	MRM 32/3265	79.8	EU074052	C6/36 cells
Murray Valley encephalitis	Alfuy	ALFV	MRM 3929	82–82.4	EU073972	C6/36 cells
Murray Valley encephalitis	Murray Valley encephalitis	MVEV	3329	80.5–80.9	EU074028	C6/36 cells
Bagaza	Bagaza	BAGV	Dak Ar B 209	82.5	EU073974	C6/36 cells
Ilheus	Ilheus	ILHV	PE 20545	81.2	EU073990	C6/36 cells
Ilheus	Rocio	ROCV	SPH 34675	81.5	EU074009	C6/36 cells
Israel Turkey meningoencephalomyelitis	*I*srael Turkey meningoencephalomyelitis	ITV	ME 30502	81.4	EU073991	C6/36 cells
Ntaya	Ntaya	NTAV	Original	80.7	EU074004	C6/36 cells
Tembusu	Tembusu	TMUV	MM1775	80.5	EU074017	C6/36 cells
Tembusu	Sitiawan	SITV	Original	80.7	EU074037	Vero cells
Yaounde	Yaounde	YAOV	Dak Ar Y276	81.9–82.2	EU074036	C6/36 cells

NKV viruses						
Entebbe bat	Entebbe bat	ENTV	UgIL-30	82.1	EU073986	SW13 cells
Entebbe bat	Sokoluk	SOKV	LEIV-400K	81.6	EU074013	BHK21 cells
Yokose	Yokose	YOKV	Oita 36	81.6–81.9	EU074026	SW13 cells
Apoi	Apoi	APOIV	22605	81.3–81.7	EU074040	BHK21 cells
Jutiapa	Jutiapa	JUTV	JG-128	81.5	EU074053	BHK21 cells
Modoc	Modoc	MODV	3321	79.8	EU074050	BHK21 cells
Sal Vieja	Sal Vieja	SVV	78TWM-106	80.9–81.3	EU074046	BHK21 cells
Carey island	Carey island	CIV	P-70 1215	78.9–79.3	EU074047	Vero cells
Montana myotis leukoencephalitis	Montana myotis leukoencephalitis	MMLV	R8313	79.5	EU074045	Vero cells
Phnom Penh bat	Phnom Penh bat	PPBV	30834 A38	80.1	EU074049	BHK21 cells
Phnom Penh bat	Batu Cave	BCV	P-70 1459	80.1–80.5	EU074044	Vero cells
Rio Bravo	Rio Bravo	RBV	US Bat 3360	79.5–79.9	EU074048	SW13 cells
Dakar bat	Dakar bat	DBV	IBAN 10487 47503	79.9–80.3	EU074042	Vero cells
Bukalasa bat	Bukalasa bat	BBV	UGBP-111	79.8	EU074043	Suckling mice brain
Tentative species						
Tentative species	Cell fusing agent	CFAV	H9-84	83.1–83.4	EU074055	C6/36 cells
Tentative species	Cell fusing agent	CFAV	Rio Piedras 23/11/02	82.7	EU074056	C6/36 cells
Tentative species £	Ngoye	NGOV	JJLV-329	82.7	EU074038	Crushed tick
Tentative species $	Kamiti River	KRV	SR-75	82.5	EU074051	C6/36 cells

* According to Grard et al. (2007)
§ According to Charrel et al. (2005)
○ According to Charrel et al. (2006)
£ According to Grard et al.(2006)
$ According to Sang et al. (2003) and Crabtree et al. (2003)

primers were performed and tested. Here, we present an improved version of the reverse primer, PF2R-bis (5′-GTG-TCC-CA I-CCN-GCN-GTR- TC) the design of which was based on an alignment of all available sequence within the genus *Flavivirus*. Several modifications of the PF1S primers were also tested, but they did not improve the detection performance (data not shown).

Real-time PCR protocol

Assay format. The primers were used to elaborate a one-step RT- PCR assay performed in a real-time PCR format, using the QuantiTect® SYBR® Green RT- PCR (Qiagen) onto an ABI Prism 7000 Sequence Detection System (Applied Biosystems).

Choice of optimized primers. This was made after testing 10-fold serial dilutions of RNA (10^{0}–10^{-13}) of 14 representative viruses of the genus in an RNA carrier solution (Qiagen, Poly-A, 30 μg/mL): DENV-2 strain Trinidad 1751, WNV strain NY-99, W-TBEV strain Hypr, YFV strain 17D, CFA V strain Rio Piedras, KRV, APOIV, DB V, MMLV, PPB V, MODV, RBV, SV V, JUTV (see Table 1 for more details) and a standard amplification protocol (1 μM for each primer, hybridization temperature at 52°C).

Optimization of the amplification protocol. This was performed using 5 viruses (DENV—2 strain Trinidad1751, YFV, WNV strain NY -99, W-TBEV strain Hypr, MMLV) and the ultimate RNA dilution that enabled detection using standard amplification parameters according to the manufacturer's instructions. Several concentrations of primers (0.3–1 μM of each primer) and of MgCl2 (5–6 mM), and different annealing temperatures (48°C–55°C) were tested.

Evaluation of the optimized protocol. The protocol was further evaluated using synthetic flavivirus RNAs. Specific PCR products carrying the sequence of the T7 promoter and the specific targeted NS5 region were prepared for 12 different flaviviruses (Stram et al. 2005) and directly transcribed using the T7-MEGAshort script kit (Ambion) (Table 2). RNA was submitted to DNAse digestion, purified using the MEGAclear columns (Ambion) according to the manufacturer's instructions, and quantified by ultraviolet spectrophotometry (1A 260 unit of single-stranded RNA = 40 μg/mL).

The RNA concentration (copies/μL) was calculated as follows: $N = (C \times 10^{-3} \times 6.023.10^{23})/(L \times 330.10^{6})$ where C represents the concentration of RNA (μg/mL) assessed by OD measurement; $6.023.10^{23}$ is the Avogadro number; L is the length of the synthetic RNA (nt); and 330 is an approximation of the molecular weight of a nucleotide (g/mol).

The limit of detection (LOD) of the assay was evaluated using 10-fold serial dilutions of synthetic RNA in an RNA carrier solution (Qiagen, Poly -A, 30 μg/mL) tested in triplicate.

Detection and characterization of different flavivirus species

Eighty-six strains of flaviviruses representing 51 species and 3 tentative species (Table 1) were submitted to RNA extraction. Viral RNA and a 10^{-1} RNA dilution were tested using the optimized protocol as described above. In addition, human sera from Saudi A rabian patients (n = 11) testing positive for Alkhurma virus infection (as determined using specific primers (Charrel et al. 2005)), human sera from either Thai (n = 12) or Senegalese (n = 1) patients who had tested positive for Dengue virus infection (as determined using specific primers), mosquito pools from Puerto Rico (Cook et al. 2006), and pools of ticks from Senegal (Grard et al. 2006) and Saudi Arabia (Charrel et al. 2007) were also tested.

All PCR products were submitted to direct sequencing using the amplification primers for initiation of the polymerization reaction. Raw nucleotide sequences were refined and analyzed with the Sequencher software version 4.7 (Gene Codes Corporation) and compared with those available in the GenBank database using the BLASTN algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

Flavivirus nucleotide sequences in the ~1.0-kb part of the NS5 region (nt 8899 to 9943 according to the YFV strain 17D ORF) previously used by Kuno et al. (Kuno et al. 1998b) for phy-

TABLE 2. SYNTHETIC RNA TEMPLATES

Virus	*Forward primer (including T7 promoter*)*	*Reverse primer*	*GenBank accession number*	*Length of synthetic RNA*	*Nb of copies detected /reaction***
W- TBEV Hypr	TAATACGACTCACTATAGGGAGAGAGAGAAAGGCACCTCA	GCTCACCCTCCATGTACCG	U39292	351	6.5
KADV	TAATACGACTCACTATAGGGAGAACGTGAGTTGCACCTCA	CGAGGTAACGGAGGATCTGT	DQ235146	342	23
YFV17D	TAATACGACTCACTATAGGGAGACTGCCAATGAGGCTGTC	CAAGATCTCCTGTTCATCATC	NC_002031	404	415
DENV-2 Trinidad 1751	TAATACGACTCACTATAGGGAGAGAAGAATTyACAAGAAArGT	TTrTGTTCTCCTTyCATGTGG	Unpublished data	480	1,200
WNV NY-99	TAATACGACTCACTATAGGGAGATGGTGGATGAGGAGCG	GCAGCTCAAGCACCTTAGC	DQ211652	350	10,500
JEV	TAATACGACTCACTATAGGGAGAAAGTCAACAGCAACGCG	TATGGCTCGAGCGAGCATG	M18370	480	3,600
SLEV	TAATACGACTCACTATAGGGAGAGAAGCTCATCTTAAAGGAG	CAATGATTGCCTCTGCAAGC	DQ359217	374	29
APOIV	TAATACGACTCACTATAGGGAGAGCGGCAATTGCATCTCA	CTCTCATCCTCAAGATCTGCT	AF160193	320	35
RBV	TAATACGACTCACTATAGGGAGAGATGCAGGGATGGAGTG	GATGCATTCATCATCCAGATC	AF144692	392	20,500
MMLV	TAATACGACTCACTATAGGGAGACAGTTACATCTCACTGGT	ATCCAGAACTGTCCCTCCA	AJ299445	440	11,500
CFAV Rio Piedras	TAATACGACTCACTATAGGGAGAAGCCATTCAGGACCAGT	AGGGCTGTTAGAAGAGCTTC	Unpublished data	380	2.5
KRV	TAATACGACTCACTATAGGGAGAGCGACGCCTTCATCTC	TGAGCTCGGTTAACAAGGTC	NC05064	359	2

* T7 promoter sequence is underlined

** The number of copies indicated corresponds to the last 10-fold dilution of a synthetic RNA template that provided reproducible positive results. It was evaluated from the 260 nm OD of an RNA solution as indicated in the text.

logenetic analysis of the genus *Flavivirus* were retrieved from GenBank and used for multiple alignments using the ClustalX version 1.8 software (Thompson et al. 1997). Phylogenetic analysis was then carried out using the neighbor-joining method and the MEGA version 3 software (Kumar et al. 2004). In brief, a phylogenetic backbone was constructed using the ~1.0-kb NS5 data set. Each of the PF1S/ PF2R-bis sequences generated in the current study (nt 8889–9120 according to the YFV strain 17D ORF) was subsequently injected in the alignment, and the localization of the sequence within the evolutionary tree was analyzed using the *p*-distance algorithm and the neighbor-joining method. The robustness of the branching pattern was tested using 500 bootstrap replications.

RESULTS

Optimized protocol

The final optimized protocol was as follows, using the PF1S sense primer and the PF2R-bis reverse primers in a 25-μL final volume: each reaction mixture contained 5 μL of RNA, 12.5 μL of 2X QuantiTect® (Qiagen France, Courtaboeuf) RT- PCR Master Mix, 0.25 μL of QuantiTect RT Mix, 0. 55 μM of each primer. The cycling program was 50°C for 30 min, 95°C for 15 min, followed by 40 cycles consisting of 94°C for 15 s, 50°C for 30 s, and 72°C for 45 s. Analysis of the melting curve of specific PCR products was performed by slowly raising the temperature from 60°C to 95°C by means of regular fluorescence measurements.

Compared to the PF1S/PF2R primers set, the PF1S/ PF2R-bis primers pair resulted in earlier detection signals as evaluated by the comparison of Ct values.

Limit of detection

The protocol was further evaluated using quantified serial dilutions of 12 synthetic flavivirus RNAs. The LOD was <21,000 copies/reaction for all RNAs tested, reaching values <50 copies/reaction for TBEV, KADV, SLEV, and APOIV, and <3 copies/reaction for CFAV and KRV (Table 2).

Spectrum of detection of flaviviruses

Flavivirus RNA could be detected from all 86 cultured strains or biological samples tested (either from undiluted samples or from a 10^{-1} RNA dilution), representing 51 species and 3 tentative species.

The amplicons obtained from the different flaviviruses were identified by analysis of dissociation plots. The melting temperature of amplification products varied from 78.9°C to 84.1°C (see Table 1 for more details), and could be unambiguously distinguished from primer dimers (dissociation temperature <74°C) (Macpherson et al. 2006).

Clinical and veterinary samples

In addition to virus strains and synthetic RNAs, a series of biological samples previously found positive for the presence of different flaviviruses based on specific PCR protocols was tested using the optimized protocol. The ALKV genome could be detected from 11 human sera, and the DENV genome from 13 sera (serotype 1, $N = 2$; serotype 2, $N = 9$; serotype 4, $N = 2$), from patients who developed clinical symptoms.

The CFAV genome could be detected from 67 pools of mosquito es from the Puerto Rico area, the NGOV genome from 2 crushed ticks from Senegal and the ALKV genome from 1 crushed tick from Saudi Arabia.

Specificity

The PF1S/PF2R-bis system was tested using nucleic acid extracts from three different mammalian cell lines (VERO, BHK -21, SW13), an insect cell line (C6/3 6), known negative human serum samples, and several pools of uninfected mosquitoes and ticks. In a further negative control, water was substituted for RNA. No false-positive results were observed, implying high specificity for the flavivirus sequences.

Sequence analysis

Identification of the different flaviviruses was tested using sequences obtained from the direct characterization of amplicons. Using the BLASTN program and the nr/nt database (at

the NCBI website), all virus species for which a reference sequence was available were precisely identified with an e value between 2e-114 and 2e-94, and a 94–100% coverage value (percentage of length allowing significant matching). A 94% coverage value was found for all viruses of the Kuno data set (Kuno et al. 1998b), for which only partial NS5 sequences are available (i.e., 13 nt at the 5′ end of PF1S/PF2R-bis sequence are missing). In the case of ITV and BAGV, the correct identification was obtained with e values at 2e-107 and 2e-114, respectively, but BAGV scored at 7e-101 and ITV at 7e -95 as a second-rank identification for ITV and BAGV, respectively. However, phylogenetic analysis unambiguously confirmed the correct taxonomic identification in both cases (see *infra*). For all other sequences tested, distinction between the first and second rank identifications was obvious, with e values between 2e -86 and 2e-32 for the latter.

A phylogenetic analysis was performed by injecting individual PF1S/ PF2R-bis sequences within an NS 5 tree based on the Kuno dataset. All the sequences produced using the PF1S/ PF2R-bis amplification system clusterized with the corresponding reference sequence, when available (Fig. 1).

In the case of WESSV and NGOV, there was no reference sequence available in the databases. The first rank identification for WESSV was SEPV with a low e value (9e-43), clearly indicating that the two sequences originated from distinct virus species. This was confirmed by analysis of genetic distances (identity = 79%) and of

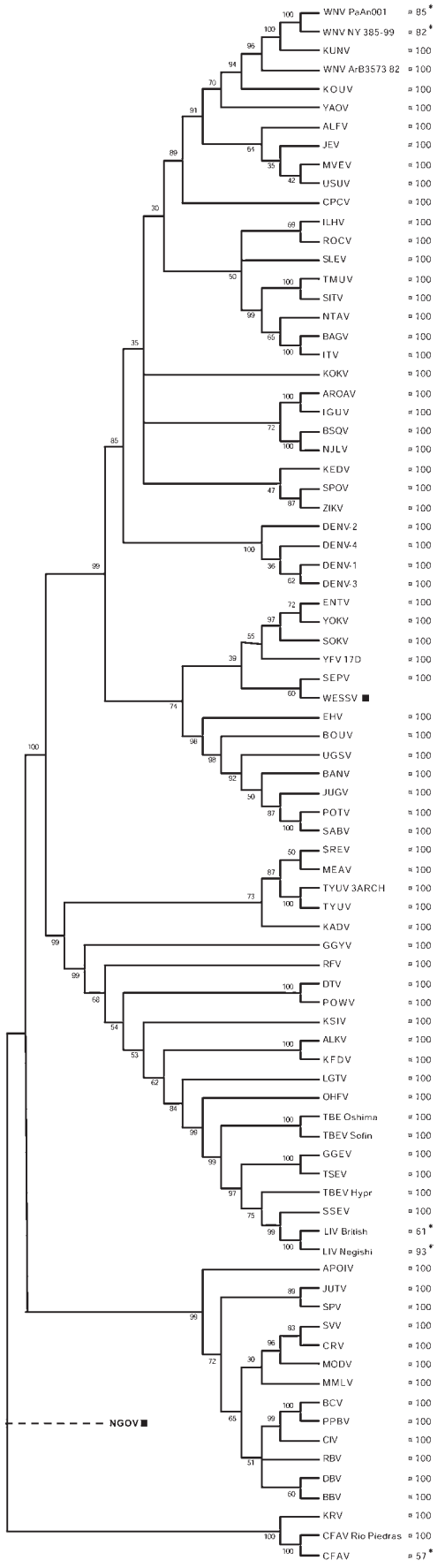

FIG. 1. Phylogenetic reconstruction in the NS5 gene. Phylogenetic reconstruction was performed using the *p*-distance algorithm and the NJ method implemented in Mega. A condensed tree is presented in which the length of horizontal lines is not proportional to genetic distances. The percent bootstrap values corresponding to 500 replications are indicated with a cutoff value of 30%. Sequences used correspond to positions 8899 to 9943 according to the YFV strain 17D ORF, except for NGOV and WESSV (■) (nt 8889–9120). The (○) sign indicates sequences generated using the PF1S/PF2R-bis system that clusterized with the corresponding viral sequence within the reference tree. The bootstrap resampling value supporting grouping is indicated (*). In the case of CFAV, LIV, and WNV, the bootstrap value supporting clustering with the precise homologous isolate was lower. However, clustering within the corresponding virus species was supported by 100% boots trap values in all cases.

the phylogenetic topology. However, the proposed genetic relationship between SEPV and WESS constituted a valuable clue for the taxonomic assignment of WESSV (both belong to the group of *Aedes*-borne flaviviruses).

For NGOV, the first rank identification was EHV, with an extremely low e value (1e-34), indicative of distant taxons. This was confirmed by phylogenetic analysis. The analysis of genetic distances showed an identity value at 61%, and the phylogenetic topology suggested a distant taxonomic relationship with other flaviviruses.

Altogether, these results demonstrate that sequences produced using this detection protocol allow a robust taxonomic identification of flaviviruses.

DISCUSSION

The elaboration of molecular assays allowing the consensus detection of flaviviruses is challenging, considering the enormous genetic diversity within this evolutionary lineage. We propose here a PCR protocol, which is the most efficient among those proposed to date for the consensus detection of flaviviruses. It combines the convenient real-time PCR format (that allows large-scale detection screening without any risk of PCR contamination) and a broad-spectrum of flavivirus detection (possible detection of all tested 50 species and 3 tentative species). By comparison, the assay proposed by Kuno and collaborators (Kuno 1998a), using closely related NS5 primers, allowed the detection of 52 species and 1 tentative species, but was based on a standard PCR format that requires electrophoresis of amplicons onto agarose gels, and sensitivity was not reported. An assay by Scaramozzino and colleagues (Scaramozzino et al. 2001) was also proposed for use in either real-time PCR or nested PCR format using the NS5 gene, but it was tested on 8 flavivirus species only. Another study (Gaunt et al. 2005) used a standard nested PCR protocol in the E gene followed by restriction enzyme analysis that allowed the detection of 40 species and 1 tentative species.

Two other aspects of the performances of this molecular assay deserve further comments. First, the test sensitivity was found to be surprisingly high, considering that it was based on the use of degenerate primers. The limit of detection of the assay cannot compare for all viruses with specific detection real-time detection techniques, but the sensitivity is good for most of the viruses tested and excellent for some of them (lower than 50 copies/reaction for TBEV, KADV, SLEV, APOIV, and CFAV–related viruses). This sensitivity allowed the detection of different flaviviruses from a series of human sera or field samples. Second, it is interesting to note that the short NS5 region amplified by the proposed protocol (229 to 232 nt—primers excluded—depending on the different virus species) is usable for the reliable taxonomic identification of flaviviruses.

In summary, the convenient format of this assay and its performances for detecting and identifying flaviviruses make it a good candidate for the identification of previously identified flaviviruses in cell culture and the investigation of field samples. Moreover, due to the design of the primers in highly conserved patterns of the viral genomes and to its ability to detect recently discovered species (e.g., Ngoye virus or Kamiti River virus), it also constitutes a promising tool for the discovery and identification of new flavivirus species, including viruses distantly related to the "classical" arthropod-borne flaviviruses.

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Sensitive and Broadly Reactive Reverse Transcription-PCR Assays To Detect Novel Paramyxoviruses[▽]

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We have developed a set of reverse transcription-PCR assays for the detection and identification of known and novel paramyxoviruses in clinical specimens. Primers were designed from the conserved motifs of the polymerase *pol* gene sequences to detect members of the *Paramyxovirinae* or *Pneumovirinae* subfamily or groups of genera within the *Paramyxovirinae* subfamily. The consensus-degenerate hybrid oligonucleotide primer design and seminested or nested PCR assay design were used to enhance the breadth of reactivity and sensitivity of the respective assays. Using expressed RNA and 10-fold dilution series of virus-infected tissue culture isolates from different members of the family or genera, these assays were able to detect on average between 100 and 500 copies of template RNA. The assays were specific to the respective group of genera or subfamily viruses. This set of primers enhances our ability to look for novel viruses in outbreaks and diseases of unknown etiology.

The paramyxoviruses are ubiquitous pathogens and have been identified in a variety of hosts, including birds (chickens and turkeys), aquatic animals (salmon, whale, seal, dolphin, and porpoise), rodents (mice and rats), dogs, cats, sheep, reptiles (snake and lizards), horses, cattle, bats, pigs, simians, and humans. In humans, paramyxoviruses have been associated with a wide range of diseases, including croup, bronchiolitis, pneumonia, encephalitis, meningitis, parotitis, orchitis, spontaneous abortion, rash illnesses, and persistent infections (7, 10, 12, 16). Recently a variety of new members of the *Paramyxoviridae* family have been identified in animals, and some, most notably Nipah and Hendra viruses, have caused serious and sometimes fatal human infections (1, 2, 4). It is likely that there are additional, as yet unidentified members of this family and some will likely infect and cause disease in humans. Therefore, we believe the *Paramyxoviridae* constitute an important target for improved methods to detect novel viruses.

Detection of most paramyxoviruses has been routinely carried out by cell culture isolation, electron microscopy, antigen detection assays (immunofluorescence assays or enzyme immunoassays [EIA]), serologic assays, and genome-based assays, such as PCR assays. Each system has limitations. Traditional genome-based, antigen-based, and antibody-based assays usually are too specific to detect novel viruses. Cell culture isolation will allow detection only of viruses that grow and replicate in the culture system used and will require further characterization, usually by antigen- or genome-based assays. Electron microscopy requires a fairly high titer of virus for visualization and also requires further characterization.

To increase our ability to detect novel viruses, we chose to develop broadly reactive PCR assays. This strategy has been used very successfully to identify and characterize a number of novel human viruses, including severe acute respiratory syndrome coronavirus (5), hepatitis G virus (17), Sin Nombre virus (8), human retrovirus 5 (3), and novel animal viruses, such as the macaque gammaherpesvirus (15) and pig endogenous retrovirus (9). The present article describes the development of a set of PCR assays that should detect all known and novel paramyxoviruses. The primers for these assays were developed from highly conserved regions of the genome. We applied the consensus-degenerate hybrid oligonucleotide primer methodology (13, 14) in primer design and seminested PCRs to optimize the specificity and sensitivity of these broadly reactive assays.

MATERIALS AND METHODS

Virus and isolation of viral RNA. The reference viruses or viral RNA used in this study are listed in Table 1 and include three strains from the genus *Avulavirus*, two strains from the genus *Henipavirus*, five strains from the genus *Morbillivirus*, seven strains from the genus *Respirovirus*, five strains from the genus *Rubulavirus*, four strains from the genus *Pneumovirus*, three strains from the genus *Metapneumovirus* and three newly isolated unclassified paramyxoviruses. RNAs were extracted from 100 μl of supernatant fluid of virus-infected cells with the QIAamp viral RNA kit (Qiagen, Santa Clarita, CA) according to the manufacturer's instructions. The RNA was eluted from the column in 50 μl of RNase-free water. RNAs for the Hendra virus, Nipah virus, Menangle virus, and Fer-de-Lance virus (FDLV) strains were obtained from Paul Rota (Centers for Disease Control and Prevention) as extracts in Trizol, which were prepared according to the instructions provided by the commercial source (Invitrogen, Carlsbad, CA).

Broadly reactive oligonucleotide primer selection. Conserved amino acid sequences for the family, subfamily, and genera were selected from alignment of deduced L-protein-coding sequences recorded in GenBank (National Institutes of Health, Bethesda, MD). A total of 33 nonredundant paramyxovirus L-protein sequences were used and aligned using the Clustal W program. We selected highly conserved domains between 8 and 10 amino acids in length and back translated into degenerate nucleotide sequences to represent all possible codons for the corresponding amino acids. To minimize the number of primers, primers were designed with mixed degenerate bases restricted to between 9 and 12 nucleotides in the 3′ portion of the primers, and inosines (maximum of four) and consensus nucleotides were used for the remaining middle and 5′ portion of the

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TABLE 1. Reference viruses used in evaluating PCR in this work

Virus and classification	Abbreviation	Source
Paramyxovirinae		
Avulavirus		
Newcastle disease virus	NDV	Jack King, Qingzhong Yu
Avian paramyxovirus-2	APMV-2	Jack King, Qingzhong Yu
Avian paramyxovirus-3	APMV-3	Jack King, Qingzhong Yu
Henipavirus		
Hendra virus	Hendra	Paul Rota
Nipah virus	Nipah	Paul Rota
Morbillivirus		
Measles virus	MV	Paul Rota
Canine distemper virus	CDV	Randy Renshaw
Phocine distemper virus	PDV	Bert Rima
Dolphin morbillivirus	DMV	Bert Rima
Porpoise morbillivirus	PMV	Bert Rima
Respirovirus		
Bovine parainfluenza virus 1	BPIV1	Dean Erdman
Bovine parainfluenza virus 3	BPIV3	Randy Renshaw
Human parainfluenza virus 1	HPIV1	Dean Erdman
Human parainfluenza virus 3	HPIV3	Dean Erdman
Sendai virus	Sendai	Lela K. Riley
Unclassified *Respirovirus*		
Pacific salmon paramyxovirus	PSPV	Gael Kurath
Guinea pig parainfluenza virus 3	CaVPIV3	Lela K. Riley
Rubulavirus		
Mumps virus	Mumps	Paul Rota
Human parainfluenza virus 2	HPIV2	Dean Erdman
Canine parainfluenza virus 2	CaPIV2	Randy Renshaw
Simian virus 5	SV5	Richard W. Compans
Simian virus 41	SV41	Yasuhiko Ito
Unclassified *Paramyxovorinae*		
Fer-de-Lance virus	FDLV	Gael Kurath
Menangle virus	Menangle	Paul Rota
Salem virus	SalV	Randy Renshaw
Pneumovirinae		
Metapneumovirus		
Avian metapneumovirus type C	AMPV-C	Jack King, Qingzhong Yu
Human metapneumovirus 75	HMPV75	Dean Erdman
Human metapneumovirus 83	HMPV83	Dean Erdman
Pneumovirus		
Bovine respiratory syncytial virus	BRSV	Randy Renshaw
Human respiratory syncytial virus A	hRSV[a] A	Dean Erdman
Human respiratory syncytial virus B	hRSV B	Dean Erdman
Pneumonia virus of mice	PVM	Joseph Domachowske

[a] hRSV, human RSV.

primers. In addition, primers were designed to achieve similar reaction conditions and an amplicon size between 200 and 500 bp. To minimize the potential for nonspecific cross-reactivity, a Blastn search analysis of GenBank was performed for similarities with known sequences. The primers were synthesized at the Biotechnology Core Facility, Division of Scientific Resources of the Centers for Disease Control and Prevention (CDC).

RT-PCR and nested RT-PCR amplification. To maximize sensitivity and specificity, we nested or seminested PCR assays and optimized reaction conditions, including primer concentration, magnesium (Mg^{2+}) concentration, and thermal cycling temperatures and profiles. For the first PCR in the seminested assay, we used the SuperScript III One-Step reverse transcription-PCR (RT-PCR) kit (Invitrogen, Carlsbad, CA). The optimized PCR mixtures contained 50 pmol each of forward and reverse primers, 1× buffer with a final concentration of 2.0 mM $MgSO_4$ and 200 μM (each) deoxynucleoside triphosphates, 20 U of RNase inhibitor, a 5-μl aliquot of RNA/DNA extracts, and 1 U of SuperScript III RT/Platinum *Taq* mix. Water was then added to achieve a final volume of 50 μl. The RT-PCR mixture was sequentially incubated at 60°C for 1 min for denaturing, 44 to 50°C for 30 min (for RT), 94°C for 2 min (for hot start), and then 40 cycles at 94°C for 15 s, 48 to 50°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 7 min. For the second amplification in the seminested PCR assay, we used 1× buffer (Platinum *Taq* kit; Invitrogen), 2 mM $MgCl_2$, 200 μM (each) of deoxynucleoside triphosphates, 50 pmol (each) of forward and reverse primers, 1 U Platinum *Taq*, one 2-μl aliquot from the first reaction, and water to achieve a final volume of 50 μl. The mixture was first heated to 94°C for 2 min. The cycling conditions were 40 cycles with the same conditions as for the first amplification: 94°C for 15 s, primer annealing at 48 to 50°C for 30 s, and 72°C for 30 s. A final extension was carried out at 72°C for 7 min. The final nested or seminested PCR products were visualized by UV light after electrophoresis on a 2% agarose gel containing 0.5 μg/ml ethidium bromide in 0.5× Tris-borate buffer (pH 8.0). A DNA VIII marker (Roche, Indianapolis, IN) was run in the gels to estimate amplicon size.

Specificity and sensitivity. Initial primer validation and selections were performed using one reference RNA template from the subfamily or genera for which the PCR assay was designed. Mumps virus RNA was used for the *Rubulavirus-Avulavirus* genus subgroup-specific primers and the *Paramyxovirinae* subfamily primers, Hendra RNA for the *Morbillivirus-Respirovirus-Henipavirus* genus subgroup-specific primers, and respiratory syncytial virus A RNA for the *Pneumovirinae* subfamily-specific primers. Following the initial screening, the selected primer pairs were then tested against the representative paramyxoviruses listed in Table 1. Finally, to test for unanticipated nonspecific reactivity, the PCR assays were tested against pooled nucleic acids of influenza A and B viruses, rhinoviruses, adenovirus, two distinct human coronaviruses, human coronavirus

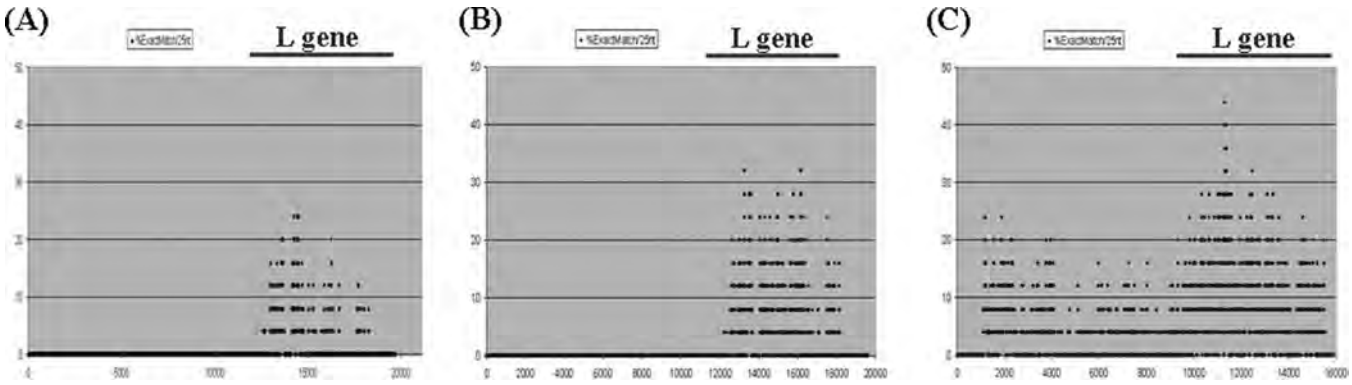

FIG. 1. Similarity plots of the aligned paramyxoviruses' genomes. The plots were obtained using an in-house program based on multiple alignments of viral genomes from 29 different strains in the *Paramyxoviridae* (A), 22 different strains in the *Paramyxovirinae* (B), and 7 different strains in the *Pneumovirinae* (C). The identity percentage score given on the *y* axis was calculated based on the exact-match percentage with a window of 25 nucleotide positions, and the window was progressively moved across the alignment in 1-nucleotide-position steps. The *x* axis shows the first position of the window in the multiple alignments of the viral genomes.

229E, and human coronavirus OC-43 and bacteria (*Chlamydia pneumoniae*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Mycoplasma pneumoniae*).

The sensitivities of the PCR assays were determined using two sources of RNA: RNA that was extracted from each dilution of a 10-fold dilution series of virus-infected cell culture with known infectivity titers (PFU) and serial dilutions of synthetic RNA that was transcribed in vitro from cloned genome fragments as previously described (18).

Sequencing. Amplicons from the final round of PCR were purified using the QIAquick PCR purification kit (Qiagen, Inc., Valencia, CA). Both strands of the amplicons were sequenced with a BigDye Terminators v1.1 ready reaction cycle sequencing kit on an ABI Prism 3100 automated sequencer (Applied Biosystems, Foster City, CA) using the corresponding PCR primers. The remaining reaction conditions were according to the manufacturer's instructions.

RESULTS

Development of consensus degenerate primers. To design family- or subfamily-specific primers, all full-length genome sequences were obtained from the *Paramyxoviridae* family viral sequences archived in the GenBank viral database. Viral sequences with more than 95% similarity were treated as identical; the resulting 29 nonredundant full-length genome sequences were used initially to design primers for this study. First, a multiple full-length genome sequence alignment was performed by using Clustal W and was scanned according to a program written in house to identify genes that have the most-conserved multiple regions from the broadest possible grouping in the family *Paramyxoviridae*. The plot (Fig. 1) showed that the RNA polymerase (L) coding sequences were the most conserved, and thus, this gene was selected as the locus for the broadly reacting PCR assays. For the final primer design, we used an additional four L-gene sequences available in GenBank for a total of 33 L-gene-specific nonredundant sequences. Since the subfamilies, *Paramyxovirinae* and *Pneumovirinae*, are so genetically distinct, primers were designed for each separately. The highly conserved amino acid regions a with minimal length of six amino acids were checked for possible presence in other nontarget organisms by a BLAST search to avoid nonspecific amplification in PCR. Primers were designed from these highly conserved regions using all codon possibilities for 9 to 12 bases at the 3′ end of the primer and consensus sequences or inosines at positions of fourfold degeneracy for a "consensus clamp" at the 5′ end. After comparative analysis including sizes of amplicons, the similarity of reaction kinetics, nonspecific cross-reactivity, and experimental evaluation, three consensus degenerate primers (two for the first PCR and the third for the second, seminested PCR) corresponding to the most-conserved motifs were selected as the pan-*Paramyxovirinae* primers and three consensus degenerate primers corresponding to the most-conserved motifs were selected as the pan-*Pneumovirinae* primers, as shown in Table 2. Since the subfamily *Paramyxovirinae* has greater diversity

TABLE 2. Consensus degenerate primers used for detection of paramyxoviruses

Primer name	Amino acid motif in RNA-dependent DNA polymerase	Targeted group
PAR-F1	GAA GGI TAT TGT CAI AAR NTN TGG AC	*Paramyxovirinae*
PAR-F2	GTT GCT TCA ATG GTT CAR GGN GAY AA	*Paramyxovirinae*
PAR-R	GCT GAA GTT ACI GGI TCI CCD ATR TTN C	*Paramyxovirinae*
RES-MOR-HEN-F1	TCI TTC TTT AGA ACI TTY GGN CAY CC	*Respirovirus, Morbillivirus, Henipavirus*
RES-MOR-HEN-F2	GCC ATA TTT TGT GGA ATA ATH ATH AAY GG	*Respirovirus, Morbillivirus, Henipavirus*
RES-MOR-HEN-R	CTC ATT TTG TAI GTC ATY TTN GCR AA	*Respirovirus, Morbillivirus, Henipavirus*
AVU-RUB-F1	GGT TAT CCT CAT TTI TTY GAR TGG ATH CA	*Avulavirus, Rubulavirus*
AVU-RUB-F2	ACA CTC TAT GTI GGI GAI CCN TTY AAY CC	*Avulavirus, Rubulavirus*
AVU-RUB-R	GCA ATT GCT TGA TTI TCI CCY TGN AC	*Avulavirus, Rubulavirus*
PNE-F1	GTG TAG GTA GIA TGT TYG CNA TGC ARC C	*Pneumovirinae*
PNE-F2	ACT GAT CTI AGY AAR TTY AAY CAR GC	*Pneumovirinae*
PNE-R	GTC CCA CAA ITT TTG RCA CCA NCC YTC	*Pneumovirinae*

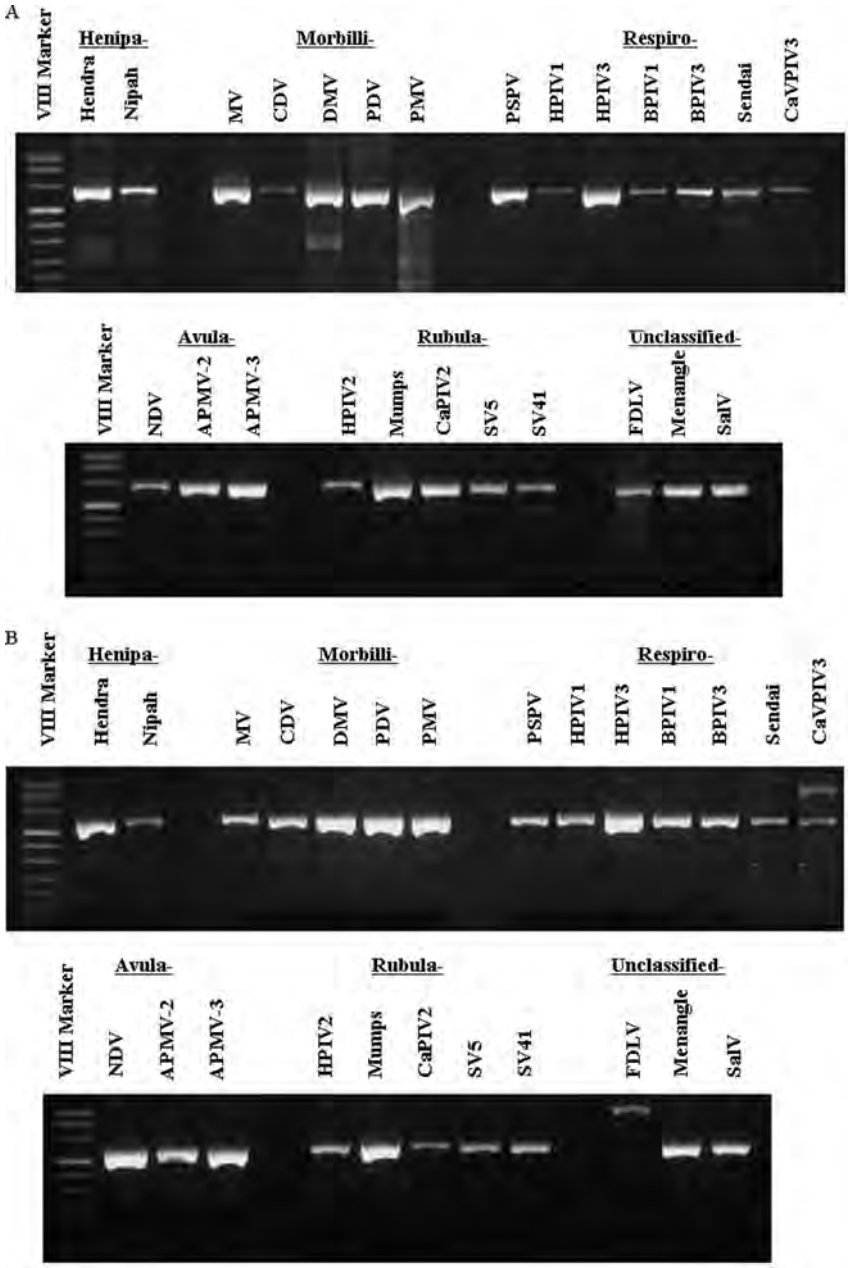

FIG. 2. Amplification of RNAs from 25 different viral members in the subfamily *Paramyxovirinae* by one-step RT-PCR using the pan-PAR-F1/PAR-R primer pair (A) or the pan-PAR-F2/PAR-R primer pair (B). Viral names are abbreviated as shown in Table 1.

among its members than the subfamily *Pneumovirinae*, we further divided the *Paramyxovirinae* into two subgroups of genera based on RNA polymerase gene relatedness, the *Morbillivirus-Respirovirus-Henipavirus* subgroup and the *Rubulavirus-Avulavirus* subgroup, and further developed primers to achieve less degeneracy and greater sensitivity, as noted in Table 2.

Standardization of optimized conditions. Different combinations of RT and PCR steps including one tube/one step, one tube/two steps, and two tubes/two steps, have a significant effect on the outcome of the assays. By comparative testing, we observed that a one-tube/one-step RT-PCR protocol was not only more convenient and more sensitive (data not shown) but also minimized risk of handling cross-contamination. RT-PCRs were optimized for band intensity and low background by evaluating combinations of primer concentration, Mg^{2+} concentration, and annealing temperatures.

Broad reactivity of consensus degenerate primers. As shown in Fig. 2A and B, all of the 25 reference viruses representing five genera from the *Paramyxovirinae* subfamily were successfully detected by pan-*Paramyxovirinae* primers. As shown in Fig. 3 and 4, members of the two groups of genera within this subfamily were also detected by the appropriate subgroup-specific primers. The observed variation in amplicon intensity was probably due at least in part to differences in amount of template RNA for the respective viruses. No amplicons were detected for the PCR assays against the pooled "other respiratory pathogen genomes" or the negative controls (see Fig. 6). Two previously unclassified paramyxoviruses, FDLV and Sa-

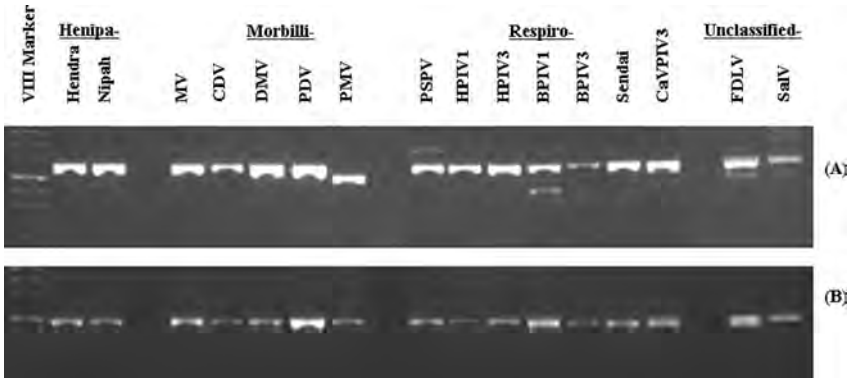

FIG. 3. Amplification of RNAs from 14 different viral members in the genera of *Henipavirus*, *Morbillivirus*, and *Respirovirus* and two unclassified viral members in the subfamily *Paramyxovirinae* by one-step RT-PCR using the pan RES-MOR-HEN-F1/RES-MOR-HEN-R primer pair (A) or the pan RES-MOR-HEN-F2/RES-MOR-HEN-R primer pair (B). Each viral name is abbreviated as shown in Table 1.

lem virus (SalV), were amplified only by the *Morbillivirus-Respirovirus-Henipavirus* genus subgroup-specific primers and Menangle virus only by the *Rubulavirus-Avulavirus* genus subgroup-specific primers. These results suggest that FDLV and SalV strains are more closely related to the *Morbillivirus-Respirovirus-Henipavirus* subgroup and the Menanagle strain to the *Rubulavirus-Avulavirus* subgroup.

As shown in Fig. 5, the *Pneumovirinae* seminested PCR assay detected all of the seven tested reference viruses, and the specificity of the amplification was confirmed by sequence studies. These primers did not amplify RNA from other common respiratory viruses or the negative controls (Fig. 6).

Sensitivity of consensus degenerate PCR assay. To test the sensitivity of the PCR assays, we used mumps virus RNA for both the *Paramyxovirinae* subfamily-specific primers and the *Rubulavirus-Avulavirus* genus subgroup-specific primers. Hendra viral RNA was used to evaluate the *Morbillivirus-Respirovirus-Henipavirus* genus subgroup-specific primers and respiratory syncytial virus RNA for the *Pneumovirinae* subfamily-specific primers. Generally, sensitivity was improved at least 10-fold with the addition of the nested or seminested step (Fig. 7). The same 10-fold serial dilutions of mumps virus RNA were used to compare the sensitivity between the *Paramyxovirinae* subfamily-specific RT-PCR and the *Rubulavirus-Avulavirus* genus subgroup-specific RT-PCR. As expected, the *Rubulavirus-Avulavirus* genus subgroup-specific primers with less degeneracy resulted in a 10-fold- to 100-fold-higher sensitivity than the *Paramyxovirinae* subfamily-specific primers. By using serial dilutions of RNA transcripts, the sensitivity limit was calculated as between 10 and 100 RNA copies for the *Rubulavirus-Avulavirus* subgroup-specific PCR, the *Morbillivirus-Respirovirus-Henipavirus* subgroup-specific PCR, and the *Pneumovirinae*

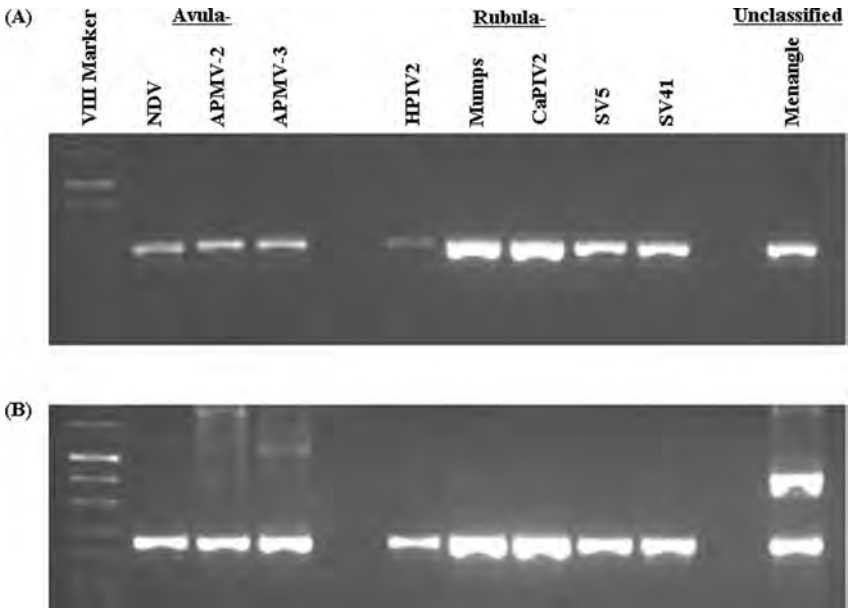

FIG. 4. Gel electrophoresis of amplification products of a one-step RT-PCR assay against RNA from eight different members of *Avulavirus* and *Rubulavirus* genera and one previously unclassified member of the subfamily *Paramyxovirinae*. The pan AVU-RUB-F1/AVU-RUB-R primer pair (A) or the pan AVU-RUB-F2/AVU-RUB-R primer pair (B) was used. Each virus gives an appropriately sized band and is identified by its abbreviation as shown in Table 1.

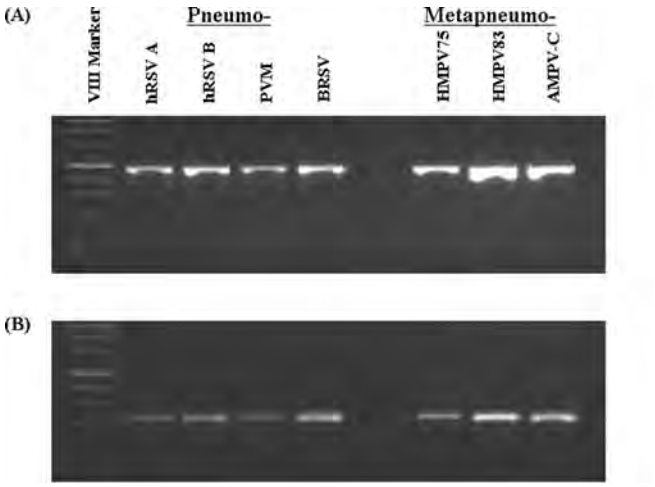

FIG. 5. Gel electrophoresis of amplification products of one-step RT-PCR assays against RNA from seven different members of the subfamily *Pneumovirinae*. (A) Pan PNE-F1/PNE-R primer pair. (B) Pan PNE-F2/PNE-R primer pair. Each virus gives an appropriately sized band and is identified by its abbreviation as shown in Table 1.

subfamily-specific PCR and between 500 and 1,000 copies for the *Paramyxovirinae* subfamily-specific PCR(data not shown).

Validation with clinical specimens. To validate further the specificity and sensitivity of each of these group-specific PCRs with clinical samples, we tested a panel of 14 blinded samples provided by Dean Erdman (CDC), consisting of paramyxovirus-containing clinical samples, non-paramyxovirus-containing clinical samples, and cell culture isolates in different dilutions, which had been tested by agent-specific assays. As noted in Table 3, the appropriate subfamily-specific or genus subgroup-specific PCR assays correctly identified each of the specimens with the correct specificity.

FIG. 6. Gel electrophoresis of amplification products with subfamily and genus group seminested RT-PCR assays showing the appropriately sized positive band for the positive control material for the respective RT-PCR assay and no appropriately sized band for negative-control material or a pool of RNA from other common respiratory pathogens, as described in Materials and Methods. The seminested RT-PCR assays are pan-PAR (*Paramyxovirinae* subfamily), pan RES-MOR-HEN (group of *Respirovirus*, *Morbillivirus*, and *Henipavirus* genera), pan-AVU-RUB (group of *Avulavirus* and *Rubulavirus* genera), and pan-PNE (*Pneumovirinae* subfamily). Lanes: 1, positive control; 2, negative control (water); 3, blank; 4, pool of RNA from other common respiratory pathogens.

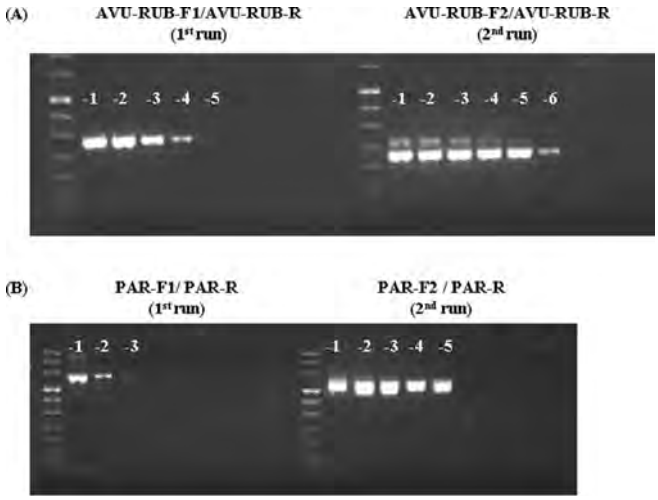

FIG. 7. Improved detection sensitivity by seminested RT-PCR and by genus subgroup primers with less degeneracy. (A) Seminested RT-PCR amplification of RNA extracted from 10-fold serial dilution of mumps virus stock using the pan-AVU-RUB-F1/AVU-RUB-R primer pair (1st run) and the pan-AVU-RUB-F2/AVU-RUB-R primer pair (2nd run). (B) Seminested RT-PCR amplification of RNA extracted from 10-fold serial dilution of mumps virus stock using the pan-PAR-F1/PAR-R primer pair (1st run) and the pan-PAR-F2/PAR-R primer pair (2nd run).

DISCUSSION

In this report, we describe successful development of a set of broadly reactive PCR assays for *Paramyxoviridae*. These assays were developed by identifying conserved sequences among members of various groups in the family *Paramyxoviridae*, using degenerate and inosine-containing primers to account for mismatches, a second, nested, PCR to improve sensitivity, and the consensus-degenerate hybrid oligonucleotide primer strategy (13) to improve sensitivity and specificity. In designing the broad-range primers, we first looked for conserved amino acid sequences in the RNA-dependent RNA polymerase protein coded by the L gene, the most conserved viral gene in the family *Paramyxoviridae*. The analysis of the RNA-dependent RNA polymerase proteins of paramyxoviruses indicated that they encompass three conserved domains (I, II, and III) separated by two nonconserved hinge regions, and strong selective constraints act against amino acid sequence changes in these three conserved domains (6, 11). They are predicted to be essential for the key functions of RNA binding, RNA replication, and protein kinase activity and may have retained the same structure, that of their putative common ancestor, as they have diverged in sequence. The invariance of these conserved sequences suggests that they may be ideal targets for the exploration of unidentified members in the *Paramyxoviridae*. The broad-range primers in this report were designed from the highly conserved domain I and II among paramyxovirus members.

The extensive variability within this family and the drop in sensitivity with increased primer degeneracy prevented us from developing a single assay for the family but instead led us to develop subfamily-specific and two genus subgroup PCR assays to achieve the desired level of sensitivity, <100 copies of RNA in the reaction mixture. The two genus subgroup assays took advantage of more closely related genera, i.e., a group of

TABLE 3. Validation with clinical specimens[a]

Specimen ID	PCR result				Sequence confirmation result	Blinded result
	Paramyxovirinae	Rubulavirus-Avulavirus subgroup	Respirovirus-Morbillivirus-Henipavirus subgroup	Pneumovirinae		
2005495091	Pos	Neg	Pos	Neg	HPIV3	HPIV3
2005495105	Pos	Neg	Pos	Neg	HPIV3	HPIV3
2005495106	Pos	Neg	Pos	Neg	HPIV3	HPIV3
2002023415	Neg	Neg	Neg	Pos	hRSV-B1	hRSV-B1[c]
2002023422	Neg	Neg	Neg	Pos	hRSV-B1	hRSV-B1
2002023428	Neg	Neg	Neg	Neg	Neg	FLU A
2002023434	Neg	Neg	Neg	Neg	Neg	FLU A
2002023436	Pos	Neg	Pos	Neg	HPIV3	HPIV3
2002023437	Pos	Neg	Pos	Neg	HPIV3	HPIV3
2002023644[b]	Neg	Neg	Neg	Neg	Neg	HMPV
2002029001	Neg	Neg	Neg	Pos	HMPV	HMPV
2002029002	Neg	Neg	Neg	Pos	HMPV	HMPV

[a] Pos, positive; Neg, negative. For other abbreviations, see Table 1.
[b] The specimen 2002023644 had a low viral load (<10 copies/5 μl template) which is below the detection limit of the pan-*Pneumovirinae* RT-PCR.
[c] hRSV-B1, human RSV-B1.

the *Rubulavirus* and *Avulavirus* genera and a group of the *Morbillivirus*, *Respirovirus*, and *Henipavirus* genera. The two genus subgroup assays reached the desired level of sensitivity, 10 and 100 copies, while the corresponding *Paramyxovirinae* subfamily assay achieved a sensitivity of 500 to 1,000 copies.

In summary, we have developed a set of seminested RT-PCR assays for detection of paramyxoviruses. The broad reactivity of these RT-PCR assays should allow us to detect known and novel members of the family *Paramyxoviridae* within genera described to date. The utility of these assays in discovery of novel members is supported by our ability to detect and classify eight recently isolated paramyxovirus species, *Porpoise morbillivirus*, *Pacific salmon paramyxovirus*, *Bovine parainfluenza virus 1*, *Guinea pig parainfluenza virus 3*, *Canine parainfluenza virus 2*, *Canine parainfluenza virus 3*, *Menangle virus*, and *Salem virus*, whose sequences were not available when the primers were designed. These pan-paramyxovirus PCR assays and similar assays for other viral families should enhance our ability to quickly identify, by virus family, subfamily, or genus, a wide range of novel viral pathogens and should enhance our ability to respond to and characterize outbreaks and diseases of unknown etiology.

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Emergence of Fatal Avian Influenza in New England Harbor Seals

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ABSTRACT From September to December 2011, 162 New England harbor seals died in an outbreak of pneumonia. Sequence analysis of postmortem samples revealed the presence of an avian H3N8 influenza A virus, similar to a virus circulating in North American waterfowl since at least 2002 but with mutations that indicate recent adaption to mammalian hosts. These include a D701N mutation in the viral PB2 protein, previously reported in highly pathogenic H5N1 avian influenza viruses infecting people. Lectin staining and agglutination assays indicated the presence of the avian-preferred SAα-2,3 and mammalian SAα-2,6 receptors in seal respiratory tract, and the ability of the virus to agglutinate erythrocytes bearing either the SAα-2,3 or the SAα-2,6 receptor. The emergence of this A/harbor seal/Massachusetts/1/2011 virus may herald the appearance of an H3N8 influenza clade with potential for persistence and cross-species transmission.

IMPORTANCE The emergence of new strains of influenza virus is always of great public concern, especially when the infection of a new mammalian host has the potential to result in a widespread outbreak of disease. Here we report the emergence of an avian influenza virus (H3N8) in New England harbor seals which caused an outbreak of pneumonia and contributed to a U.S. federally recognized unusual mortality event (UME). This outbreak is particularly significant, not only because of the disease it caused in seals but also because the virus has naturally acquired mutations that are known to increase transmissibility and virulence in mammals. Monitoring the spillover and adaptation of avian viruses in mammalian species is critically important if we are to understand the factors that lead to both epizootic and zoonotic emergence.

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Fatal pulmonary epizootics of influenza have been observed previously in seal populations, including outbreaks of H7N7 in 1979 to 1980 (1, 2), H4N5 in 1983 (3) and H4N5 and H3N3 in 1991 to 1992 (4). Such outbreaks are significant not just because of the detriment they pose to animal health but because influenza in mammals can act as a source for human pandemics (5).

In a <4-month period beginning in September 2011, 162 harbor seals (*Phoca vitulina*) were found dead or moribund along the New England coast. This number is approximately four times the expected mortality for this period. Most of the affected individuals were less than 6 months old, and common causes of death (including malnourishment) were ruled out. Five of the affected animals were investigated to identify a causative agent, and here we demonstrate that avian influenza virus subtype H3N8 was responsible for the observed clinical and pathological signs in these animals. Unlike any previous outbreak in seals, this H3N8 virus has naturally acquired mutations that reflect adaptation to mammalian hosts and that are known to increase virulence and transmissibility in avian H5N1 viruses infecting mammals. The virus has further acquired the ability to use the SAα-2,6 receptor commonly found in the respiratory tracts of mammals, including humans. The existence of a transmissible and pathogenic influenza is of obvious public concern.

RESULTS AND DISCUSSION

Five animals were submitted for anatomical and microbiological analysis. All were collected from the peak of the outbreak (late September to October) and had pneumonia and ulcerations of the skin and oral mucosa (Fig. 1). Nucleic acids extracted from lung, trachea, liver, kidney, thoracic lymph node, mesenteric lymph node, spleen, skin lesion, and oral mucosa were tested by PCR for the presence of a wide range of pathogens, including herpesviruses, poxviruses, adenoviruses, polyomaviruses, caliciviruses, paramyxoviruses, astroviruses, enteroviruses, flaviviruses, rhabdoviruses, orbiviruses, and influenza viruses. Influenza A virus was detected in several tissues from all five animals, and PCR cloning and sequencing of genes for hemagglutinin (HA) and neuraminidase (NA) revealed the subtype to be H3N8, a subtype

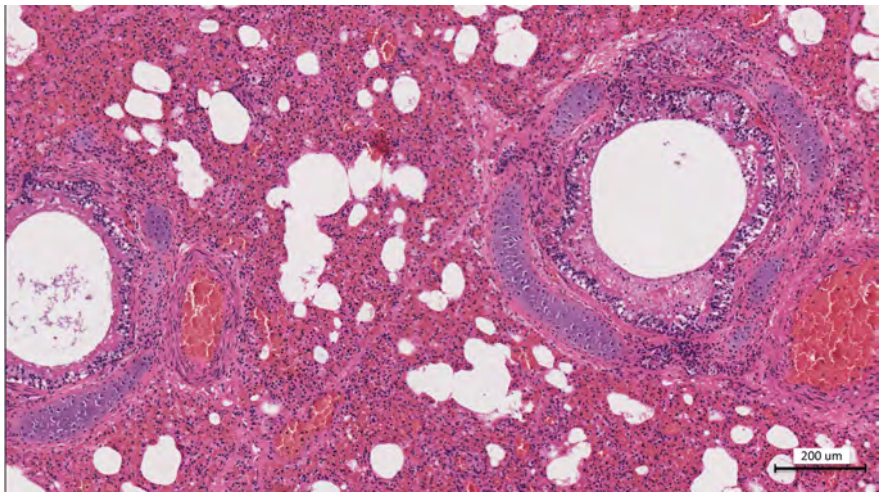

FIG 1 Hematoxylin and eosin staining of the lung at a ×10 magnification. There is diffuse acute interstitial pneumonia and a mix of acute hemorrhagic alveolitis with necrotizing bronchitis. Multifocally, alveoli are either filled with hemorrhage and scant inflammatory cells or expanded with emphysema. There is irregularity to the bronchial mucosa due to necrosis, a mild to moderate edema, and mucous partially filling the bronchial lumen. There is mild to moderate expansion of the interlobular septa, with edema and hemorrhage.

typically associated with infection of avian, equine, and canine hosts (6-8). Influenza virus was isolated from the allantoic fluid of specific-pathogen-free (SPF) eggs inoculated with homogenates of PCR-positive tissues, including lung, lymph nodes, tonsil, and kidney, and all isolates were reconfirmed to be H3N8. In accord with conventional nomenclature, the virus is provisionally named A/harbor seal/Massachusetts/1/2011.

In situ hybridization (ISH) using oligonucleotide probes for influenza virus H3N8 segments 4 (HA) and 7 (matrix) and immunohistochemistry using polyclonal antibodies against H3N8 HA antigen confirmed the presence of influenza virus in lung, where signal was concentrated in the bronchiole epithelium and mucosa of the pulmonary parenchyma (Fig. 2 and see Fig. S1 in the supplemental material). The average load of HA and NA RNAs in lung was 300 copies/100 ng of extracted RNA. ISH staining in nonrespiratory tissues was limited to sporadic infection of single cells in intestine, kidney, and lymph node, and the average HA/NA RNA load was five copies/100 ng. These results are consistent with the histological observation that the main site of viral replication is the respiratory tract. Cellular morphology and terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) consistent with apoptosis was observed in virus-infected pulmonary epithelial cells (Fig. 3). TUNEL staining also revealed the widespread presence of apoptotic cells in areas where no virus was observed but not in negative-control tissue, suggesting an additional host-mediated response to the infection.

Full-genome sequencing was completed following PCR cloning of all eight influenza genome segments directly from infected tissue. Sequences were submitted to GenBank and assigned accession numbers JQ433879 to JQ433882. Phylogenetic analyses of these nucleotide sequences with avian, canine, and equine H3N8 influenza virus genomes demonstrated the closest relationship to a virus identified in North American waterfowl in all 8 segments (Fig. 4). These data are consistent with the recent transmission of the H3N8 virus from wild birds to seals. The closest avian relative, A/blue-winged teal/Ohio/926/2002, had an overall 96.07% nucleotide sequence identity across the genome (Hamming distance), with no individual segment having less than 94% identity. This level of similarity across all segments with an isolate separated by a span of 10 years suggests that this virus has been circulating in the aquatic bird populations since at least 2002.

A total of 37 amino acid substitutions separate the seal H3N8 and avian H3N8 viruses, which are summarized in Table 1. The corresponding amino acids found in other seal influenza viruses, in the canine and equine H3N8 viruses, and in selected human influenza viruses are included for comparison. Of these, mutations PB2-701N and HA-260M are shared exclusively by the seal and mammalian (canine/equine) H3N8 viruses, while mutations NA-399R, PB2-382V, and PA-184N are shared by the seal H3N8 and human H3N2 viruses, all of which suggests adaptation to mammalian hosts. Mutations PB2-60N, PB2-376R, PB1-174I, PB1-309G, PB1-359G, PB1-376V, PB1-377G, PB1-464E, NP-63V, NP-128G, and NP-296H are all exclusively found in the seal H3N8 virus (Table 1). Future studies will be required to assess the functional significance of many of these mutations, especially in seal H3N8 PB1, where a significant number of exclusive mutations were observed. Given the importance of PB1 in viral replication, it is probable that these mutations represent adaptive selection to accommodate host-specific differences in intracellular replication.

The seal H3N8 genome was interrogated for any genetic features that might contribute to enhanced transmissibility and virulence in seals. Expression of a second peptide (PB1-F2) from segment 2 has been associated with an increase in pathogenicity by inducing apoptosis and increasing both inflammation and secondary bacterial pneumonia (9, 10). The seal H3N8 virus contains an intact open reading frame for the pathogenic version of this accessory protein, which includes a serine at amino acid position 66 (9). All five seals had evidence of apoptosis and secondary bacterial pneumonia.

Glycosylation can also affect pathogenicity in influenza viruses (11-14). Six potential glycosylation sites were detected in the seal H3N8 HA, based on the sequence $X_{-2}X_{-1}NX(S/T)X_{+1}$, at amino acid positions 24, 38, 54, 181, 301, and 499. None had features

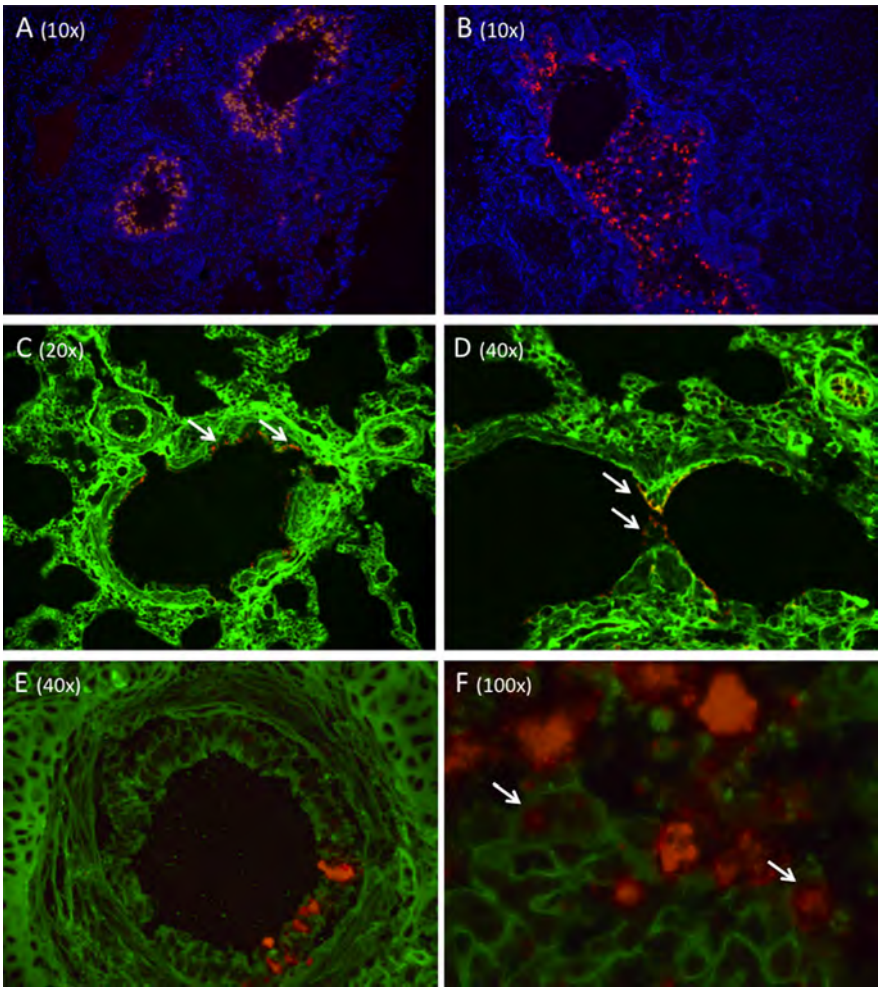

FIG 2 (A and B) Fluorescent *in situ* hybridization (ISH) of H3N8 virus-infected seal cells with DAPI counterstaining. A probe targeting the viral hemagglutinin demonstrates diffuse infection of the bronchial mucosal epithelium. ISH was also performed using probes for the matrix gene. Staining was identical to that shown here for HA. (C and D) Lectin staining to demonstrate the distribution of SAα-2,3 and SAα-2,6 in seal pulmonary parenchyma. The SAα-2,6 (green) was detected using fluorescein-labeled *Sambucus nigra* agglutinin (SNA) lectin, while SAα-2,3 (red) was detected using *Maackia amurensis* II (MAL II) lectin. Both infected and uninfected control tissues were stained, and the results were consistent for both. High levels of SAα-2,6 are observed on bronchiole and alveolar epithelial cells and on endothelial cells. The images in panels C and D were selected because they show staining for both sialic acids; however, the expression of SAα-2,3 was rarely observed (arrows) and limited to bronchiole luminal (C) and occasional alveolar (D) epithelia. (E and F) Costaining of SAα-2,6 and H3N8 HA. SAα-2,6 (green) is expressed on the respiratory epithelium of an intrapulmonary bronchus (E). H3N8 virus-infected cells (red) are present. A serial section was also stained for SAα-2,3, and none was detected. A high-magnification image of infected mucosa clearly shows H3N8 virus infection of cells expressing SAα-2,6 (arrows). All composite images are presented separately (single stains) in Fig. S1 to S3.

suggestive of inactivity or reduced efficiency, as was previously demonstrated for H5N2 (12). Many H5N1 viruses have an additional glycosylation site at positions 158 to 160, and previous work demonstrated that the deletion of this site is critical for H5N1 viruses to bind to human-SAα2,6-like receptors and to transmit between mammals (15). This glycosylation site is missing in the seal H3N8 HA.

In order to investigate the specificity of sialic acid binding, the seal virus was compared to avian H5 and swine H9, both of which bind to the sialyloligosaccharide SAα2,3 ligand in a structural-homology model (Fig. 5). All structures confirmed the presence of a highly conserved serine at position 152 (corresponding to S136 by H3 numbering [16, 17]), which lies in a binding pocket, where its hydroxyl group contacts the axial carboxylate of sialic acid (17). While the seal virus contains the same conserved S152, it also harbors a neighboring G151E mutation (Table 1), which introduces a large residue capable of both donating and receiving hydrogen bonds with residues in close proximity to the ligand-binding pocket. Rotamer hydrogen bond analysis of the modeled seal structure indicates that HA's altered conformation results in reduced hydrogen bonding between the conserved serine and SAα2,3 compared to that of H5 and H9 influenza viruses. Such changes in sialic acid binding play important roles in novel host adaptation (18).

Mutations at positions 226 and 228 (H3 numbering) in the H3 HA can also affect receptor-binding preferences and can either completely abrogate (Q226L) or reduce (G228S) affinity for the avian-preferred SAα-2,3 interaction (18, 19). Seal H3N8 virus maintains the avian phenotype at positions 226 (Q) and 228 (G), which correlates with a continued ability to use SAα-2,3. Together, these findings suggest that the seal virus may still be able to use SAα2,3, but perhaps with less efficiency than in its original

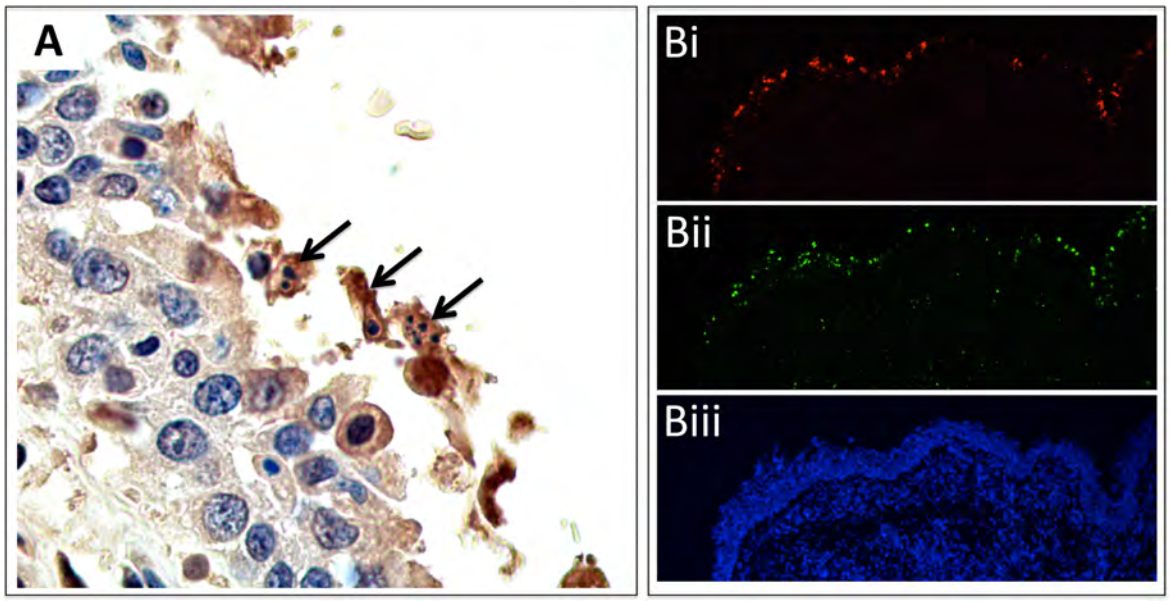

FIG 3 (A) Immunohistochemistry (IHC) of seal bronchus. Polyclonal antibodies were raised against H3N8 virus-specific HA antigen. Brown staining (DAB reporter system) indicates the presence of viral antigen. There is irregularity of the mucosal surface, with sloughed epithelium. IHC demonstrates the presence of viral antigen, pyknosis, and apoptosis (arrows). No viral antigen or apoptosis was seen on negative-control tissue. (Bi) ISH staining of H3N8 virus in lung epithelium (HA probe). (Bii) TUNEL staining (green) in the same region (serial section) of the lung, showing the presence of apoptotic cells. Comparison of virus and TUNEL staining shows localization of apoptosis to virally infected cells. (Biii) DAPI (with dihydrochloride) staining for cell nuclei.

avian host. Given this, we investigated whether seal H3N8 may have adapted and acquired an additional or increased affinity for the SAα-2,6 receptors that are more prevalent in mammalian respiratory tissue (20-23).

The pulmonary distribution of SAα-2,3 and SAα-2,6 influenza receptors was investigated using the receptor-specific lectins *Sambucus nigra* agglutinin (SNA) for SAα-2,6 and *Maackia amurensis* lectin II (MAL II) for SAα-2,3. SAα-2,6 was widely expressed in both infected and noninfected pulmonary parenchyma, with the highest concentration seen on endothelial cells, followed by alveolar/bronchiole epithelia (Fig. 2 and see Fig. S2 in the supplemental material). SAα-2,3 was also observed, but less frequently, and was generally limited to the luminal surfaces of epithelial cells of the bronchioles. This broadly agrees with the expression of these SA saccharides in humans and pigs (20-23) and demonstrates that seals do express receptors that would allow avian viruses to initiate infection. This observation is supported by the H3N3 seal virus from the 1991 epizootic (4), which was shown to preferentially bind SAα-2,3 *in vitro* (19). However, the limited prevalence of SAα-2,3 in the lower lung suggests that the process of infection is inefficient and may help to explain why epizootics of avian influenza occur but are infrequent in harbor seals.

Importantly, the rare expression of SAα-2,3 is insufficient to explain the diffuse infection seen throughout the pulmonary parenchyma (Fig. 2). In contrast, the wide distribution of SAα-2,6 is far more consistent with the level of infection observed. Costaining of infected lung with viral HA and SAα-2,3 or SAα-2,6 demonstrated clear infection of SAα-2,6-positive cells, in which no SAα-2,3 was seen (Fig. 2 and S3).

Hemagglutination assays were also performed to confirm sialic acid binding preferences. Seal H3N8 isolates were first sequenced to confirm that passage in eggs had not altered the HA phenotype detected in the infected tissues, and the viruses were then tested for their ability to agglutinate erythrocytes that preferentially express SAα-2,3 (horse) or SAα-2,6 (guinea pig, pig) (24, 25), relative to several avian H3N8 viruses (Fig. 6). Average agglutination titers for seal H3N8 virus with horse erythrocytes (1:48) show that the virus can still bind to SAα-2,3. However, titers were appreciably higher with guinea pig (1:192) and pig (1:144) erythrocytes, demonstrating a preference for SAα-2,6. These findings show that seal H3N8 can use both avian and mammalian receptors and add to previous studies that have demonstrated changes in receptor preferences following a host switch event (26). The patterns of SAα-2,3 and SAα-2,6 binding to seal H3N8 virus also agree with the patterns observed for H3 avian viruses adapting to humans (18).

A further mutation was observed in HA, this time at position 110 (Table 1). In avian H3 viruses, phenylalanine (Phe/F) is consistently seen, while seal H3N8 uses Ser (F110S). The significance of this (if any) is currently unknown; however, previous work has suggested that this amino acid (position 110) is a critical component of the influenza fusion peptide (27), and given the essential role of fusion in viral replication and the host-specific differences that presumably exist in this process, the F110S substitution may well represent further adaption of this virus to mammalian replication.

The ability of avian influenza viruses to adapt to SAα-2,6-mediated cell entry and replication is regarded as a significant driving force in the emergence of global pandemics (19, 28-30), especially for viruses with phenotypes that confer increased virulence. Such phenotypes are often, though not exclusively, dictated by mutations in segment 1 (PB2), which is an important determinant of host range for influenza viruses. Previous studies have experimentally demonstrated the effect of various PB2 substitutions on virulence and transmissibility in mammalian hosts (15, 31-38), including the modification of the aspartic acid (D) avian phenotype to an asparagine (N) mammalian phenotype at amino

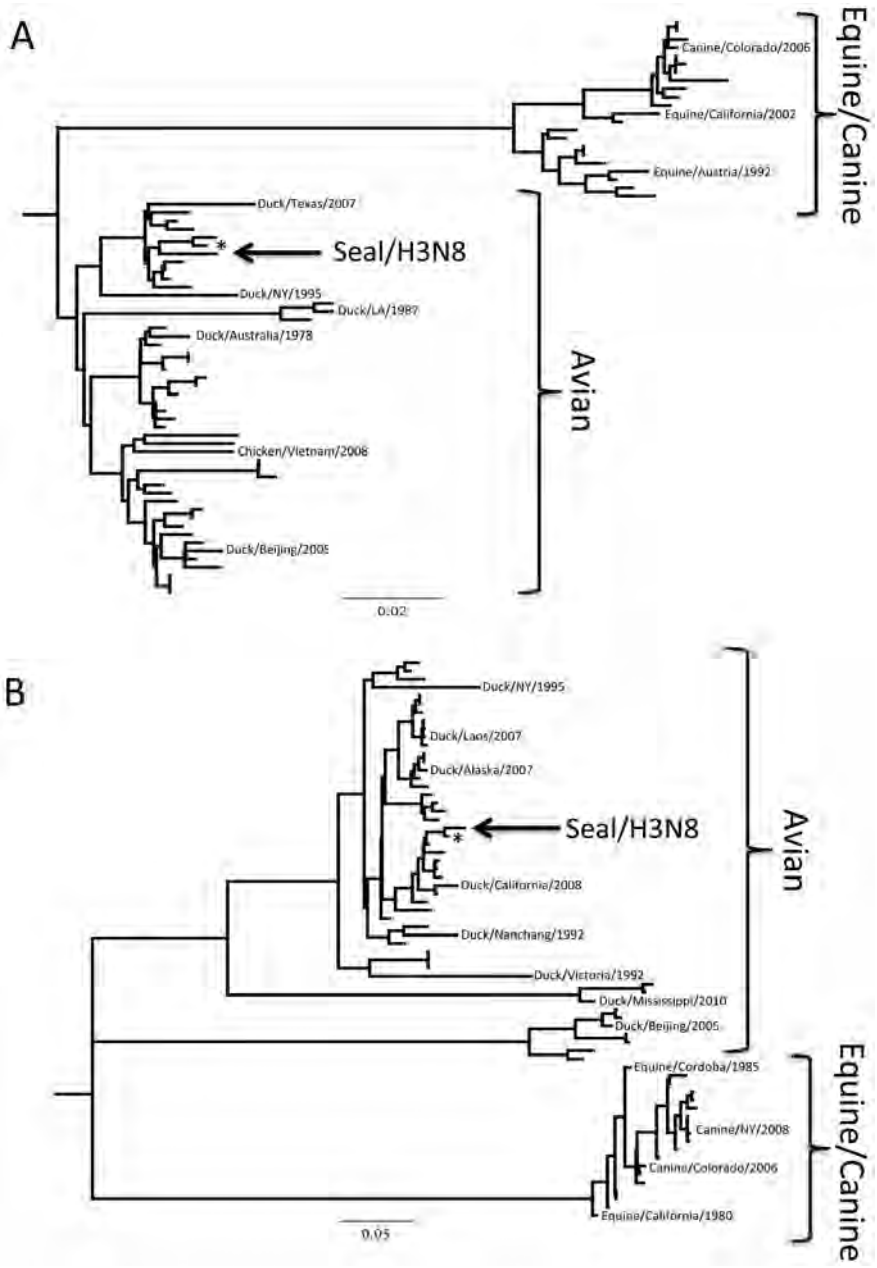

FIG 4 Phylogenetic trees of representative influenza H3N8 genome segments. Nucleotide sequence alignments for all genome segments were created using ClustalW, and trees were produced using neighbor-joining, maximum-likelihood, and Bayesian algorithms. Models of evolution were selected using ModelTest, and a tree was selected based on a consensus of the results of the three algorithms. Only the trees for HA and NA are shown; however, all eight segments showed strong association with sequences of avian origin. Trees are constructed with H3N8 viruses only, and published sequences were selected to represent variation in the year, host, and location of isolation. *, A/blue-winged teal/Ohio/926/2002; NY, New York; LA, Louisiana.

acid 701 (15, 31, 32, 36, 39, 40). This D701N mutation has been experimentally introduced into an adapted version of the H7N7 seal influenza virus isolated in 1982 (1, 2) and was shown to increase the pathogenicity of the virus to mice (32).

The seal H3N8 virus from the 2011 outbreak has naturally acquired this D701N substitution (Table 1), which was confirmed by clonal sequencing directly from infected tissue (50 clones/animal) to be the only phenotype present in all five animals. None of the previous outbreaks of influenza in seals showed this 701N phenotype, but it is consistently found in H3N8 viruses from horses and dogs, demonstrating further adaptation to replication in mammalian hosts. These observations raise significant concern about the virulence and transmission of this virus between mammals. Interestingly, analysis of HA sequences over the course of the outbreak show the introduction and maintenance of two nucleotide polymorphisms (Table 2), and while this is insufficient to convincingly demonstrate seal-to-seal transmission, it leads us to postulate that mammalian spread might already have occurred.

Together, the adaptations observed in A/harbor seal/Massachusetts/1/11 suggest that it may be able to persist within the seal

TABLE 1 List of amino acid substitutions between seal H3N8 and avian H3N8 viruses[a]

Segment (protein)	nt position	aa position	Amino acid substitution									
			Seal H3N8 (2011)	Avian H3N8	Seal H7N7 (1980)	Seal H4N5 (1982)	Seal H3N3 (1992)	Equine H3N8	Canine H3N8	Human H3N2	Human H1N1 (seasonal)	Human H1N1 (pandemic)
1 (PB2)	178	60	N	D	D	D	D	D	D	D	D	D
	441	147	M	I	I	I	I	V	V	I	I	T
	1127	376	R	K	K	K	K	K	K	K	K	K
	1144	382	V	I	I	I	I	I	I	V	I	I
	2101	701	N	D	D	D	D	N	N	D	D	D
2 (PB1)	522	174	I	M	M	M	M	M	M	M	M	M
	925	309	G	W	W	W	W	W	W	W	W	W
	1075	359	G	S	S	S	S	S	S	S	S	S
	1126	376	V	I	I	I	I	I	I	I	I	I
	1130	377	G	D	D	D	D	D	D	D	D	D
	1392	464	E	D	D	D	D	D	D	D	D	D
3 (PA)	253	85	A	T	T	T	T	T	T	T	T	I
	551	184	N	S	S	S	S	S	S	N	S	S
	794	265	L	P	P	T	P	P	P	P	P	P
4 (HA)	242	81 (65)	K	T	G	D	T	T	T	T	S	N
	323	108 (92)	S	N	E	T	N	S	N	K	N	S
	329	110 (94)	S	F	S	V	F	F	F	Y	E	D
	452	151 (135)	E	G	A	K	G	R	R	T	V	V
	527	176 (160)	V	A	A	A	A	S	S	K	L	S
	713	238 (222)	L	W	Q	W	W	W	L	R	K	K
	778	260 (244)	M	V	T	V	V	M	M	L	I	T
	802	268 (252)	V	I	I	I	I	V	V	I	I	V
	859	287 (271)	N	D	D	A	D	D	D	D	N	D
	1114	372	K	Q	Q	Q	Q	Q	Q	Q	Q	Q
	1247	416	L	S	T	E	S	S	S	S	N	N
5 (NP)	187	63	V	I	I	I	I	I	I	I	I	I
	383	128	G	D	D	D	D	D	D	D	D	D
	886	296	H	Y	Y	Y	Y	Y	Y	Y	Y	Y
	1336	446	G	R	R	R	R	R	R	R	K	R
6 (NA)	440	147	E	V	I	None	I	V	I	V	V	I
	849	283	D	E	N	None	D	E	E	Y	T	S
	937	313	R	G	Q	None	G	G	G	S	G	G
	958	320	S	P	L	None	H	P	P	V	F	F
	1186	396	D	N	N	None	N	N	D	R	I	I
	1195	399	R	W	W	None	W	W	W	R	W	W
	1295	432	A	E	A	None	N	E	E	E	R	K
8 (NS1/NS2)	263	88	H	R	R	R	R	R	R	R	R	R

[a] A total of 40 amino acid substitutions were observed in a comparison of seal H3N8 virus with avian H3N8 virus. Sequences of other seal influenza viruses, canine and equine H3N8 viruses, and selected human influenza viruses were included for comparison. Amino acid positions presented in parentheses represent corresponding H3 numbering. None, no sequence available for comparison; nt, nucleotide; aa, amino acid.

population and evolve into a new clade within the H3N8 group, as happened with the canine and equine viruses. An additional concern is the potential zoonotic threat that this virus poses, as it has already acquired mutations in both PB2 and HA that are often, though perhaps not exclusively, regarded as prerequisites for pandemic spread (19-23, 28, 30, 37) and it is uncertain how any persistence of the virus in mammals may continue to alter its phenotype. A comparison of A/harbor seal/Massachusetts/1/11 with human H3N2 viruses revealed three substitutions that are already common to both seal H3N8 and human H3N2 viruses. These are NA-W399R, PB2-I382V, and PA-S184N (Table 1). In all cases, these substitutions are shared by the seal H3N8 and human H3N2 viruses but are not found in influenza viruses isolated previously from seals, in avian, equine, or canine H3N8 viruses, or in either seasonal or pandemic H1N1 viruses. Further studies will be required to establish the functional significance of these substitutions; however, the natural epizootic emergence at this time of a pathogenic virus that can transmit between mammals, found in a species that can become infected with multiple influenza virus subtypes, must be considered a significant threat to both wildlife and public health.

MATERIALS AND METHODS

Extractions, PCR, and sequencing. RNA was extracted from all tissues using Trizol reagent, and cDNA was synthesized using Superscript III (Invitrogen) according to the manufacturer's instructions. PCR for the detection of influenza A virus was performed using primers FLUAV-M-U44 (GTCTTCTAACCGAGGTCGAAACG) and FLUAV-M-L287 (GCA TTTTGGACAAAGCGTCTACG), to produce a 243-bp product of segment 7 (coding for matrix protein). For full-genome sequencing, full-length cDNAs were amplified for all eight influenza segments. Primers were designed to target terminal sequences for each segment, based on alignments of avian, canine, and equine H3N8 sequences from the Influenza Research Database (http://www.fludb.org). All PCRs were performed using fast-cycling chemistry (Qiagen), according to the manufac-

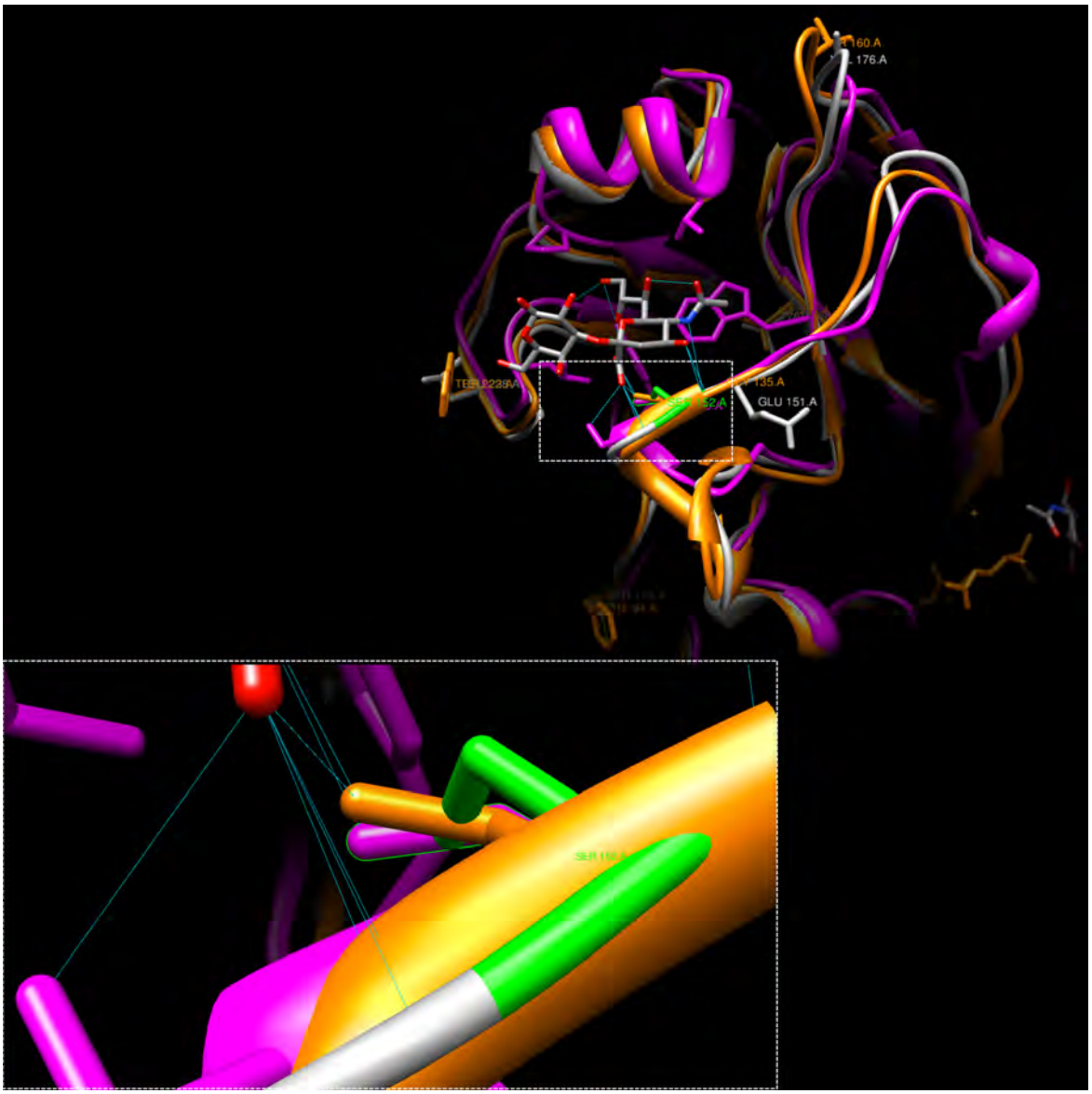

FIG 5 Structural-homology model showing the interaction of influenza HA with the SAα2,3 ligand. Seal H3 (gray), avian H5 (orange) (Protein Data Bank [PDB] accession number 1JSO), and swine H9 (pink) (PDB accession number 1JSH) were compared. The mutation G151E causes a conformational shift and interrupts H bonding between seal H3 S152 and SAα2,3, which suggests a reduction in SAα2,3 binding efficiency. A lost H bond in seal H3 is depicted in green.

turer's instructions. Amplified products were cloned into the pGEM T-easy vector (Promega) and sent for commercial sequencing.

Virus isolation and intravenous pathogenicity index test. Homogenates from PCR-positive tissues were inoculated into SPF embryonated chicken eggs, and virus growth was determined by PCR. Tissue homogenates were also used to infect the Vero and MDCK cell lines in the presence of trypsin. Virus isolates were sent to the National Veterinary Services Laboratory (Ames, IA), where the chicken intravenous pathogenicity index test was performed according to the OIE manual (41).

Molecular pathology. Fluorescent *in situ* hybridization (FISH) was performed using the Quantigene ViewRNA ISH tissue assay (Affymetrix), according to the manufacturer's instructions. FISH conditions were optimized to include a 10-min boiling and 20-min protease treatment. Oligonucleotide probes were designed commercially by Affymetrix using sequences of HA and M (accession numbers JQ433879 and JQ433882, respectively). Immunohistochemistry (IHC) was performed by pretreating deparaffinized tissue sections with a 1:10 dilution of antigen retrieval solution (DAKO) for 20 min in a steamer. Samples were then washed three times in distilled water (dH_2O), incubated in 3% hydrogen peroxide (in phosphate-buffered saline [PBS]) for 10 min, washed again twice in dH_2O and once in PBS, and then blocked (10% normal goat serum, 0.1% bovine serum albumin [BSA]) for 20 min. Sections were treated with HA polyclonal H3N8 antibody (Novus Biologicals; catalogue number NBP1-46796) at a 1:250 dilution for 2 h at room temperature. Following three washes in PBS, sections were incubated in Signal Stain Boost IHC reagent (Cell Signaling; catalogue number 8112) for 30 min at room temperature. Sections were again washed three times in PBS, stained with 3,3-diaminobenzidine (DAB; Dako), and counterstained with hematoxylin. TUNEL staining was performed using the *in situ* cell death detection kit and fluorescein (Roche) with deparaffinization and protease treatment as described for the FISH protocol.

Simultaneous detection of SAα-2,3 and SAα-2,6 glycans. Deparaffinized tissue sections (5 μM) were blocked with 1× Carbo-Free solution (Vector Laboratories; catalogue number SP-5040) for 1 h at room tem-

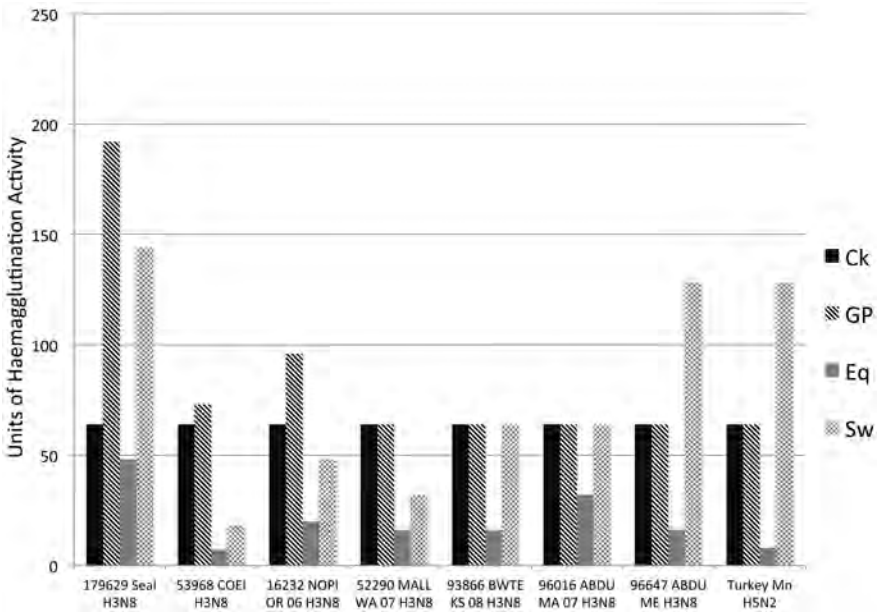

FIG 6 Hemagglutination assays were performed on an isolate of the H3N8 seal virus (A/harbor seal/New Hampshire/179629/2011) to confirm sialic acid binding preferences. Viruses were tested for their ability to agglutinate erythrocytes that preferentially express SAα-2,3 (horse) or SAα-2,6 (guinea pig, pig). Average agglutination titers for seal H3N8 with horse erythrocytes (1:48) show that the virus can still bind to SAα-2,3, though weakly. Titers were appreciably higher with guinea pig (1:192) and pig (1:144) erythrocytes, demonstrating a preference for SAα-2,6. Given that horse erythrocytes express SAα-2,3, it is interesting that the avian H3N8 viruses did not agglutinate with horse RBCs (red blood cells) efficiently, even following repeated attempts. Ito et al. (25) showed that avian H3N8 viruses from Asia in the early 1980s could bind to horse RBCs, while Wiriyarat et al. (43) gave examples of avian viruses (albeit not H3 viruses) that did not bind to horse RBCs. It is not known whether the avian viruses included here simply have a preference for the *N*-acetyl (NeuAc) sialic acid species, which is not found on horse RBCs, while the seal virus uses *N*-glycolyl (NeuGc) SAα-2,3. Ck, chicken; GP, guinea pig; Eq, equine; Sw, swine; 179629 Seal H3N8, A/harbor seal/New Hampshire/179629/2011; 53968 COEI H3N8, A/common eider/Massachusetts/20507-001/2007 (H3N8) virus; 16232 NOPI OR 06 H3N8, A/northern pintail/Oregon/44249-547/2006 (H3N8) virus; 52290 MALL WA 07 H3N8, A/mallard/Washington/44338-052/2007 (H3N8) virus; 93866 BWTE KS 08 H3N8, A/blue-winged teal/Kansas/44440-003/2008 (H3N8) virus; 96016 ABDU MA 07 H3N8, A/American black duck/Maine/44411-174/2008 (H3N8) virus; 96647 ABDU ME H3N8, A/American black duck/Maine/44411-532/2008 (H3N8) virus; Turkey Mn H5N2, A/turkey/Minnesota/3689-1551/1981 (H5N2) virus.

perature. Sections were then stained for SAα-2,6 using fluorescein SNA (Vector Laboratories; catalogue number FL-1301) at 10 μg/ml for 30 min at room temperature, and rinsed twice for 3 min each time in PBS. Sections were then reblocked in 1× Carbo-Free solution for 30 min, before being stained for SAα-2,3 with 10 μg/ml of biotinylated MAL II (Vector Laboratories; catalogue number B-1265) for 30 min at room temperature. The MAL II was poured off, and Texas Red streptavidin (Vector Laboratories; catalogue number SA-5006) was laid over the sections at 10 μg/ml for a further 30 min. Sections were rinsed twice for 3 min each time in PBS and mounted with Vectashield hard-set mounting solution with DAPI (4′,6-diamidine-2-phenylindole) counterstaining.

TABLE 2 Sequence analysis of the HA genes isolated from various tissues[a]

Animal	Sample	Date	Nucleotide at position:	
			1347	1499
278-Pv	Kidney	28 Sept 2011	C	C
286-Pv	Trachea	29 Sept 2011	C	C
	Mes LN		C	C
	Kidney		C	C
295-Pv	Mes LN	3 Oct 2011	C	C
	Lung		C	C
	Tonsil		T	C
	Kidney		T	A
	Tonsil	3 Oct 2011	T	A
	Trachea		T	A
	Cerv LN		T	A

[a] Two polymorphisms were observed in HA at positions 1347 and 1499, relative to avian H3N8 sequence CY041887. Isolates from animals earlier in the outbreak showed C at position 1347 and C at position 1499. The variations C1347T and C1499A were observed in animal 295-Pv, in addition to the wild-type sequence. Animal 294-Pv showed only the variant genotype.

Hemagglutination assays. Hemagglutination assays were performed according to the WHO diagnostic manual (42). Briefly, red blood cells (RBCs) from rooster chicken, guinea pig, horse, and pig were obtained from Lampire Biological Laboratories (Ottsville, PA). The RBCs were washed in PBS and resuspended to 0.5% (chicken) or 0.75% (guinea pig and pig). Equine RBCs were resuspended to 1% in PBS with 0.5% BSA (43). Viruses were diluted to 64 HA units using chicken red blood cells. Serial dilutions were then made, added to equal volumes of washed RBCs of each species, and incubated in U-bottom plates, with the exception of chicken RBCs, which were incubated in V-bottom plates. Reaction mixtures were incubated at room temperature for 1 h, with the exception of those with chicken RBCs, which were incubated for 30 min. The HA titer endpoint is the reciprocal of the highest dilution which causes complete hemagglutination. The seal H3N8 virus was compared with several avian H3N8 isolates, including A/common eider/Massachusetts/20507-001/2007 (H3N8), A/northern pintail/Oregon/44249-547/2006 (H3N8), A/mallard/Washington/44338-052/2007 (H3N8), A/blue-winged teal/Kansas/44440-003/2008 (H3N8), A/American black duck/Maine/44411-174/2008 (H3N8), and A/American black duck/Maine/44411-532/2008 (H3N8). An H5N2 virus was also included: A/turkey/Minnesota/3689-1551/1981 (H5N2) virus.

Sequence analysis. Nucleotide sequences were aligned using ClustalW. Phylogenetic trees were constructed using neighbor-joining, maximum-likelihood, and Bayesian algorithms. Models of evolution were selected using ModelTest, and a representative tree was selected based on

a consensus of the results of the three algorithms. Published H3N8 sequences included in the analyses were selected to represent the diversity of year, host, and location of isolation.

Structural modeling. To create a homology model of the seal 2012 outbreak HA sequence, 10 template models were selected based on their super-secondary structures, with use of the LOMETS meta-threading approach (44, 45). Continuous fragments excised from these templates were then reassembled into full-length models by replica exchange Monte Carlo simulations (44). *Ab initio* modeling of threaded unaligned regions was then used to complete the structure. Low free-energy states were subsequently identified through clustering of simulation decoys by the SPICKER near-native model selection algorithm (46). Chimera was utilized for structural analysis and visualization (47). Hydrogen bonding analysis was based on geometric criteria established through survey of small-molecule crystal systems and Dunbrack rotamer libraries (48, 49).

Nucleotide sequence accession numbers.

The sequences of all eight influenza genome segments were submitted to GenBank and assigned accession numbers JQ433879 to JQ433882.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00166-12/-/DCSupplemental.

Figure S1, JPG file, 0.1 MB.

Figure S2, JPG file, 0.2 MB.

Figure S3, JPG file, 0.2 MB.

ACKNOWLEDGMENTS

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From:	Cadhla Firth on behalf of Cadhla Firth <firth@ecohealthalliance.org>
To:	Laing, Eric
Cc:	Kevin Olival, PhD
Subject:	Re: SEARCH seq approaches
Date:	Wednesday, May 12, 2021 11:12:57 PM
Attachments:	PREDICTII Consensus PCR Lab Protocols_Mar_2017 - Priority Viral Families.pdf

Hi Eric,

No problem! The strategy is super simple - there are standardized consensus PCR protocols targeting <700nt of the RdRp gene that were used by all of the PREDICT labs (attached) for each virus family. Recently there was a new paper that came out that suggested that the CoV consensus PCR assay we are all using misses Sars-CoV-2 and some other newly described viruses, and updated the primers. These are the ones were were talking about testing and potentially switching over to: https://www.mdpi.com/1999-4915/13/4/599

Most of the PREDICT work used these cPCR assays and kind of stopped there so we have quite a large number of viruses that were detected and ID'd as being something novel based on short RdRp sequences, but no further genomic characterization was done.

Let me know if you have any other questions or want to chat further about any of this :)

Best wishes,

Cadhla

On Thu, May 13, 2021 at 11:55 AM Laing, Eric <eric.laing@usuhs.edu> wrote:

> Hi Cadhla and Kevin,
>
> I have not been involved in PREDICT and don't know the nucleic acid strategies in use for SEARCH. Can someone send me some literature, figures or a brief explanation so I can get up to speed?
>
> - Eric
>
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Specific Aims

An important and frequently asked scientific question is whether bats are better hosts for lethal viruses than other mammals. There are a number of zoonotic viruses that cause high mortality in humans and originate in bats: SARS coronaviruses (SARS-CoV and SARS-CoV-2), Ebola virus, Marburg virus, Nipah virus, Hendra virus, and Middle East respiratory syndrome coronavirus (MERS-CoV). Further, there is increasing evidence that many other human viruses may have originated in bats, including measles, mumps and hepatitis C viruses. However, there is a lack the bat animal models necessary to study basic bat immunology and to conduct controlled and sophisticated viral infection studies. **These studies are invaluable to answering why bats appear to host viruses lethal to humans without any apparent outward disease.** Additionally, given the ability of bats to tolerate viral infections, studies elucidating the mechanisms behind these unusual traits could lead to new clinical approaches and therapeutics for humans affected by bat-borne viral diseases. Finally, the order chiroptera is diverse, comprising of over 1,300 species. A comprehensive understanding of ba immunology and physiology, as related to viral infection, will require bat animal models from multiple species.

We propose to establish sustainable breeding colonies of intermediate horseshoe bats (*Rhinolophu affinis*) and Indian flying foxes (*Pteropus medius*) in the United States that will facilitate research requiring liv bats and bat-derived reagents, such as cell lines. In this project, we will import and establish breeding colonie of these species, creating two novel bat models that represent natural reservoirs of SARS-related coronaviruse and henipaviruses. This will create unprecedented opportunities to study host susceptibility and tolerance to hig consequence pathogens such as SARS-CoV, SARS-CoV-2, and Nipah virus. Hundreds of SARS-CoV-lik viruses have been detected in rhinolophid bats, ~~thus susceptibility testing will allow us to identify susceptibl species~~but only a few have been isolated and none have been used to challenge rhinolophid bats. While th exact host species of SARS-CoV and SARS-CoV-2 are currently unknown, multiple species of bats within the genus *Rhinolophus* have been associated with SARS-related coronaviruses, making any representative bat o this genus a high-value model for studying coronaviruses. Nipah virus is an emerging zoonotic virus wit pandemic potential that causes near-annual outbreaks of fatal encephalitis in humans. The Indian flying fox i the natural reservoir of Nipah virus in South Asia, and is also host to more than 50 other viruses, includin uncharacterized coronaviruses, paramyxoviruses, and filoviruses. This species is robust and readily colonized

Aim 1. To establish colonies of horseshoe bats and Indian flying foxes for the study of coronavirus and henipavirus infections. Bats will be captured and quarantined in Bangladesh and tested for coronaviruses henipaviruses and filoviruses ~~and lyssaviruses~~. Those that are negative will be housed in free-flight holding pen for adaptation to captivity. Offspring of these bats will be imported to Colorado State University for establishmen of breeding colonies that will be provided to investigators performing infectious disease research.

Aim 2. To develop cell lines, reagents and methodologies for the study of viral infections in horsesho bats and Indian flying foxes. Primary bat cells will be isolated from bats for viral susceptibility testing, and stocks of primary cells will be generated for the research community. Susceptible cells will be immortalized t provide indefinite propagation. Organoids of virus targets, such as intestines, will also be generated for viru studies. Recombinant proteins, including cytokines and soluble cell surface antigens, will be generated for i vitro and in vivo use. Monoclonal antibodies to these proteins will also be generated so that investigators ca perform in vitro testing and generation of primary cells that require such growth factors, such as antigen-specifi T cells, and for the in vivo neutralization of cytokines upon challenge with viruses. Methodologies fo transcriptomic, metabolomic and proteomic studies will also be developed.

Aim 3. To perform experimental infections of bats with SARS-CoV-2, bat RaTG13-CoV and Nipah virus to develop laboratory models of bat reservoir hosts. Bats will be challenged with viruses under controlle laboratory conditions to perform kinetic and temporal studies of how these viruses behave in bats, and how the bats control infections without developing disease. We will also perform studies to assess durability of immunity in bats to determine whether immunity wanes and leads to repeated susceptibility to infection.

Commented [MK1]: Added this because we want to do proteomics and metabolomics. Also because my current understanding leads me to believe that bat physiology and flight are connected to bat immunology.

Commented [MK2]: I don't quite understand what this means.

Commented [S3R2]: Hopefully, this is clearer.

Commented [MK4]: Should we expand upon this a bit more – adding something about what goes in to developing these colonies and how we are prepared to do it.

Commented [MK5]: T-cells specifically mentioned here - I would add BM derived monocytes – those also require specific stimulatory cytokines and there is precedent for their successful isolation/development (Zhou et al., 2016). Facts I know you are aware of, I just thought they could be alluded to here.

Commented [S6R5]: We typically ship fresh or frozen bone marrow to others who then make make macrophages and dendritic cells. I'll go into detail in the grant.

Commented [MK7]: To make this we require sequence information, correct? Thes can be synthesized?

Commented [S8R7]: The IFF genome is done. Getting cytokine sequences is very easy – we did it for Artibeus by culturing splenocytes overnight with concanavalin A and pokeweed mitogen, then did RNA-Seq. This led to dozens of full-length cytokine sequences (Kingfisher is making some of these for us now).

Commented [MK9]: Maybe something about single-cell sequencing (– I know everyone is doing it). We will have a bevy of different cell types and can pin point what is and isn't activated upon infection of susceptible cells. This could be critical for understanding viral maintenance.

Sort of an aside, but I think the possibility of trained immunity is quite interesting in relation to bats. (If this is a naïve/bad idea – feel free to ignore! I am an immunology novice.) IF there is any epigenetic or chromatin-remodeling that influences the innate immune response, single cell seq technologies that combine looking at chromatin changes or methylation, particularly for myeloid progenitors, could be revealing.

Commented [S10R9]: Lin-fa is designing those studies so if something needs to go in this Aim page we need to wait to see what he writes.

Research Strategy (must address sustainability of projects and resources)

A. Significance

Bats are found on every continent other than Antarctica and they are reservoirs or suspected reservoirs of many viruses, including coronaviruses, paramyxoviruses, filoviruses and lyssaviruses. In recent years, several bat-borne viruses have emerged that have caused substantial morbidity and mortality among humans. The COVID-19 pandemic is caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), a virus that likely originated in horseshoe bats (*Rhinolophus* species) commonly found in Asia. Frequent outbreaks of Nipah virus, hosted by *Pteropus* species bats, also occur in southern Asia, causing encephalitis that is usually fatal. In each of these virus/reservoir host relationships, virtually no disease occurs in the bats. Although numerous studies have been conducted on bats and viruses, many critical questions, such as the role of bats in maintaining and spreading potential zoonotic viruses and how bat immune responses control infections without disease, remain poorly defined. There is an urgent need to study these emerging zoonotic pathogens in their natural hosts to better understand their threats to humans. Unfortunately, there currently are few natural reservoir host models for the study of bat-borne viruses that are highly consequential to humans. This project aims to address this deficiency.

A.1. *Bats as reservoir hosts of zoonotic viruses.*

Generally, in zoonotic virus/reservoir host relationships, which have been best-studied in rodents and primates, each virus is hosted by one or a few related species {Kitchen, 2011 #44}. Viruses, like all other biological entities, are subject to evolutionary pressures and are likely genetically and biochemically "optimized" to circulate within their reservoir host populations. The process of "optimization" or co-evolution often results in persistent infection (for the life of the host) or replication and shedding of virus that allows for continual transmission to other susceptible hosts {Mandl, 2015 #43}. In both cases, the virus does not cause substantial disease within the reservoir host population. This is unsurprising, as a robust immune response in the reservoir could lead to viral clearance or immunopathology. When spillover of pathogenic viruses occurs, such viruses are not biochemically optimized for human hosts, which can have unexpected outcomes. In some cases, viral infection leads to disease and death, or alternatively, immune clearance. Because of the occurrence of severe human diseases caused by some of the bat-borne viruses, an important question is: *how do bats host these viruses without becoming diseased?* The answer to this question is likely complicated and will vary between species of bats and species of viruses. Each of these viruses encodes immune modulating proteins that often target the innate antiviral responses of infected cells of the host. It is thought that these proteins are contributory factors of human disease ("virulence factors"). However, because they evolved in their bat reservoirs (i.e., "optimized"), their impacts on the orthologous target proteins of humans must somehow be different, otherwise there would not be differential outcomes in bats (no disease) and humans (disease). Although bats share many immunological features with other mammals, little research into their immune systems and responses during infection with these viruses has been conducted. This is necessary to clarify why the response to viral infection differs between humans and bats.

A.2. *Coronaviruses*. Historically, coronavirus infections of humans have been associated with mild colds that are typically resolved within several days. Unlike most other viral infections, coronaviruses fail to induce durable immunity, thus repeated infections of common cold coronaviruses can occur. However, since 2002, three novel coronaviruses have emerged from bats that have caused significant impacts on humans: SARS-CoV, MERS-CoV and SARS-CoV-2. SARS-CoV emerged in 2002 and caused a significant disease with high mortality in humans (11% case fatality rate - WHO); however, because most patients exhibited signs of disease prior to virus shedding, it was controlled quickly and ended within two years. In 2012, MERS-CoV emerged in the Middle East and causes a disease in humans similar to SARS-CoV but with a higher mortality rate (35% case fatality rate - WHO). Unlike SARS-CoV, MERS-CoV established in a secondary reservoir host, dromedary camels, in North Africa prior to its spillover into humans. Consequently, MERS outbreaks occur many times each year and nearly all outbreaks can be traced to contact with dromedary camels. SARS-CoV-2 emerged in late 2019 and is the etiologic agent of the ongoing COVID-19 pandemic. Both SARS-CoV and SARS-CoV-2 use ACE2 as cellular

Commented [MK11]: I like the gist of this section, but the first part could really apply to any reservoir host. The idea of unexpected outcomes is one I like, and I want to keep that, but maybe we can add more specifics about the unique immunological features of bats?

Commented [MK12]: This goes from immunity straight to co-evolution, making it a bit abrupt. It doesn't quite connect the dots until the end. So, I rearranged it. You can obviously ignore what I did, and put it back, but I will stand by my comment about needing a better transition from immunity to co-evolution.

Commented [MK13]: I know this is a "lame' sentence.

entry receptors, and SARS-CoV-2 has been shown to infect many species. It is now evident that thousands of distinct coronaviruses circulate in bats throughout the world, and that hundreds of these are SARS-related CoV (SARSr-CoV) that circulate in rhinolophid bats. There are 78 known species of bats within the genus *Rhinolophus* and they are found throughout Asia and Europe. Recombination frequently occurs among coronaviruses, and this has likely contributed to the diverse pool of coronaviruses circulating in rhinolophid bats. Only a few bat coronaviruses have been isolated; nearly all are represented by sequences detected in bats. One bat coronavirus, RaTG13, shares a high degree of sequence similarity to SARS-CoV-2 (32015507). Its sequences have been detected in several intermediate horseshoe bats (*R. affinis*) that are found throughout southern Asia, including Bangladesh. Although there are no isolates of RaTG13, its complete genome has been determined (GenBank MN996532). Examination of ACE2 sequences from various rhinolophid species suggests intermediate horseshoe bats have the most "human-like" ACE2 for spike binding to the 20 ACE2 residues that are thought to be important (32239522, 31996437). Because of these features, we will focus our efforts on colonizing intermediate horseshoe bats.
A.3. Nipah virus (Jon).
A.4. Sustainability of the resource.

B. Innovation
B1. This project will be the first to establish bat breeding colonies for the infectious disease research community with species relevant to SARS coronaviruses and henipaviruses.
B2. We will determine basic clinical attributes of these bats to provide baseline readings, including body temperatures, hematology, serum chemistry and metabolic profiles.
B3. We will establish bat primary cells and immortalized cell lines for distribution to the research community.
B4. We will develop recombinant bat cytokines for the derivation of primary cell cultures, including endothelial cells, dendritic cells and T cells, and to use these cytokines for in vivo studies to manipulate immune responses during infection.
B5. We will generate monoclonal antibodies for the detection of cytokines, cell surface antigens and for in vivo cytokine neutralization and cellular depletion studies
B6. We will

C. Approach
C1. Preliminary Studies

C1.1. *Fruit bat colony at Colorado State University*. Dr. Schountz established a breeding colony of Jamaican fruit bats (*Artibeus jamaicensis*) in 2006 with previous NIH support (AI25489, AI089419) (Fig 1). This species is found in northern South America, the Caribbean Islands, Central America, and the Florida Keys. Adult bats have an average weight of 42 gm, a wingspan of 45 cm and body length of about 10 cm. Mature females produce two offspring per year, carry pups for about 40 days after birth, and can live up to 15 years captivity. There are about 400 bats currently in the colony and about 100 to 150 new pups are born at 6-month intervals. Colorado State University has extensive animal expertise by virtue of its College of Veterinary Medicine and Biomedical Sciences, including veterinarians with experience with bats. **Thus, we have extensive investigator and institutional experience with bat husbandry and colony management.**

Fig. 1. Jamaican fruit bats in the CSU colony.

C.1.2. *Candidate SARS-like coronavirus reservoirs*. Receptor incompatibility can pose a potent barrier t infection, preventing a virus from gaining access to the cell; a necessary step to initiate the replication cycle There is ample evidence that one or a few amino acid substitutions at critical sites of interaction in cellula receptors can affect viral entry {Demogines, 2013 #45;Kaelber, 2012 #47;Ng, 2015 #42;Yan, 2010 #46} Angiotensin converting enzyme 2 (ACE2) is the reported receptor for SARS-CoV and SARS-CoV-2. Therefore to initially assess the potential of rhinolophid bats as a reservoir for SARS-like coronaviruses we performed a

Commented [MK14]: Is this more about finding a bat model that will support SARS-CoV and SARS-CoV-2 infections?

Commented [MK15]: There is a paper that looks at polymorphisms within R.sinicus ACE2 and their effect on susceptibility to SARS-rCoV strains (Guo, 2020, JVI). The data is ok, and I believe it, but they do a wildly inappropriate evolution analysis (it's meant for between species and they do it on an a within species set, which violates all the assumptions). The virology data is ok, their 0 timepoint of their qPCR is high (start with a lot of genomes) but they increase nicely over time, so it's alright. I sort of don't want to cite it because of the aforementioned issues, but it supports the idea that variation at the receptor can change susceptibility to a SARS-rCoV. Does anyone know of any other papers that do something similar with regards to bat ACE2?

Table 1. ACE2 amino acid identity amongst Rhinolophid bats and hosts supporting SARS-CoV-2.

		Amino Acid in *ACE2*																				
Species name	Common name	24	27	28	30	31	34	35	37	38	41	42	45	82	83	330	353	354	355	357	393	Amino Acid Identity
Homo sapiens	Human (dbSNP)	*Q*	*T/A*	*F*	*D*	*K*	*H*	*E/D/K*	*E*	*D*	*Y*	*Q*	*L*	*M/I*	*Y*	*N*	*K*	*G*	*D/N*	*R*	*R*	100%
Mesocricetus auratus	Syrian Hamster (N=1)	**Q**	T	**F**	**D**	**K**	Q	**E**	**E**	**D**	Y	Q	L	N	Y	**N**	**K**	G	D	R	R	85%
Rhinolophus sinicus	Chinese rufous horseshoe bat (N=25)	R/E/L	**T/M/I**	**F**	**D**	**K/E/T**	S/T/F	**E/K**	**E**	D/N	Y/H	Q/K	L	N	Y	**N**	**K**	G	D	R	R	85%
Felis catus	Domestic cat (N=1)	L	**T**	**F**	E	**K**	**H**	**E**	**E**	E	Y	Q	L	T	Y	**N**	**K**	G	D	R	R	80%
Rhinolophus affinis	Intermediate horseshoe bat (N=23)	R	**I**	**F**	**D**	N	**H/R**	**E**	**E**	**E/D**	Y	Q	L	N	**Y/H**	**N**	**K**	G	D	R	R	80%
Rhinolophus pearsonii	Pearson's horseshoe bat (N=2)	**R/Q**	**T/I**	**F**	**D**	**K**	**H/R**	**E**	**E**	**D**	H	E	L	D	Y	**N**	**K**	D	D	R	R	80%
Mustela putoris	Ferret (N=1)	L	**T**	**F**	E	**K**	Y	**E**	**E**	E	Y	Q	L	T	Y	**N**	**K**	R	D	R	R	70%
Rhinolophus pusillus	Least horseshoe bat (N=1)	K	K	**F**	N	D	S	**E**	**E**	**D**	Y	Q	L	N	Y	**N**	**K**	G	D	R	R	70%
Rhinolophus macrotis	Big-eared horseshoe bat (N=1)	E	K	**F**	**D**	**K**	S	**K**	**E**	**D**	Y	E	L	N	Y	K	**K**	G	D	R	R	70%
Rhinolophus landeri	Lander's horseshoe bat (N=1)	L	**T**	**F**	**D**	D	S	A	**E**	N	Y	Q	L	N	F	**N**	**K**	G	D	R	R	65%

alignment of all nine *Rhinolophus* species ACE2 sequences available in GenBank with Human ACE2 and ACE2 of known susceptible mammalian species. We specifically compared amino acid identities at 20 sites involved in spike binding (Table 1). Where available, sequences from multiple individuals were included.

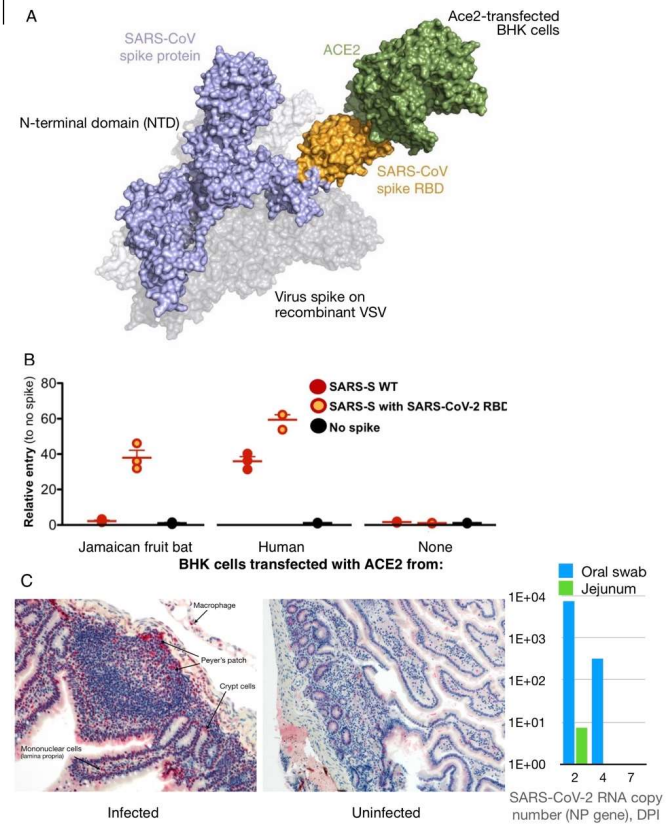
Strikingly, for those species where multiple ACE2 haplotypes are reported (*R. sinicus, R. pearsonii,* and *R .affinis*), multiple different amino acid identities are encoded at sites important for ACE2 binding (Table 1). In cases where there are multiple amino acids possible (e.g. Table 1, site 34), occasionally one amino acid matches the residue(s) encoded by humans. This apparent genetic variation reveals two important implications: 1) certain bat individuals may encode ACE2 receptors that are permissive to coronaviruses infecting humans while others of the same species do not, and 2) certain bat individuals may be more likely to harbor a SARSr-CoV strain able to infect humans. We can capitalize on this naturally occurring variation through widespread capture, genotyping at loci of importance for coronavirus infection, and selective breeding to develop a colony of bats ideally poised for controlled viral infection and immunology studies.

Based on current ACE2 sequence data, the intermediate horseshoe bat and Pearson's horseshoe bat (*R. pearsonii*) encode the same amino acids as human at 16 of the 20 residues important for ACE2 binding. The Least horseshoe bat (*R. pusillus*) has 14 matching residues. These three species are found in Bangladesh (Table 1). While no bat species encodes the same 20 residues as human, both domestic cats (16 matches) and ferrets (14 matches) are susceptible to SARS-CoV-2, suggesting that all three Bangladeshi species may harbor permissive ACE2 receptors. Intermediate horseshoe bats (16 matches) are widely distributed in southern Asia, including Bangladesh as well as southern China where most of the SARSr-CoV sequences have been detected in rhinolophid bats. For this reason, we believe this is the most logical species to capture for susceptibility testing and model development. However, because there are 78 rhinolophid species, we will also capture the other species that are found in Bangladesh (*R. lepidus*, *R. malayanus*, *R. monoceros*, *R. humanus*, *R. luctus*, *R. subbadius*) for importation and SARS-CoV-2 susceptibility testing at CSU.

Commented [MK16]: Maybe a figure showing the species ranges and our proposed site of the Bangladeshi bat colonies?

C1.3. *Experimental bat coronavirus infections*. Drs. Munster and Schountz have extensive experience with experimental infection models of bats, including with MERS-CoV, SARS-CoV-2, H18N11 bat influenza virus, Zika virus and Tacaribe virus. Challenge of Egyptian fruit bats with an infectious clone of WIV-CoV showed limited susceptibility, despite the ability of its spike protein to bind to Egyptian fruit bat ACE2 (30572566). Recent work by our groups has shown that Jamaican fruit bat ACE2 is bound by SARS-CoV-2 (but not SARS-CoV) spike protein expressed by a VSV pseudotype virus and that challenge of Jamaican fruit bats with SARS-CoV-2 leads to a limited infection that is confined to the small intestine, particularly mononuclear cells of the Peyer's patches and lamina propria, and crypt cells (Fig 2). Viral RNA was detected in oral swabs for 4 days and low

Fig. 2. Infection of Jamaican fruit bats with SARS-CoV-2. Model of SARS-CoV-2/ACE2 receptor (A). BHK cells transfected with Jamaican fruit bat ACE2 cDNA are susceptible to pseudotyped VSV expressing SARS-CoV-2 spike protein (B). Experimental challenge of Jamaican fruit bats with SARS-CoV-2 showed abundant viral antigen in mononuclear cells of the Peyer's patches and lamina propria that are likely macrophages, as well as intestinal epithelial cells and crypt cells, with detectable vRNA for up to 4 days in oral swabs (C).



levels in the small intestine on day 2. However, no neutralizing antibody or nucleoprotein antibody (ELISA) was detected, suggesting abortive infection or robust control of the virus by the innate immune response.

C1.4. *Proteomic detection of bat cytokines*. We used mass spectrometry to detect several cytokines in trypsinized supernatants of Jamaican fruit bat spleen cells incubated overnight with concanavalin A, including IL-4, IFNγ, IL-2, IL-6, IL-10 and TNF. Assays were conducted on the Acquity UPLC (M class) coupled to a Xevo TQ-S (Waters) MS (housed in the Proteomics and Metabolomics Facility at CSU). The results (Fig. 3.) demonstrate our ability to identify 5 peptides from 4 cytokines in a single experiment and proof of feasibility for the work proposed herein.

C1.5. Detection of NK cells in Jamaican fruit bats. We have screened more than two dozen monoclonal antibodies for cross reactivity to bat cells and have identified only a few. One of these recognizes NK1.1 on bat spleen cells (Fig 4) and this clone has been used to deplete NK cells in mice (9570522). If this antibody also depletes NK cells in bats, it will allow us to scrutinize the role of NK cells during infection. Of particular interest, bats have a substantially higher number of NK activation and repression genes relative to other mammals, suggesting they may play a more important role in bat immunology than in other species (29706541).

C2. Specific Aims

Outreach. We will alert potentially-interested investigators about the resources established by this R24 by personal communications with those publishing in coronavirus/henipavirus research, announcements at conferences, and establish a web site that provides information about the colonies. For security reasons, the web site will be restricted to investigators who request access and will be password protected. Need more here

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C2.1 Aim 1. To establish colonies of horseshoe bats and Indian flying foxes for the study of coronavirus and henipavirus infections. Bats will be captured and quarantined in Bangladesh and tested for coronaviruses, henipaviruses, filoviruses and lyssaviruses. Those that are negative will be imported to Colorado State University for establishment of breeding colonies.

C2.1.1. *Establishment of captive breeding colony of Pteropus medius in Bangladesh*. We will establish a captive breeding colony of Pteropus medius in Bangladesh. Temporary holding cages used for quarantine prior to shipment to the US will be erected at the Sheikh Kamal Wildlife Center (SKWC) Purabari, adjacent to Bhawal National Park, Gazipur, north of Dhaka. The land is managed by the Forest Department under the direction of the Conservator of Forests (see Kabir letter of support) Ministry of Environment (Govt of Bangladesh). The bats will be cared for and maintained by Forest Department staff. Staging bats in Bangladesh prior to shipment to the US will occur in X phases.

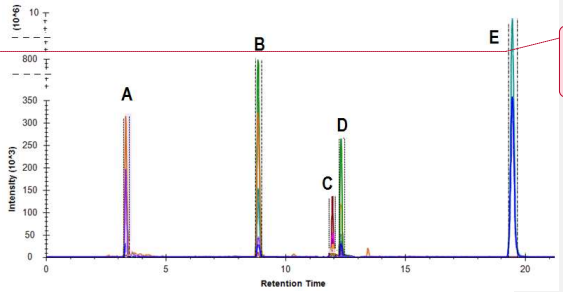

Fig. 4. Detection of bat cytokines using unrefined targeted MS assay. The following peptides were detected in bat cell supernatant after mitogen-stimulation: A. IL-10: FLP**C**ENK, B. IL-4: SLSGLAGQNT, C. IL-6: RDG**C**FQSGFNEK, D. IFN-γ: AINELIRVMHELTPGSNLR, E. IL-10: AAFSMVK.

Commented [MK17]: I assume the holding cages will be different for the rhinolophid bats, but the capture methods will be the same?

Phase 1: Holding cage development. We will build four temporary holding cages [octagonal shape, approx. 6m diameter and 6ft tall] that are large enough to allow bats to fly, and low enough that project personnel can easily repeatedly catch bats for testing during the screening process. Each cage will be built to hold 30 bats, with a heavy retractable curtain subdividing each cage in half, which will allow us to separate bats into smaller groups. One of the cages will be left empty to allow for isolation of bats that test positive for Nipah virus on PCR during the course of quarantine. Each cage will be made from a metal frame with rubber-coated mesh screening enclosing the cage. An outer cage will be built around each cage, with 6" of space between the fencing to exclude bats inside from contact with bats outside. A solid roof will be built over each cage to prevent excreta from wild bats from entering the cage area and potentially exposing captive bats to henipaviruses. Each cage will also have a door and an anti-chamber to allow staff to safely enter and exit, don and doff PPE, and then enter the flight cage without bats being able to escape.

Facilities at the wildlife park where the bats will be held will include a food preparation station, PPE storage, first aid and disinfection supplies, and a mobile veterinary lab that includes electricity, a portable gas anesthesia machine, and supplies for collecting biological specimens from the bats.

Phase 2: Bat capture. We will capture wild bats from colonies located in Gazipur. Our goal is to capture a total of 81 bats: 6 males and 75 females comprising three groups of 25 females and 2 males - all Nipah virus negative (by PCR and Luminex). We will capture bats 20 at a time. With each group of 20 bats, we will transport them to the cages from the field site, with each bat held in an individual cotton bag suspended from a post in the back of a vehicle. The driver will be wearing PPE, including an N95 respirator, and all vehicle windows will be open during transport. A plastic sheet will line the floor of the vehicle under the bats to allow for disinfection following transport. Bats will be sampled in the vet lab and then immediately transferred into a holding cage and blood samples and swabs collected. Samples will be screened that day for IgG antibodies using a Luminex, and swabs will be screened for Nipah virus RNA using a qRT-PCR assay at icddr,b. Seropositive and or PCR positive individuals will be released at the park. Seronegative and PCR negative individuals will be moved into a second staging cage, where they will be held for 21 days, with a second and third round of testing on day 10 and 21 post capture. Any bats subsequently testing positive will be released. Negative bats will be retained.

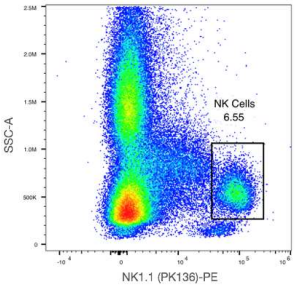

Fig. 3. Detection of NK cells in Jamaican fruit bats. Anti-mouse NK1.1 (clone PK136) was used to stain Jamaican fruit bat splenocytes and allows identification of NK cells in bats. In mice, this antibody depletes NK cells, thus it may be useful for depleted bat NK cells to allow examination of their roles in viral infections.

Phase 3: Breeding colony. We will maintain separate groups of 27 bats in the three flight cages. Pteropus medius is a synchronous seasonal breeder, with a 6-month gestation period. We will aim to capture bats during the third trimester of pregnancy (January/February) and allow them to give birth in captivity. Pups will be weaned at 3 months and placed

into a fourth cage, where they will be maintained until 1 year of age. Pups will be screened every 2 weeks for Nipah virus and antibodies. Maternal antibodies are expected to wane at around 6-7 months of age. By isolating the pups, born to seronegative, Nipah-negative dams, we will create an SPF (specific-pathogen free) colony that will be used to found the colony at CSU. We will also allow the bats in Bangladesh to breed, creating an F1 generation that will be maintained in captivity and used to support the US-based colony.

C2.1.2. *Experimental challenge of rhinolophid bats.* We will determine whether Ace2 sequences from various *Rhinolophus* species bats render BHK cells susceptible to SARS-CoV and SARS-CoV-2 using our VSV pseudotype system (Fig XX). Rhinolophid species will be captured and quarantined in Bangladesh and tested for evidence of SARS-like CoV infection (PCR of rectal swabs and serology). Naïve bats will be shipped to Colorado State University for challenge with SARS-CoV and SARS-CoV-2 to determine susceptibility. Those species that are susceptible will be collected in Bangladesh and after quarantine and testing, up to 50 founders will be shipped to CSU to establish breeding colonies. In the event that more than one species is susceptible, we will determine which is most suitable based upon infection kinetics of each virus.

Upon receipt, bats will be immediately examined by veterinary staff at CSU and manually fed meal worms.

C2.2 Aim 2. To develop cell lines, reagents and methodologies for the study of viral infections in horseshoe bats and Indian flying foxes. Primary bat cells will be isolated from bats for susceptibility testing, and stocks of primary cells will be generated for the research community. Susceptible cells will be immortalized to provide indefinite propagation. Organoids of virus targets, such as intestines, will also be generated for virus studies. Recombinant proteins, including cytokines and soluble cell surface antigens, will be generated for in vitro and in vivo use. Monoclonal antibodies to these proteins will also be generated so that investigators can perform in vitro testing and generation of primary cells that require such growth factors, such as antigen-specific T cells, and for the in vivo neutralization of cytokines upon challenge with viruses. Methodologies for transcriptomic, metabolomic and proteomic studies will also be developed.

C2.2.1. *Establishment of primary and immortalized bat cell lines*. We will generate primary cells from (Tony)

C2.2.2. Production of recombinant bat cytokines and monoclonal antibodies. Recombinant horseshoe bat and Indian flying fox cytokines will be generated. Codon optimized genes will be designed from bat genomic sequences and available splicing data and synthesized by Genscript and sequence verified by Genewiz. Cytokine genes will be produced in *E. coli*, baculovirus infected SF9 cells or human 293-Freestyle cells, depending on existing literature from other systems. For example, soluble active human CD4 has been successfully produced in *E. coli* (**2187501**), providing a starting point for production of bat CD4. Recombinant his-tagged human CD20 is commercially available from *E. coli* and human 293T cells (Sinobiologicals), indicating that bat CD20 may be producible in a similar fashion. Cytokine genes will be cloned into appropriate expression vectors (pET28a for bacteria, pFastBac for baculovirus expression, pcDNA3.1 for mammalian expression) with purification tags (6xHis/GST) and fusion partners (thioredoxin/Sumo) to aid folding as necessary. Proteins will be expressed in appropriate cell types (e.g. BL21 pLysS *E. coli*, SF9 insect cells for baculovirus expression, 293Freestyle for mammalian expression). Expressed protein will be purified on AKTA Start FPLC systems using appropriate affinity chromatography columns including nickel columns, ion/anion exchange columns, or glutathione columns. Affinity purified proteins will be further purified using Superdex 200 size exclusion chromatography to select for monomers or dimers of the recombinant protein as needed. Following purification, proteins will be tested for activity and storage/stability of each protein will be optimized to maximize activity and protein life-span. The identity of purified proteins will also be verified by Orbitrap mass spectrometry at the CSU ARC-Bio proteomics core facility.

We will also produce soluble versions of CD4, CD8β and CD20 proteins for both bat species; these will be used to generate monoclonal antibodies as described below so to provide reagents for detection of helper T cells, cytotoxic T cells and B cells, respectively, by flow cytometry on splenocytes. We will also screen these antibodies for the abilities to deplete these subsets in vivo so that examination of the role of these cells can be performed.

Monoclonal antibodies will be generated as we previously described (32908982). Briefly, recombinant cytokines and soluble CD antigens will be emulsified in Sigma Adjuvant System and used to immunize female BALB/c mice using the CSU Veterinary Service Core. After biweekly boosting and determination of high antibody

titers by ELISA (cytokines) and flow cytometry (CD antigens), mice will be given a final dose of respective antigens in PBS, then euthanized 4 days later for fusion to Sp2/0 Ag14 myeloma cells using the ClonaCell™-HY Hybridoma Kit (Stem Cell Sciences). Cells are plated on methylcellulose containing HAT medium, which allows concurrent selection and cloning during 2 weeks of incubation. Clones will be picked and screened for production of antibodies specific to cytokines (ELISA) and CD antigens (flow cytometry). For cytokine-specific antibodies, we will identify suitable pairs of monoclonal antibodies that can be used for capture ELISA (pg/ml sensitivity) and neutralization (for in vitro and in vivo use). Antibodies to CD antigens will be tested in live bats for their abilities to deplete CD4+ helper T cells, CD8β+ cytotoxic T cells, and CD20+ B cells.

C2.2.3. Development of metabolomic profiles. (Lin-fa and Rushika)
C2.2.4. Development of proteomic profiles. (Lin-fa and Rushika)

C2.3 Aim 3. To perform experimental infections of bats with SARS-CoV-2, bat RaTG13-CoV and Nipah virus to develop laboratory models of bat reservoir hosts. Bats will be challenged with viruses under controlled laboratory conditions to perform kinetic and temporal studies of how these viruses behave in bats, and how the bats control infections without developing disease. We will also perform studies to assess durability of immunity in bats to determine whether immunity wanes and leads to repeated susceptibility to infection.

C2.3.1. *Experimental infection of horseshoe bats with SARS-CoV-2.* We have previously performed MERS-CoV and SARS-CoV-2 infection experiments with Jamaican fruit bats (26899616) (Fig XX). In year 1, we will capture rhinolophid bats in Bangladesh for quarantine, testing for evidence of coronavirus infection, and identification of coronaviruses that might be harbored by the bats. Those that are uninfected and seronegative will be shipped to CSU for immediate challenge with SARS-CoV-2 to determine species susceptibility and inform species of interest for colonization. Only one reference genome for a *Rhinolophus* species is available (*R. ferremuquium*); however, it is likely that most genes will be of high enough similarity for use with the species that we expect to capture in Bangladesh.

Six bats of each species will be challenged with SARS-CoV-2, whereas three bats of each species will be held as negative controls. At two-day intervals, rectal and oral swabs will be collected in virus transport medium from each bat for RNA extraction and virus isolation. At seven-day intervals, 100 ul of blood will be collected from the wing vein for serological analysis using a microbead flow cytometric assay we have developed for SARS-CoV-2 (33083807). Bats will be euthanized at day 21 for necropsy, thus providing 4 serological time points per bat (days 0, 7, 14, 21). We previously determined that MERS-CoV replicates in the lungs, liver, spleen and intestines of Jamaican fruit bats, and that SARS-CoV-2 replicates in the intestines only (Fig XX). We will extract RNA from each tissue for detection of vRNA and attempt virus isolation from those tissues that possess vRNA.

Based upon this preliminary study, we will assess the susceptibility of each species to SARS-CoV-2 and use the information from this study to determine which species is most likely a suitable model for the study of SARS-CoV-2 reservoir host. This species will be used to establish the breeding colony as a resource for the coronavirus research community. We will also assess host immunity on bats by using immunosuppressants that target the type I IFN response (fludarabine), T cell response (cyclosporine A) and general immunosuppression (dexamethasone).

In years 3-5, challenge studies will be performed to determine target cells of SARS-CoV-2 infection and immunosuppress bats during SARS-CoV-2 challenge. It is evident that coronaviruses replicate in the intestines of bats; however, it is unknown which cells are targets. Thus, it will be necessary to perform single cell RNA-Seq of infected cells to identify the gene expression profiles of target cells, which will clarify susceptible cells. Once target organs are identified in the Year 2 study described above, we will infect bats and collect targeted tissues and organs to prepare single cell suspensions as we describe with SARS-CoV-2 challenged African green monkeys (33431511). Briefly, target organs will be minced and digested with DNase and Liberase TM, subjected to AKC RBC lysis, and filtered for individual cells for use with 10X Genomics Chromium platform (10,000 cells per sample). The RNA Seq data will be processed through the cellRanger pipeline for gene expression analysis and cell identification with the FindMarkers component of Seurat R package (31178118).

We will use dexamethasone (Dex), fludarabine and cyclosporine A (CyA) to immunosuppress bats and challenge with SARS-CoV-2. (Our experience is that a combination of Dex and cyclophosphamide leads to pathological expansion of the microbiota and compromises the health of bats.) Dex is a general immunosuppressant that acts upon a variety of immune cells, including all T cell subsets, B cells and impairment of macrophage maturation; fludarabine is a STAT1 inhibitor that suppresses type I IFN receptor signaling; and cyclosporine A disrupts T cell activation. We will use these immunosuppressants to assess how they influence SARS-CoV-2 infection, with an expectation that they will each lead to increased virus replication and shedding and, perhaps, a disease phenotype. Samples will be collected to assess infection kinetics and host responses during infection.

We will also perform challenge studies with the rescued RaTG13 virus described below. Because this virus genome was discovered in *R. affinis* bats, a species commonly found in Bangladesh, we are confident that this species will be susceptible upon experimental challenge. Importantly, with the development of an infectious clone, we will solicit R01 funding to develop this project independently of the R24 once we have demonstrated susceptibility.

Caveats and Extensions. The principal concern is that none of the bat species are susceptible to SARS-CoV-2. We think this is unlikely, given the high similarity of the important ACE2 residues (Table 1) that mediate spike binding. As extensions, we can also challenge the bats with SARS-CoV (Dr. Schountz's lab staff and BSL-3 are Tier 1 Select Agent approved) and MERS-CoV.

C2.3.2. *Experimental infections of Indian flying foxes with Nipah virus*. In year 1, pregnant Indian flying foxes bats will be captured and held in Bangladesh to verify they are not infected to Nipah virus (as described above). Pups born in captivity will be shipped to RML for use in initial infection studies in Year 2 of the grant. This work will be supervised by Dr. Munster. After baseline infection kinetics have been determined, we will perform subsequent challenge studies to better assess virus distribution using mCherry-labeled virus (which retains fluorescence after fixation, 26781134), to identify specific target cells by single-cell sequencing (24023909) and impact of immunosuppression on the response to Nipah virus. An annotated reference genome for the Indian flying fox is available (32849415) and will be used for transcript identification.

We will follow our previous experimental strategies for experimental infection of bats (31527796, 30716104, 26899616, 22379103, 31682727). The first experimental challenge of Indian flying foxes will occur in year 2. Bats born in captivity at the Bangladesh holding facility will be shipped directly to RML, which has animal BSL-4 capacity. For this initial study, 18 bats of both sexes will be used. Bats will be housed in cages specifically designed for BSL-4 use at RML. They will be anesthetized and inoculated intranasally with 10^5 $TCID_{50}$ of a Bangladesh isolate of NiV that is maintained as a stock at RML. Bats will be recumbently positioned during inoculation to ensure delivery of virus to the trachea and esophagus (i.e., lung and GI delivery). Three unchallenged bats will be cohoused with inoculated bats beginning 2 days after challenge (contact transmission study) and three bats will be held as uninfected negative controls. At 2 day intervals, oral and rectal swabs, and urine samples will be collected for virus detection. On days 2, 6, 10 and 20, three bats will be euthanized for necropsy, and the contact and negative control bats will be euthanized on day 21. At euthanasia, whole blood and serum will be collected for hematology, serum chemistry and serology studies. Tissues will be partitioned, with some flash-frozen in liquid nitrogen for virus isolation and RNA extraction, and others in buffered formalin for immunohistochemistry, RNA-Scope for vRNA and histopathology.

As described above, we will also assess the role of the immune response with use of immunosuppressants to determine if viral loads are elevated and whether clinical signs of disease occur.

Caveats and Extensions.

C2.3.3. *Generation of bat RaTG13-CoV infectious clone*. Reverse genetics have been used as a powerful tool to generate infectious clones of SARS-CoV, SARS-CoV-2 and MERS-CoV (32526206, 28377531, 24043791, 32978313, 32289263). To rescue recombinant RaTG13 we will follow methods previously described to generate SARS/MERS-CoV full-length genome cDNA (24043791, 14569023, 12368349, 18818320, 19036930). Six contiguous RaTG12 cDNA fragments will be synthesized and the contiguous cDNA fragments will then be ligated

into a full-length cDNA using a subcloning strategy with *Bgl*I class II restriction endonuclease sites. The ligated cDNA product under T7 promoter control will then be electroporated into a co-culture of mammalian cell lines (Vero, BSR-T7) along with T7 RNA polymerase and a nucleoprotein expressing vector to enhance replication efficiency. To construct neonGreen fluorescent protein (GFP) and red fluorescent protein (RFP) reporter viruses, the ORF7a/7b sequence will be replaced with GFP or RFP sequences as previously described (16306622, 32289263). The same simultaneous ligation strategy will be employed to generate the full-length cDNA of RaTG13 GFP/RFP variants. Cytopathic effects and plaques will be monitored for 24 – 72 hours post-transfections; duplicate wells will be a) stained with anti-SARS-CoV nucleoprotein secondary antibodies for intracellular detection of RaTG13 infectious clone replication and b) lysed and cells/cell-culture supernatant will be passaged to fresh Vero cells for amplification of the virus stock. Replication kinetics and titers of infectious clone RaTG13 and reporter virus variants will be monitored by standard plaque assays and/or measurement of fluorescent plaques. Recombinant RaTG13 infectious clones will be sequenced with at least 2X coverage to ensure fidelity after passaging on Vero cells. A mammalian cell line will be transfected with human and bat ACE-2 orthologues to allow functional characterization of infectious clones of RaTG13 receptor-usage for cellular entry. Virus genome sequencing will be performed after passage to verify sequence integrity, as we have previously described (32793912).

Caveats and Extensions. In the event a novel SARSr-CoV sequence is discovered in quarantined rhinolophid bats in Bangladesh, the virus will be considered as a candidate for infectious clone development, especially if it is commonly found in a particular species of *Rhinolophus* bat (i.e., putative reservoir host/virus model).

C2.3.4. *Detection of virus specific antibodies in experimentally-challenged flying foxes and horseshoe bats*. Whether bats develop long-lasting adaptive immune responses to viruses remains unknown. Egyptian fruit bats, natural hosts of Marburg virus and Ravn virus (19649327), challenged with Marbug virus seroreverted to undetectable levels three months after infection (28821722). Virus-specific antibody responses are surrogates of predictive protection or correlates of protection for nearly all vaccines. Here, we will establish primary and secondary humoral responses to virus infections in two virus-host models: NiV-flying foxes, RaTG13-horseshoe bats. Utilizing multiplex serology strategies that our groups have developed for NiV and coronaviruses (32034942, 31671094, 33083807) we will capture bat immunoglobulin (Ig) G and IgM with virus envelope/spike glycoprotein antigen-based assays. The kinetics, magnitude and duration of virus-specific flying fox and horseshoe IgG/IgM will be monitored longitudinally for seroconversion, seroreversion and anamnestic boosting after secondary infection. Population-level biosurveillance of NiV and other emerging zoonotic viruses relies on susceptible-infected-recovered models to understand the spread of these viruses among wildlife hosts. Experimental establishment of longevity of virus-specific bat IgG-responses will provide valuable information for ongoing biosurveillance projects focused on the transmission/spillover dynamics of emerging zoonotic viruses in wildlife host populations and communities.

Caveats and Extensions.

Vertebrate Animals

Description of Procedures:

Housing of Bats at Colorado State University

Indian flying foxes. During year 1 of the grant, CSU will renovate a building at the Foothills Campus to house the flying foxes. This building is approximately 300 meters from the Center for Vector-borne Infectious Diseases, where Drs. Schountz and Perera laboratories are located. It has approximately 2500 ft^2 and will include an anteroom where a sink, food prep area, refrigerator and freezer will be used for food preparation. The diet for the bats will be composed of apples, bananas, cantaloupe pear, grapes, carrots, sweet potato and kale, sprinkled with HMS Bat Supplement, which contains essential vitamins and minerals. This dietary formulation has been provided to by Brian Pope at the Lubee Bat Conservancy in Gainesville, FL who will also provide expert guidance for maintaining the breeding colony (letter of support provided). Free-choice salt licks will be provided. Enrichment, including artificial tree branches, landscape fabric, skewers for hanging fruit (i.e., stimulation of foraging behavior) will be included. The facility will have 4 meter ceilings to permit free-flight, with a capacity to house 25 bats for the breeding colony, with room for an additional 20 bats for short-term holding. This will allow us to import additional bats that will not be used for breeding, but instead for acclimation and veterinary health check after importation, and prior to shipment from CSU to other investigators that will require bats for their research projects (e.g., RML, UTMB, NEIDL, etc.).

For breeding purposes, we will have 5 males and 20 females. Flying foxes produce one offspring per year and these offspring will be distributed to the research community for experimental work. When breeders are retired, offspring will be used to replenish the breeding colony.

Horseshoe bats. Horseshoe bats are insectivorous and usually require dietary adjustments to maintain in breeding colonies. Elizabeth Falendysz, a veterinarian with the US Geological Survey in Madison, WI, will provide guidance for colonizing horseshoe bats (letter of support provided). She has maintained a breeding colony of insectivorous big brown bats (*Myotis lucifugus*) for several years as part of the USGS's white nose syndrome research. Bats will be maintained in a free-flight room at the Center for Vector-borne Infectious Diseases at CSU. This facility was constructed in 2020 and includes three rooms dedicated for bat research. Similar to our Jamaican fruit bat colony room, the horseshoe bat room has approximately 240 ft^2 of space with 8 ft ceilings. We will provide enrichment, including landscape fabric (for hiding) draped on walls and handing from rope lines across the room. Live meal worms will be provided daily as a food source and water provided ad libitum.

Although little is known about horseshoe bat reproduction, females typically become reproductive by age 2. For species found in temperate climates, gestation is tied hibernation. However, the species found in Bangladesh typically do not hibernate because of the nation's tropical and subtropical climates. Therefore, females may give birth every 4 to 6 months (similar to the Jamaican fruit bats in the CSU colony). Because of this low fecundity, we will typically use more males than females for experiments and distribution to the research community.

Justifications:
Indian flying foxes are a natural reservoir host of Nipah virus. More than 100 SARS-related coronaviruses have been detected in horseshoe bats and are, thus, likely reservoir hosts SARSr-CoV. For these reasons, we will colonize these species.

Minimization of Pain and Distress:

Housing of bats at CSU (Aim 1). Bats will be provided free-flight rooms at CSU, similar to how we have housed Jamaican fruit bats since 2006 (for details, see 25494448). No procedures involving pain will be employed, other than temporary pain associated with minor procedures, such as blood collection for hematology and serum chemistry studies. To minimize distress, bats are provided enrichment, including landscape fabric dangling from

the ceiling for horseshoe bats (social gathering and sequestration), and horizontal ropes attached to the ceiling for flying fox roosts. Handling of bats will be minimized and limited to collection for basic health studies (e.g., hematology and serum chemistry), training of personnel, veterinary care that may arise, and for removal from the colony (e.g., euthanasia for generation of cell lines in Aim 2, infection studies in Aim 3, shipment to other investigators in the USA). As per standard CSU veterinary care, bats will be monitored and fed daily, and rooms will be sanitized twice weekly by CSU Laboratory Animal Resources staff. We will abide by the US Animal Welfare Act for providing care and handling of all bats.

Select Agent Research

Coronaviruses are not select agents, other than the original SARS-CoV. Nipah virus is also a select agent and requires BSL-4 containment. Both CSU and RML are Tier 1 select agent facilities and Dr. Schountz's laboratory staff have select agent approvals for work at CSU. Dr. Schountz also has approval to work in the BSL-4 at RML should it become necessary for him to work there for Nipah virus infection experiments. Dr. Munster is the Director of the Virus Ecology laboratory at RML and has extensive experience with BSL-4 work with henipaviruses, including Nipah virus.

Multiple PD/PI Leadership Plan

Consortium/Contractual Arrangements

Letters of Support

Resource Sharing Plans

Data Sharing Plan:

Sharing Model Organisms:

Genomic Data Sharing (GDS):

Authentication of Key Biological Resources

Appendix (maximum of 10 PDF documents)

Literature Cited

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From: Schountz,Tony on behalf of Schountz,Tony <Tony.Schountz@colostate.edu>
To: Maria Kaczmarek; Eric Laing
Subject: Re: R24 - questions about your section
Date: Wednesday, January 20, 2021 10:59:55 AM
Attachments: R24 Grant_tsJAN15.docx

Hi Maria and Eric,

I think I have incorporated the changes but please check on the attached. This is the current snap-shot of the proposal, but it has a ways to go, yet.

Thanks,

Tony
—
Tony Schountz, PhD
Associate Professor
Center for Vector-borne Infectious Diseases
Department of Microbiology, Immunology and Pathology
College of Veterinary Medicine
Colorado State University
200 West Lake Street
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> On Jan 19, 2021, at 6:05 PM, Maria Kaczmarek <kaczmarek@ecohealthalliance.org> wrote:
>
> Hello!
>
> Attached you will find some edits from Eric. See the message below.
>
> Cheers,
> Maryska
>
> ---------- Forwarded message ---------
> From: **Laing, Eric** <eric.laing@usuhs.edu>
> Date: Tue, Jan 19, 2021 at 8:02 PM
> Subject: Re: R24 - questions about your section
> To: Maria Kaczmarek <kaczmarek@ecohealthalliance.org>
>
> Hi Maria,
>
> Nice to meet you. Thanks for the ORF5/ORF7 catch. I changed that and added the correct citations. Will you pass this along to Tony? He has probably updated the GFP text to neonGreen.

- Eric

Eric D. Laing, Ph.D.
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cell: (301) 980-8192
office: (301) 295-9884
lab: (301) 295-9618

eric.laing@usuhs.edu

On Tue, Jan 19, 2021 at 7:47 PM Maria Kaczmarek <kaczmarek@ecohealthalliance.org> wrote:

> Hi Dr. Laing,
>
> My name is Maryska Kaczmarek and I am working with Jon Epstein at EcoHealth.
> I had two quick questions about your section in the R24. I have attached just that section here with them included in the word document.
>
> Let me know if my comment/questions make sense.
>
> Cheers,
> Maryska
>
> --
> **Maria "Maryska" Kaczmarek, PhD**
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> ***EcoHealth Alliance develops science-based solutions to prevent pandemics and promote conservation.***

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

<R24_Edl_mekQuestions-edl.docx>

Specific Aims

An important and frequently asked scientific question is whether bats are better hosts for lethal viruses than other mammals. There are a number of zoonotic viruses that cause high mortality in humans and originate in bats: SARS coronaviruses (SARS-CoV and SARS-CoV-2), Ebola virus, Marburg virus, Nipah virus, Hendra virus, and Middle East respiratory syndrome coronavirus (MERS-CoV). Further, there is increasing evidence that many other human viruses may have originated in bats, including measles, mumps and hepatitis C viruses. However, there is a lack the bat animal models necessary to study basic bat immunology and to conduct controlled and sophisticated viral infection studies. **These studies are invaluable to answering why bats appear to host viruses lethal to humans without any apparent outward disease.** Additionally, given the ability of bats to tolerate viral infections, studies elucidating the mechanisms behind these unusual traits could lead to new clinical approaches and therapeutics for humans affected by bat-borne viral diseases. Finally, the order chiroptera is diverse, comprising of over 1,300 species. A comprehensive understanding of ba immunology and physiology, as related to viral infection, will require bat animal models from multiple species.

We propose to establish sustainable breeding colonies of intermediate horseshoe bats (*Rhinolophu affinis*) and Indian flying foxes (*Pteropus medius*) in the United States that will facilitate research requiring liv bats and bat-derived reagents, such as cell lines. In this project, we will import and establish breeding colonie of these species, creating two novel bat models that represent natural reservoirs of SARS-related coronaviruse and henipaviruses. This will create unprecedented opportunities to study host susceptibility and tolerance to hig consequence pathogens such as SARS-CoV, SARS-CoV-2, and Nipah virus. Hundreds of SARS-CoV-lik viruses have been detected in rhinolophid bats, ~~thus susceptibility testing will allow us to identify susceptibl species~~but only a few have been isolated and none have been used to challenge rhinolophid bats. While th exact host species of SARS-CoV and SARS-CoV-2 are currently unknown, multiple species of bats within the genus *Rhinolophus* have been associated with SARS-related coronaviruses, making any representative bat o this genus a high-value model for studying coronaviruses. Nipah virus is an emerging zoonotic virus wit pandemic potential that causes near-annual outbreaks of fatal encephalitis in humans. The Indian flying fox i the natural reservoir of Nipah virus in South Asia, and is also host to more than 50 other viruses, includin uncharacterized coronaviruses, paramyxoviruses, and filoviruses. This species is robust and readily colonized

Aim 1. To establish colonies of horseshoe bats and Indian flying foxes for the study of coronavirus and henipavirus infections. Bats will be captured and quarantined in Bangladesh and tested for coronaviruses henipaviruses and filoviruses ~~and lyssaviruses~~. Those that are negative will be housed in free-flight holding pen for adaptation to captivity. Offspring of these bats will be imported to Colorado State University for establishmen of breeding colonies that will be provided to investigators performing infectious disease research.

Aim 2. To develop cell lines, reagents and methodologies for the study of viral infections in horsesho bats and Indian flying foxes. Primary bat cells will be isolated from bats for viral susceptibility testing, and stocks of primary cells will be generated for the research community. Susceptible cells will be immortalized t provide indefinite propagation. Organoids of virus targets, such as intestines, will also be generated for viru studies. Recombinant proteins, including cytokines and soluble cell surface antigens, will be generated for i vitro and in vivo use. Monoclonal antibodies to these proteins will also be generated so that investigators ca perform in vitro testing and generation of primary cells that require such growth factors, such as antigen-specifi T cells, and for the in vivo neutralization of cytokines upon challenge with viruses. Methodologies fo transcriptomic, metabolomic and proteomic studies will also be developed.

Aim 3. To perform experimental infections of bats with SARS-CoV-2, bat RaTG13-CoV and Nipah virus to develop laboratory models of bat reservoir hosts. Bats will be challenged with viruses under controlle laboratory conditions to perform kinetic and temporal studies of how these viruses behave in bats, and how the bats control infections without developing disease. We will also perform studies to assess durability of immunity in bats to determine whether immunity wanes and leads to repeated susceptibility to infection.

Commented [MK1]: Added this because we want to do proteomics and metabolomics. Also because my current understanding leads me to believe that bat physiology and flight are connected to bat immunology.

Commented [MK2]: I don't quite understand what this means.

Commented [S3R2]: Hopefully, this is clearer.

Commented [MK4]: Should we expand upon this a bit more – adding something about what goes in to developing these colonies and how we are prepared to do it.

Commented [MK5]: T-cells specifically mentioned here - I would add BM derived monocytes – those also require specific stimulatory cytokines and there is precedent for their successful isolation/development (Zhou et al., 2016). Facts I know you are aware of, I just thought they could be alluded to here.

Commented [S6R5]: We typically ship fresh or frozen bone marrow to others who then make make macrophages and dendritic cells. I'll go into detail in the grant.

Commented [MK7]: To make this we require sequence information, correct? Thes can be synthesized?

Commented [S8R7]: The IFF genome is done. Getting cytokine sequences is very easy – we did it for Artibeus by culturing splenocytes overnight with concanavalin A and pokeweed mitogen, then did RNA-Seq. This led to dozens of full-length cytokine sequences (Kingfisher is making some of these for us now).

Commented [MK9]: Maybe something about single-cell sequencing (– I know everyone is doing it). We will have a bevy of different cell types and can pin point what is and isn't activated upon infection of susceptible cells. This could be critical for understanding viral maintenance.

Sort of an aside, but I think the possibility of trained immunity is quite interesting in relation to bats. (If this is a naïve/bad idea – feel free to ignore! I am an immunology novice.) IF there is any epigenetic or chromatin-remodeling that influences the innate immune response, single cell seq technologies that combine looking at chromatin changes or methylation, particularly for myeloid progenitors, could be revealing.

Commented [S10R9]: Lin-fa is designing those studies so if something needs to go in this Aim page we need to wait to see what he writes.

Research Strategy (must address sustainability of projects and resources)

A. Significance

Bats are found on every continent other than Antarctica and they are reservoirs or suspected reservoirs of many viruses, including coronaviruses, paramyxoviruses, filoviruses and lyssaviruses. In recent years, several bat-borne viruses have emerged that have caused substantial morbidity and mortality among humans. The COVID-19 pandemic is caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), a virus that likely originated in horseshoe bats (*Rhinolophus* species) commonly found in Asia. Frequent outbreaks of Nipah virus, hosted by *Pteropus* species bats, also occur in southern Asia, causing encephalitis that is usually fatal. In each of these virus/reservoir host relationships, virtually no disease occurs in the bats. Although numerous studies have been conducted on bats and viruses, many critical questions, such as the role of bats in maintaining and spreading potential zoonotic viruses and how bat immune responses control infections without disease, remain poorly defined. There is an urgent need to study these emerging zoonotic pathogens in their natural hosts to better understand their threats to humans. Unfortunately, there currently are few natural reservoir host models for the study of bat-borne viruses that are highly consequential to humans. This project aims to address this deficiency.

A.1. *Bats as reservoir hosts of zoonotic viruses.*

Generally, in zoonotic virus/reservoir host relationships, which have been best-studied in rodents and primates, each virus is hosted by one or a few related species {Kitchen, 2011 #44}. Viruses, like all other biological entities, are subject to evolutionary pressures and are likely genetically and biochemically "optimized" to circulate within their reservoir host populations. The process of "optimization" or co-evolution often results in persistent infection (for the life of the host) or replication and shedding of virus that allows for continual transmission to other susceptible hosts {Mandl, 2015 #43}. In both cases, the virus does not cause substantial disease within the reservoir host population. This is unsurprising, as a robust immune response in the reservoir could lead to viral clearance or immunopathology. When spillover of pathogenic viruses occurs, such viruses are not biochemically optimized for human hosts, which can have unexpected outcomes. In some cases, viral infection leads to disease and death, or alternatively, immune clearance. Because of the occurrence of severe human diseases caused by some of the bat-borne viruses, an important question is: *how do bats host these viruses without becoming diseased?* The answer to this question is likely complicated and will vary between species of bats and species of viruses. Each of these viruses encodes immune modulating proteins that often target the innate antiviral responses of infected cells of the host. It is thought that these proteins are contributory factors of human disease ("virulence factors"). However, because they evolved in their bat reservoirs (i.e., "optimized"), their impacts on the orthologous target proteins of humans must somehow be different, otherwise there would not be differential outcomes in bats (no disease) and humans (disease).

Although bats share many immunological features with other mammals, little research into their immune systems and responses during infection with these viruses has been conducted. This is necessary to clarify wh the response to viral infection differs between humans and bats.

A.2. *Coronaviruses*. Historically, seasonal coronavirus infections of humans have been associated with mil colds that are typically resolved within several days. Most humans have been infected with seasona coronaviruses, yet the frequency of re-infections with these coronaviruses has created a perception tha immunity is short-lived~~Unlike most other viral infections, coronaviruses fail to induce durable immunity, thus repeated infections of common cold coronaviruses can occur~~. However, since 2002, three novel coronaviruses have emerged from bats that have caused significant impacts on humans: SARS-CoV, MERS-CoV and SARS-CoV-2. SARS-CoV emerged in 2002 and caused a significant disease with high mortality in humans (11% case fatality rate - WHO); however, because most patients exhibited signs of disease prior to virus shedding, the pandemic~~it~~ was controlled quickly and ended within two years. In 2012, MERS-CoV emerged in ~~the Middle East~~Saudi Arabia and causes a disease in humans similar to SARS-CoV but with a higher mortality rate (35% case fatality rate - WHO). Unlike SARS-CoV, MERS-CoV established in a secondary reservoir host, dromedary camels, in North Africa prior to its spillover into humans. Consequently, MERS outbreaks occur many times each

Commented [MK11]: I like the gist of this section, but the first part could really apply to any reservoir host. The idea of unexpected outcomes is one I like, and I want to keep that, but maybe we can add more specifics about the unique immunological features of bats?

Commented [MK12]: This goes from immunity straight to co-evolution, making it a bit abrupt. It doesn't quite connect the dots until the end. So, I rearranged it. You can obviously ignore what I did, and put it back, but I will stand by my comment about needing a better transition from immunity to co-evolution.

Commented [MK13]: I know this is a "lame' sentence.

Commented [EL14]: I tried to rework this, but in general over the last year I've found previous HCoV literature lacking. There's no evidence that seasonal fluctuations in HCoV-specific Ab result in loss of immunity; most of the HCoV surveillance papers did not really show that waning Ab to one HCoV resulted in symptomatic "re-infection" with a homologous or heterologous HCoV.

year and nearly all outbreaks can be traced to contact with dromedary camels. SARS-CoV-2 emerged in late 2019 and is the etiologic agent of the ongoing COVID-19 pandemic. Both SARS-CoV and SARS-CoV-2 utilize humanse ACE2 as cellular entry receptors, and SARS-CoV-2 has been shown to infect many species demonstrating broad host tropism. It is now evident that thousands of distinct coronaviruses circulate in bats throughout the world, and that hundreds of these are SARS-related CoV (SARSr-CoV) that circulate in rhinolophid bats. There are 78 known species of bats within the genus *Rhinolophus* and they are found throughout Asia and Europe. Recombination frequently occurs among coronaviruses, and this has likely contributed to the diverse pool of coronaviruses circulating in rhinolophid bats. Although, oOnly a few bat coronaviruses have been isolated; nearly all are represented by sequences detected in bats. One bat coronavirus, RaTG13, shares a high degree of sequence similarity to SARS-CoV-2 (32015507). Its sequences have been detected in several intermediate horseshoe bats (*R. affinis*) that are found throughout southern Asia, including Bangladesh. Although no isolates of RaTG13 have been made?RaTG13 has not been isolated, its complete genome has been determined (GenBank MN996532). Examination of ACE2 sequences from various rhinolophid species suggests intermediate horseshoe bats have the most "human-like" ACE2 orthologue for spike binding to the 20 ACE2 residues that are thought to be important for receptor-binding interactions (32239522, 31996437). Because of these features, we will focus our efforts on colonizing intermediate horseshoe bats.

A.3. Nipah virus (Jon).

A.4.

B. Innovation

B1. This project will be the first to establish bat breeding colonies for the infectious disease research community with species relevant to SARS coronaviruses and henipaviruses.

B2. We will determine basic clinical attributes of these bats to provide baseline readings, including body temperatures, hematology, serum chemistry and metabolic profiles.

B3. We will establish bat primary cells and immortalized cell lines for distribution to the research community.

B4. We will develop recombinant bat cytokines for the derivation of primary cell cultures, including endothelial cells, dendritic cells and T cells, and to use these cytokines for in vivo studies to manipulate immune responses during infection.

B5. We will generate monoclonal antibodies for the detection of cytokines, cell surface antigens and for in vivo cytokine neutralization and cellular depletion studies

B6. We will

C. Approach

C1. Preliminary Studies

C1.1. *Fruit bat colony at Colorado State University*. Dr. Schountz established a breeding colony of Jamaican fruit bats (*Artibeus jamaicensis*) in 2006 with previous NIH support (AI25489, AI089419) (**Fig 1**). These bats are found in northern South America, the Caribbean Islands, Central America, and the Florida Keys. Adult bats have an average weight of 42 gm, a wingspan of 45 cm and body length of about 10 cm. Mature females produce two offspring per year and carry pups for about 40 days after birth. This species can live up to 15 years captivity. There are about 400 bats currently in the colony and about 100 to 150 new pups are born at 6-month intervals. Colorado State University has extensive animal expertise by virtue of its College of Veterinary Medicine and Biomedical Sciences, including veterinarians with experience with bats. **Thus, we have extensive investigator and institutional experience with bat husbandry and colony management.**

Fig. 1. Jamaican fruit bats in the CSU colony.

C.1.2. *Candidate SARS-like coronavirus reservoirs*. Receptor incompatibility can pose a potent barrier to infection, preventing a virus from gaining access to the cell; a necessary step to initiate the replication cycle. There is ample evidence that one or a few amino acid substitutions at critical sites of interaction in cellular receptors can affect viral entry {Demogines, 2013 #45;Kaelber, 2012 #47;Ng, 2015 #42;Yan, 2010 #46}. Angiotensin-2 or ACE2 is the reported receptor for SARS-CoV and SARS-CoV-2. Therefore, to initially assess the potential of rhinolophid bats as a reservoir for SARS-like coronaviruses we performed an alignment of all nine *Rhinolophus* species ACE2 sequences available in GenBank with Human ACE2 and ACE2 of known susceptible mammalian species. We specifically compared amino acid identities at 20 sites involved in spike binding (Table 1). Where available, sequences from multiple individuals were included.

Strikingly, for those species where multiple ACE2 haplotypes are reported (*R.sinicus, R.pearsonii,* and *R.affinis*) multiple different amino acid identities are encoded at sites important for ACE2 binding (Table 1). In cases where there are multiple amino acids possible (e.g. Table 1, site 34), occasionally one amino acid matches the residue(s) encoded by humans. This apparent genetic variation reveals two important implications: 1) certain bat individuals may encode ACE2 receptors that are permissive to coronaviruses infecting humans while others of the same species do not, and 2) certain bat individuals may be more likely to harbor a SARSr-CoV strain able to infect humans. We can capitalize on this naturally occurring variation through widespread capture, genotyping at loci of importance for coronavirus infection, and selective breeding to develop a colony of bats ideally poised for controlled viral infection and immunology studies.

Based on current ACE2 sequence data, the intermediate horseshoe bat and Pearson's horseshoe bat (*R. pearsonii*) encode the same amino acids as human at 16 of the 20 residues important for ACE2 binding. The Least horseshoe bat (*R. pusillus*) has 14 matching residues. These three species are found in Bangladesh (Table 1). While no bat species encodes the same 20 residues as human, both domestic cats (16 matches) and ferrets (14 matches) are susceptible to SARS-CoV-2, suggesting that all three Bangladeshi species may harbor permissive ACE2 receptors. Intermediate horseshoe bats (16 matches) are widely distributed in southern Asia, including Bangladesh as well as southern China where most of the SARSr-CoV sequences have been detected in rhinolophid bats. For this reason, we believe this is the most logical species to capture for susceptibility testing and model development. However, because there are 78 rhinolophid species, we will also capture the other species that are found in Bangladesh (*R. lepidus*, *R. malayanus*, *R. monoceros*, *R. humanus*, *R. luctus*, *R. subbadius*) for importation and SARS-CoV-2 susceptibility testing at CSU.

Table 1. ACE2 amino acid identity amongst Rhinolophid bats and hosts supporting SARS-CoV-2

Commented [MK15]: Is this more about finding a bat model that will support SARS-CoV and SARS-CoV-2 infections?

Commented [MK16]: There is a paper that looks at polymorphisms within R.sinicus ACE2 and their effect on susceptibility to SARS-rCoV strains (Guo, 2020, JVI). The data is ok, and I believe it, but they do a wildly inappropriate evolution analysis (it's meant for between species and they do it on an a within species set, which violates all the assumptions). The virology data is ok, their 0 timepoint of their qPCR is high (start with a lot of genomes) but they increase nicely over time, so it's alright. I sort of don't want to cite it because of the aforementioned issues, but it supports the idea that variation at the receptor can change susceptibility to a SARS-rCoV. Does anyone know of any other papers that do something similar with regards to bat ACE2?

Commented [EL17R16]: •Also this article in PLoS Pathogen from 2017. PLoS Path 2017 Nov 30;13(11):e1006698.
•Michael and Vincent's Cell Report DPP4 paper is a good example of this for MERS-CoV. PMID: 30110630

Commented [MK18]: Maybe a figure showing the species ranges and our proposed site of the Bangladeshi bat colonies?

		Amino Acid in *ACE2*																				
Species name	Common name	24	27	28	30	31	34	35	37	38	41	42	45	82	83	330	353	354	355	357	393	
Homo sapiens	Human (dbSNP)	*Q*	*T/A*	*F*	*D*	*K*	*H*	*E/D/K*	*E*	*D*	*Y*	*Q*	*L*	*M/I*	*Y*	*N*	*K*	*G*	*D/N*	*R*	*R*	
Mesocricetus auratus	Syrian Hamster (N=1)	Q	T	F	D	K	Q	E	E	D	Y	Q	L	N	Y	N	K	G	D	R	R	
Rhinolophus sinicus	Chinese rufous horseshoe bat (N=25)	R/E/L	T/M/I	F	D	K/E/T	S/T/F	E/K	E	D/N	Y/H	Q/K	L	N	Y	N	K	G	D	R	R	85%
Felis catus	Domestic cat (N=1)	L	T	F	E	K	H	E	E	E	Y	Q	L	T	Y	N	K	G	D	R	R	80%
Rhinolophus affinis	Intermediate horseshoe bat (N=23)	R	I	F	D	N	H/R	E	E	E/D	Y	Q	L	N	Y/H	N	K	G	D	R	R	80%
Rhinolophus pearsonii	Pearson's horseshoe bat (N=2)	R/Q	T/I	F	D	K	H/R	E	E	D	H	E	L	D	Y	N	K	D	D	R	R	80%
Mustela putoris	Ferret (N=1)	L	T	F	E	K	Y	E	E	E	Y	Q	L	T	Y	N	K	R	D	R	R	70%
Rhinolophus pusillus	Least horseshoe bat (N=1)	K	K	F	N	D	S	E	E	D	Y	Q	L	N	Y	N	K	G	D	R	R	70%
Rhinolophus macrotis	Big-eared horseshoe bat (N=1)	E	K	F	D	K	S	K	E	D	Y	E	L	N	Y	K	K	G	D	R	R	70%
Rhinolophus landeri	Lander's horseshoe bat (N=1)	L	T	F	D	D	S	A	E	N	Y	Q	L	N	F	N	K	G	D	R	R	65%

Commented [MK19]: Since viruses move through populations, I thought collecting all available complete ACE2 sequences on ncbi (skipped uniprot, but I can add, if necessary) for each of the species listed would be more representative. Also, I edited the text to reflect these changes.

C1.3. *Experimental bat coronavirus infections*. Drs. Munster and Schountz have extensive experience with experimental infection models of bats, including with MERS-CoV, SARS-CoV-2, H18N11 bat influenza virus, Zika virus and Tacaribe virus. Challenge of Egyptian fruit bats with an infectious clone of WIV-CoV showed limited susceptibility, despite the ability of its spike protein to bind to Egyptian fruit bat ACE2 (30572566). Recent work by our groups has shown that Jamaican fruit bat ACE2 is bound by SARS-CoV-2 (but not SARS-CoV) spike protein expressed by a VSV pseudotype virus and that challenge of Jamaican fruit bats with SARS-CoV-2 leads to a limited infection that is confined to the small intestine, particularly mononuclear cells of the Peyer's patches and lamina propria, and crypt cells (Fig 2). Viral RNA was detected in oral swabs for 4 days and low levels in the small intestine on day 2. However, no neutralizing antibody or nucleoprotein antibody (ELISA) was detected, suggesting abortive infection or robust control of the virus by the innate immune response.

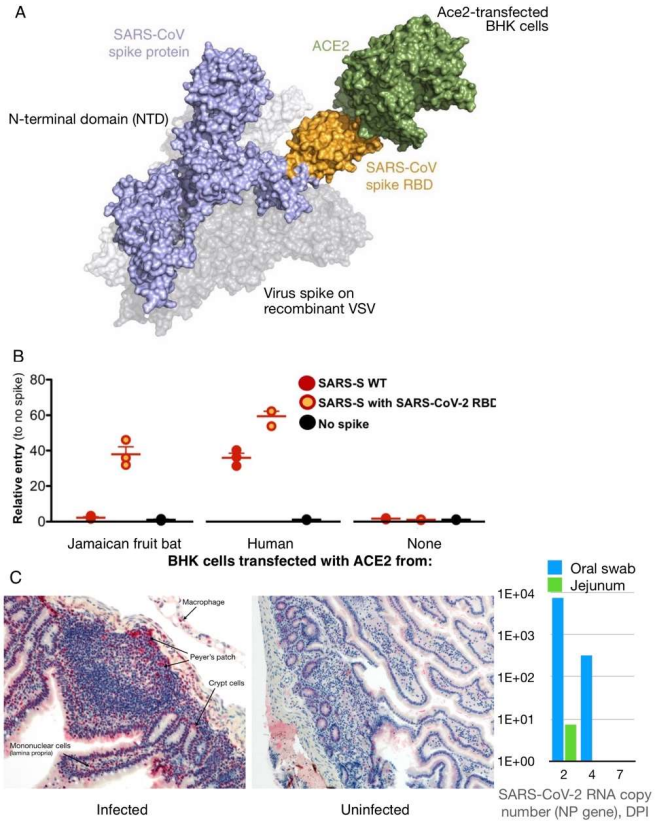
C1.4.

C2. Specific Aims

C2.1 Aim 1. To establish colonies of horseshoe bats and Indian flying foxes for the study of coronavirus and henipavirus infections. Bats will be captured and quarantined in Bangladesh and tested for coronaviruses, henipaviruses, filoviruses and lyssaviruses. Those that are negative will be imported to Colorado State University for establishment of breeding colonies.

C2.1.1. *Establishment of captive breeding colony of Pteropus medius in Bangladesh*. We will establish a captive breeding colony of Pteropus medius in Bangladesh. Temporary holding cages used for quarantine prior to shipment to the US will be erected at the Sheikh Kamal Wildlife Center (SKWC) Purabari, adjacent to Bhawal National Park, Gazipur, north of Dhaka. The land is managed by the Forest Department under the direction of

Commented [MK20]: I assume the holding cages will be different for the rhinolophid bats, but the capture methods will be the same?



the Conservator of Forests (see Kabir letter of support) Ministry of Environment (Govt of Bangladesh). The bats will be cared for and maintained by Forest Department staff. Staging bats in Bangladesh prior to shipment to the US will occur in X phases.

Phase 1: Holding cage development. We will build four temporary holding cages [octagonal shape, approx. 6m diameter and 6ft tall] that are large enough to allow bats to fly, and low enough that project personnel can easily repeatedly catch bats for testing during the screening process. Each cage will be built to hold 30 bats, with a heavy retractable curtain subdividing each cage in half, which will allow us to separate bats into smaller groups. One of the cages will be left empty to allow for isolation of bats that test positive for Nipah virus on PCR during the course of quarantine. Each cage will be made from a metal frame with rubber-coated mesh screening enclosing the cage. An outer cage will be built around each cage, with 6" of space between the fencing to exclude bats inside from contact with bats outside. A solid roof will be built over each cage to prevent excreta from wild bats from entering the cage area and potentially exposing captive bats to henipaviruses. Each cage will also have a door and an anti-chamber to allow staff to safely enter and exit, don and doff PPE, and then enter the flight cage without bats being able to escape.

Facilities at the wildlife park where the bats will be held will include a food preparation station, PPE storage, first aid and disinfection supplies, and a mobile veterinary lab that includes electricity, a portable gas anesthesia machine, and supplies for collecting biological specimens from the bats.

Phase 2: Bat capture. We will capture wild bats from colonies located in Gazipur. Our goal is to capture a total of 81 bats: 6 males and 75 females comprising three groups of 25 females and 2 males - all Nipah virus negative (by PCR and Luminex). We will capture bats 20 at a time. With each group of 20 bats, we will transport them to the cages from the field site, with each bat held in an individual cotton bag suspended from a post in the back of a vehicle. The driver will be wearing PPE, including an N95 respirator, and all vehicle windows will be open during transport. A plastic sheet will line the floor of the vehicle under the bats to allow for disinfection following transport. Bats will be sampled in the vet lab and then immediately transferred into a holding cage and blood samples and swabs collected. Samples will be screened that day for IgG antibodies using a Luminex, and swabs will be screened for Nipah virus RNA using a qRT-PCR assay at icddr,b. Seropositive and or PCR positive individuals will be released at the park. Seronegative and PCR negative individuals will be moved into a second staging cage, where they will be held for 21 days, with a second and third round of testing on day 10 and 21 post capture. Any bats subsequently testing positive will be released. Negative bats will be retained.

Phase 3: Breeding colony. We will maintain separate groups of 27 bats in the three flight cages. Pteropus medius is a synchronous seasonal breeder, with a 6-month gestation period. We will aim to capture bats during the third

trimester of pregnancy (January/February) and allow them to give birth in captivity. Pups will be weaned at 3 months and placed into a fourth cage, where they will be maintained until 1 year of age. Pups will be screened every 2 weeks for Nipah virus and antibodies. Maternal antibodies are expected to wane at around 6-7 months of age. By isolating the pups, born to seronegative, Nipah-negative dams, we will create an SPF (specific-pathogen free) colony that will be used to found the colony at CSU. We will also allow the bats in Bangladesh to breed, creating an F1 generation that will be maintained in captivity and used to support the US-based colony.

C2.1.2. *Experimental challenge of rhinolophid bats.* We will determine whether Ace2 sequences from various *Rhinolophus* species bats render BHK cells susceptible to SARS-CoV and SARS-CoV-2 using our VSV pseudotype system (Fig XX). Rhinolophid species will be captured and quarantined in Bangladesh and tested for evidence of SARS-like CoV infection (PCR of rectal swabs and serology). Naïve bats will be shipped to Colorado State University for challenge with SARS-CoV and SARS-CoV-2 to determine susceptibility. Those species that are susceptible will be collected in Bangladesh and after quarantine and testing, up to 50 founders will be shipped to CSU to establish breeding colonies. In the event that more than one species is susceptible, we will determine which is most suitable based upon infection kinetics of each virus.

Upon receipt, bats will be immediately examined by veterinary staff at CSU and manually fed meal worms.

C2.2 Aim 2. To develop cell lines, reagents and methodologies for the study of viral infections in horseshoe bats and Indian flying foxes. Primary bat cells will be isolated from bats for susceptibility testing, and stocks of primary cells will be generated for the research community. Susceptible cells will be immortalized to provide indefinite propagation. Organoids of virus targets, such as intestines, will also be generated for virus studies. Recombinant proteins, including cytokines and soluble cell surface antigens, will be generated for in vitro and in vivo use. Monoclonal antibodies to these proteins will also be generated so that investigators can perform in vitro testing and generation of primary cells that require such growth factors, such as antigen-specific T cells, and for the in vivo neutralization of cytokines upon challenge with viruses. Methodologies for transcriptomic, metabolomic and proteomic studies will also be developed.

C2.2.1. Establishment of primary and immortalized bat cell lines.(Tony)
C2.2.2. Production of recombinant bat cytokines and monoclonal antibodies. (Brian cytokines, Tony Mab)
C2.2.3. Development of metabolomic profiles. (Lin-fa and Rushika)
C2.2.4. Development of proteomic profiles. (Lin-fa and Rushika)

C2.3 Aim 3. To perform experimental infections of bats with SARS-CoV-2, bat RaTG13-CoV and Nipah virus to develop laboratory models of bat reservoir hosts. Bats will be challenged with viruses under controlled laboratory conditions to perform kinetic and temporal studies of how these viruses behave in bats, and how the bats control infections without developing disease. We will also perform studies to assess durability of immunity in bats to determine whether immunity wanes and leads to repeated susceptibility to infection.

C2.3.1. Experimental infection of horseshoe bats with SARS-CoV-2. (Tony, Lin-fa, Vincent)
C2.3.2. Experimental infections of Indian flying foxes with Nipah virus. (Tony, Lin-fa, Vincent)

C2.3.3. Generation of bat RaTG13-CoV infectious clone. ~~(Eric)~~ Reverse genetics have been used as a powerful tool to generate infectious clones of SARS-CoV and MERS-CoV (32526206, 28377531, 24043791, 32978313). To rescue recombinant RaTG13 we will follow methods previously described to generate SARS/MERS-CoV full length genome cDNA (24043791, 14569023, 12368349, 18818320, 19036930). Six contiguous RaTG12 cDNA fragments will be synthesized and the contiguous cDNA fragments will then be ligated into a full-length cDNA using a subcloning strategy with BglI class II restriction endonuclease sites. The ligated cDNA product under T7 promoter control will then be electroporated into a co-culture of mammalian cell lines (Vero, BSR-T7) along with T7 RNA polymerase and a nucleoprotein expressing vector to enhance replication efficiency. To construct green fluorescent protein (GFP) and red fluorescent protein (RFP) reporter viruses, the ORF5 sequence will be replaced with GFP or RFP sequences. The same simultaneous ligation strategy will be employed to generate

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the full-length cDNA of RaTG13 GFP/RFP variants. Cytopathic effects and plaques will be monitored for 24 – 72 hours post-transfections; duplicate wells will be a) stained with anti-SARS-CoV nucleoprotein secondary antibodies for intracellular detection of RaTG13 infectious clone replication and b) lysed and cells/cell-culture supernatant will be passaged to fresh Vero cells for amplification of the virus stock. Replication kinetics and titers of infectious clone RaTG13 and reporter virus variants will be monitored by standard plaque assays and/or measurement of fluorescent plaques. Mammalian cell line will be transfected with human and bat ACE-2 orthologues to allow functional characterization of infectious clones of RaTG13 receptor-usage for cellular entry.

C2.3.4. Detection of virus specific antibodies in experimentally-challenged flying foxes and horseshoe bats. Whether bats develop long-lasting adaptive immune responses to viruses remains unknown. Egyptian fruit bats, natural hosts of Marburg virus and Ravn virus (19649327), challenged with Marbug virus seroreverted to undetectable levels three months after infection (28821722). Virus-specific antibody responses are surrogates of predictive protection or correlates of protection for nearly all vaccines. Here, we will establish primary and secondary humoral responses to virus infections in two virus-host models: NiV-flying foxes, RaTG13-horseshoe bats. Utilizing multiplex serology strategies that our groups have developed for NiV and coronaviruses (32034942, 31671094, 33083807) we will capture bat immunoglobulin (Ig) G and IgM with virus envelope/spike glycoprotein antigen-based assays. The kinetics, magnitude and duration of virus-specific flying fox and horseshoe IgG/IgM will be monitored longitudinally for seroconversion, seroreversion and anamnestic boosting after secondary infection. Population-level biosurveillance of NiV and other emerging zoonotic viruses relies on susceptible-infected-recovered models to understand the spread of these viruses among wildlife hosts. Experimental establishment of longevity of virus-specific bat IgG-responses will provide valuable information for ongoing biosurveillance projects focused on the transmission/spillover dynamics of emerging zoonotic viruses in wildlife host populations and communities.

Commented [EL21]: Jon do you have a reference for the most recent NiV seasonal transmission study (PNAS)?

Commented [EL22]: Just threw these in here

Vertebrate Animals

Description of Procedures:

Justifications:

Minimization of Pain and Distress:

Select Agent Research

Multiple PD/PI Leadership Plan

Consortium/Contractual Arrangements

Letters of Support

Resource Sharing Plans

Data Sharing Plan:

Sharing Model Organisms:

Genomic Data Sharing (GDS):

Authentication of Key Biological Resources

Appendix (maximum of 10 PDF documents)

Literature Cited

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Specific Aims

An important and frequently asked scientific question is whether bats are better hosts for lethal viruses than other mammals. There are a number of zoonotic viruses that cause high mortality in humans and originate in bats: SARS coronaviruses (SARS-CoV and SARS-CoV-2), Ebola virus, Marburg virus, Nipah virus, Hendra virus, and Middle East respiratory syndrome coronavirus (MERS-CoV). Further, there is increasing evidence that many other human viruses may have originated in bats, including measles, mumps and hepatitis C viruses. However, there is a lack the bat animal models necessary to study basic bat immunology and to conduct controlled and sophisticated viral infection studies. **These studies are invaluable to answering why bats appear to host viruses lethal to humans without any apparent outward disease.** Additionally, given the ability of bats to tolerate viral infections, studies elucidating the mechanisms behind these unusual traits could lead to new clinical approaches and therapeutics for humans affected by bat-borne viral diseases. Finally, the order chiroptera is diverse, comprising of over 1,300 species. A comprehensive understanding of ba immunology and physiology, as related to viral infection, will require bat animal models from multiple species.

We propose to establish sustainable breeding colonies of intermediate horseshoe bats (*Rhinolophu affinis*) and Indian flying foxes (*Pteropus medius*) in the United States that will facilitate research requiring liv bats and bat-derived reagents, such as cell lines. In this project, we will import and establish breeding colonie of these species, creating two novel bat models that represent natural reservoirs of SARS-related coronaviruse and henipaviruses. This will create unprecedented opportunities to study host susceptibility and tolerance to hig consequence pathogens such as SARS-CoV, SARS-CoV-2, and Nipah virus. Hundreds of SARS-CoV-lik viruses have been detected in rhinolophid bats, ~~thus susceptibility testing will allow us to identify susceptibl species~~but only a few have been isolated and none have been used to challenge rhinolophid bats. While th exact host species of SARS-CoV and SARS-CoV-2 are currently unknown, multiple species of bats within the genus *Rhinolophus* have been associated with SARS-related coronaviruses, making any representative bat o this genus a high-value model for studying coronaviruses. Nipah virus is an emerging zoonotic virus wit pandemic potential that causes near-annual outbreaks of fatal encephalitis in humans. The Indian flying fox i the natural reservoir of Nipah virus in South Asia, and is also host to more than 50 other viruses, includin uncharacterized coronaviruses, paramyxoviruses, and filoviruses. This species is robust and readily colonized

Aim 1. To establish colonies of horseshoe bats and Indian flying foxes for the study of coronavirus and henipavirus infections. Bats will be captured and quarantined in Bangladesh and tested for coronaviruses henipaviruses and filoviruses ~~and lyssaviruses~~. Those that are negative will be housed in free-flight holding pen for adaptation to captivity. Offspring of these bats will be imported to Colorado State University for establishmen of breeding colonies that will be provided to investigators performing infectious disease research.

Aim 2. To develop cell lines, reagents and methodologies for the study of viral infections in horsesho bats and Indian flying foxes. Primary bat cells will be isolated from bats for viral susceptibility testing, and stocks of primary cells will be generated for the research community. Susceptible cells will be immortalized t provide indefinite propagation. Organoids of virus targets, such as intestines, will also be generated for viru studies. Recombinant proteins, including cytokines and soluble cell surface antigens, will be generated for i vitro and in vivo use. Monoclonal antibodies to these proteins will also be generated so that investigators ca perform in vitro testing and generation of primary cells that require such growth factors, such as antigen-specifi T cells, and for the in vivo neutralization of cytokines upon challenge with viruses. Methodologies fo transcriptomic, metabolomic and proteomic studies will also be developed.

Aim 3. To perform experimental infections of bats with SARS-CoV-2, bat RaTG13-CoV and Nipah virus to develop laboratory models of bat reservoir hosts. Bats will be challenged with viruses under controlle laboratory conditions to perform kinetic and temporal studies of how these viruses behave in bats, and how the bats control infections without developing disease. We will also perform studies to assess durability of immunity in bats to determine whether immunity wanes and leads to repeated susceptibility to infection.

Commented [MK1]: Added this because we want to do proteomics and metabolomics. Also because my current understanding leads me to believe that bat physiology and flight are connected to bat immunology.

Commented [MK2]: I don't quite understand what this means.

Commented [S3R2]: Hopefully, this is clearer.

Commented [MK4]: Should we expand upon this a bit more – adding something about what goes in to developing these colonies and how we are prepared to do it.

Commented [MK5]: T-cells specifically mentioned here - I would add BM derived monocytes – those also require specific stimulatory cytokines and there is precedent for their successful isolation/development (Zhou et al., 2016). Facts I know you are aware of, I just thought they could be alluded to here.

Commented [S6R5]: We typically ship fresh or frozen bone marrow to others who then make make macrophages and dendritic cells. I'll go into detail in the grant.

Commented [MK7]: To make this we require sequence information, correct? Thes can be synthesized?

Commented [S8R7]: The IFF genome is done. Getting cytokine sequences is very easy – we did it for Artibeus by culturing splenocytes overnight with concanavalin A and pokeweed mitogen, then did RNA-Seq. This led to dozens of full-length cytokine sequences (Kingfisher is making some of these for us now).

Commented [MK9]: Maybe something about single-cell sequencing (– I know everyone is doing it). We will have a bevy of different cell types and can pin point what is and isn't activated upon infection of susceptible cells. This could be critical for understanding viral maintenance.

Sort of an aside, but I think the possibility of trained immunity is quite interesting in relation to bats. (If this is a naïve/bad idea – feel free to ignore! I am an immunology novice.) IF there is any epigenetic or chromatin-remodeling that influences the innate immune response, single cell seq technologies that combine looking at chromatin changes or methylation, particularly for myeloid progenitors, could be revealing.

Commented [S10R9]: Lin-fa is designing those studies so if something needs to go in this Aim page we need to wait to see what he writes.

Research Strategy (must address sustainability of projects and resources)

A. Significance

Bats are found on every continent other than Antarctica and they are reservoirs or suspected reservoirs of many viruses, including coronaviruses, paramyxoviruses, filoviruses and lyssaviruses. In recent years, several bat-borne viruses have emerged that have caused substantial morbidity and mortality among humans. The COVID-19 pandemic is caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), a virus that likely originated in horseshoe bats (*Rhinolophus* species) commonly found in Asia. Frequent outbreaks of Nipah virus, hosted by *Pteropus* species bats, also occur in southern Asia, causing encephalitis that is usually fatal. In each of these virus/reservoir host relationships, virtually no disease occurs in the bats. Although numerous studies have been conducted on bats and viruses, many critical questions, such as the role of bats in maintaining and spreading potential zoonotic viruses and how bat immune responses control infections without disease, remain poorly defined. There is an urgent need to study these emerging zoonotic pathogens in their natural hosts to better understand their threats to humans. Unfortunately, there currently are few natural reservoir host models for the study of bat-borne viruses that are highly consequential to humans. This project aims to address this deficiency.

A.1. *Bats as reservoir hosts of zoonotic viruses.*

Generally, in zoonotic virus/reservoir host relationships, which have been best-studied in rodents and primates, each virus is hosted by one or a few related species {Kitchen, 2011 #44}. Viruses, like all other biological entities, are subject to evolutionary pressures and are likely genetically and biochemically "optimized" to circulate within their reservoir host populations. The process of "optimization" or co-evolution often results in persistent infection (for the life of the host) or replication and shedding of virus that allows for continual transmission to other susceptible hosts {Mandl, 2015 #43}. In both cases, the virus does not cause substantial disease within the reservoir host population. This is unsurprising, as a robust immune response in the reservoir could lead to viral clearance or immunopathology. When spillover of pathogenic viruses occurs, such viruses are not biochemically optimized for human hosts, which can have unexpected outcomes. In some cases, viral infection leads to disease and death, or alternatively, immune clearance. Because of the occurrence of severe human diseases caused by some of the bat-borne viruses, an important question is: *how do bats host these viruses without becoming diseased?* The answer to this question is likely complicated and will vary between species of bats and species of viruses. Each of these viruses encodes immune modulating proteins that often target the innate antiviral responses of infected cells of the host. It is thought that these proteins are contributory factors of human disease ("virulence factors"). However, because they evolved in their bat reservoirs (i.e., "optimized"), their impacts on the orthologous target proteins of humans must somehow be different, otherwise there would not be differential outcomes in bats (no disease) and humans (disease).

Although bats share many immunological features with other mammals, little research into their immune systems and responses during infection with these viruses has been conducted. This is necessary to clarify wh the response to viral infection differs between humans and bats.

A.2. *Coronaviruses*. Historically, coronavirus infections of humans have been associated with mild colds that ar typically resolved within several days. Unlike most other viral infections, coronaviruses fail to induce durabl immunity, thus repeated infections of common cold coronaviruses can occur. However, since 2002, three nove coronaviruses have emerged from bats that have caused significant impacts on humans: SARS-CoV, MERS-CoV and SARS-CoV-2. SARS-CoV emerged in 2002 and caused a significant disease with high mortality in humans (11% case fatality rate - WHO); however, because most patients exhibited signs of disease prior to virus shedding, it was controlled quickly and ended within two years. In 2012, MERS-CoV emerged in the Middle East and causes a disease in humans similar to SARS-CoV but with a higher mortality rate (35% case fatality rate - WHO). Unlike SARS-CoV, MERS-CoV established in a secondary reservoir host, dromedary camels, in North Africa prior to its spillover into humans. Consequently, MERS outbreaks occur many times each year and nearly all outbreaks can be traced to contact with dromedary camels. SARS-CoV-2 emerged in late 2019 and is the etiologic agent of the ongoing COVID-19 pandemic. Both SARS-CoV and SARS-CoV-2 use ACE2 as cellular

Commented [MK11]: I like the gist of this section, but the first part could really apply to any reservoir host. The idea of unexpected outcomes is one I like, and I want to keep that, but maybe we can add more specifics about the unique immunological features of bats?

Commented [MK12]: This goes from immunity straight to co-evolution, making it a bit abrupt. It doesn't quite connect the dots until the end. So, I rearranged it. You can obviously ignore what I did, and put it back, but I will stand by my comment about needing a better transition from immunity to co-evolution.

Commented [MK13]: I know this is a "lame' sentence.

entry receptors, and SARS-CoV-2 has been shown to infect many species. It is now evident that thousands of distinct coronaviruses circulate in bats throughout the world, and that hundreds of these are SARS-related CoV (SARSr-CoV) that circulate in rhinolophid bats. There are 78 known species of bats within the genus *Rhinolophus* and they are found throughout Asia and Europe. Recombination frequently occurs among coronaviruses, and this has likely contributed to the diverse pool of coronaviruses circulating in rhinolophid bats. Only a few bat coronaviruses have been isolated; nearly all are represented by sequences detected in bats. One bat coronavirus, RaTG13, shares a high degree of sequence similarity to SARS-CoV-2 (32015507). Its sequences have been detected in several intermediate horseshoe bats (*R. affinis*) that are found throughout southern Asia, including Bangladesh. Although no isolates of RaTG13 have been made?, its complete genome has been determined (GenBank MN996532). Examination of ACE2 sequences from various rhinolophid species suggests intermediate horseshoe bats have the most "human-like" ACE2 for spike binding to the 20 ACE2 residues that are thought to be important (32239522, 31996437). Because of these features, we will focus our efforts on colonizing intermediate horseshoe bats.

A.3. Nipah virus (Jon).

A.4.

B. Innovation

B1. This project will be the first to establish bat breeding colonies for the infectious disease research community with species relevant to SARS coronaviruses and henipaviruses.

B2. We will determine basic clinical attributes of these bats to provide baseline readings, including body temperatures, hematology, serum chemistry and metabolic profiles.

B3. We will establish bat primary cells and immortalized cell lines for distribution to the research community.

B4. We will develop recombinant bat cytokines for the derivation of primary cell cultures, including endothelial cells, dendritic cells and T cells, and to use these cytokines for in vivo studies to manipulate immune responses during infection.

B5. We will generate monoclonal antibodies for the detection of cytokines, cell surface antigens and for in vivo cytokine neutralization and cellular depletion studies

B6. We will

C. Approach

C1. Preliminary Studies

C1.1. *Fruit bat colony at Colorado State University*. Dr. Schountz established a breeding colony of Jamaican fruit bats (*Artibeus jamaicensis*) in 2006 with previous NIH support (AI25489, AI089419) (**Fig 1**). These bats are found in northern South America, the Caribbean Islands, Central America, and the Florida Keys. Adult bats have an average weight of 42 gm, a wingspan of 45 cm and body length of about 10 cm. Mature females produce two offspring per year and carry pups for about 40 days after birth. This species can live up to 15 years captivity. There are about 400 bats currently in the colony and about 100 to 150 new pups are born at 6-month intervals. Colorado State University has extensive animal expertise by virtue of its College of Veterinary Medicine and Biomedical Sciences, including veterinarians with experience with bats. **Thus, we have extensive investigator and institutional experience with bat husbandry and colony management.**

Fig. 1. Jamaican fruit bats in the CSU colony.

C.1.2. *Candidate SARS-like coronavirus reservoirs*. Receptor incompatibility can pose a potent barrier to infection, preventing a virus from gaining access to the cell; a necessary step to initiate the replication cycle. There is ample evidence that one or a few amino acid substitutions at critical sites of interaction in cellular receptors can affect viral entry {Demogines, 2013 #45;Kaelber, 2012 #47;Ng, 2015 #42;Yan, 2010 #46}. Angiotensin-2 or ACE2 is the reported receptor for SARS-CoV and SARS-CoV-2. Therefore, to initially assess the potential of rhinolophid bats as a reservoir for SARS-like coronaviruses w

Commented [MK14]: Is this more about finding a bat model that will support SARS-CoV and SARS-CoV-2 infections?

Commented [MK15]: There is a paper that looks at polymorphisms within R.sinicus ACE2 and their effect on susceptibility to SARS-rCoV strains (Guo, 2020, JVI). The data is ok, and I believe it, but they do a wildly inappropriate evolution analysis (it's meant for between species and they do it on an a within species set, which violates all the assumptions). The virology data is ok, their 0 timepoint of their qPCR is high (start with a lot of genomes) but they increase nicely over time, so it's alright. I sort of don't want to cite it because of the aforementioned issues, but it supports the idea that variation at the receptor can change susceptibility to a SARS-rCoV. Does anyone know of any other papers that do something similar with regards to bat ACE2?

performed an alignment of all nine *Rhinolophus* species ACE2 sequences available in GenBank with Human ACE2 and ACE2 of known susceptible mammalian species. We specifically compared amino acid identities at 20 sites involved in spike binding (Table 1). Where available, sequences from multiple individuals were included.

Strikingly, for those species where multiple ACE2 haplotypes are reported (*R.sinicus, R.pearsonii,* and *R.affinis*), multiple different amino acid identities are encoded at sites important for ACE2 binding (Table 1). In cases where there are multiple amino acids possible (e.g. Table 1, site 34), occasionally one amino acid matches the residue(s) encoded by humans. This apparent genetic variation reveals two important implications: 1) certain bat individuals may encode ACE2 receptors that are permissive to coronaviruses infecting humans while others of the same species do not, and 2) certain bat individuals may be more likely to harbor a SARSr-CoV strain able to infect humans. We can capitalize on this naturally occurring variation through widespread capture, genotyping at loci of importance for coronavirus infection, and selective breeding to develop a colony of bats ideally poised for controlled viral infection and immunology studies.

Based on current ACE2 sequence data, the intermediate horseshoe bat and Pearson's horseshoe bat (*R. pearsonii*) encode the same amino acids as human at 16 of the 20 residues important for ACE2 binding. The Least horseshoe bat (*R. pusillus*) has 14 matching residues. These three species are found in Bangladesh (Table 1). While no bat species encodes the same 20 residues as human, both domestic cats (16 matches) and ferrets (14 matches) are susceptible to SARS-CoV-2, suggesting that all three Bangladeshi species may harbor permissive ACE2 receptors. Intermediate horseshoe bats (16 matches) are widely distributed in southern Asia, including Bangladesh as well as southern China where most of the SARSr-CoV sequences have been detected in rhinolophid bats. For this reason, we believe this is the most logical species to capture for susceptibility testing and model development. However, because there are 78 rhinolophid species, we will also capture the other species that are found in Bangladesh (*R. lepidus*, *R. malayanus*, *R. monoceros*, *R. humanus*, *R. luctus*, *R. subbadius*) for importation and SARS-CoV-2 susceptibility testing at CSU.

Commented [MK16]: Maybe a figure showing the species ranges and our proposed site of the Bangladeshi bat colonies?

Commented [MK17]: Since viruses move through populations, I thought collecting all available complete ACE2 sequences on ncbi (skipped uniprot, but I can add, if necessary) for each of the species listed would be more representative. Also, I edited the text to reflect these changes.

Table 1. ACE2 amino acid identity amongst Rhinolophid bats and hosts supporting SARS-CoV-2

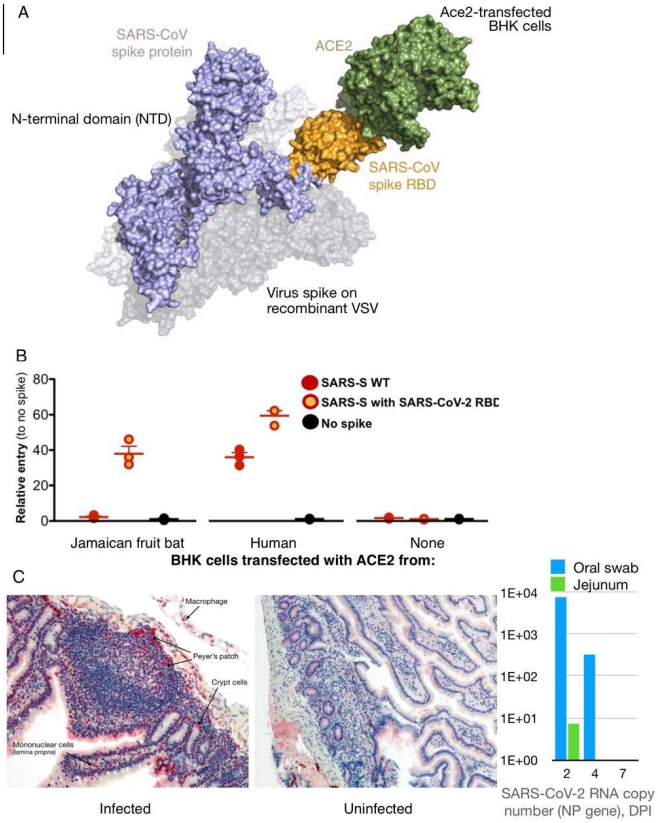
		Amino Acid in *ACE2*																				
Species name	**Common name**	**24**	**27**	**28**	**30**	**31**	**34**	**35**	**37**	**38**	**41**	**42**	**45**	**82**	**83**	**330**	**353**	**354**	**355**	**357**	**393**	
Homo sapiens	**Human (dbSNP)**	***Q***	***T/A***	***F***	***D***	***K***	***H***	***E/D/K***	***E***	***D***	***Y***	***Q***	***L***	***M/I***	***Y***	***N***	***K***	***G***	***D/N***	***R***	***R***	
Mesocricetus auratus	Syrian Hamster (N=1)	**Q**	T	**F**	**D**	**K**	Q	**E**	**E**	**D**	**Y**	**Q**	L	N	**Y**	**N**	**K**	**G**	**D**	**R**	**R**	
Rhinolophus sinicus	Chinese rufous horseshoe bat (N=25)	R/E/L	**T/M/I**	**F**	**D**	**K/E/T**	S/T/F	**E/K**	**E**	**D/N**	**Y/H**	**Q/K**	**L**	N	**Y**	**N**	**K**	**G**	**D**	**R**	**R**	85%
Felis catus	Domestic cat (N=1)	L	**T**	**F**	E	**K**	**H**	**E**	**E**	E	**Y**	**Q**	**L**	T	**Y**	**N**	**K**	**G**	**D**	**R**	**R**	80%
Rhinolophus affinis	Intermediate horseshoe bat (N=23)	R	I	**F**	**D**	N	**H/R**	**E**	**E**	**E/D**	**Y**	**Q**	**L**	N	**Y/H**	**N**	**K**	**G**	**D**	**R**	**R**	80%
Rhinolophus pearsonii	Pearson's horseshoe bat (N=2)	**R/Q**	**T/I**	**F**	**D**	**K**	**H/R**	**E**	**E**	**D**	H	E	**L**	D	**Y**	**N**	**K**	D	**D**	**R**	**R**	80%
Mustela putoris	Ferret (N=1)	L	**T**	**F**	E	**K**	Y	**E**	**E**	E	**Y**	**Q**	**L**	T	**Y**	**N**	**K**	R	**D**	**R**	**R**	70%
Rhinolophus pusillus	Least horseshoe bat (N=1)	K	K	**F**	N	D	S	**E**	**E**	**D**	**Y**	**Q**	**L**	N	**Y**	**N**	**K**	**G**	**D**	**R**	**R**	70%
Rhinolophus macrotis	Big-eared horseshoe bat (N=1)	E	K	**F**	**D**	**K**	S	**K**	**E**	**D**	**Y**	E	**L**	N	**Y**	K	**K**	**G**	**D**	**R**	**R**	70%
Rhinolophus landeri	Lander's horseshoe bat (N=1)	L	**T**	**F**	**D**	D	S	A	**E**	N	**Y**	**Q**	**L**	N	F	**N**	**K**	**G**	**D**	**R**	**R**	65%

C1.3. *Experimental bat coronavirus infections*. Drs. Munster and Schountz have extensive experience with experimental infection models of bats, including with MERS-CoV, SARS-CoV-2, H18N11 bat influenza virus, Zika virus and Tacaribe virus. Challenge of Egyptian fruit bats with an infectious clone of WIV-CoV showed limited susceptibility, despite the ability of its spike protein to bind to Egyptian fruit bat ACE2 (30572566). Recent work by our groups has shown that Jamaican fruit bat ACE2 is bound by SARS-CoV-2 (but not SARS-CoV) spike protein expressed by a VSV pseudotype virus and that challenge of Jamaican fruit bats with SARS-CoV-

2 leads to a limited infection that is confined to the small intestine, particularly mononuclear cells of the Peyer's patches and lamina propria, and crypt cells (Fig 2). Viral RNA was detected in oral swabs for 4 days and low levels in the small intestine on day 2. However, no neutralizing antibody or nucleoprotein antibody (ELISA) was detected, suggesting abortive infection or robust control of the virus by the innate immune response.
C1.4.

C2. Specific Aims

C2.1 Aim 1. To establish colonies of horseshoe bats and Indian flying foxes for the study of coronavirus and henipavirus infections. Bats will be captured and quarantined in Bangladesh and tested for coronaviruses, henipaviruses, filoviruses and lyssaviruses. Those that are negative will be imported to Colorado State University for establishment of breeding colonies.



C2.1.1. *Establishment of captive breeding colony of Pteropus medius in Bangladesh*
We will establish a captive breeding colony of Pteropus medius in Bangladesh. Temporary holding cages used for quarantine prior to shipment to the US will be erected at the Sheikh Kamal Wildlife Center (SKWC) Purabari, adjacent to Bhawal National Park, Gazipur, north of Dhaka. The land is managed by the Forest Department under the direction of the Conservator of Forests (see Kabir letter of support) Ministry of Environment (Govt of Bangladesh). The bats will be cared for and maintained by Forest Department staff. Staging bats in Bangladesh prior to shipment to the US will occur in X phases.
Phase 1: Holding cage development. We will build four temporary holding cages [octagonal shape, approx. 6m diameter and 6ft tall] that are large enough to allow bats to fly, and low enough that project personnel can easily repeatedly catch bats for testing during the screening process. Each cage will be built to hold 30 bats, with a heavy retractable curtain subdividing each cage in half, which will allow us to separate bats into smaller groups. One of the cages will be left empty to allow for isolation of bats that test positive for Nipah virus on PCR during the course of quarantine. Each cage will be made from a metal frame with rubber-coated mesh screening enclosing the cage. An outer cage will be built around each cage, with 6" of space between the fencing to exclude bats inside from contact with bats outside. A solid roof will be built over each cage to prevent excreta from wild bats from entering the cage area and potentially exposing captive bats to henipaviruses. Each cage will also have a door and an anti-chamber to allow staff to safely enter and exit, don and doff PPE, and then enter the flight cage without bats being able to escape.

Commented [MK18]: I assume the holding cages will be different for the rhinolophid bats, but the capture methods will be the same?

Facilities at the wildlife park where the bats will be held will include a food preparation station, PPE storage, first aid and disinfection supplies, and a mobile veterinary lab that includes electricity, a portable gas anesthesia machine, and supplies for collecting biological specimens from the bats.

Phase 2: Bat capture. We will capture wild bats from colonies located in Gazipur. Our goal is to capture a total of 81 bats: 6 males and 75 females comprising three groups of 25 females and 2 males - all Nipah virus negative (by PCR and Luminex). We will capture bats 20 at a time. With each group of 20 bats, we will transport them to the cages from the field site, with each bat held in an individual cotton bag suspended from a post in the back of a vehicle. The driver will be wearing PPE, including an N95 respirator, and all vehicle windows will be open during transport. A plastic sheet will line the floor of the vehicle under the bats to allow for disinfection following transport. Bats will be sampled in the vet lab and then immediately transferred into a holding cage and blood samples and swabs collected. Samples will be screened that day for IgG antibodies using a Luminex, and swabs will be screened for Nipah virus RNA using a qRT-PCR assay at icddr,b. Seropositive and or PCR positive individuals will be released at the park. Seronegative and PCR negative individuals will be moved into a second staging cage, where they will be held for 21 days, with a second and third round of testing on day 10 and 21 post capture. Any bats subsequently testing positive will be released. Negative bats will be retained.

Phase 3: Breeding colony. We will maintain separate groups of 27 bats in the three flight cages. Pteropus medius is a synchronous seasonal breeder, with a 6-month gestation period. We will aim to capture bats during the third trimester of pregnancy (January/February) and allow them to give birth in captivity. Pups will be weaned at 3 months and placed into a fourth cage, where they will be maintained until 1 year of age. Pups will be screened every 2 weeks for Nipah virus and antibodies. Maternal antibodies are expected to wane at around 6-7 months of age. By isolating the pups, born to seronegative, Nipah-negative dams, we will create an SPF (specific-pathogen free) colony that will be used to found the colony at CSU. We will also allow the bats in Bangladesh to breed, creating an F1 generation that will be maintained in captivity and used to support the US-based colony.

C2.1.2. *Experimental challenge of rhinolophid bats.* We will determine whether Ace2 sequences from various *Rhinolophus* species bats render BHK cells susceptible to SARS-CoV and SARS-CoV-2 using our VSV pseudotype system (Fig XX). Rhinolophid species will be captured and quarantined in Bangladesh and tested for evidence of SARS-like CoV infection (PCR of rectal swabs and serology). Naïve bats will be shipped to Colorado State University for challenge with SARS-CoV and SARS-CoV-2 to determine susceptibility. Those species that are susceptible will be collected in Bangladesh and after quarantine and testing, up to 50 founders will be shipped to CSU to establish breeding colonies. In the event that more than one species is susceptible, we will determine which is most suitable based upon infection kinetics of each virus.

Upon receipt, bats will be immediately examined by veterinary staff at CSU and manually fed meal worms.

C2.2 Aim 2. To develop cell lines, reagents and methodologies for the study of viral infections in horseshoe bats and Indian flying foxes. Primary bat cells will be isolated from bats for susceptibility testing, and stocks of primary cells will be generated for the research community. Susceptible cells will be immortalized to provide indefinite propagation. Organoids of virus targets, such as intestines, will also be generated for virus studies. Recombinant proteins, including cytokines and soluble cell surface antigens, will be generated for in vitro and in vivo use. Monoclonal antibodies to these proteins will also be generated so that investigators can perform in vitro testing and generation of primary cells that require such growth factors, such as antigen-specific T cells, and for the in vivo neutralization of cytokines upon challenge with viruses. Methodologies for transcriptomic, metabolomic and proteomic studies will also be developed.

C2.2.1. Establishment of primary and immortalized bat cell lines.(Tony)

C2.2.2. Production of recombinant bat cytokines and monoclonal antibodies. (Brian cytokines, Tony Mab)

C2.2.3. Development of metabolomic profiles. (Lin-fa and Rushika)

C2.2.4. Development of proteomic profiles. (Lin-fa and Rushika)

C2.3 Aim 3. To perform experimental infections of bats with SARS-CoV-2, bat RaTG13-CoV and Nipah virus to develop laboratory models of bat reservoir hosts. Bats will be challenged with viruses under controlled laboratory conditions to perform kinetic and temporal studies of how these viruses behave in bats, and

how the bats control infections without developing disease. We will also perform studies to assess durability of immunity in bats to determine whether immunity wanes and leads to repeated susceptibility to infection.

C2.3.1. Experimental infection of horseshoe bats with SARS-CoV-2. (Tony, Lin-fa, Vincent)
C2.3.2. Experimental infections of Indian flying foxes with Nipah virus. (Tony, Lin-fa, Vincent)
C2.3.3. Generation of bat RaTG13-CoV infectious clone. (Eric)

Vertebrate Animals

Description of Procedures:

Justifications:

Minimization of Pain and Distress:

Select Agent Research

Multiple PD/PI Leadership Plan

Consortium/Contractual Arrangements

Letters of Support

Resource Sharing Plans

Data Sharing Plan:

Sharing Model Organisms:

Genomic Data Sharing (GDS):

Authentication of Key Biological Resources

Appendix (maximum of 10 PDF documents)

Literature Cited

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From:	Laing, Eric on behalf of Laing, Eric <eric.laing@usuhs.edu>
To:	Schountz,Tony
Cc:	Wang Linfa; Munster, Vincent (NIH/NIAID) [E]; Perera,Rushika; Geiss,Brian; Maria Kaczmarek; epstein
Subject:	Re: Grant status
Date:	Friday, January 15, 2021 9:21:27 PM
Attachments:	R24 Grant_MEK_ts010721 SPECIFIC AIMS-edl.docx

Hi Tony and Jon,

I added two sections to C.3.3.

- Eric

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On Tue, Jan 12, 2021 at 9:46 AM Schountz,Tony <Tony.Schountz@colostate.edu> wrote:

> All, attached is the very rough skeleton of the R24 proposal. I've noted areas for each of you to work on, but of course please feel free to edit any other places you want. It's certainly going to change a bit before the final submission, but Jon and I want to get it to you sooner rather than later. Keep in mind that R24s are not supposed to be hypothesis-driven; they are really about resource development. The NIAID program officers gave us permission to do experimental challenges (Aim 3) so that's the place where we can do some science. We need to keep the Research Strategy to 12 pages (like an R01), so that means that means your sections should be a page to a page and a half. For your references, please just insert the PMID number and I'll get it the references inserted and formatted. It would be great if you could get your edits to us by Friday, but no later than Monday morning so that Jon and I can tidy up the proposal in preparation for submission on January 22.
>
> For R24s, the Resource Sharing plan is critically important since the mechanism is directed to distribution of materials to the greater research community. We haven't started on that but I expect that once the proposal starts to shape up, this section will be mostly self-populating (but of course everone will get to see it before it is submitted).
>
> Susan Rogers here at CSU is assembling the budgets and I think it will start routing through CSU's internal review process. If there are any problems, I'll be sure to let you know ASAP.
>
> Jon and I have lined up several people who will provide letters of support for the proposal and I will be working on draft letters for each of them today.

Let me know if you have questions.

Thanks,

Tony

—

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ORIGINAL RESEARCH

WILEY Ecology and Evolution Open Access

Pteropus lylei primarily forages in residential areas in Kandal, Cambodia

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European Commission Innovate program (ComAcross project), Grant/Award Number: DCI-ASIE/2013/315-047; Centre National d'Etudes Spatiales (TeleNipah project), Grant/Award Number: DAR 4800000780

Abstract

1. Bats are the second most species-rich Mammalian order and provide a wide range of ecologically important and economically significant ecosystem services. Nipah virus is a zoonotic emerging infectious disease for which pteropodid bats have been identified as a natural reservoir. In Cambodia, Nipah virus circulation has been reported in *Pteropus lylei*, but little is known about the spatial distribution of the species and the associated implications for conservation and public health.
2. We deployed Global Positioning System (GPS) collars on 14 *P. lylei* to study their movements and foraging behavior in Cambodia in 2016. All of the flying foxes were captured from the same roost, and GPS locations were collected for 1 month. The habitats used by each bat were characterized through ground-truthing, and a spatial distribution model was developed of foraging sites.
3. A total of 13,643 valid locations were collected during the study. Our study bats flew approximately 20 km from the roost each night to forage. The maximum distance traveled per night ranged from 6.88–105 km and averaged 28.3 km. Six of the 14 bats visited another roost for at least one night during the study, including one roost located 105 km away.
4. Most foraging locations were in residential areas (53.7%) followed by plantations (26.6%). Our spatial distribution model confirmed that residential areas were the preferred foraging habitat for *P. lylei*, although our results should be interpreted with caution due to the limited number of individuals studied.
5. *Synthesis and applications*: Our findings suggest that the use of residential and agricultural habitats by *P. lylei* may create opportunities for bats to interact with humans and livestock. They also suggest the importance of anthropogenic habitats for conservation of this vulnerable and ecologically important group in Cambodia. Our mapping of the probability of occurrence of foraging sites will help identification of areas where public awareness should be promoted regarding the

*These authors contributed equally to this work.

ecosystem services provided by flying foxes and potential for disease transmission through indirect contact.

KEYWORDS
distribution model, ecology, epidemiology, flying fox, GPS, habitat use, interface, Nipah virus, telemetry

1 | INTRODUCTION

Bats are the second most species-rich Mammalian order with over 1,300 species worldwide (Voigt & Kingston, 2016) and provide a wide range of ecologically important and economically significant ecosystem services (Kunz, Torrez, Bauer, Lobova, & Fleming, 2011). They are also recognized as reservoir hosts for highly pathogenic viruses such as Nipah virus (NiV; Calisher, Childs, Field, Holmes, & Schountz, 2006).

Nipah virus was first identified in pigs and people in Malaysia in 1998 (Chua, 2000) and has reemerged annually in Bangladesh since 2001 (Luby et al., 2009). NiV causes lethal encephalitis in people, and bats in the *Pteropus* genus are the reservoir (Epstein, Field, Luby, Pulliam, & Daszak, 2006). Transmission of the virus in Malaysia is presumed to have occurred as a result of pigs consuming bat-contaminated fruits, followed by contamination of humans working with pigs (Chua, 2003). In Bangladesh, direct bat-to-human transmission of the virus occurs through the consumption of date palm sap (Luby et al., 2006). NiV has been isolated or detected in several *Pteropus* species in Southeast Asia, including *P. medius* in Bangladesh, *P. lylei* in Thailand and Cambodia, and *P. vampyrus* and *P. hypomelanus* in Malaysia. However, despite its detection in *P. hypomelanus*, a serological study on Tioman Island did not find the virus in any of the local people (Chong, Tan, Goh, Lam, & Bing, 2003) that the bats live among and regularly interact with (Aziz, Clements, Giam, Forget, & Campos-Arceiz, 2017). Seasonal NiV shedding patterns have been suggested for *P. lylei* in Thailand, with peak shedding occurring in May (Cappelle, Hul, Duong, Tarantola, & Buchy, 2014; Wacharapluesadee et al., 2010).

Understanding the capacity of a reservoir to spread the virus at local and regional levels to humans and domestic animals is fundamental to surveillance and prevention initiatives. Knowledge about the distribution and movement patterns of these bat species is thus required, and telemetry (measurement and transmission of data from remote sources) is a valuable tool to monitor the drivers and characteristics of fruit bat movements (Smith et al., 2011). This can be used to develop appropriate host management strategies that maximize the conservation of bat populations and minimize the risk of disease outbreaks in domestic animals and humans.

Telemetry studies have been undertaken on several *Pteropus* species in Asia and Australia. In Australia, tracking of fourteen *P. poliocephalus* males revealed that these are highly mobile between roosts and regularly travel long distances (Roberts, Catterall, Eby, & Kanowski, 2012). For instance, one *P. alecto* was tracked between Papua New Guinea and Australia and traveled more than 3,000 km over 11 months (Breed, Field, Smith, Edmonston, & Meers, 2010). In Southeast Asia, the movements of seven *P. vampyrus* males encompassed Malaysia, Indonesia, and Thailand, indicating the need for regional management plans for such species (Epstein et al., 2009). These studies highlight the difference between migratory and nomadic flying foxes and the need to adapt management strategies to relevant geographic scales.

At a local scale, telemetry studies indicate that *Pteropus* bats make foraging flights on a nightly basis, with distances from the roost ranging from a few kilometers to 20–30 km. Depending on species, foraging sites range from apparently intact forest to cultivated areas. In Bangladesh, the roosting ecology of *P. giganteus* is associated with forest fragmentation, likely because fragmented forests offers more foraging options to the bats, including fruit species cultivated by humans (Hahn et al., 2014). Conversely, in the Philippines, most foraging locations of eight *Acerodon jubatus* were situated in closed forest remote from areas of evident human activity (de Jong et al., 2013). Another study on *A. jubatus* and *P. vampyrus* in the Philippines suggested these species prefer undisturbed forest types and select against disturbed and agricultural areas (Mildenstein, Stier, Nuevo-Diego, & Mills, 2005). Foraging also repeatedly occurred 15–30 km from the roost on average. Similarly, movements of *P. alecto* were very similar between nights with most foraging sites located less than 6 km from roost sites. In Thailand, *P. lylei* also undertakes relatively short foraging movements (2.2–23.6 km) on a nightly basis to fields, plantations, backyards, and mangroves (Weber et al., 2015).

In Cambodia, three flying fox species are thought to occur, large flying fox *P. vampyrus* which is listed as "near threatened" by IUCN, Lyle's flying fox *P. lylei* which is listed as "vulnerable," and island flying fox *P. hypomelanus*, which is listed as "least concern" (IUCN, 2008; Kingsada et al., 2011). Most of the known flying fox roost sites in Cambodia are located on the grounds of pagodas, where hunting is limited by the presence of the monks (Ravon, Furey, Hul, & Cappelle, 2014). Consequently, these are often located in the middle of villages close to human and domestic animal populations, and available foraging areas mostly comprise anthropogenic landscapes. Flying foxes in Cambodia are likely to interact frequently with humans and to depend on human activities for their subsistence. As a consequence, understanding of their preferred foraging areas is important to inform public health and conservation actions.

The objective of our study was to use telemetry data to determine and characterize foraging locations visited by flying foxes

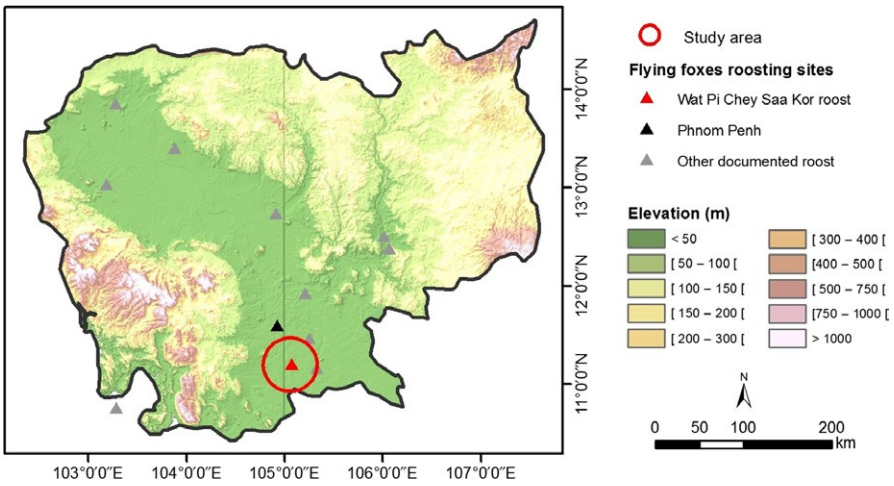

FIGURE 1 Location of the study area and other flying fox roost sites known in Cambodia

inhabiting a *P. lylei* roost in Koh Thom District, Kandal Province, Cambodia.

2 | MATERIALS AND METHODS

2.1 | Study site

The *P. lylei* roost selected for this study was located at Wat Pi Chey Saa Kor (11.200 N, 105.058 E), Kom Poung Kor village, Koh Thom District, Kandal Province (Figure 1). The site comprises a grove of trees on the grounds of a Buddhist pagoda which encompasses 21 roost trees with an estimated population of 4,000 flying foxes (Ravon et al., 2014). The village is bisected by a road with houses on either side and is characterized by a mosaic of agriculture that lacks significant areas of natural vegetation/forest. Land uses in the region include cultivation of rice and other crops, backyards, plantations, and various backyard animal farming activities.

2.2 | Study period

Our study was conducted from April 18, 2016 to May 17, 2016 when shedding of the NiV by *P. lylei* is believed to peak in Cambodia (Cappelle et al., 2014), similar to Thailand (Wacharapluesadee et al., 2010). Nine Global Positioning System (GPS) collars were deployed from April 18, 2016 to April 21, 2016 and five GPS collars from May 3, 2016 to May 6, 2016. Data were collected from these every day for two weeks after each collaring, related to the lifespan of individual collars.

2.3 | Bat collaring

Bats were captured using mist nets between 6 p.m. and 5 a.m. using methods described in (Newman, Field, Epstein, & De Jong, 2011). Weight, forearm length, sex, age, and reproductive status were documented for each bat. Animals were selected for collaring based on weight. Adult males and females without pups weighing at least 400 g were selected so that collars, weighting 20 g, would comprise <5% of body mass (Brigham, 1988). Pregnant and lactating female bats were excluded to avoid adding extra burdens.

Selected bats were anesthetized by injecting medetomidine into the pectoral muscle (Epstein, Zambriski, Rostal, Heard, & Daszak, 2011). GPS devices (FLR V, Telemetry Solutions™, www.telemetrysolutions.com) attached to nylon bands were secured around the neck of each bat using catgut suture (1.0) and three surgical knots (Figure 2), which were presumed to last for at least 30 days. Following collar attachment, atipamezol was injected intramuscularly. Each collared bat was kept in a separate cage during recovery from anesthesia and offered pieces of mango ad libitum prior to release.

We deployed 14 GPS collars on 13 adult males and one adult female (Table 1). Collars 1–5 were programed to transmit one location every 30 min from 5 p.m. to 6 a.m. while collars 6–14 were programed to transmit one location every 30 min for the first night only and one location every 5 min from 5 p.m. to 6 a.m. on following nights. As a consequence, collars 1–5 were expected to last for 1 month and allow observations of foraging behavior across the expected annual excretion peak of NiV. Collars 6–14 were expected to last for 10 days and provide detailed information on *P. lylei* foraging sites, including night roosts. Data were remotely downloaded each morning from active collars with a base station, which automatically connected to the GPS collars when within reading distance (10–20 m).

2.4 | Spatial data and site characterization

Global Positioning System data were transferred each morning to a computer, converted into KML format (QGIS, version 2.14), and mapped to identify foraging locations visited by bats the previous night (Google Earth, version 7.1). Foraging sites were identified based on clusters of two or more locations obtained from individual bats and as many as possible were visited depending on accessibility. Tree species visited by bats and evidence of foraging such as partially

eaten fruits were recorded to facilitate identification of roosting and feeding trees. Nonfruiting trees were also recorded.

2.5 | Habitat use

All locations were classified in three major categories: roost locations (all points less than 30 m from the roost site), foraging locations (a cluster of ≥2 two points separated by <30 m where the bat spent at least 10 min at night (i.e., from 6 p.m. to 6 a.m.)), and commuting locations (isolated points connecting the roost and foraging sites located >30 m from a foraging or roost location). Based on patterns visible in Google Earth, five habitat types were recognized for foraging locations: plantations (including fruit trees within the plantation and trees around the plantation), residential areas (locations within 50 m of human settlements, including pagodas, backyards, roads), agricultural lands (any cultivated land not included in "plantations" and "residential areas"), rivers, and uncultivated areas (all locations not included in the preceding categories).

FIGURE 2 Collared *Pteropus lylei*, southern Cambodia

2.6 | Spatial analysis

The home range of an animal illustrates spatial and temporal use of an area and is defined as the area commonly used for normal activities such as foraging for food, breeding, and caring for young (Burt, 1943). We used the kernelUD() function of the Adehabitat package in R software (Version 3.2.3) to estimate the home range for all bats, using the kernel density method (Calenge, 2006). The function computes the different percentage levels of home range estimation, for example the 50% home range identifies the areas where an individual is likely to occur 50% of the time.

We used QGIS to analyze the trajectories of each bat and to generate heatmaps based on kernel density estimation. The density was calculated based on the number of points in a location, with larger numbers of clustered points resulting in larger values. We also used the sp package in R software to calculate the maximum linear distance traveled from the roost per night.

The spatial distribution of foraging sites in the study area was modeled using the GPS data collected, a set of generated background data and land cover data. We created a map which classified habitats according to their expected influence on foraging site selection by the bats. This map was the product of a classification procedure based on Landsat images (30 m spatial resolution) acquired in 2015 and ground-truthing. Details of the classification are provided as Appendix (Supporting information Appendix S1: Table S1), and the result is illustrated by (Supporting information Appendix S1: Figure S1). The eight different habitats identified in this classification were

TABLE 1 Characteristics of *Pteropus lylei* studied and GPS device performance, southern Cambodia. The proportion of valid data corresponds to the proportion of locations recorded with valid geographic coordinates

Bat ID	Sex	Reproductive Status	Weight (g)	Forearm (mm)	Collar lifespan (nights)	Total recorded data	Proportion of valid data (%)
Bat01	Male	Mature	560	169	26	760	32
Bat02	Male	Mature	565	152.9	3	247	90
Bat03	Male	Mature	540	165.5	11	439	81
Bat04	Male	Mature	435	NA	9	394	40
Bat05	Male	Mature	490	149.4	23	716	88
Bat06	Male	Mature	430	151.9	13	1,904	95
Bat07	Male	Mature	425	149.5	9	1,747	41
Bat08	Male	Mature	420	144.9	12	1,675	95
Bat09	Male	Mature	532	145.9	1	22	41
Bat10	Male	Mature	425	144.5	8	1,200	89
Bat11	Male	Mature	590	153.7	13	1,768	98
Bat12	Male	Mature	414	148.3	12	1,752	99
Bat13	Female	Adult	430	149.4	12	1,592	96
Bat14	Male	Mature	550	152.4	13	1,912	97

speculated to have the following impacts on the distribution of foraging sites. Plantations were expected to be highly attractive to bats because of the high density of fruit available. Tree vegetation was expected to be attractive because of the potential presence of fruit consumed by bats. Water bodies such as rivers were also expected to attract the bats due to the presence of fruit trees on their banks. Residential areas were expected to have mixed effects as a source of disturbance for the bats and a potential source of fruit in backyards. The four remaining habitats in the classification (rice field, bare soil, flooded vegetation, and shrubland) were not expected to attract the bats.

To train and validate the model, we used all GPS locations of foraging sites and generated an equivalent number of background locations in the study area which were used as pseudoabsences by the model. Half of the data were randomly assigned to a training dataset and the other half to a validation dataset. We used a generalized linear model with the training dataset as the response variable with a binomial distribution (1 for presence and 0 for pseudoabsence) and habitat type as an explanatory qualitative variable. To deal with the discrepancy between the spatial resolution of our classification (30 m) and GPS points (1–5 m), we calculated the distances of all data points to the closest habitats with an expected influence on bat habitat selection: plantations, tree vegetation, water bodies, and residential areas. Because of this discrepancy and landscape fragmentation in the study area, GPS locations of bats foraging in attractive habitats could be recorded in an adjacent nonattractive habitat. We therefore generated four explanatory variables (dPlant, dTree, dWater, and dResid) to allow us to capture the spatial structure of the study area. Using the distance to these attractive habitats as explanatory variables in the model would then help take into account the limited spatial resolution of our habitat classification as well as spatial autocorrelation. As a consequence, no further variable was added to the model to deal with the latter. Finally, distance to the roost (dRoost) was added to the explanatory variables as this should be minimized by the bats to optimize their energy efficiency while foraging. We used the results of the model, which was based on data from 14 individuals, to map the probability of presence of the foraging sites of *P. lylei* in the study area. The validation dataset was used to estimate the performance of the model through the calculation of the area under the ROC curve (AUC).

3 | RESULTS

3.1 | Collar performance

A total of 84 bats were caught, 14 of which were selected for collaring (Table 1). Our GPS devices transmitted from 1 to 26 nights, with an average of 11.8 nights (Table 1). A total of 13,646 valid locations were collected over 27 nights from the 14 collared bats. The proportion of valid data (i.e., data with an actual geographic location provided) varied from 32% to 99% of the data provided by each collar. Overall, 84.6% of the data generated were valid locations (n = 13,646/16,128).

3.2 | Habitat use

Tree species identified during visits to foraging sites are listed in Table 2. Partially eaten mango (*Mangifera indica*, n = 15) and sapodilla (*Manilkara zapota*, n = 3) were found at exact GPS foraging locations (Figure 3). It was not possible to detect whether leaves or flowers were also consumed.

TABLE 2 Tree species identified at foraging sites of 14 GPS-collared *Pteropus lylei*, southern Cambodia

Common name	Scientific name	Species at GPS locations (5 m precision)	Species ≤30 m from GPS locations	Known to be consumed by flying foxes[a]
Banana	*Musa paradisiaca*		X	Direct
Banyan	*Ficus benghalensis*		X	Unknown
Custard apple	*Annona reticulate*		X	Direct
Eucalyptus	*Eucalyptus exserta*	X	X	Indirect
Jack fruit	*Artocarpus heterophyllus*		X	Direct
Java apple	*Syzgium malaccense*		X	Unknown
Kapok	*Ceiba pentandra*	X	X	Direct
Longan	*Dimocarpus longan*		X	Indirect
Mango	*Mangifera indica*	X	X	Direct
Neem	*Azadirachta indica*	X	X	Direct
Papaya	*Carica papaya*		X	Direct
Sacred fig	*Ficus religiosa*	X	X	Direct
Sapodilla	*Manikara zapota*	X	X	Direct
Sugar palm tree	*Borassus flabellifer*	X	X	Indirect

[a]Direct means direct evidence from feces or feeding remains, indirect means information based on evidence from location data but with no direct evidence from feces or feeding remains. Based on (Aziz, Clements, Peng et al., 2017; Hahn et al., 2014; Weber et al., 2015; Win & Mya, 2015).

Among the valid data, 29% of the locations (*n* = 3,959/13,646) corresponded to the roost site where the bats were captured, 20.3% (*n* = 2,774) to commuting locations, and 50.7% (*n* = 6,913) to foraging locations and night roosts. Most of the foraging locations were in residential areas: 53.7% (*n* = 3,714/6,913), 26.6% (*n* = 1,836) in plantations, 16.2% (*n* = 1,118) in uncultivated areas, 3.2% (*n* = 219) in agricultural lands, and 0.4% (*n* = 26) on rivers (Table 3). (Supporting information Appendix S1: Figure S2) shows the spatial distribution of the foraging sites in the study area.

3.3 | Movement patterns and flight distances

The maximum distance traveled per bat/night ranged from 6.88 to 105.14 km and averaged 28.3 km (Table 3). All individuals showed fidelity to at least one foraging site, returning on 3–11 nights to the same site (all locations <30 m from the previous one were counted as the same foraging site) during the study period. Thirty-six foraging sites were shared by at least two bats. All bats (excluding bat #9 due to lack of data) shared at least one and as many as eight foraging locations with another bat. Shared foraging locations or night roosts were relatively close to the roost, with an average and maximum distance of 2.85 and 7.75 km, respectively. Eight bats returned to the study roost every night (bats #1–3, #6, #9, #11, #13–14). Of the six remaining bats, four went to a nearby *P. lylei* roost in Prey Veng Province (28 km east, Wat Veal Lbang, Prey Veng, 700 flying foxes), whereas two went to more distant and previously unknown roosting sites: 65 km in one night (site A) and 105 km over two nights (site B) for bat #8 and 50 km during one night (site C) for bat #10 (Figure 4).

3.4 | Spatial analysis

The complete results of the home range estimations for all bats are shown in (Supporting information Table S2). The estimated home ranges were maximal for bats #08 and #10 which went to distant roosts, with 95% home range of respectively 5,984 and 1,158 km^2. For the eight bats that did not join another roost, the 95% home range ranged from 29.5 to 316.8 km^2 with an average 95% home range of 104.5 km^2 (*SD* = 115.5 km^2). The 50% home range of these same eight bats ranged from 4.3 to 41.1 km^2 with an average 95% home range of 14.9 km^2 (*SD* = 13.4 km^2). Our heatmap of GPS locations showed that most foraging sites and night roosts were located <15 km from the roost (Figure 5). The spatial distribution model showed that foraging locations were significantly negatively correlated with the distance to the roost, residential areas, and water bodies. Conversely, foraging locations were significantly and positively correlated with distance to plantations. Residential areas, trees, and plantations were the main foraging habitats used by the bats (Table 4). Our map of the probability of *P. lylei* foraging sites highlights areas close to the roost but also helps to identify further areas where bat–human interfaces could occur (Figure 6). Model performance was very good with a cross-validated AUC of 0.93.

FIGURE 3 Partially consumed mangoes at a GPS foraging location of *Pteropus lylei*, Kandal Province, southern Cambodia

4 | DISCUSSION

Our study yielded two main results. First, our study bats mostly foraged in residential areas (53.7% of foraging locations) rather than in plantations (25.6%) and our spatial model indicated that residential areas were the preferred foraging habitat (Table 4). While other studies have shown that *P. lylei and P. giganteus* can primarily forage in anthropogenic landscapes (Hahn et al., 2014; Luskin, 2010; Weber et al., 2015), our data indicate a particularly strong interface through residential backyards where the potential for contact between bats and humans would be higher due to continuous human presence. This could potentially facilitate NiV transmission to humans and domestic animals and two transmission routes have been documented in previous outbreaks of NiV. The first is directly from bats to humans due to consumption of raw palm sap contaminated by flying foxes, which has led to recurrent outbreaks in Bangladesh (Luby et al., 2009). The second route was suggested for the Malaysian outbreak where pigs were likely infected after consuming fruit contaminated by flying foxes (Chua, 2003) and supported by isolation of the virus from fruit partially eaten by bats in Malaysia (Chua et al., 2002). Consistent with this second route, a direct bat-to-human transmission via ingestion of fruit has been suggested for another paramyxovirus in Malaysia (Yaiw et al., 2007). Thus, by frequently foraging in residential areas, *P. lylei* could contaminate fruit where humans and domestic animals live, increasing the chance of indirect contact. As such, further information on the use by local residents of fruit partially eaten by bats would help to characterize transmission risks and inform preventative actions including promotion of public awareness. Similarly, palm sap collectors in the study area reported seeing flying foxes on palm trees and urine and feces on collection containers. As our data also indicate that *P. lylei* visits these trees (Table 2), research on palm sap collection in the area is needed to assess the risk associated with this potential transmission route.

TABLE 3 Maximum distances traveled per night by *Pteropus lylei* and proportion of foraging areas per category, southern Cambodia

Bat ID	No. of foraging locations and night roosts	Max distance/night (km)	Residential area (%)	Plantation area (%)	Agricultural land area (%)	Uncultivated area (%)	River (%)
Bat01	111	8.95	32	41	17	0	11
Bat02	145	7.91	15	75	10	0	0
Bat03	189	10.28	99	1	0	0	0
Bat04	100	29.60	75	9	16	0	0
Bat05	190	29.35	89	4	0	7	0
Bat06	1,109	23.35	32	31	4	32	1
Bat07	411	27.39	50	2	4	44	0
Bat08	798	105.14	62	17	2	19	0
Bat09	3	6.88	0	100	0	0	0
Bat10	628	52.11	18	60	2	21	0
Bat11	761	10.39	4	76	0	20	0
Bat12	964	50.33	79	8	4	9	0
Bat13	421	25.45	62	29	4	4	2
Bat14	1,083	9.03	93	2	2	2	0
Total	6,913	28.3[a]	54[b]	27[b]	3[b]	16[b]	0[b]

[a]mean of the maximal distance per night for all bats. [b]Proportion of foraging area for all locations of all bats.

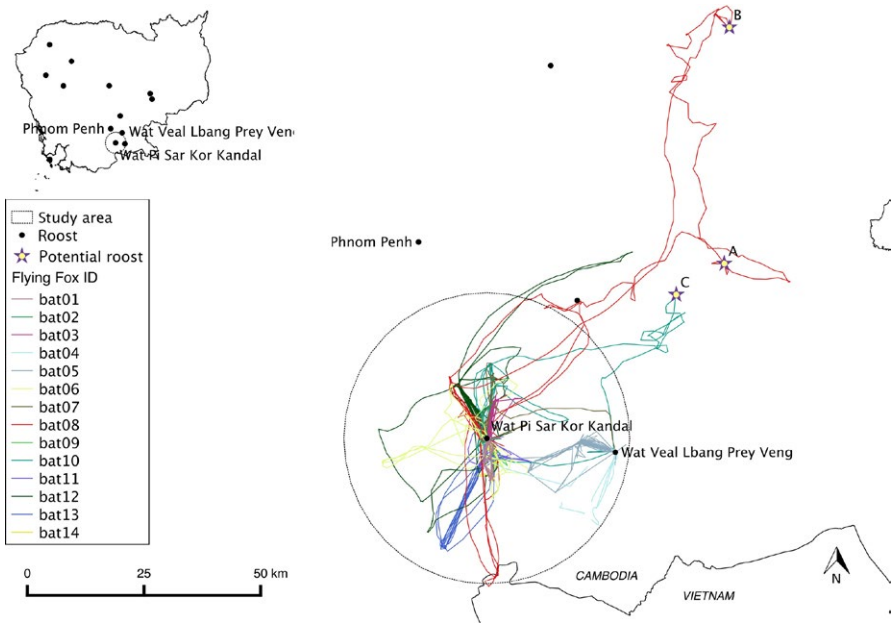

FIGURE 4 Movements of 14 GPS-collared *Pteropus lylei* during the study period in southern Cambodia

Our finding that *P. lylei* mostly forages in residential areas—which mostly correspond to backyards—rather than in plantations was unexpected because human disturbance would likely be higher in the former and food availability greater in the latter. Since our data indicate that *P. lylei* feeds on a variety of fruit in April–May, the greater diversity of fruit typically found in backyards compared to plantations could possibly explain this. More generally, the link between flying fox foraging behavior and the greater diversity of fruits in anthropogenic versus natural environments has been reported elsewhere (Hahn et al., 2014; Luskin, 2010; Weber et al., 2015). All foraging sites in our study were located in anthropogenic landscapes and all individuals showed fidelity to foraging areas, indicating repeated utilization once a food resource was located. This is presumably more energy-efficient than random foraging and is consistent with studies of *A. jubatus* in the Philippines (de Jong et al., 2013) and *P. alecto* in Australia (Palmer & Woinarski, 1999). From an epidemiological standpoint, an infectious flying fox repeatedly shedding virus in the same area could facilitate site contamination and increase the risk of transmission to humans or animals. Indeed, all of our 14 bats shared at least one foraging site during the study. Repeated shedding

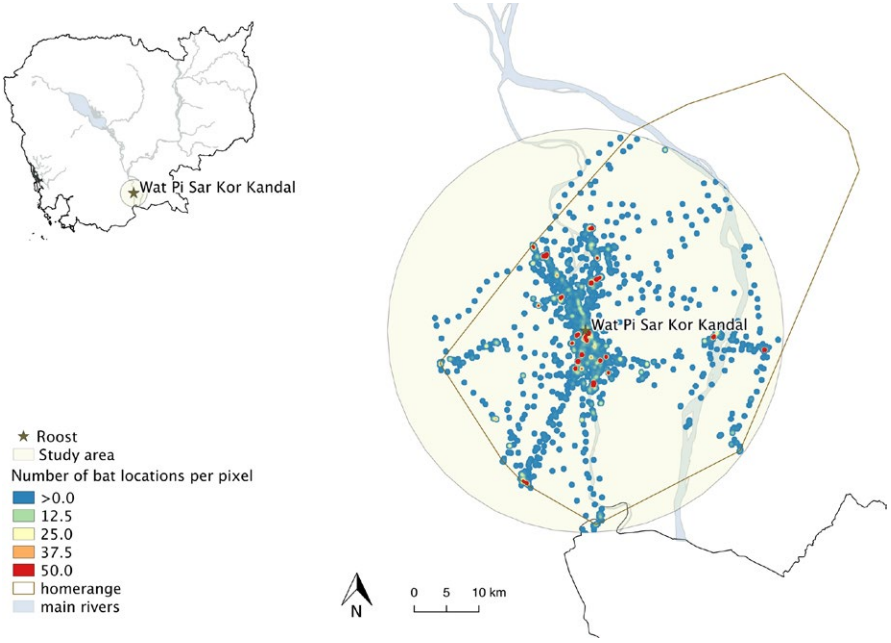

FIGURE 5 Heatmap of *Pteropus lylei* movements and home range (minimum convex polygon) in southern Cambodia

at a shared foraging site or night roost could also increase pathogen transmission in the bat population through fruit contamination. In future analyses, we will use a hidden Markov model to determine different phases of nightly movements and attempt to differentiate foraging sites from night roosts.

From a conservation perspective, the apparent preference for backyards and plantations suggest that our *P. lylei* population is highly dependent on human activities for foraging. As such, understanding of community knowledge, attitudes, and practices regarding bats will be important to develop appropriate conservation and public awareness strategies and is now underway. Nevertheless, that residential backyards were the most strongly selected foraging habitat suggests that conflict with humans may be limited in our study area. This is consistent with the fact that other patches of trees were also attractive to our study bats ("Tree vegetation" in Table 4), albeit less than backyards and plantations. Were major bat–human conflicts to occur in our study area, the few attractive non-human-dominated habitats present could possibly become overselected by the bats. However, our results must of course be interpreted with caution as only 14 bats in the same population were studied.

Second, because six of our 14 study bats visited at least one other roost during our 28-day study, it would appear that movements to other roost sites are relatively frequent. However, these movements were limited in time and the fidelity shown to the day roost by all of our study bats is consistent with the non-nomadic ecology attributed to *P. lylei*. Similar to observations for *P. vampyrus* (Epstein et al., 2009) and *P. medius* (Epstein, unpublished), visits to four other roosts including one 105 km from the study site were observed. These frequent exchanges between roosts are consistent with a regional circulation of different NiV strains in Southeast Asia suggested in previous studies (Epstein, 2017; Wacharapluesadee et al., 2016). From a conservation perspective, they also suggest that *P. lylei* in Cambodia is likely a metapopulation and that conservation strategies should be planned on a regional scale. This is consistent with the results of another telemetry study on the migratory *P. vampyrus*, calling for a comprehensive protection by regional management plans across their international range (Epstein et al., 2009).

The main limitation of our research is the small number of individuals we could study. With only 14 nonrandomly selected individuals tracked out of an estimated 4,000–6,000, our data are unlikely to be representative of the roost population as a whole. Additionally, because foraging behavior is highly dependent on local environments, our results should not be extrapolated to all *P. lylei* colonies in Cambodia. Furthermore, our study group had a strong male bias, with only one female tagged with the GPS device. Though

TABLE 4 Results of generalized linear model. Significant explanatory variables with a *p*-value $<10^{-3}$ are given in bold

Variable	Coefficient (*SE*)	*p*-Value
Intercept	2.844 (0.355)	$1.10\ 10^{-15}$
Habitat type		
Residential area	**2.853 (0.385)**	**$1.34\ 10^{-13}$**
Tree vegetation	**2.178 (0.296)**	**$1.77\ 10^{-13}$**
Plantation	**1.865 (0.519)**	**$3.26\ 10^{-4}$**
Bare soil	0.695 (0.345)	0.044
Water	0.289 (0.670)	0.666
Flooded vegetation	−0.598 (0.499)	0.231
Shrubland	−13.879 (486.4)	0.977
Rice field	Reference	
dResid	**−0.337 (0.111)**	**$2.28\ 10^{-3}$**
dTree	−0.519 (0.411)	0.206
dWater	**−0.599 (0.135)**	**$9.38\ 10^{-6}$**
dPlant	**0.133 (0.040)**	**$8.91\ 10^{-4}$**
dRoost	**−0.220 (0.016)**	**$<2\ 10^{-16}$**

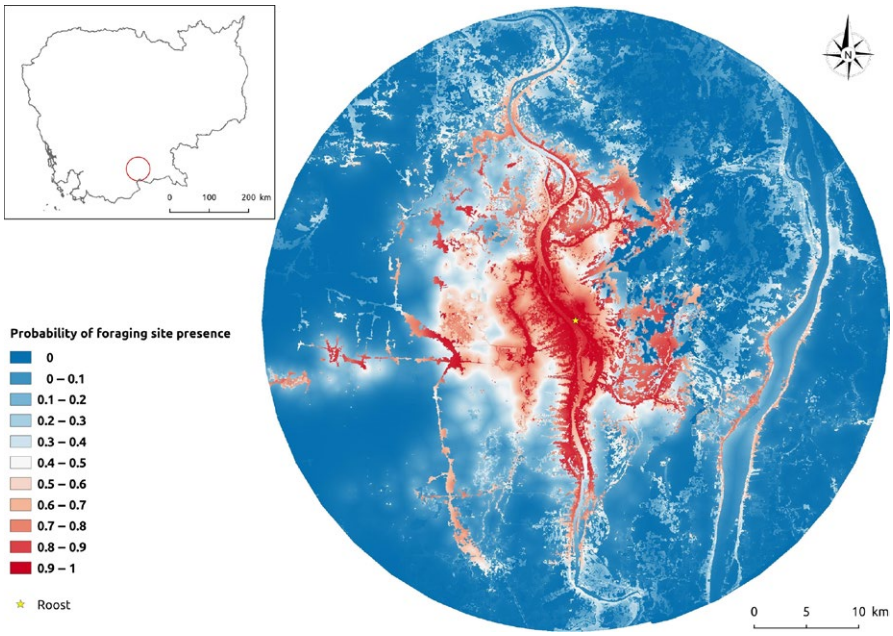

FIGURE 6 Probability of occurrence of *Pteropus lylei* foraging sites based on spatial distribution modeling, southern Cambodia. The model was trained and validated with GPS locations from 14 tracked individuals

other females were caught, these were excluded as they were pregnant or lactating and because limited data are available for female *P. lylei*, it remains unclear if the sexes differ in their foraging behavior. For instance, while female and male *P. poliocephalus* are similar in their movement patterns (Roberts et al., 2012; Tidemann & Nelson, 2004), lactating females of *P. alecto* travel greater distances between roosts and foraging sites than males (Palmer & Woinarski, 1999; Roberts et al., 2012). Nine of the 14 GPS collars we deployed lasted for at least 10 nights (average 11.8 nights), and 80% of the data were valid. Three other collars provided relatively few valid locations, and only one failed to transmit meaningful data. This performance rate was probably influenced by extended battery life due to high temperatures during the study period, while the open agricultural landscape of our study area probably facilitated the acquisition of GPS locations, saving further battery power. We deployed GPS devices on a limited number of individuals, preventing us from any generalization of the observed patterns at the population level. However, the results were consistent between the different individuals and provided useful information on the movement and foraging ecology of *P. lylei* in Cambodia. The GPS devices we used were battery-powered, and the size of the battery was limited by the body weight of the flying foxes. By programing five GPS devices to record locations every 30 min instead of 5 min for the nine other devices, we expected them to last for a month. However, data for only two of these bats were collected for more than 20 days, limiting our capacity to observe any change in foraging patterns over this period. Further studies should then be implemented to assess any variability of foraging patterns over time.

While our data represent a brief snapshot in time, they nonetheless illustrate the potential for foraging behavior to potentially facilitate NiV transmission to humans and domestic animals. To date, no transmission from *P. lylei* to human or animals has been recorded despite the circulation of NiV in this species in Cambodia and Thailand (Cappelle et al., 2014; Reynes et al., 2005; Wacharapluesadee et al., 2010). The presence of a hazard such as the NiV in a reservoir population does not necessarily lead to an emergence (Hosseini et al., 2017). Indeed, despite NiV being detected in *P. hypomelanus* on Tioman Island, no outbreak has occurred there, and no evidence of the virus has been found in people on the island (Chong et al., 2003). As such, close and frequent interfaces between bats and humans, including bats roosting in the middle of villages and feeding on cultivated fruit in residential backyards and orchards (Aziz, Clements, Giam et al., 2017) may not be sufficient to lead to an emergence. Other factors such as cultural and agricultural practices must be taken into account.

Different agricultural practices may lead to different levels of exposure in the countries of Southeast and South Asia. Conditions specific to intensive pig farming in Malaysia or palm sap collection in Bangladesh may explain why the virus emerged in these countries. Nevertheless, understanding the ecology of *P. lylei* may significantly improve our ability to target limited resources for interventions, and educational campaigns that discuss the risks of NiV to people and their domestic animals (Nahar et al., 2014; Parveen et al., 2016). In particular, while based on only 14 individuals, our mapping of the probability of occurrence of foraging sites for the *P. lylei* will help targeting prevention measures to areas where contact between flying foxes and humans can be expected.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

JC and AT conceived the study and designed methodology; JC and TH coordinated the capture of the bats with the help of KC, SR, NF, VH, and CN; JHE coordinated the deployment of the GPS collars; AT, MG, and AJ collected environmental data and produced the land cover map of the study area; KC and SR collected the GPS data from the collars; KC and SR analyzed the data and led the writing of the manuscript. KC, SR, and JC drafted the first version of the manuscript and all authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

The data used in this study are available on Movebank (movebank.org, study name "Foraging movements of Lyle's flying foxes in Cambodia (data from Choden et al. 2019)") and are published in the Movebank Data Repository (Choden et al., 2019). https://doi.org/10.5441/001/1.j25661td.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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From: Jon Epstein on behalf of Jon Epstein <epstein@ecohealthalliance.org>
To: Laing, Eric
Subject: Re: EHA NIH West Africa EIDRC_technical narrative_d1 - Invitation to edit
Date: Wednesday, June 12, 2019 4:45:35 PM
Attachments: Pteropus lylei priarily forages in residential areas in Kandal Cambodia_2019.pdf

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I don't. I think it's just an abstract.
Here's the telemetry paper.

On Wed, Jun 12, 2019 at 2:37 PM Laing, Eric <eric.laing@usuhs.edu> wrote:

> Thanks Jon, do you have an electronic copy of this article that you could share with me?
> http://agritrop.cirad.fr/585738/
>
> Eric D. Laing, Ph.D.
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> On Wed, Jun 12, 2019 at 2:29 PM Jon Epstein (via Google Docs) <drive-shares-noreply@google.com> wrote:
>
> Jon Epstein has invited you to **edit** the following document:
>
> EHA NIH West Africa EIDRC_technical narrative_d1
>
> Eric, Vincent - looking for your help writing preliminary data sections describing Luminex system, how it differentiates filoviruses, and experimental data supporting our ability to assess bat infections with MarV, RAVN, and Ebola viruses.
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BOHRN

The Bat / One Health Research Network

The 1st Annual Bat / One Health Research Network Workshop

8-9 November 2018 • Vienna, Austria

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Workshop Overview

Executive Summary

The Bat/One Health Research Network (BOHRN) convened its 1st Annual Research Workshop in Vienna, Austria, 7-8 November 2018, in advance of the International Meeting on Emerging Diseases and Surveillance (IMED). This two-day workshop was organized and hosted by the Defense Threat Reduction Agency, Cooperative Threat Reduction Directorate, Biological Threat Reduction Program (BTRP) in its capacity as a sponsor of life-sciences research-based Threat Reduction Networks (TRNs). This event provided an opportunity to advance BOHRN's core agenda of enabling interdisciplinary collaboration at the interface of biological threat reduction, research, and conservation.

The BOHRN initiative was organized at a side-meeting of the 2nd International Symposium on Infectious Diseases of Bats in Fort Collins, CO on 29 June 2017. During this meeting participants established a Steering Committee and began preliminary actions to build a multi-disciplined, self-sustainable network to better characterize global threats of bat-borne pathogens and formalize community standards and conservation- conscientious practices for One Health disease research. During a series of follow-on meetings, members of the BOHRN Steering Committee identified objectives and developed a research strategy to prioritize and target common needs. The BOHRN 1st Annual Research Workshop in Vienna provided an opportunity to validate its research strategy with a wider audience.

The workshop began with a series of introductory presentations from Dr. Martha Stokes, DTRA BTRP, who provided background on her organization and the BOHRN effort. There were also a series of presentations from other subject matter experts who provided short lectures on areas were identified as knowledge gaps by members of the Steering Committee at previous BOHRN meetings (note: the full agenda may be found here). Next, workshop attendees participated in two breakout sessions. The first session focused on the research focus areas within the four (4) BOHRN Working Groups and aimed to solicit feed-back in real time on the short and long-term objectives within each network working group.

The second breakout session was initiated by an interactive exercise, facilitated by Dr. Tigga Kingston (Texas Tech University) and Dr. Jon Epstein (EcoHealth Alliance), mapping the intersectional of ecological and epidemiological research questions. Participants were then divided into regional groups with diverse and varying levels of expertise to sketch out hypothesis-driven research projects that mapped to BOHRN working group focus areas. Members of the BOHRN Steering Committee and other experts were on-hand to provide mentorship and guidance. At the end of the workshop, each project was presented orally by a member of the project team in a mock peer review session for feed-back and discussion.

The output and recommendations gathered from the small-group sessions will inform BOHRN next steps, which Dr. Stokes described at the conclusion of the workshop as a series of special grant awards for project proposals under BOHRN. She described the process as 'still under construction' but affirmed her leadership's commitment to maintain the network's initial momentum. While the exact mechanism and criteria for award are still being discussed, all interested parties may anticipate a call for proposals via the BOHRN website at some point in the spring of 2019.

There are a number of factors that make bats unique disease reservoirs, including their social behavior, distinct physiology and metabolism, ability to travel long distance, nocturnal activity, species diversity, and long life-span. BTRP anticipates that by taking a lead in funding bat-associated pathogen research, their organization can play a significant role in better characterizing the role of bats in global zoonotic disease ecology, coupled with assessing the impact of human-mediated interactions and environmental changes, to better understand threat reduction value of surveillance and intervention efforts.

Previous BOHRN Events

BOHRN Kick-off Meeting

- Concurrent with 2nd International Symposium on EID
- Sponsored by BTRP
- Took place in Fort Collins, CO – 29 June 2017
- **Outcomes:** (1) established a steering committee; (2) drafted terms of reference; (3) identified research areas of interest

BOHRN Steering Committee Strategy Mapping Meeting – 1

- Concurrent with Prince Mahidol Award Ceremony
- Sponsored by BTRP
- Took place in Bangkok, Thailand – 30 January 2018
- **Outcomes:** (1) prioritized research focus areas; (2) developed targeted action plans; (3) drafted associated workplans and timelines

BOHRN Steering Committee Strategy Mapping Meeting – 2

- Concurrent with International One Health Congress
- Sponsored by BTRP
- Took place in Saskatoon, Canada – 20-21 June 2018
- **Outcomes:** (1) completed workplans and timelines for research focus areas; (2) established BOHRN branding and website; (3) drafted communication and outreach strategy

BOHRN Biological Threat Characterization Discussion

- Concurrent with Western Asia Bat Network (WABNet) Kickoff Meeting
- Sponsored by BTRP, organized by EcoHealth Alliance
- Took place in Tbilisi, Georgia – 20 September 2018
- **Outcomes:** (1) identified and characterized regionally-focused gaps and needs (2) activated communication and outreach strategy;

Workshop Outcomes

Presentation Summaries

The following subject matter experts were invited to present on areas that were identified as knowledge gaps in BOHRN. Event participants received a pdf copy of each presenter's slides.

Dr. Jon Epstein

Dr. Jon Epstein, EcoHealth Alliance, presented on *Understanding the Ecology of Emerging Zoonoses*. His presentation focused on the three stages of disease emergence to help understand the complexities of spillover. Starting with the first stage, wildlife and domestic animal interactions, Dr. Epstein explained the movement of microbes into domestic animals. Human's increasing interactions with domestic animals leads to the second stage where the microbe has spilled over into the human population causing widespread outbreaks. The third and final stage of disease emergence is the outbreak reaching pandemic levels. Dr. Epstein proceeded to present two cases Nipah Virus spillover from Pteropid Bats and Nipah Virus spillover from date palm sap harvesting. Both cases were used to support evidence that the driver of spillover is human activity. However, as Dr. Epstein explained, this does not account for why human infections occur a small areas of these bat's known habitats. Therefore, it is important to understand why spillover is only occurring in these small areas, whether it is a rare event or there is a need for more broad spread surveillance.

Dr. Jonathan Towner

Dr. Jonathan Towner, from the Center for Disease Control and Prevention, presented on *Filovirus Maintenance in Nature: Potential Lessons Learned from Studying Marburg Virus*. Dr. Towner's presentation focused on the persistence of Marburg Virus (MARV) in nature supported by the recent study findings that Egyptian Rousette bats are identified as a natural reservoir for MARV. The study looked at bats during birthing and breeding seasons in the Python Cave of Uganda and focused on the impact seasonal pluses have on human spillover. From this study, Dr. Towner presented on the need for messaging to miners and the community to emphasize the importance of bats to the ecosystem and the effects of culling the bats in Python Cave. In addition, the presentation focused on discussing virus transmission from bat to bat, long-term immunity in bats, and the potential to recreate the study with Ebola Virus.

Dr. Brian Bird

Dr. Brian Bird, from the University of California-Davis, presented on *Synergies Between the Bench and the Field for Virus Discovery and Capacity Building*. Dr. Bird's focused on the work of the USAID PREDICT program and the Ebola Host Project. Dr. Bird began his presentation by explaining the challenges of targeted, risk-based surveillance the PREDICT program focuses on. He led into a discussion on virus discovery and detection from identifying viruses by consensus polymerase chain reaction (PCR) supplemented by high-throughput screening (HTS) to performing experiments to understand and rank the potential risk of the virus. Dr. Bird then explained the process PREDICT uses to strengthening laboratory efforts and used the Ebola Host Project in Sierra Leone as an example of

these efforts. The Ebola Host project has led to the training of numerous local scientists and the development of community outreach materials. Dr. Bird's presentation summarized the efforts in Sierra Leone to focus on Filoviruses which has led to identifying new Ebola viruses in insect eating bats before known human or animal sickness.

Dr. Susan Tsang

Dr. Susan Tsang presented on *Flying Foxes as Bushmeat in Sulawesi Indonesia, Building Community Outreach Initiatives Based on Novel Understanding of Who, Where, and Why*. Dr. Tsang began by identifying the common challenges in institutional capacity, identification of stakeholders, interagency coordination, and funding. Her presentation then focused on the flying foxes as bushmeat and the cultural understanding of the drivers for how and why bats are hunted. Dr. Tsang used the outreach initiatives in Sulawesi, Indonesia to emphasize that outreach must include regional level coordination to allow for national level communication at both the front and tail end of any project. In addition, outreach should be designed for the community and the importance of assessing effective ways to disseminate information. Dr. Tsang explained potential resolutions to the common challenges could include providing training on outreach, incorporating voices from all levels of policy, and demonstrating the value to other sectors for interdisciplinary funding.

Breakout Session 1 Overview

In advance of the first breakout session, Dr. Jon Epstein (EcoHealth Alliance) and Dr. Tigga Kingston (Texas Tech University) provided an update from the Steering Committee, summarizing a year's worth of Steering Committee Strategy Sessions. They presented two - three slides per Working Group, summarizing the group's mission, focus areas, objectives, measurements of success, challenges, and timelines. The new participants, who had not been part of previous BOHRN strategy sessions, were able to discuss the slides as a large group, before breaking out into smaller groups to provide constructive feedback and guidance based on their knowledge and experiences. Breakout session discussions led to the development of cross-cutting recommendations on capacity for in region repositories and curation of voucher material and the implementation of a data-sharing culture. The outcome of these suggested recommendations will ultimately build an additional working group.

Steering Committee Presentations

BOHRN planners collated and drafted the following material from the BOHRN strategy sessions, to provide a visual tool to solicit feedback from a group of new stakeholders. This information was presented in slide-form as an introduction to the large-group discussions and break-out group sessions.

Working Group 1: researching host-pathogen biology and interactions

MISSION: EXPLAIN THE DETERMINANTS OF PATHOGEN TOLERANCE, TRANSMISSION, AND SPILLOVER FROM BATS AT INDIVIDUAL AND POPULATION LEVELS

Established Working Group 1 Research Focus Areas

Bat physiology and immunology	Distributions of pathogen amongst species	Bat pathogen community biology	Modeling approaches for host dynamics and epidemiology

Objective		Outcomes
Complete a systematic review of knowledge gaps on model systems	→	Publish a systematic review of modeling systems and knowledge gaps that were defined
Identify modeling systems that are representatives of all geographic and phylogenetic areas	→	Modeling systems are defined, characterized, and validated
Evaluate the transmission risks and spillover pathogens to another animal host	→	Intrinsic and extrinsic risk factors are identified for major diseases and geographic areas

Overall Challenges: (1) Objectives require multidisciplinary team: (2) consortia would be needed for modeling systems review and validation

Established Working Group 1 Research Projects and Activities Priority Timeline

Short-term project / activity pipeline *6 -12 months*	Long-term project / activity pipeline *12 months +*
Map funding landscape ⇒ Identify funders ⇒ Host a funders meeting	Conduct long-term lab and field studies ⇒ Develop cell lines and bat animal models ⇒ IgM immunoassay ⇒ Develop methods for determining the age of bats ⇒ Determine the timing of viral shedding and the effects of environmental stresses ⇒ Determine co-infection in bat species ⇒ Determine temperate versus tropical variables associated with infection (hibernation periods / viral replication) ⇒ Understand climate change with respect to physiology ⇒ Develop heat stable preservatives ⇒ Develop smaller telemetry and physiology sensors

Working Group 2: researching pathogen surveillance, diagnostic capacity and epidemiology

MISSION: FORM REGIONAL NETWORKS TO ESTABLISH A COMMON METHODOLOGY FOR SURVEILLANCE OF HUMAN AND ANIMAL HEALTH; BETTER UNDERSTAND SPILLOVER RISKS AND EPIDEMIOLOGY OF BAT PATHOGENS

Established Working Group 2 Research Focus Areas

Molecular epidemiology	Geographic and phylogenetic distribution of pathogens	Detection, diagnosis, and reporting of bat-borne pathogens	Established guidance and protocols for sampling

Objective		Outcomes
Conduct a gap analysis of existing diagnostic tools	→	Publish review of epidemiology of known bat-borne pathogens
Conduct outreach to various groups of researchers and build awareness amongst public and science community	→	Established and linked regional networks of practice and expertise
Establish a common methodology for surveillance	→	A better understanding of the risks associated with spillover and established standards for surveillance and reporting

Overall Challenges: (1) The logistics and bureaucracy of creating a multidisciplinary team of international experts; (2) funding to support and sustain efforts to standardize surveillance

Established Working Group 2 Research Projects and Activities Priority Timeline

Short-term project / activity pipeline *6 -12 months*	Long-term project / activity pipeline *12 months +*
Conduct a gap analysis of diagnostic tools ⇒ Identify list of labs and contacts ⇒ Create a list for priority interventions / assistance ⇒ Analyze return data; publish resource lists	Conduct surveillance platform assessment ⇒ Conduct a literature review of previous surveillance platform assessments ⇒ Identify most beneficial platform for animal and human health data information sharing ⇒ Identify most logical platform for low resource settings ⇒ Identify the best field-forward platforms

Working Group 3: researching ecology (bat, domesticated animals and wildlife interface)

MISSION: DEFINE HOW AND TO WHAT EXTENT THE ECOLOGICAL CONTEXT OF BATS, AND THE HUMAN INFLUENCE ON THAT CONTEXT, INFLUENCE PATHOGEN DYNAMICS AND SPILLOVER THREATS

Established Working Group 3 Research Focus Areas

Bat behavior, distribution and movement	Effect of anthropogenic disturbance and modification on pathogen dynamics	Domesticated animals and wildlife behavior, distribution, and movement impact on interaction with bats

Objective		Outcomes
Engage the ecological community to define system uniqueness and interdependencies	→	Pathogen research community acknowledges and integrates ecological systems and interdependencies
Advocate for ecological design and analysis frameworks to pathogen research	→	BOHRN research projects are designed using a framework for well-balanced outcomes
Build capacity for disease researchers to gather ecological data to provide context for their studies	→	More funded studies return ecological data
Define emerging ecological principles that could inform spillover threats	→	Emerging ecological principles become widely accepted governing principles for practice
Establish key messages and conduct efforts to promote a culture of conservation amongst One Health researchers, practitioners, and stakeholders	→	BOHRN establishes itself as a consistent and unbiased perspective from the community and its statements are widely accepted and distributed

Overall Challenges: (1) Science communities have polarized and insular view of bats and diseases; (2) lack of collaboration and communication efforts

Established Working Group 3 Research Projects and Activities Priority Timeline

Short-term project / activity pipeline *6 -12 months*	**Long-term project / activity pipeline** *12 months +*
Conduct conservation / One Health literature review ⇒ Establish parameters ⇒ Conduct literature review ⇒ Quantify interdisciplinary relationships w/ assessment of numbers of publications ⇒ Publish results Establish ecology tool / training aid kits ⇒ Identify and source materials	N/A ⇒ N/A

- ⇒ Collect and build case-control studies for training
- ⇒ Develop training plans
- ⇒ Distribute through BOHRN

Working Group 4: researching human-bat interactions

MISSION: FULLY DEVELOP, UNDERSTAND, AND COMMUNICATE THE BAT AND HUMAN INTERFACE TO KEY STAKEHOLDERS AND COMMUNITIES

Established Working Group 4 Research Focus Areas

Hunting and commodity chain	Human behavioral risk characterization	Interactions in human dwellings	Ecotourism

Objective		Outcomes
Develop and test policy interventions for specific human-bat interfaces	→	Policy interventions for human-bat interfaces are developed and put into place
Communicate key findings to stakeholders	→	Effectively communicate and publish findings of studies
Develop global risk maps to assess existing data and validate risk maps	→	Publish global risk maps highlighting geographic areas of risk
Identify high-risk groups and develop education platforms to measure knowledge, attitude and practices	→	Getting community buy-in and understanding of concepts

Overall Challenges: (1) Truthful responses in behavior research on bat-human interactions; (2) accuracy of risk map and models; (3) cultural barriers and beliefs

Established Working Group 4 Research Projects and Activities Priority Timeline

Short-term project / activity pipeline *6 -12 months*	Long-term project / activity pipeline *12 months +*
⇒ Develop global risk maps ⇒ Survey high-risk groups for their KAP	Conduct research studies / support for ecology ⇒ Develop and validate education platforms ⇒ Research to measure changes in KAP ⇒ Validate ground-truth risk maps

⇒ Adapt education platforms / materials

Recommendations

BOHRN organizers invited many researchers from diverse backgrounds at varying levels of professional experience to the Vienna workshop. This approach facilitated lively discussions and prompted the Steering Committee to consider new objectives, priorities, and perspectives within their previously established Working Group bounds. The following recommendations were captured by note-takers, observers, and other members of the Steering Committee, and will be marked for further discussion and adjudication during BOHRN's next Steering Committee meeting which will be held at International Bat Research Conference (IBRC) in Phuket, Thailand 2019.

Working Group 1: researching host-pathogen biology and interactions

Breakout Session Recommendations: members of the breakout group accepted the overall mission and objectives that Working Group 1 had established and proposed adding two additional research focus areas: (1) the role of bat taxonomy in host-pathogen coevolution and (2) host specificity in bat-borne pathogens. Members of the breakout group also proposed the following additions to the priority timeline:

- ⇒ Establish species identification consensus tools and techniques – such as the role of bar coding and other methods
- ⇒ Host or link to public-facing databases (e.g., Vertnet, National Science Foundation digitized database)
- ⇒ Identify regional resource repositories for voucher materials
- ⇒ Establish sustainable freezer network
- ⇒ Develop funding models for in-country collection curation capacity building / field sample collection transfer (business plans, logistics, maintenance, training)
- ⇒ Establish a database of reagents
- ⇒ Establish a list of international regulatory experts for transport of select agent materials (e.g., Bombali ebolavirus discovery and the issues they had with reporting and transfer)

Working Group 2: researching pathogen surveillance, diagnostic capacity and epidemiology

Breakout Session Recommendations: members of this breakout group generally accepted the mission and objectives that Working Group 2 had established. They proposed amending the research focus area for "Molecular Epidemiology" to include "Molecular and Serological Epidemiology". They also proposed the following additions to the priority timeline:

- ⇒ Establish a set of common research questions and topics related to biosurveillance data-type (syndromic, diagnostic, environmental) associated with bat-borne pathogen threats
- ⇒ Establish a catalog of surveillance models
- ⇒ Develop a sera and antibody collection with a standardized pool of collection
- ⇒ Conduct studies that integrate bat ecology and pathogen research (One Health research team that collects virology and ecological data at the same time)

- ⇒ Establish a list of minimum biosecurity / biosafety protocols for research (lab / field) and recommended sample sets / study
- ⇒ Establish a list of laboratories with bat sample repositories (by region and country
- ⇒ Establish a registry of "Bat Experts" by region and country
- ⇒ Identify diagnostic capabilities (person / institution)
- ⇒ Develop a hypothesis map
- ⇒ Outline funding mechanisms for other BOHRN stakeholders

The breakout group also recommended that any efforts to seek "standardization" (surveillance platforms) should use the phrase "common framework" as methods and implementation will vary in different countries and regions.

Working Group 3: researching ecology (bat, domesticated animals and wildlife interface)

Breakout Sessions Recommendations: during the breakout session, members of this group did not have any substantial modifications to the Working Group's mission, focus areas, or objectives. They did provide several ideas long-term timeline priorities, which included:

- ⇒ Conduct ecological and taxonomic studies that support disease research (and threat reduction), this will create a demand for ecologists to collect samples and will ultimately capacity for ecology through training and networking
- ⇒ Identify ecological and taxonomic gaps at local levels

Since much of Working Group 3's approach was built around the development of training modules, the group discussed training and the importance of tailoring existing projects / programs. They talked about sustainability in bat research programs and mechanisms for incentivization, offering ideas such as scholarships at the end of a short research project or using a training workshop as a research candidate selection opportunity.

Working Group 4: researching human-bat interactions

Breakout Sessions Recommendations: members of this breakout group did not have any major changes to the Working Group's mission, focus areas, or objectives. They did, however, want to emphasize the importance determining *where* human behavioral risks are the highest and *what* drives specific human bat interactions and the need to map these interactions accordingly. With regard to the timeline priorities, they made the following recommendations:

- ⇒ Characterize the risk map with priorities
  - DTRA (BTRP) priority pathogens, USG priority pathogen threats, WHO regional threats
  - Chart recent pandemics with drivers (e.g., bush meat markets overlaid with outbreaks)
- ⇒ For database define the approach to obtain data; Bat Conservation International (example), Bat-Plant.com for ecology interactions

Breakout Session 2 Overview

The first breakout session provided a foundation for the second breakout session during which participants formed into regional teams to craft research projects within the bounds of the BOHRN Working Group research focus areas. BTRP intends to fund several high priority threat reduction projects in FY19-FY20 and developed this exercise to test the viability of the network's strategy thus far. The

projects that were developed will not be summarized in this report, as they may be part of future project proposal; however, the images below show the work, collaboration, and collegial spirit of this session.

Participant Feedback

After the BOHRN Workshop participants were sent an anonymous feedback survey via SurveyMonkey. The participants were asked the following six questions:

1. What did you like about this Workshop?
2. Do you think the objectives for the BOHRN Workshop were achieved? Please explain your answer.
3. What do you wish we did differently?
4. What does success of this network look like to you, for your field of study?
5. What do you think was the most important aspect of this Workshop?
6. Any other comments or suggestions?

Overall, the participants responded positively to the efforts accomplished at the first annual BOHRN Workshop. An appreciation for the multidisciplinary networking opportunities, the potential opportunities the network presents, and the alignment of breakout group work with the Workshop presentations was convey by all participants. One participant's comment reflects this in saying "the multidisciplinary networking opportunities for engaging the ecological context of emerging infectious disease and breakout sessions were a nice complement to the big group discussions." Participant feedback indicated that the BOHRN Workshop objectives were achieved but there was a need for further information on next steps and more opportunity for discussion after the final small group session. Suggestions for change were to extend the workshop for two whole days and provide more focus on funding the discussed research.

BOHRN Path Forward

As a result of this workshop, BTRP intends to release an announcement for research project funding in the early part of 2019. The official announcement will be released on the BOHRN website (www.BOHRN.net) and emailed to anyone who has participated in a BOHRN activity.

At the conclusion of the workshop, Dr. Martha Stokes presented draft criteria for project award consideration, which included:

- Performed in BTRP engagement countries
- Demonstrated commitment to capacity building in BTRP mission areas (biosafety and biosecurity, and biosurveillance)
- Demonstrated commitment to open science
    - Transparent sharing of knowledge and information
    - Should include a data curation plan and broad statement on information access
    - Sample sharing not require, but strongly encouraged and preferred
- Demonstrated commitment to One Health
    - Inter-disciplinary research teams
    - Local engagement plans or educational outreach

- Include early to mid-career project investigators
- Address cross-cutting themes of BOHRN
    - Projects should be tied to no less than two working groups
    - Projects should be tied to no less than one focus area within each working group
- Include mentorship from member of steering committee or a Steering Committee/Executive Committee-approved designee (correlates to respective working group(s))

These factors are still under consideration and BTRP may change any or all. The only information regarding "Criteria for Eligibility" for a BOHRN grant/project award will be released on BOHRN.net. The timeline for award will also be released on BOHRN.net.

Annex 1: Agenda

Day 1

Time	Session	Objectives
0700 – 0800	**Closed-door Steering Committee Meeting**	
0730 – 0810	**Photo Registration (for non-steering committee members)**	
0810 – 0845	**Welcome and Introductions** Marty Stokes *Biological Threat Reduction Program (BTRP)*	Welcome all participants, provide four slides about BTRP and TRNs All participants go around the room and introduce name and organization
0845 – 0900	**BOHRN Overview** Marty Stokes *Biological Threat Reduction Program (BTRP)*	Provide an overview about BOHRN, its mission and objectives; make sure to discuss (1) the funding opportunity; (2) the principles of capacity building / mentorship
0900 – 0910	**BOHRN Workshop Agenda, Objectives, and Housekeeping** Katie Leahy *Global Systems Engineering*	Provide overview of meeting objectives, scheme of maneuver, and other housekeeping items
Session 1: BOHRN Focus Group Progress and Work		
0910 – 0930	**Understanding the Ecology of Viruses** Jon Epstein *EcoHealth Alliance*	Discuss the challenges and understanding of the ecology of viruses such as Nipah and Ebola
0930 – 0950	**Host/Pathogen Interaction** Jon Towner *CDC- Division of High-Consequence Pathogens and Pathology*	Present on work focusing on viruses in the national reservoir hosts and determine the mechanisms by which the viruses are maintained in nature
0950 – 1010	**Laboratory Response** Brian Bird *UC Davis, School of Veterinary Medicine*	Synergies between the Bench and the Field: Rift Valley Fever and Ebola
1010 – 1030	**Building Policy and Community Outreach Initiatives Based on a Novel Understanding of Who, What, and Why** Susan Tsang *American Museum of Natural History and National Museum of the Philippines*	Discuss efforts to bridge policy gaps between local, national, regional, and international efforts
1030 – 1110	**Focus Area Research Mentor Progress Reports**	A representative or mentor from each group will present their Focus Area

		objectives, its long / short-term goals schedule, and progress Group 1: 10 min Group 2: 10 min Group 3: 10 min Group 4: 10 min
1110 – 1215	**Breakout Groups**	Participants will be broken into the focus area groups; their placement will be pre-arranged by decision of the steering committee and they will have a sticker on the back of their name card; they will be asked to listen in on the focus area group discussion, see if they could contribute to the group's direction
1215 – 1320	**Working Lunch**	
1320 – 1400	**Breakout Group Open Discussion**	Each group will present any changes to their schedules or objectives Group 1: 10 min Group 2: 10 min Group 3: 10 min Group 4: 10 min
Session 2: BOHRN Project Development Work		
1400 – 1430	**Doing Business with BTRP: Pathways to Contracts, Objectives for BOHRN and Beyond** Dr. Martha Stokes *BTRP*	20 Minute presentation of slides that Lance gave in Georgia, plus 1-2 developed with Scott V., plus 10 minutes for questions from the audience; this presentation will queue funding project development for focus area-specific RFPs
1430 – 1600	**Interactive Illustration Hypothesis Mapping Exercise**	Dr. Kingston and Dr. Epstein will facilitate an interactive hypothesis mapping session for the group
1600 – 1715	**Breakout Groups**	Breakout into blended project development groups. These groups will be based on seating arrangement (e.g., tables 1 and 2 will work together) to ensure that we have multi-disciplinary efforts.
1715 – 1730	**Close-out Day 1 and Review Day 2**	
1830 – 2000	**Dinner / Social Event** **Quad Chart / Poster Presentations**	

Day 2

Time	Session	Objectives
0900 – 1130	**Small Group Project Development Work**	Groups will come back to the main room to continue work in the smaller group project development
1000 – 1030	**Working Tea Break**	
1130 – 1300	**Working Lunch Break / Small Group Brief-outs**	
1300 – 1315	**Close-out / Group Discussion**	
TBD	**Steering Committee Meeting**	

Annex 2: Participant list

Name	Country	Organization
Abel Wade	Cameroon	National Veterinary Laboratory, Cameroon
Wanda Markotter	South Africa	University of Pretoria, Dept of Microbiology and Plant Pathology
Benneth Obitte	Nigeria	Texas Tech University
Iroro Tanshi	Nigeria	Texas Tech University
Joram Buza	Tanzania	Nelson Mandela African Institute of Science and Technology
Robert Kityo	Uganda	Makerere University, Kampala
Julian Lutwama	Uganda	Uganda Virus Research Institute
Astghik Ghazaryn	Armenia	Yerevan State University
Ioseb Natradze	Georgia	Ilia State University
Keti Sidamonideze	Georgia	National Center for Disease Control and Public Health -Georgia
Lela Urushadze	Georgia	National Center for Disease Control and Public Health - Georgia
Nesreen Alhmoud	Jordan	Royal Scientific Society
Meryem Lemrani	Jordan	Pasteur Institute in Morocco
Ehab Abu-Basha	Jordan	Jordan University of Science and Technology
Shusmita Dutta	Bangladesh	University of North Bengal
Ariful Islam	Bangladesh	EcoHealth Alliance
Shahanaj Shano	Bangladesh	Jahangirnagar Univeristy
Pilot Dovih	India	National Centre for Biological Sciences
Juliana Senawi	Malaysia	University of Kebangsaan Malaysia
Philip Alviola	Philippines	University of the Philippines-Los Banos
Catalino Demetria	Philippines	Research Institute for Tropical Medicine
Benjamin Lee	Singapore	Duke-NUS, Singapore
Sara Bumrungsri	Thailand	Prince of Songkla University
Pipat Soisook	Thailand	Princess Maha Chakri Sirindhorn Natural History Museum
Supaporn Wacharapluesadee	Thailand	WHO CC for Research and Training in Viral Zoonoses, King Chulalongkorn Memorial Hospital, Thailand
Vu Dinh Thong	Vietnam	Institute of Ecology and Biological Resources
Patrick Ayscue	United States	Metabiota
Brian Bird	United States	University of California- Davis
Bradford Brooks	United States	Metabiota
Jon Epstein	United States	EcoHealth Alliance
Jason Farlow	United States	Metabiotia
Tracey Goldstein	United States	University of California- Davis
Andreas Handel	United States	University of Georgia

Rebekah Kading	United States	Colorado State University
Tigga Kingston	United States	Texas Tech University
Eric Laing	United States	Uniformed Services Health Service University
Kendra Phelps	United States	EcoHealth Alliance
Mariano Sanchez-Lockhart	United States	United States Army Medical Research Institute for Infectious Diseases - Genomic Center
Jonathan Towner	United States	Center for Disease Control and Prevention, Viral Special Pathogens Branch
Susan Tsang	United States	Royal Scientific Society
Marty Stokes	United States	DTRA Biological Threat Reduction Program
Steve Becker	United States	DTRA A&AS
Katie Leahy	United States	Global Systems Engineering
Chris Russell	United States	Global Systems Engineering
Jason Hudson	United States	Global Systems Engineering
Megan Hudson	United States	Global Systems Engineering

From:	Megan Hudson on behalf of Megan Hudson <megan.hudson@globalsyseng.com>
To:	nisreen.hmoud@rss.jo; joram.buza@nm-aist.ac.tz; tigga.kingston@ttu.edu; kityrob@gmail.com; spwa@hotmail.com; abelwade@gmail.com; wanda.markotter@up.ac.za; pilotdovih@gmail.com; abubasha@just.edu.jo; meryem.lemrani@pasteur.ma; wava_38@hotmail.com; c_demetria@yahoo.com.ph; paalviola@up.edu.ph; phelps@ecohealthalliance.org; epstein@ecohealthalliance.org; rebekah.kading@colostate.edu; sara.b@psu.ac.th; sarabumrungsri@gmail.com; julianas@ukm.edu.my; wiantoro@gmail.com; benjamin_lee@nparks.gov.sg; lisamariep10@gmail.com; pipat66@gmail.com; vudinhthong@hotmail.com; iroro.tanshi@ttu.edu; benneth.obitte@ttu.edu; jjlutwama03@yahoo.com; jit8@cdc.gov; astghik.ghazaryan@ysu.am; ioseb.natradze@iliauni.edu.ge; ksidamonidze@gmail.com; l.urushadze@ncdc.ge; Katie Leahy; Megan Hudson; Chris Russell; Jason Hudson; mariano.sanchez-lockhart.ctr@mail.mil; farlowscience@gmail.com; payscue@metabiota.com; bbrooks@metabiota.com; docshusmitadutta@gmail.com; shahanajshano@gmail.com; arif@ecohealthalliance.org; eric.laing.ctr@usuhs.edu; stsang@amnh.org; bhbird@ucdavis.edu; ahandel@uga.edu; tgoldstein@ucdavis.edu
Cc:	Stokes, Martha M CIV (USA); Becker, Stephen M CTR DTRA J3-7 (US); Katie Leahy
Subject:	Bat One Health Research Network
Date:	Wednesday, February 20, 2019 12:00:39 PM
Attachments:	BOHRNVienna_FinalReport[1].pdf image001.png

Dear all,

On behalf of Dr. Marty Stokes, please find the final report for the BOHRN Workshop in Vienna.

As discussed in Vienna, there are several action items for the BOHRN network. In order to move forward on several of these items, we ask that you take a few moments to answer the following questionnaire. This survey will help us to identify BOHRN's efforts and progress towards its overarching goals and evaluate the networks threat reduction efforts. Please follow the link and complete the survey no later than 28 February: https://www.surveymonkey.com/r/6FQPQR3

Additionally, please use the following Drop Box link for access to the BOHRN Workshop participant list with pictures and the quad charts submitted by all participants. You may also access the video of the BOHRN Workshop here.

We had hoped to make a more formal announcement regarding solicitation for BOHRN special projects around this time; however, BTRP is internally still reviewing necessary criteria for award and will not be ready to make a more formal announcement until the April / May timeframe. The announcement will be released via the www.bohrn.net website.

Please let us know if you have any questions or concerns.

Kind Regards,

Megan

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