From: Rocke, Tonie E
To: Luke Hamel; Daszak Peter
Subject: Fwd: [EXTERNAL] PREEMPT - A few important items
Date: Tuesday, March 27, 2018 12:36:26 PM
Attachments: DARPA PREEEMPT DEFUSE full v2 JU TR 032618.docx

Hi Luke: Just checking to make sure this email got to you yesterday with my additions (deliverables, bio) and corrections. Thanks -Tonie

---------- Forwarded message ----------
From: **Rocke, Tonie** <trocke@usgs.gov>
Date: Mon, Mar 26, 2018 at 3:54 PM
Subject: Re: [EXTERNAL] PREEMPT - A few important items
To: Luke Hamel <hamel@ecohealthalliance.org>
Cc: Daszak Peter <daszak@ecohealthalliance.org>, Tonie Rocke <terocke@gmail.com>, "Abbott, Rachel" <rabbott@usgs.gov>, "Richgels, Katherine" <krichgels@usgs.gov>, Jonathon Musser <musser@ecohealthalliance.org>, Evelyn Luciano <luciano@ecohealthalliance.org>

Hi Luke: Attached are my comments on the full draft; I added my comments to Jerome's draft. I corrected a few errors, deleted at least one sentence in my section, and also added my deliverables for Task 7. I'm not certain what you want for project metrics. A timeline or something? Just repeating the deliverables doesn't seem appropriate. Also, I have someone checking on the CAGE code. Let me know if you need anything else. Thanks! -Tonie

On Mon, Mar 26, 2018 at 2:32 PM, Luke Hamel <hamel@ecohealthalliance.org> wrote:

> Hi Tonie,
>
> Thank you for the facility description. There won't be a need for us to have a call, but please review the draft and look for ways to reduce the text of your technical section.
>
> Additionally, I was hoping to confirm the following two points:
> (1) Is NWHC's CAGE code the following? **52Y40**
> (2) Which of the following 'organization types', best describes NWHC?
> -"LARGE BUSINESS", "SMALL DISADVANTAGED BUSINESS", "OTHER SMALL BUSINESS", "HBCU", "MI", "OTHER EDUCATIONAL", OR "OTHER NONPROFIT";
>
> Best,
>
> **Luke Hamel**
> *Program Assistant*
>
> EcoHealth Alliance
> 460 West 34th Street – 17th floor
> New York, NY 10001
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> ***EcoHealth Alliance leads cutting-edge scientific research into the critical connections between human and wildlife health and delicate ecosystems. With this science, we develop solutions that prevent pandemics and promote conservation.***

On Mon, Mar 26, 2018 at 3:16 PM, Rocke, Tonie <trocke@usgs.gov> wrote:

> Hello again Luke: Here is a brief description of our facility. Let me know if this is sufficient. Also, will we be having a call or not? I have not yet had a chance to review the technical proposal but will do so shortly.
>
> On Mon, Mar 26, 2018 at 2:01 PM, Luke Hamel <hamel@ecohealthalliance.org> wrote:
>
> > Excellent. Thank you for all of your work on this, Tonie! I will speak with Jonathon and address your question regarding the DARPA kick-off meeting.
> >
> > Best,
> >
> > **Luke Hamel**
> > *Program Assistant*
> >
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>
> On Mon, Mar 26, 2018 at 2:58 PM, Rocke, Tonie <trocke@usgs.gov> wrote:
>
> > Hello Luke: Attached is my budget justification (word and updated excel files) and my final budget. I found several mistakes in the original budget (miscalculations in the travel budget) which I fixed and also when time was added to my salary, the fringe was not adjusted. Thus the budget is slightly different but not by much. I think I have caught everything and it all adds up now, but feel free to check. I will send facility description along soon. Thanks -Tonie
> >
> > On Mon, Mar 26, 2018 at 8:28 AM, Luke Hamel <hamel@ecohealthalliance.org> wrote:
> >
> > > Hi Tonie,
> > >
> > > I hope you had a great trip. If you are able to begin drafting a budget justification, that would be very helpful. Whatever you cannot complete, we will be sure to get done.
> > >
> > > Regarding the budget justification, I have reattached a template with appropriate headings and language that is already correctly formatted. **I would just ask you to insert the appropriate name/cost amount, substituting CAPITALIZED words and filling in gaps (indicated by underscores).**
> > >
> > > Each section in the budget (e.g. Personnel, fringe, travel, etc.) should have a corresponding section in the budget justification (as shown in the template). Essentially, any line item that is listed in the budget needs to be justified in the 'budget justification' document.
> > >
> > > Best,

Luke Hamel
Program Assistant

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On Mon, Mar 26, 2018 at 8:00 AM, Rocke, Tonie <trocke@usgs.gov> wrote:

> Hi Luke: I have returned from Mexico and just wading through email. Do you still need me to prepare a budget justification in a word document (everything was in the excel file) this AM? I'll get on it right away if it hasn't already been done. Please advise. Best -Tonie
>
> On Sat, Mar 24, 2018 at 1:29 PM, Luke Hamel <hamel@ecohealthalliance.org> wrote:
>
> > Hi Tonie,
> >
> > It looks as though we have a detailed budget from you, but we still will need a budget justification (essentially a Word doc. that provides justification for each line item of the budget).
> >
> > Rachel and Katie - If you have time this weekend to get a start on the budget justification doc, that would be very helpful. If you're not available, which I understand may very well be the case, we will be happy to take this on. Please let us know.
> >
> > Thank you,
> >
> > **Luke Hamel**
> > *Program Assistant*
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On Fri, Mar 23, 2018 at 4:46 PM, Tonie Rocke <terocke@gmail.com> wrote:

Hello all: I sent my budget justification to Ana several days ago. Thanks for addressing safety issues Rachel!

Sent from my iPhone

On Mar 23, 2018, at 2:38 PM, Abbott, Rachel <rabbott@usgs.gov> wrote:

> Hi Luke,
> I have added some paragraphs to the page you sent. It just deals with safety of RCN, so I hope that is enough. Most of the text came out of documents we have to write to get approval to use our RCN vaccines in the field (risk analysis for USDA CVB and environmental assessment for USGS). Unfortunately, as I said before, I'll be unavailable until next Thursday, but Tonie should be back in her office on Monday.
> --Rachel
>
> On Fri, Mar 23, 2018 at 1:45 PM, Luke Hamel <hamel@ecohealthalliance.org> wrote:
>
> Hi Rachel and Katherine,
>
> I wanted to address a few important PREEMPT items with you:
>
> - Regarding **NWHC budget justification**
>   - If you have not already done so (and I apologize for not knowing the answer), could you please send us your budget justification document? We are hoping to have all collaborator budgets and budget justifications as soon as possible.
> - Regarding language on **'Long-term safety and efficacy'**
>   - In the PREEMPT proposal, we must state how we will establish methods to assess the 'long-term safety and efficacy of our preemptive approaches'
>   - Given your field of work, do you have any existing language on how to address potential negative impacts of intervention approaches on non-target species?
>   - I have attached language from the BAA to provide you with further guidance on what DARPA requires us to include.
>   - This being said - Rachel and Katherine, could you please write-up a short section (a paragraph or so), that addresses this issue 'long-term safety and efficacy'?
>   - I apologize for the extremely short notice, but **we would greatly appreciate it if you could return this to us by tomorrow afternoon, Sat.**

(3/24).

- Regarding '**pricing assumptions**' for NWHC facilities
  - Previously, we had asked you to identify any 'pricing assumptions' that may correspond with use of government facilities. Due to confusion about what exactly was being asked for, we reached out to DARPA staff, asking them to clarify the matter:
    - We asked: "EcoHealth Alliance has a USG entity listed as a subcontractor in our proposal. Is the USG entity required to identify any pricing assumptions beyond those within their fully detailed and documented budget?
    - To which they responded: "No"
      - Long story short...there is NO need for you to identify any additional pricing assumptions.

Thank you and please let me know if you have any questions. I will be available by email and phone (mobile number listed below) over the weekend, should you need to contact me.

Best,

Luke Hamel
Program Assistant

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--
Rachel Abbott
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Fax: (608) 270-2415

<PREEMPT_Eco_Impacts_Risk_Plan RCA.docx>

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A. EXECUTIVE SUMMARY

Technical Approach: Our goal is to defuse the potential for spillover of novel bat-origin high-zoonotic risk SARS-related coronaviruses in Southeast Asia. **In TA1** we will develop **host-pathogen ecological niche models** to predict the species composition of bat caves across Southeast Asia. We will parameterize this with a full inventory of host and virus distribution at our field sites, three caves in Yunnan Province, China and a series of unique datasets on bat host-viral relationships. By the end of Y1, we will use these to create a prototype app for the warfighter that identifies the likelihood of bats harboring dangerous viral pathogens at any site across Asia. We will intensively sample bats at our field sites to sequence SARSr-CoV spike proteins, reverse engineer them to conduct binding assays, and insert them into SARS-CoV backbones to infect humanized mice to assess capacity to cause SARS-like disease. Our modeling team will use these data to build **machine-learning genotype-phenotype models** of viral evolution and spillover risk. We will uniquely validate these with human serology data through LIPS assays designed to assess which spike proteins allow spillover into people.

In TA2, we will evaluate two approaches to reduce SARSr-CoV shedding in cave bats: (**1) Broadscale Immune Boosting**, in which we will inoculate bats with immune modulators to upregulate their innate immune response and downregulate viral replication; **(2) Targeted Immune Priming**, in which we will inoculate bats with novel chimeric polyvalent recombinant spike proteins to enhance innate immunity against specific, high-risk viruses. We will trial inoculum delivery methods on captive bats including automated aerosolization, transdermal nanoparticle application and edible, adhesive gels. We will use stochastic simulation modeling informed by field and experimental data to characterize viral dynamics in our cave sites, to maximize timing, inoculation protocol, delivery method and efficacy of viral suppression. The most effective delivery method and treatments will be trialed in our experimental cave sites in Yunnan Province, with reduction in viral shedding as proof-of-concept.

Management Approach: Members of our collaborative group have worked together on bats and their viruses for over 15 years. The lead organization, EcoHealth Alliance, will oversee all modeling, lab, and fieldwork. EHA staff will develop models to evaluate the probability of specific SARS-related CoV spillover, and identify the most effective strategy for delivery of both immune boosting and immune targeting inocula. Specific work will be subcontracted to the following organizations:

- Prof. Ralph Baric, UNC, will lead the immune priming work, building on his track record in reverse-engineering and manipulating SARS-CoV, MERS-CoV and other virus spike proteins over the last two decades.
- Prof. Linfa Wang, Duke-NUS, will lead work on immune boosting, building from his groups' pioneering work on bat immunity.

- Dr. Zhengli Shi, Wuhan Institute of Virology will conduct viral testing on all collected samples, binding assays and some humanized mouse work.
- Dr. Tonie Rocke, USGS National Wildlife Health Center will develop a delivery method for immunological countermeasures, following from her work on vaccine delivery in wildlife, including bats.
- Dr. Jerome Unidad, PARC will develop an innovative aerosol technology that could work with a wide-range of formulations into a field-deployable device that can be used for large-scale inoculation of bats.

B. EXECUTIVE SUMMARY SLIDE

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C. GOALS AND IMPACT

Overview

The overarching goals of DEFUSE are:

- **Identify and model the spillover risk** of novel SARS-related CoVs in South and SE Asia
- Design and demonstrate proof-of-concept that interventions to upregulate the naturally low innate immunity of bats to viruses (**immune boosting**) and to high risk SARSr-CoVs in particular (**immune priming**) will transiently reduce spillover risk.

We will analyze, design and field-test a novel strategy to reduce risk of viral emergence from bats that will help protect the warfighter within SACOM and SEACOM, and will be scalable to other systems including Ebola virus, rabies and other bat-origin pathogens.

Innovation and uniqueness:

Bats harbor more emerging zoonoses than any other group of mammals, and are ubiquitous, abundant, wide-ranging and often overlooked. Despite this, other than PPE, there is no available current technology to reduce the risk of exposure to novel coronaviruses from bats. Models of bats' capacity to harbor viruses, of ecological and environmental drivers of their emergence, and of the evolutionary potential of different strains to spillover are rudimentary. No vaccines or therapeutics exist for SARSr-CoVs, and exposure mitigation strategies are non-existent. SARSr-CoVs are enzootic in Asian, African[1], and European bats[2] that roost in caves but forage widely at night, shedding virus in their feces and urine. The limitations of this lack of capacity are significant – we have recently shown evidence of spillover of SARSr-CoVs into people in China, unrelated to the original SARS pandemic, and have isolated strains capable of producing SARS-like disease in humanized mice that don't respond to antibody treatment or vaccination. These viruses are a clear-and-present danger to our military and to global health security because of their continuous circulation and evolution in bats and periodic spillover into humans in locations where surveillance is virtually nonexistent.

EcoHealth Alliance leads the world in predictive models of viral emergence. We will build on our machine-learning models of spillover hotspots, host-pathogen ecological niche and genotype-phenotype mapping by incorporating unique datasets to validate and refine hotspot risk maps of viral emergence in SE Asia and beyond. We have shown that bats are able to carry otherwise lethal viruses by virtue of dampened innate immunity (e.g. inflammatory) pathways, which likely evolved as an adaptation to the physiologic stress of flight. We will use this insight to design strategies, like small molecule Rig-like receptor (RLR) or Toll-like receptor (TLR) agonists, to upregulate bat immunity and down-regulate viral replication in their cave roosts, thereby significantly reducing the frequency and magnitude of viral shedding and spillover **(broadscale immune boosting strategy)**. We will complement this by treating bats with novel chimeric polyvalent recombinant spike proteins to enhance their adaptive immune response against specific, high-risk coronaviruses **(targeted immune priming strategy)**, especially when their innate immune response is boosted as above. We will design novel automated application methods, based on our previous work delivering wildlife vaccines, to apply these interventions in a way that eliminates the need for a person to enter a cave and potentially get exposed to bat borne viruses or other hazards.

Technical Area 1

Our strategy to reduce spillover risk of bat SARS-related CoVs begins with modeling to predictively assess spillover risk across South and SE Asia using baseline genotype-phenotype analysis of host and strain diversity from the literature, from surveillance in our designated model caves in China, and across the region in other projects. In TA1, the DEFUSE modeling and analytics team, will build joint species distribution models (JSDM) of environmental and

ecological correlates and traits of cave bat communities to predict species composition of bat caves across Southern China, South and SE Asia. Dr. Epstein at EHA will coordinate animal experimental work with the teams at NWHC, Duke-NUS and Wuhan and radio telemetry studies with the field surveillance team. We will then use a series of datasets we have built to produce host-virus risk models for the region. These include our comprehensive database of bat host-viral relationships and estimates of zoonotic viral richness per bat species[3]; biological inventory data on all bat caves in Southern China; and modeled species distribution data for all bats. We will parameterize the model with data from three cave sites in Yunnan, China (one with high-risk SARSr-CoVs, two other control/comparison sites), including: radio- and GPS-telemetry to identify home range and additional roost sites for each bat species; inventory of bat population density, distribution and segregation and their daily, weekly and seasonal changes; viral prevalence and individual viral load; shedding of low- and high-risk SARSr-CoV strains among bat species, age classes, genders; and telemetry and mark-recapture data to assess metapopulation structure and inter-cave connectivity. We will test and validate model predictions of a cave's viral spillover potential with data from prior PREDICT sampling in 7 other Asian countries. At the end of Yr 1, we will produce a prototype app for the warfighter that identifies the likelihood of bats harboring dangerous viral pathogens in a region. **The 'Spatial viral spillover risk' app** will be updated real-time with surveillance data (e.g. field-deployable iPhone and android compatible echolocation data) from our project and others, to ground-truth and fine-tune its predictive capacity.

The Wuhan Institute of Virology team will test bat fecal, oral, and blood samples for SARSr-CoVs. We will collect viral load data using fresh fecal pellets from individually sampled bats and from tarps laid on cave floors deployed where necessary to reduce roost disturbance. SARSr-CoV spike proteins will be sequenced, analyzed phylogenetically for recombination events, and high-risk viruses (spike proteins close to SARS-CoV) characterized and isolated. The UNC team will reverse-engineer spike proteins to conduct binding assay to human ACE2 (the SARS-CoV receptor). They will culture SARS-like bat coronaviruses to distinguish high-risk strains that can replicate in primary human cells and low risk strains that require exogenous enhancers. Viral spike glycoproteins that bind receptors will be inserted into SARS-CoV backbones, inoculated into human cells and humanized mice to assess capacity to cause SARS-like disease, and to be blocked by monoclonal therapies, the nucleoside analogue inhibitor GS-5734[4] or vaccines against SARS-CoV[4-8].

The EHA modeling team will use these data to **build models of risk of viral evolution and spillover**. These genotype-to-phenotype machine-learning models will predict viral ability to infect human host cells based on genetic traits and results of receptor binding and mouse infection assays. Using data on diversity of spike proteins, recombinant CoVs, and flow of genes within each bat cave via bat movement and migration, we will estimate evolutionary rates, rates of recombination, and capacity to generate novel strains capable of human infection.

Finally, virus-host relationship and bat home range data will be used to estimate spillover potential - extending models well beyond our field sites. We will then **validate model predictions of viral spillover risk** by **1)** conducting spike protein-based binding and cell culture experiments, and **2)** identifying spillover strains in people near our bat cave sites. Our preliminary work on this shows ~3% seroprevalence to SARSr-CoVs, using a specific ELISA [REF]. We will design LIPS assays to the specific high- and low- zoonotic-risk SARSr-CoVs identified in this project as we have done previously [REF]. We will use previously collected and newly collected human sera from these populations to test for presence of antibodies to the high- and low-risk SARSr-CoVs identified by our modeling. We will then **model optimal strategies to maximize treatment efficacy** for TA2, using stochastic simulation modeling informed by field and experimental data to characterize viral circulation dynamics in bats. We will estimate frequency and population coverage required for our intervention approaches to suppress viral spillover. We will determine the seasons, locations within a cave, and delivery methods (spray, swab, or automated cave mouth or drone) that will be most effective. Finally we will determine the time period treatment will be effective for, until re-colonization or evolution leads to return of a high-risk SARSr-CoV.

Technical Area 2

In TA2, we will **develop scalable approaches that target and suppress the animal virus in its reservoir(s)and/or vector(s), to reduce the likelihood of virus transmission into humans**. We will evaluate two approaches to defuse SARS-related CoV spillover potential**: 1) Broadscale Immune Boosting:** using the unique immune damping in bats that our group has discovered, we will apply immune modulators like bat interferon to live bats, to up-regulate their naïve immunity and then assess their ability to suppress viral replication and shedding; **2) Targeted Immune Priming:** building on preliminary development of polyvalent chimeric recombinant SARSr-CoV spike proteins, we will conduct application trials with live bats to assess suppression of replication and shedding of a broad range of dangerous SARS-related CoVs.

Both lines of work will begin in Yr 1 and run parallel. Prof. Linfa Wang (Duke-NUS) will lead the immune boosting work, building on his pioneering work on bat immunity[9] which shows that the long-term coexistence of bats and their viruses has led to equilibrium between viral replication and host immunity. This is likely due to down-regulation of their innate immune system as a fitness cost of flight[9]. The weakened functionality of bat innate immunity factors like STING, a central DNA-interferon (IFN) sensing molecule, may allow bats to maintain an effective, but not over-response to viruses[10]. A similar finding was observed for bat IFNA, which is less abundant but constitutively expressed without stimulation[11]. Given high native SARSr-CoV load in bats, we aim to boost bat innate immunity through the IFN pathway, break the host-virus equilibrium to suppress bat SARSr-CoV replication and shedding.

We will trial the following, concurrently and competitively, for efficiency, cost and scalability: **i)** Universal bat interferon. Aerosol spraying or intranasal application of IFN or other small molecules reduces viral loads in humans, ferrets and mouse models[12,13]. Interferon has been used clinically when antiviral drugs are unavailable, e.g. against filoviruses[14]. Replication of SARSr-CoV is sensitive to interferon treatments, as shown in our previous work[13] ; **ii)** Boosting bat IFN by blocking bat-specific IFN negative regulators. Uniquely, bat IFNA is naturally constitutively expressed but cannot be induced to a high level[11], indicating a negative regulatory factor in the bat interferon production pathway. We will use CRISPRi to identify the negative regulator and then screen for compounds targeting this gene; **iii)** Activating dampened bat-specific IFN production pathways which include DNA-STING-dependent and ssRNA-TLR7-dependent pathways. Our work showing that mutant bat STING restores antiviral functionality suggests these pathways are important in bat-viral coexistence[10]. By identifying small molecules to directly activate downstream of STING, we will activate bat interferon and promote viral clearance. A similar strategy will be applied to ssRNA-TLR7-dependent pathways; **iv)** Activating functional bat IFN production pathways, e.g. polyIC to TLR3-IFN pathway or 5'ppp-dsRNA to RIG-I-IFN pathway. A similar strategy has been demonstrated in a mouse model for SARS-CoV, IAV and HBV[12,15]; **v)** Inoculating crude coronavirus fragments to upregulate innate immune responses to specific CoVs – a partial step towards the targeted immune priming work below.

Prof. Ralph Baric (UNC) will lead the immune priming work. He will develop recombinant chimeric spike-proteins[16]from our known SARSr-CoVs, and those we characterize during project DEFUSE. The structure of the SARS-CoV spike glycoprotein has been solved and the addition of two proline residues at positions V1060P and L1061P stabilize the prefusion state of the trimer, including key neutralizing epitopes in the receptor binding domain[17]. In parallel, the spike trimers or the receptor binding domain can be incorporated into alphavirus vectored or nanoparticle vaccines for delivery, either as aerosols, in baits, or as large droplet delivery vehicles[6,18-21]. We will test these in controlled lab conditions, taking the best candidate forward for testing in the field. We have built recombinant spike glycoproteins harboring structurally defined domains from SARS epidemic strains, pre-epidemic strains like SCH014 and zoonotic strains like HKU3. It is anticipated that recombinant S glycoprotein based vaccines harboring immunogenic blocks across the group 2B coronaviruses will induce broad scale immune responses that simultaneously reduce genetically heterogeneous virus burdens in bats, potentially reducing disease risk (and transmission risk to people) in these animals for longer periods[22,23].

The immune dampening features are highly conserved in all bat species tested so far. Duke-NUS has established the only experimental breeding colony of cave bats (*Eonycteris spelaea*) in SE Asia. This genus is evolutionarily related to *Rhinolophus* spp. (the hosts of SARSr-CoVs), so we have confidence that results will be transferable. Our initial proof-of-concept tests will be in this experimental colony, extended to a small group of wild-caught *Rhinolophus*

sinicus bats at Wuhan Institute of Zoology. We (Prof. Wang) have previous experience conducting SARS-CoV infection experiments with *Rhinolophus* sp. bats in the BSL-4 facility at CSIRO, AAHL (L.Wang, unpublished results).

Finally, work on a delivery method for our immune boosting and priming molecules will be developed and implemented by Dr. Tonie Rocke at the USGS, National Wildlife Health Center who has previously developed animal vaccines through to licensure[24]. Using locally acquired insectivorous bats[25,26], we will assess delivery vehicles and methods including: 1) transdermally applied nanoparticles; 2) series of sticky edible gels that bats will groom from themselves and each other; 3) aerosolization via sprayers that could be used in cave settings; 4) automated sprays triggered by timers and movement detectors at critical cave entry points, and 5) sprays delivered by remote controlled drone. We have already used simple gels to vaccinate bats against rabies in the lab[25], and hand delivered these containing biomarkers to vampire bats in Peru and Mexico to show they are readily consumed and transferred among bats. In our bat colony, we will trial delivery vehicles using the biomarker rhodamine B (which marks hair and whiskers upon consumption) to assess uptake. The most optimal approaches will then be tested on wild bats in our three cave sites in Yunnan Province with the most successful immunomodulators from TA2. Fieldwork will be conducted under the auspices of Dr. Yunzhi Zhang (Yunnan CDC, Consultant at EcoHealth Alliance). A small number of bats will be captured and assayed for viral load and immune function after treatment, but so as not to disturb the colony, most viral load work will be conducted on fresh fecal pellets collected daily on the cave floor. EHA has had unique access to these sites for around 10 years, under the guidance of Drs. Shi and Zhang. In year 1 of project DEFUSE, we will seek permission for experimental trials from the Provincial Forestry Department. We expect to be successful, as we have worked with the Forestry Department collaboratively for 10 years, with support of the Yunnan CDC, and we are releasing molecules that are not dangerous to people or wildlife. EHA has a proven track record of rapidly obtaining IACUC and DoD ACURO approval for bat research.

Deliverables:

- App identifying geographical risk of spillover for novel SARSr-CoVs in SE Asia
- Identified indicators (modeled and validated) of spillover capacity for different viral strains.
- Proven mechanistic approach to modulating bat innate immunity to reduce viral shedding
- Tested and validated delivery mechanism for bat cave usage including vaccines in other bat host-pathogen systems (e.g. rabies, WNS).
- Proof-of-concept approach to transiently reducing viral shedding in wild bats that can be adapted for other systems including Ebola virus.

D. TECHNICAL PLAN

Technical Area I:

Choice of site and model host-virus system. For the past 14 years, our team has conducted coronavirus surveillance in bat populations across Southern China, resulting in <150 CoV identifications in ~10,000 samples[27-29]. Bat SARSr-CoVs are genetically diverse, especially in the S gene, and most are highly divergent from SARS-CoV. However, in a cave site complex in Yunnan Province, we have found bat SARSr-CoVs with S genes extremely similar to SARS-CoV, and which, as a quasispecies population assemblage contain all the genetic components of epidemic SARS-CoV[30].

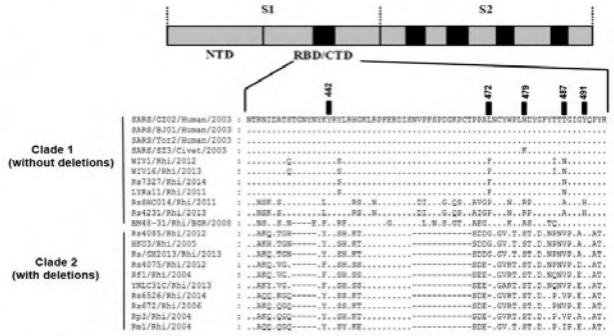

Fig. 1: Alignment of amino acid sequence of the receptor-binding motif in the spike protein of SARSr-CoVs and SARS-CoV[30]. Numbered amino acid is the key residues which is responsible for SARS-CoV S and human ACE2 interaction[31].

We have isolated three strains at this site (WIV1, WIV16 and SHC014) that unlike other SARSr-CoVs, do not contain two deletions in the receptor-binding domain (RBD) of the spike, and share substantially higher sequence identity to SARS-CoV (Fig. 1). These viruses have been demonstrated to use human ACE-2 receptor for cell entry as SARS-CoV does (Fig. 2), and replicate efficiently in various animal and human cells[27,29,30,32,33] including primary human lung airway cells, similar to epidemic SARS-CoV[7,8].

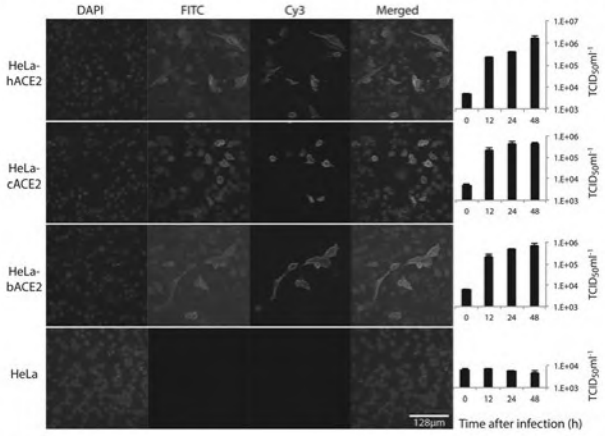

Fig. 2: Bat SARSr-CoV WIV1 replicates efficiently in HeLa cells expressing human, civet and bat ACE2[29].

Chimeras (recombinants) with these SARSr-CoV S genes inserted into a SARS-CoV backbone, as well as synthetically reconstructed full length SHCO14 and WIV-1 bat viruses cause SARS-like illness in humanized mice (a model that expresses human ACE2 receptor), with clinical signs that are not reduced by SARS-CoV monoclonal antibody therapy or vaccination[7,8]. We have now shown that people living up to 6 kilometers from this cave have SARSr-CoV antibodies (3% seroprevalence in 200+ cohort)[34], suggesting active spillover. These data, phylogeographic analysis of SARSr-CoVs (Fig. 3), and coevoutionary analysis of bats and their CoVs (unpubl. data), suggest that bat caves in SW

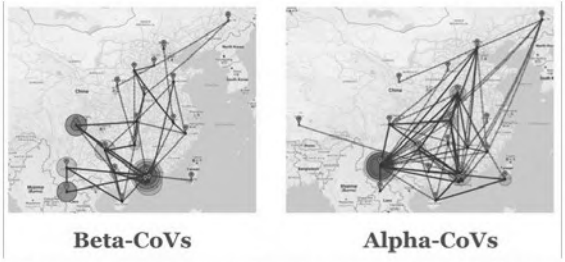

Figure 3. Ancestral location reconstruction for Beta- and Alpha-CoVs. The bigger the circle is, the more ancestral the corresponding node is.

China, and *Rhinolophus* spp. bats are the likely origin of the SARS-CoV clade, and **therefore a clear-and-present danger for the re-emergence of SARS-CoV or a similar pathogenic virus.** The *Rhinolophus* spp. bats that harbor these viruses occur throughout SE Asia, across S. and W. Asia. **Thus, the geographic focus of DEFUSE is to use our research at this site to reduce the risk for the warfighter of these viruses spilling over across the region (West, South and SE Asia).**

Spatial models of bat origin high-risk viruses across S and SE Asia. We will build models that predict regional-scale bat and viral diversity in cave sites across South and SE Asia to enable warfighters and planners to estimate regional-scale risk from viral spillover based on locations. This will provide preliminary assessments for areas requiring greater on-the ground risk characterization to target deployment of viral suppression technologies. These regional-scale joint species distribution models (JSDM) will predict the composition of bat communities in caves in South Southern China, South and SE Asia. JSDMs use environmental and habitat data to predict the distributions of many species simultaneously, producing more accurate predictions than individual, separate species predictions by explicitly modeling positive and negative interactions between species and hidden factors such as shared habitat preferences. We will use a stochastic feedforward neural network to implement JSDMs that has proven effective at making predictions across multiple scales, with incomplete observations (as occurs for bats and their viruses), and explicitly accounting for bat species co-occurrence driven by shared environmental responses or evolutionary processes[35]. We will fit our JSDM to biological inventory data on over 200 caves in the region[36], using a combination of climatic and topographic variables including physiologically relevant bioclimatic variables (BIOCLIM) drawn from public, open source data sets[37], as well as proxies for subterranean habitat such as ruggedness and habitat heterogeneity. We will refine these models using regional-scale environmental variables (land-use, distance to roads, forest cover, degree of human disturbance etc.) and cave-specific variables (cave length, availability of roosting area, entrance dimensions, cave complexity, microclimate etc.). Our previous work has shown that these factors are predictors of bat species presence/absence at a given site[38]. Remote-sensing data and physical models will be used to estimate cave structures and microclimates where they are not available from biological inventory studies. We will validate our regional-scale species models using independent occurrence estimates and observations[39,40], including our extensive database on bat species occurrence in Southeast Asia [REF].

We will extend our predictions of bat communities to predictions of zoonotic disease risk using our unique species-level database of all known bat host-viral relationships[3] (Fig. 4); our >1800 viral detections from >20,000 individual bat samples in China and 7 other Asian countries (NIAID and USAID PREDICT); and results as they become available from a new 5-year DTRA-CBEP grant for field and lab investigations to characterize bat CoV diversity in Western Asia (Turkey, Jordan, Georgia, Pakistan, and Arabian Peninsula – EHA, Olival) to extend the

geographic scope of our predictive models. We will use two strategies to predict presence of viruses at sites. Firstly, as a base case, we will assume that species have equal probability of carrying their known viral species across their range. Second, we will include viral species as additional outputs in our JSDM. We will fit this host-viral JSDM using data restricted to a smaller set of sites where both host species composition and viral detections are available. Based on performance of both models on hold-out data, we will determine which provides the best predictive power. For species composition and viral presence predictions, we will validate our models against a 20% validation subset of data that is held out for model validation, as well as data collected at our field sites in Task 3.

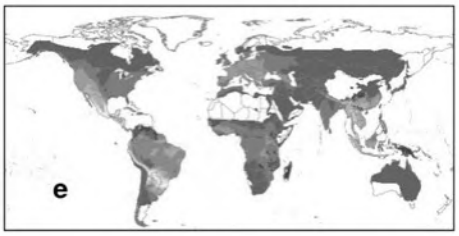

*Fig. 4: Predictive global map of total (known and unknown) viral diversity in bats (*Chiroptera *species). Based on EHA's unique database of all known mammal virus-host relationships[3].*

Prototype app for the warfighter. Drawing on experience building applications for data collection and analysis (e.g. https://flirt.eha.io/, https://eidr-connect.eha.io/, https://mantle.io/grrs), we will produce a prototype app for the warfighter that identifies the likelihood of dangerous viral pathogens spilling over from bats at a site. **The 'Viral spillover risk' app** will use outputs from our spatial risk modeling, data from EHA's extensive host-pathogen database, open-source species and pathogen ontologies, and app-directed crowd-sourced ultrasonic audio recordings to ground-truth and fine-tune its predictive capacity. This app will be updated in Y2 and Y3 to incorporate additional information on bat species-specific risk based on assays of host-virus binding and surveys of CoV prevalence. We will use risk-ranking algorithms developed by EHA (https://ibis.eha.io/) that use geolocation features, recency of information, and host and pathogen characteristics to display critical areas of high risk. The app will collect user GPS location data and preload bat species distribution and community composition estimates from our JSDMs. These will be refined with real-time surveillance data collected without the need to enter cave sites using field-deployable high-frequency microphones for bat detection[41]. We will combine reference acoustic calls from all bat species captured during proposed field work with existing data from bat call libraries globally to train species identification algorithms using bat echolocation call signatures. New algorithms using deep learning methods (e.g. convolutional neural networks[42]) will be developed, or adapted and externally validated on samples collected by the application to characterize bat species based on trained audio features. These models will be deployed on the mobile platform as they become available[42]. Bat species directly identified or estimated to occur within a scalable distance from the user will be automatically linked with viral diversity data from EHA's extensive host-pathogen database and with CoV sequence data from this project to deliver high-risk pathogen lists. The application will have 3 primary views; pathogens-

centric, bat-centric and map-centric. The pathogen-centric view will show a ranked list of likely pathogens in the user's current or selected location. The bat-centric view will show a ranked list of bat species for the user's location. The map-centric view will allow users to select a location for the other rank views, and will display a variety of map layers of interest, including heat map or distribution map layers profiling modeled or collected species occurrences around the user. Elements of the interface will be interactive, presenting popovers with more details when selected and displaying other map elements as appropriate. Alerts and notifications will give users a flexible way to monitor the app data passively, with the app proactively reaching out when critical information is received. The application will also offer a data collection module and accompanying interface elements to collect samples in the field and integrate collected data into the application database. The schemas, APIs, and protocols developed as part of this effort will be designed with principles of simplicity, interoperability, and usability in mind, including using RESTful URL schemes, and standardized data types and ontologies. Datasets will be hosted via cloud services from which the app will download updated information. Build and deployment processes will be reproducible, auditable, and transparent. All code modules will be continually available on EHA's GitHub page (LINK), be documented via README files in root directory of code repositories, and .zip archives containing code, datasets, and instructions for deployment will be made available. This will pave the way future incorporation of new structured biosurveillance data feeds and new species, viral, or host ontologies. This app will be designed for remote use (desktop platform) to assess specific sites in advance of personnel deployment on the ground, or in the field via mobile systems. This technology will improve overall situational awareness of existing and novel infectious agents found in bats, allowing DoD personnel to quickly identify areas that may pose the most significant risk for zoonotic spillover and rapidly deploy resources to respond to and mitigate their impact preemptively when necessary. The 'viral spillover risk' app will then be available to adapt for viral threats from other wildlife host species (e.g. rodents, primates) and ultimately for global use.

Full inventory of bat SARSr-CoV quasispecies at our cave test sites, Yunnan, China.
DEFUSE fieldwork will focus on three model cave test sites within a cave complex in Yunnan Province, SW China (MAP), where we have previously identified and isolated high-risk SARSr-CoVs able to infect human cells and cause SARS-like illness in mice[7,27,29,30]. At these sites, we will determine the baseline risk of SARSr-CoV spillover, prior to, during, and after our proof-of-concept field trials to reduce that risk. We will conduct longitudinal surveillance of bat populations to detect and isolate SARSr-CoVs, determine changes in viral prevalence over time, measure bat population demographics and movement patterns, to definitively characterize their SARSr-CoV host-viral dynamics. We will sample *Rhinolophus, Hipposideros*, and *Myotis* species, all of which carry SARSr-CoVs, and co-roost in the same caves[3,36]. Surveillance will be conducted before, during, and after deployment of our intervention field trial (Task X) to

establish baseline viral shedding detection rates and measure the impact of treatment on these. Field data will allow us to test the accuracy of our model predictions and compare the efficacy of laboratory trials in animal models with in-the-field trials.

Our test caves near Kunming, Yunnan Province, contain multiple co-roosting *Rhinolophus*, *Hipposideros*, and *Myotis* spp., although our preliminary data demonstrate that *R. sinicus* and *R. ferrumequinum* (which co-roost at our sites) are the SARSr-CoV primary reservoir, with *Hipposideros* and *Myotis* playing an insignificant role in viral dynamics. We will capture bats using harp traps and mist nets during evening flyout. Rectal, oral, and whole blood samples (×**2** per bat) will be collected for viral discovery using sterile technique to avoid cross-contamination. 2-mm wing tissue punch biopsies will be collected from each bat for host DNA bar-coding, sequencing of host ACE-2 receptor genes (interface site), and cophylogeny analyses. Standard morphological and physiological data will be collected for each bat (age class, sex, body weight, reproductive status etc.). In Phase I we will **sample 60** *Rhinolophus sinicus* and **60** *R. ferrumequinum*, our primary target species, (**120** bats total) **every three months** for non-lethal viral specimen collection over an **18 month period** of the project from all three cave sites. Given the average prevalence of SARSr-CoV in these species in our previous investigations in S. China (~6-9%, n=3304 *Rhinolophus* spp.), this sample size would enable to detect changes of 10% fluctuation in prevalence between sampling periods. Early in the sampling we will trial the efficacy of tarp collection of fresh feces and urine as a way of collecting viral dynamics data while reducing roost disturbance (REFS). To identify seasonal or reproductive cycle variation in viral dynamics, we will conduct repeated sampling of individuals and of tarps placed under the same roost site portion of a cave and examine roost-site fidelity (see below) to measure how well tarp-collected samples will track the general population. *Rhinolophus* species have a 7-week gestation period and generally give birth in the spring. Colony composition may change over the year, with bats aggregating during mating periods. These changes will affect viral dynamics and our sampling strategy will allow us to collect data over two mating and gestation periods and assess changes in viral prevalence. Additionally, we will conduct **pre-intervention (3 months prior to deployment) and post-intervention (3 months following deployment) CoV monitoring from these sites in Phase II (see Fig. X -Gantt chart)** to assess efficacy of our field intervention deployment. During months without physical bat trapping (2 months each quarter of sampling), fresh fecal pellets will be collected by placing clean polyethylene sheets measuring 2.0m x 2.0m beneath roosting bats. We will use infrared spotlights and digital infrared imaging to record the number and species of individuals above each plastic sheet. Fecal pellets may also be genetically barcoded to confirm species identification[43] as we routinely do for other bat surveillance projects. All specimens will be preserved in viral transport medium and immediately frozen in liquid nitrogen dry shippers in the field, then transported to partner laboratories with maintained cold chain and strict adherence to biosafety protocols. Each bat will be marked with a subcutaneous microchip (PIT tag) containing

a unique ID number (**see below**). **Study caves and bat roosts will be surveyed using portable LiDAR technology**[44-46], to give a 3-D image of the roost area which will provide data on species composition and volume/surface area that needs to be covered when applying the immune treatments in TA2 (Fig. XX). We will adjust individual sampling quotas per species to optimize viral detection based on host-specific prevalence of previous and ongoing host-pathogen models, as well as ongoing lab results from bat sampling.

Our team has more than 30 years of collective experience in safe and humane handling of bats for biological sampling. This project will operate under appropriate IACUC/ACURO and PPE guidelines. EHA has several ongoing DTRA-supported projects and is familiar with the process of obtaining ACURO approval for animal research from the DoD. The EHA team also currently maintains IACUC protocols through Tufts University (via inter-institutional agreement) and will obtain IACUC approval through this mechanism for DEFUSE.

Phase I																							
Year 1												**Year 2**											
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
						Initial Longitudinal CoV Surveillance, 3 cave sites (120 bats per sites, per event)																	
						Capture	Tarps	Tarps	Capture	Tarps	Tarps	Capture	Tarps	Tarps	Capture	Tarps	Tarps	Capture	Tarps	Tarps	Capture	Tarps	Tarps
Phase II																							
Year 3												**Year 4**											
Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun						
					Pre-intervention surveillance			Field Intervention	Post-intervention surveillance														
					Captur	Tarps	Tarps		Capture	Tarps	Capture												

Bats are highly mobile and little is known of inter-cave migration/emigration rates. To **monitor bat roost fidelity and movement** we will mark *Rhinolophid* bats with individual Passive Integrated Transponder (PIT) tags to track individual bats' entry and exit from roost caves. Tags will be inserted subcutaneously between the bats' scapulae by trained personnel. The identities of individually tagged bats inhabiting roost caves will be recorded using radio frequency identification (RFID) data loggers and antennae at the roost entrances. Time-stamped data from individual bats collected by data loggers will be downloaded every 3 days to examine temporal roost site fidelity and rates of inter-cave immigration/emigration. Infrared video cameras will record the total number of bats flying out each night. Recapture data will be collected continuously throughout the project. We will attach radio transmitters (1.2g, Advanced Telemetry Systems, MN USA), to the back of 20 individual *Rhinolophus sinicus* and *Rhinolophus ferrumequinum* from each study roost (60 total) to determine nightly foraging patterns and local dispersal patterns. Telemetry data and PIT tag data will be used to calculate home range, to determine the degree of mixing among our three sites, and parameterize our dynamic models. We will use fine scale data on roost fidelity to determine the population mix at the specific roost sites (e.g. a side pocket of a cave where only one species roosts) for our

intervention. Radio transmitters that weigh <3% of bat body weight will be attached to the fur on the back using a veterinary dermatological adhesive (Vet Bond 3M, USA). We will collect location data from 60 bats (30 males, 30 females) every day for 10 days, 3 times per year for the 18 months of Phase 1. This will provide seasonal data to assess movement, including mating and gestation periods when higher levels of mixing and aggregation in the caves are expected.

High-risk SARSr-CoV quasispecies discovery, isolation and S. gene characterization. We will screen samples for SARSr-CoV nucleic acid using our pan-coronavirus consensus one-step hemi-nested RT-PCR (Invitrogen) assay targeting a 440-nt fragment in the RNA-dependent RNA polymerase gene (RdRp) of all known alpha- and betacoronaviruses assay[47,48], as well as specific assays for known SARSr-CoVs[27-30]. PCR products will be gel purified and sequenced with an ABI Prism 3730 DNA analyzer and quantitative PCR will be performed on SARSr-CoV-positive samples to determine viral load. Full-length genome of all detected SARSr-CoVs will be sequenced by high throughput sequencing method followed by genome walking. The sequencing libraries are constructed using NEBNext Ultra II DNA Library Prep Kit for Illumina and sequenced on a MiSeq sequencer, with PCR and Sanger sequencing used to fill gaps in the genome[29,30,32]. We will build phylogenetic trees using the Maximum Likelihood algorithm in the PhyML software, then scan for recombination events using Recombination Detection Program (RDP), confirmed using similarity plot and bootscan analyses in Simplot. We will analyze the S gene (which encodes the spike protein and determines receptor binding and cross-species transmission) of each sequence to identify a virus' potential to use human molecule ACE2 as a receptor. SARSr-CoVs with high similarity with SARS-CoV in full-length genomic sequences or with S proteins likely able to use human ACE2 as receptor will be identified as potential high-risk strains. We will then attempt isolation, cell culture, and infectious clone construction for further study *in vivo* and *in vitro* analysis. We have had success isolating and culturing SARSr-CoVs using Vero E6 monolayers in DMEM medium with 10% FCS, confirmed by RT-PCR and electron microscopy[29]. For SARSr-CoVs which we are not able to culture, we will construct recombinant viruses with the S gene of new bat SARSr-CoVs and the backbone of the infectious clone of SARSr-CoV WIV1 or of SARS-CoV, using the reverse genetic system described previously, and detailed below[28]. Initial assays of receptor usage and cell tropism will use various cell lines expressing human ACE2 incubated with isolated bat SARSr-CoVs or pseudotype viruses as previously shown[29].

Approach to predicting bat SARSr-CoV spillover risk. Our approach is to combine state-of-the-art genotype-phenotype modeling with detailed step-wise experimental characterization of each bat SARSr-CoV we identify at our test cave sites.

Flow chart here:

Sample testing/screening/Isolation – phylogenetic analysis/ACE2 binding modeling – ACE2

binding assays (all from Fig A) – chimera production – mouse model – SARS vaccines protect - cross neut humAB – full length recovery (all from Fig b)-) – Data into predictive modeling (additional box)

This flow chart should use some elements of Ralph's figures A and B as indicated. Ask Ralph to send you Figs A and B in editable format so you can fuse them in the way above (a chimera!), and without the text. The flow chart needs to have less detail so the flow is visible when shrunk down.

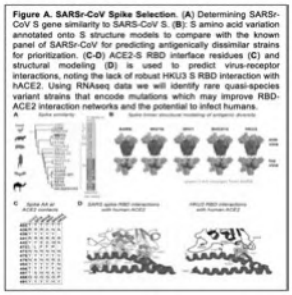

Figure A. SARSr-CoV Spike Selection. (**A**) Determining SARSr-CoV S gene similarity to SARS-CoV S. (**B**): S amino acid variation annotated onto S structure models to compare with the known panel of SARSr-CoV for predicting antigenically dissimilar strains for prioritization. (**C-D**) ACE2-S RBD interface residues (**C**) and structural modeling (**D**) is used to predict virus-receptor interactions, noting the lack of robust HKU3 S RBD interaction with hACE2. Using RNAseq data we will identify rare quasi-species variant strains that encode mutations which may improve RBD-ACE2 interaction networks and the potential to infect humans.

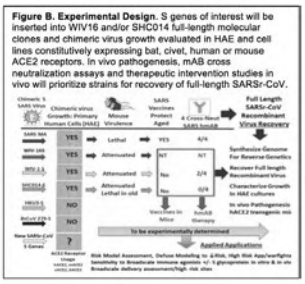

Figure B. Experimental Design. S genes of interest will be inserted into WIV16 and/or SHC014 full-length molecular clones and chimeric virus growth evaluated in HAE and cell lines constitutively expressing bat, civet, human or mouse ACE2 receptors. In vivo pathogenesis, mAB cross neutralization assays and therapeutic intervention studies in vivo will prioritize strains for recovery of full-length SARSr-CoV.

Our models will be parameterized with the experimental data from a series of assays on the S genes of bat SARSr-CoVs, with experimental and modeling work flowing together in iterative steps. The Baric laboratory pioneered many of the experimental approaches, the SARSr-CoV reverse genetic platforms, and full length S chimeric recombinant virus recovery from in silico sequence databases[7,8,23,49]. Full length recombinant strains reconstructed using reverse genetics in our lab include human epidemic strains, civet and raccoon dog SARS-CoV strains, and bat SARSr-CoVs (WIV16, WIV1, SHC014 and HKU3-SRBD repaired RBD interface). These strains will be used in the Baric, Shi and Wang laboratories for initial work on immune boosting and priming, and act as baseline data to parameterize the spillover risk modeling[7,8,23,49]. They will be supplemented by viruses we isolate under DEFUSE (worked on in the Shi lab) **and approximately 15-20 bat SARSr-CoV spike proteins/year** from DEFUSE (Baric, Shi labs). Most of the ~150 bat SARSr-CoV strains sequenced by us in prior work have not yet been examined for spillover potential and these will also be assessed in the following pipeline:

Experimental assays of SARSr-CoV spillover potential: *Ability to enter human cells:* Viral entry represents the key first step to evaluating the disease potential of SARSr-CoVs, with CoV species-specific restriction occurring primarily at entry[23,49]. To assess this we first will use structural modeling of SARSr-CoV S protein to ACE2 receptors. The structure of the SARS trimer prefusion S and the bound SARS-CoV S RBD to human and civet ACE2 have been solved, providing a platform for structural modeling and mapping hot spots of antigenic variation[50,51]. Mutations in the RBD[23,49,52,53], and host proteases and S glycoprotein proteolytic processing[54-56], regulate SARSr-CoV cell entry and cross-species infectivity. Mismatches in the S-RBD-ACE2 molecules or S proteolytic processing will prevent cell entry of SARS-CoV[23,49]. We will also

conduct *in vitro* pseudovirus binding assays, as we have done previously for WIV1 and others[29], as well as live virus binding assays for strains we are able to isolate. This work will be done in China (Shi lab), to prevent delays and unnecessary dissemination of viral cultures. *Novel SARSr-CoV Virus Recovery:* We will commercially synthesize select SARSr-CoV S glycoprotein genes, designed for insertion into our SHC014 or WIV16 molecular clone backbones (these viruses are 88% and 97% identical to epidemic SARS-Urbani in the S glycoprotein). These are BSL-3, not select agents, and pathogenic in hACE2 transgenic mice. Different backbone strains provide increased opportunities for recovery of viable viruses, and to identify potential barriers for RNA recombination-mediated gene transfer between strains[30]. Chimeric viruses will be recovered in Vero cells, or in mouse cells over-expressing human, bat or civet ACE2 receptors to support cultivation of viruses with a weaker RBD-human ACE2 interface. All chimeric viruses will be sequence verified and evaluated for: **i**) human, civet and bat ACE2 receptor usage *in vitro*, **ii**) growth in primary HAE, **iii**) sensitivity to broadly cross neutralizing human monoclonal antibodies (mAB) S215.17, S109.8, S227.14 and S230.15 and a mouse antibody (435) that recognize unique epitopes in the RBD[57,58] and **iv**) *in vivo* pathogenesis studies in hACE2 transgenic mice, using our well established approaches[7]. Should some isolates prove highly resistant to our mAB panel, we will evaluate cross neutralization against a limited number of human SARS-CoV serum samples from the Toronto outbreak in 2003 (n=10). Chimeric viruses that encode novel S genes with spillover potential (e.g. growth in HAE, use of multiple species ACE2 receptor for entry, antigenic variation) will be used to identify SARSr-CoV strains for recovery as full genome length viable viruses. *Recovery of Full length SARSr-CoV:* We will compile sequence/RNAseq data from a panel of closely related strains (e.g.<5% nucleotide variation) and compare the full length genomes, scanning for unique SNPs representing sequencing errors[59-61]. The genome of consensus candidates will be synthesized commercially (e.g. BioBasic), as six contiguous cDNA pieces linked by unique restriction endonuclease sites for full length genome assembly. Full length genomes will be transcribed into genome-length RNA and electroporation used to recover recombinant viruses[22,62]. We will re-evaluate virus growth in primary HAE cultures at low and high multiplicity of infections and *in vivo* in hACE2 transgenic mice, testing whether backbone genome sequence alters full length SARSr-CoV spillover potential. All experiments will be performed in triplicate and data provided to the Modeling Team in real time. We anticipate recovering ~3-5 full length genomes/yr, reflecting strain differences in antigenicity, receptor usage, growth in human cells and pathogenesis. *In vivo Pathogenesis:* We generated a mouse that expresses human ACE2 receptor under control of HFH4, a lung ciliated epithelial cell promoter[7]. Infection of this model with wildtype SARS-CoV results in lethal disease, but transient disease with bat SARSr-CoV WIV1, suggesting that WIV1 is less efficient at using hACE2 *in vivo* and less likely to produce severe disease in people initially on spillover. However, single amino acid variations in the SARS-CoV RBD of related strains could dramatically alter

these phenotypes, hence we will evaluate the impact of low abundant, high consequence micro-variation in the RBD. Groups of 10 animals will be infected intranasally with 1.0 x 10^4 PFU of each vSARSr-CoV, then clinical disease (weight loss, respiratory function by whole body plethysmography, mortality, etc.) followed for 6 days p.i.. Animals will be sacrificed at day 2 or 6 p.i. for virologic analysis, histopathology and immunohistochemistry of the lung and for 22-parameter complete blood count (CBC) and bronchiolar alveolar lavage (BAL) using the Vetscan HM5 (an instrument that measures parameters used for human clinical determination). *Identification of high risk/low abundant variants:* We will use RNAseq to identify low abundant quasispecies (QS) variants encoding mutations in RBD and/or residues that bind ACE2. These would alter risk assessment calculations as strains identified as low risk, might actually have low abundant, high risk variants circulating in the QS. To test this the Shi and Baric lab will structurally model and identify highly variable residue changes in the SARSr-CoV S RBD and use commercial gene blocks to introduce these changes singly and then in combination into the S glycoprotein gene of the low risk, highly abundant parental strain. We will examine the capacity of these low abundance chimeric viruses to use human, bat, civet and mouse ACE2 receptors, and to replicate in HAE cultures. *RBD deletions:* Small deletions at specific sites in the SARSr-CoV RBD leave the key RBD-ACE2 interface residues intact, such that Clade 1 strains represent higher risk of human infection (Fig. 5). We will analyze the functional consequences of these RBD deletions on SARSr-CoV hACE2 receptor usage, growth in HAE cultures and *in vivo* pathogenesis. First, we will delete these regions, sequentially and then in combination, in SHC014 and SARS-CoV Urbani, anticipating that the introduction of both deletions will prevent virus growth in Vero cells and HAE. We hypothesize that the smaller deletion may be tolerated, given its location in the RBD structure, so *in vivo* passage in the presence of receptor will restore growth, while identifying 2nd site reversions that restore efficient hACE2 usage[49]. In parallel, we will evaluate whether RBD deletion repair restores the ability of low risk strains to use human ACE2 and grow in human cells. To test this we will synthesize full length rs4237, a highly variable SARSr-CoV that encodes a few of the SHC014 RBD contact interface residues but also encodes a mutation at 479 (N479S) and has two deletions and hence, is not recoverable *in vitro*. Using the SHC014 backbone sequence, we will sequentially and then in tandem repair the deletions in the presence and absence of the S479N. We anticipate that the S479N mutation is critical given its key role in establishing the RBD-ACE2 interface, and that restoration of the RBD deletions will enhance virus recognition of hACE2

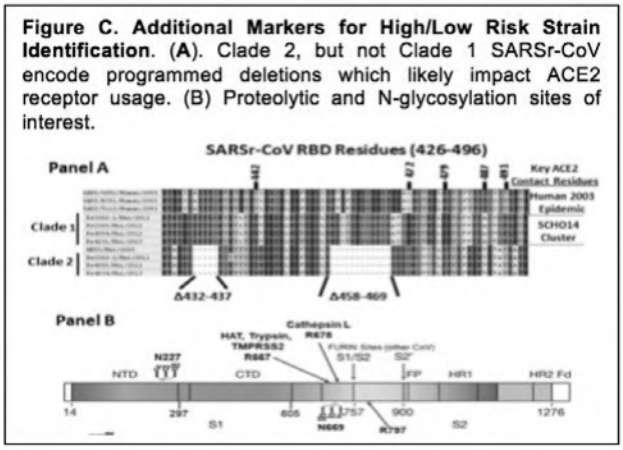

Figure C. Additional Markers for High/Low Risk Strain Identification. (**A**). Clade 2, but not Clade 1 SARSr-CoV encode programmed deletions which likely impact ACE2 receptor usage. (B) Proteolytic and N-glycosylation sites of interest.

receptors and growth in Vero cells and HAE cultures *S2 Proteolytic Cleave and Glycosylation Sites:* After receptor binding, a variety of cell surface or endosomal proteases[63-66] cleave the SARS-CoV S glycoprotein causing massive changes in S structure [67] and activating fusion-mediated entry[55], which is prevented in the absence of S cleavage[68] (Fig. 5). Tissue culture adaptations sometimes introduce a furin cleavage site which can direct entry processes, usually by cleaving S at positions 757 and 900 in S2 of other CoV, but not SARS[66]. For SARS-CoV, a variety of key cleavage sites in S have also been identified and we will analyze all SARSr-CoV S gene sequences for appropriately conserved proteolytic cleavage sites in S2 and for the presence of potential furin cleavage sites[69,70]. SARSr-CoV S with mismatches in proteolytic cleavage sites can be activated by exogenous trypsin or cathepsin L. Where clear mismatches occur, we will introduce the appropriate human-specific cleavage sites and evaluate growth potential in Vero cells and HAE cultures. In SARS-CoV, we will ablate several of these sites based on pseudotyped particle studies and evaluate the impact of select SARSr-CoV S changes on virus replication and pathogenesis (e.g. R667, R678, R797). We will also review deep sequence data for low abundant high risk SARSr-CoV that encode functional proteolytic cleavage sites, and if so, introduce these changes into the appropriate high abundant, low risk parental strain. *N-linked glycosylation:* SARS-CoV S has 23 potential N-linked glycosylation sites and 13 of these have been confirmed biochemically. Several of these regulate SARS-CoV particle binding DC-SIGN/L-SIGN, alternative entry receptors for SARS-CoV entry into macrophages/monocytes[71,72]. Mutations that introduced two new N-linked glycosylation sites may have been involved in the emergence of human SARS-CoV from civet and raccoon dogs[72]. While the sites are absent from civet and raccoon dog strains as well as clade 2 SARSr-CoV, they are present in WIV1, WIV16 and SHC014, supporting a potential role for these sites in host jumping. To evaluate this, we will sequentially introduce clade 2 residues at positions N227 and N699 of SARS-CoV and SHC014 and evaluate virus growth in Vero cells, nonpermissive cells ectopically expressing DC-SIGN and in HAE cultures, as well as in human monocytes and macrophages anticipating reduced virus growth efficiency. Using the clade 2 rs4237 molecular clone, we will introduce the clade I mutations that result in N-linked glycosylation sites at positions 227 and N699 and in rs4237 RBD deletion repaired strains, evaluating virus growth efficiency in HAE, Vero cells, or nonpermissive cells ± ectopic DC-SIGN expression[72]. *In vivo*, we will evaluate pathogenesis in transgenic ACE2 mice.

Models to predict viral spillover potential and evolution of high-risk SARSr-CoV strains. *Structural equation model of spillover potential:* We will use data from the experimental assays above to **build genotype-phenotype models of bat SARSr-CoV spillover potential**. We will use Bayesian Structural Equation Models (SEM), fit via MCMC methods[73], to predict spillover potential from the genetic traits of bat SARSr-CoVs and the ecological traits of hosts. SEMs have successfully analyzed the drivers of, and predicted stochastic species interactions[74,75]. They will

enable us to integrate multiple, interrelated tests of strain spillover potential into a common framework, while restricting relationships to plausible causal pathways. This prevents the over-fitting associated with a black-box approach. A Bayesian approach allows fitting with unbalanced and non-independent data, as per the larger number of cell-binding and cell-entry assays we will run to determine candidates for a smaller number of humanized mouse trials and LIPS assays (below). The viral traits derived from the experimental assays of spillover risk laid out above will be our primary set of predictor variables: presence of deletions in the RBD region, proteolytic binding sites, glycosylation sites, neutralization escape mutations, indeterminate mutations at high-variation sites found in low-abundance strains. We will include genetic similarity of each strain's RBD to the reference pandemic SARS-CoV genomes to test these aggregate measures as predictive proxies. To control for experimental conditions we will include whether assays were performed on live viral isolates, full-genome or synthetic chimeric viruses, and the molecular backbone used in the latter. These traits will be used as inputs to SEM's causal graph, and used to predict latent variables representing the interconnected processes that contribute to SARSr-CoV QS spillover potential: receptor binding, cell entry with and without the presence of exogenous proteases, immune system interaction, and intracellular growth, all measured by our laboratory assay. These, in turn will **act as predictors for the ultimate outcomes of host pathogenesis (Fig. 6)**. We will use previous work on these genetic traits to put informative priors on strength and direction of interactions in the causal graph. We will use **prior-knowledge model simulations to select target sequences from our sampling for characterization and genome-sequencing**, to collect data that maximally enhances the predictive power of our model. We will use regularizing priors to reduce over-fitting and help select the most predictive variables in the final predictive model.

Evolutionary modeling and simulation to predict potential strains: Our SEM modeling will generate estimates of the spillover potential of SARSr-CoV sequences from DEFUSE fieldwork and prior work. To examine risk associated with the total viral population at our test sites, we will model and simulate evolutionary processes **to identify likely viral QS that our sampling has not captured, as well as viral QS likely to arise in the future**. By estimating the spillover potential of these simulated QS, we can better characterize the risk associated with the total viral population. We will use a large dataset of S protein sequences and full-length genomes generated from prior work and DEFUSE fieldwork to estimate SARSr-CoV substitution rate and its genome-wide variation using coalescent and molecular clock models within a Bayesian MCMC framework[76]. We will then estimate SARSr-CoV recombination rates at the cave population level using the same dataset and Bayesian inference[77,78]. We will apply various methods (RDP[79], similarity plots, bootscan) to identify recombination breakpoints and hotspots within the SARSr-CoV genome. Using these estimates of substitution and recombination rates, we will simulate the evolution of the SARSr-CoV QS virome using a forward-time approach implemented in simulators that model specific RNA virus functions (e.g. VIRAPOPS[80]). This will

allow us to predict the rate at which new combinations of genetic traits can spread in viral populations and compare recombination rates among caves and bat communities. Our forward-simulated results **will provide a pool of likely unknown and future QS species**. Using these and our SEM model for spillover risk, **we will predict the QS that are most likely to arise *and* have pathogenetic and spillover potential.** We will use the evolutionary simulation results to iteratively improve our SEM model results. The number of genetic traits of interest for prediction of pathogenicity is potentially large, so we will perform variable reduction using tree-based clustering, treating highly co-occurring traits as joint clusters for purposes of prediction. We will generate these clusters from our full set of SARSr-COV sequences from DEFUSE fieldwork and prior work. However, as trait clusters may be modified in future virus evolution due to recombination, we will use our forward-evolutionary modeling to predict how well trait clusters will be conserved, retaining only those trait clusters unlikely to arise in unknown or future viral QS genomes. This will enable a good trade-off between increased predictive power based on current samples and generalizability to future strains that have not yet evolved.

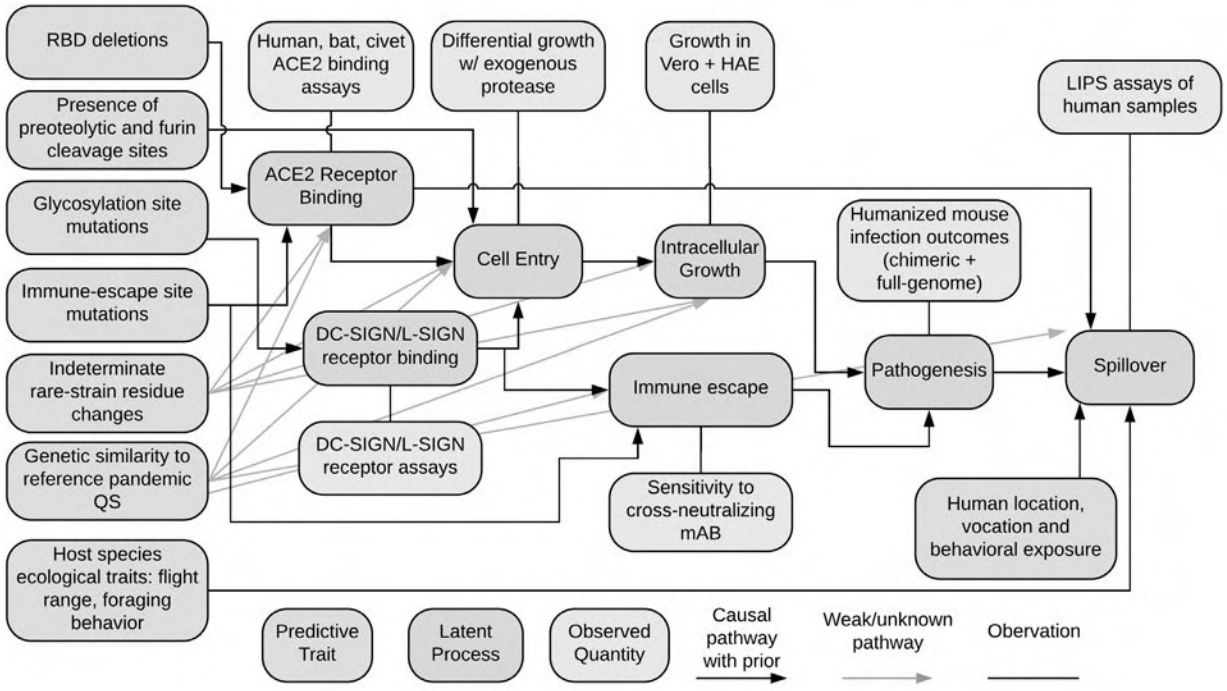

Figure 6: A simplified directed graph of a structural equation model representing the causal relationships between predictors and measures of viral pandemic potential.

Validation by LIPS assay on previously-collected human sera: Following our proof-of-concept field trial we will update these models to include not only pathogenesis but spillover probability validated with data on viral QS antibodies found in the local human population detected via Luciferase immunoprecipitation system (LIPS) assays on previously-collected human sera (NIAID project, Daszak PI). This includes >2,000 samples collected from people living close to our test

cave sites in Yunnan Province, and is the basis of a recent paper demonstrating 2.7% seropositivity to bat SARSr-CoVs in an initial sampling of this population[34] (Fig. 7). In addition to serum samples, extensive behavioral and wildlife contact data has been collected from this population, under an IRB that can be easily extended to cover DEFUSE work.

Fig. 7. Human sera were collection from villages (red dots) near bat caves where CoV positive samples have been isolated (Yanzi Cave and Shitou Cave, triangle).

Our ability to extend and validate these models with data on actual human contact and spillover allows us to fit and test models of actual, not just potential, spillover probability. Our previous work has shown that both host and viral traits predict zoonotic spillover from models[3], so in addition to viral traits, we will include key ecological traits of the host bat species in which viral QS were detected. These include flight ranges, foraging, roosting, demographic, and social behavior. To will use the extensive data on each person's behavioral exposure to wildlife, and their work, travel and occupational history, to correct for varying human exposure to bat species. We will design LIPS assays for specific high- and low-spillover risk SARSr-CoVs, to identify people who've been exposed to them, and test our model's validity. The LIPS uses viral antigens tagged with luciferase, from crude lysate, thereby eliminating the requirement for antigen purification and significantly reducing the time required for assay development and producing a more sensitive test than traditional ELISA[81]. Prof. Zhengli Shi (Wuhan Institute of Virology) will lead the LIPS serological work based on her 15 years SARSr-CoV human serological surveillance experience [82-84] and the recent success in SADS-CoV zoonotic risk study using LIPS[85]. To establish SARSr-CoV LIPS assays, we will: **1)** Insert different high- and low-risk SARSr-CoV N genes into pREN-2 vector (LIPS vector). We will first assess N gene similarity to determination their potential cross-reactivity in a LIPS assay. From our previous experience, SARSr-CoV maintain 80% similarity in the N protein, thus should be detectable using a universal SARSr-CoV N based LIPS assay; **2)** determine specificity of the LIPS assay by producing polyclonal sera via injection of recombinant protein or attenuated virus into rabbits. Selected SARSr-CoV N proteins or viral particles will be used as the immunogen for antibody production; **3)** validate SARS-CoV, MERS-CoV and SADS-CoV N protein LIPS assays by incubating antigens with their respective positive serum samples and the antigen antibody complex eluted using protein A/G beads. Luminescence is measured upon adding coelentrazine, a substrate of renilla luciferase. In a preliminary assay, LIPS successfully detected high strong antibody titer in the positive control serum sample, while the vector control did not show any response. Cut off was set as the average luminescence plus

three standard deviation from the control. We have used this to demonstrate efficacy for MERS-CoV and SADS-CoV (Fig. 8); **4)** validate LIPS positive sera results by spike protein based LIPS and viral neutralization assay. Similarly, S gene from high/low risk SARSr-CoV will be engineered into the pREN-2 vector and an S-LIPS assay produced, as above. As a confirmatory test the positive samples from LIPS, will be validated by viral neutralization assay. The data from LIPS and neutralization will be collected and analysis to validate the model.

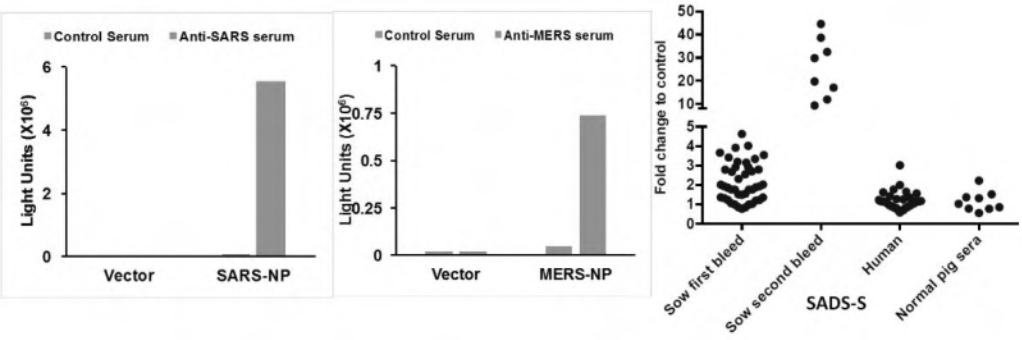

Fig. 8. LIPS assay was tested successful for SARS, MERS and SADS coronavirus N or S antibodies.

Thematic Area 2

Immune modulation approach to reducing bat SARSr-CoV spillover risk. There is no available technology to reduce the risk of exposure to novel CoVs from bats which carry zoonotic precursors to many emerging viruses including filoviruses (Ebola), CoV (SARS-CoV, MERS-CoV, etc.), paramyxoviruses (Nipah/Hendra), rhabdoviruses (rabies) and others. No vaccines or therapeutics exist for emerging CoVs, filoviruses and paramyxoviruses and exposure mitigation strategies are non-existent. We have shown that bats have unique immunological features that may explain why they coexist with viruses and rarely show clinical signs of infection. Our long-term studies demonstrate: a) bats maintain constitutively high expression of IFNα that may respond to and thus restrict, viral infection immediately[11]; b) several bat interferon activation pathways are dampened, e.g. STING (a central cytosolic DNA-sensor molecule to induce interferon) dependent and TLR7 dependent pathways[10]; c) the NLRP3 dependent inflammasome pathway is dampened, and some of the key inflammation response genes like AIM2 have been lost in bats[86,87]. The dampened IFN and inflammasome response suggest bats maintain a fine balance between IFN response and detrimental over-response. This is likely due to an adaptation of their immune-sensing pathways as a fitness cost of flight[9]. We hypothesize that the bat innate/adaptive immune responses are quite different from that of human and mouse. Firstly, virus replication will likely be restricted quickly by constitutively expressed IFNα in bats, resulting in lower B/T cell stimulation due to lower viral stimuli. Second, dampened interferon and inflammasome responses will result in lower cytokine responses that are

required to trigger T/B cell dependent adaptive immunity (e.g. antibody response). The strong innate immune response, due to the lack of an efficient antibody response, will clear the virus. **We and others have demonstrated proof-of-concept of this phenomenon:** Experimental Marburg virus infection of Egyptian fruits bats, a natural reservoir host, resulted in wide tissue distribution yet low to moderate viral loads, brief viremia, low seroconversion and a low antibody titer that waned quickly, suggesting no long-term protection is established[88-90]. Similarly, poor neutralizing antibody responses occur after experimental infection of bats with Tacaribe virus[91] and in our studies with SARS-CoV experimentally infected bats (L-F Wang, unpublished data). Indeed, we successfully showed bat interferon can inhibit bat SARSr-CoVs[28]. We hypothesize that if we can use immune modulators that upregulate the naturally low innate immunity of bats to their viruses, we will be able to transiently suppress viral replication and shedding, reducing the risk of spillover. We will evaluate two immune modulation approaches to defuse spillover of SARSr-CoVs from bats to humans: **1) Broadscale Immune Boosting strategies (Wang, Duke-NUS):** we will apply immune modulators like TLR-ligands, small molecule Rig like receptor (RLR) agonists or bat interferon in live bats, to up-regulate their innate immunity and assess suppression of viral replication and shedding; **2) Targeted Immune Priming (Baric, UNC):** the broadscale immune boosting approach will be applied in the presence and absence of chimeric immunogens to boost clearance of high-risk SARSr-CoVs. Building on preliminary development of polyvalent chimeric recombinant SARSr-CoV spike proteins, we will use novel chimeric polyvalent recombinant S proteins in microparticle encapsidated gels and powders for oral delivery and/or virus adjuvanted immune boosting strategies where chimeric recombinant SARSr-CoV S are expressed by raccoon poxvirus, which has been used extensively to deliver rabies immunogens in bats and other animals. We will conduct application trials with live bats to assess suppression of replication and shedding of a broad range of pathogenic SARS-related CoVs. Both lines of work will begin in Year 1 and run parallel, be assessed competitively for efficiency, cost, and scalability, and successful candidates used in our live bat trials at our test sites in Yunnan, China. We believe an immune boosting/priming strategy is a superior approach for this challenge because solutions are likely to be broadly applicable to many bat species, and across many viral families.

Broadscale immune boosting (led by Wang, Duke-NUS). We will work on the following key leads to identify the most effective approach to up-regulate innate immunity an suppress viral loads. *Toll-like receptor (TLR)/Rig-I Like Receptor (RLR) ligands:* We have begun profiling bat innate immune activation *in vivo*, in response to various stimuli. Our work indicates a robust response to TLR-stimuli like polyI:C when delivered *in vivo*, as measured by transcriptomics on spleen tissue (Fig. 7). We have performed transcriptomics on spleen, liver, lung and lymph node, with matched proteomics to characterize immune activation *in vivo*. These activation profiles will be used to assess the bat immune response to different stimuli and direct the

response to favor those which lower the viral load in our experimental system at Duke-NUS (below). In addition to the ligands already tested, we will stimulate the Rig-I pathway with 5'pppDSRNA, a mimetic of the natural RIG-I stimulant. These stimulants will activate functional bat IFN production pathways, and a similar strategy has been demonstrated in a mouse model for clearance of SARS-CoV, influenza A virus and Hepatitis B virus[12,15].

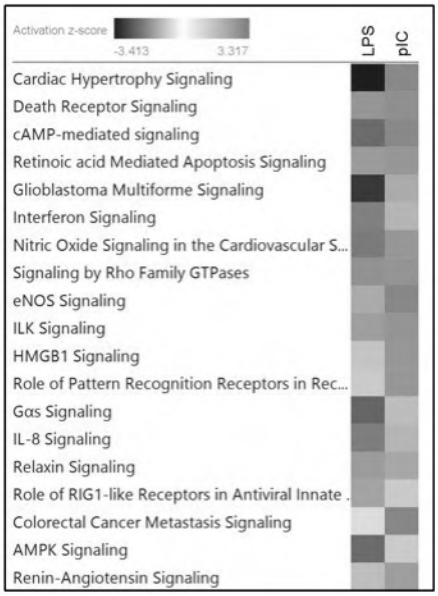

Fig. 7. Pathway analyses from Ingenuity Pathway Analysis (IPA) of whole spleen NGS after stimulation with either LPS or polyI:C. Z-score increase over control bats is indicated as per scale, and suggests strong activation of many pathways.

<u>*Universal bat interferon:*</u> To overcome any complications arising from species-specificity, we will design a conserved universal bat interferon protein sequence and produce purified protein. Utilization of a universal IFN for bats will overcome species-dependent response to the ligand, allowing the use of IFN throughout broad geographical and ecological environments and across many bat species. As a starting point, we have produced recombinant non-universal, tagged, bat IFN that are effective at inducing appropriate immune activation (Fig. 8). This ligand can be **delivered by aerosol or intranasal application** as has been shown to reduce viral titers in humans, ferrets and mouse models[12,13,15]. Interferon has been used clinically in humans as an effective countermeasure when antiviral drugs are unavailable, e.g. against filoviruses[14]. Replication of SARSr-CoV is sensitive to IFN treatments, as shown in our previous work[28]. The successful delivery, immune activation and outcome on the host will be characterized thoroughly to optimize rapid immune activation.

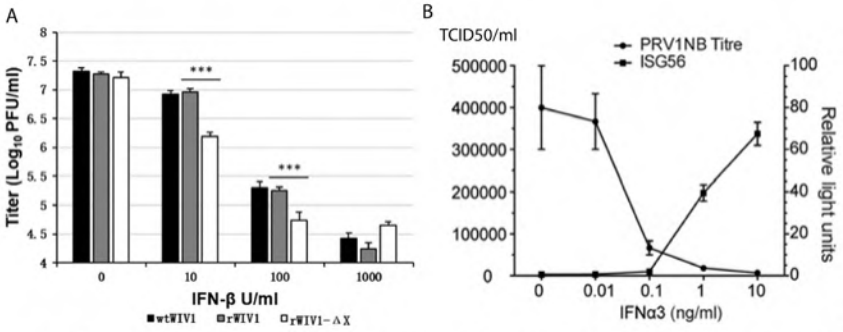

Fig. 8: Bat viruses are sensitive to IFN treatments. A) Recombinant bat SARS-related coronavirus WIV1 replication was inhibited by human IFN-β in a dose dependent manner in Vero cells. B) Bat reovirus PRV1NB replication was inhibited by recombinant bat IFNα3 in a dose dependent manner in bat PakiT03 cells.

<u>*Boosting bat IFN by blocking bat-specific IFN negative regulators:*</u> Uniquely, bat IFNα is naturally constitutively expressed but cannot be induced to a high level, indicating a negative regulatory factor in the bat interferon production pathway[92]. To fast-track the identification of this target

we will utilize a *Pteropus alecto* CRISPRi library pool that we have created covering multiple RNA targets in every gene in the *P. alecto* genome. The library has already been produced and genes affecting influenza replication in bat cells have been identified. Using CRISPRi we can identify negative regulator genes and then screen for compounds targeting these genes to boost the inducibility of the IFN system in a shorter time-frame. Based on previous work, it is highly likely this will be a conserved pathway throughout the order *Chiroptera*. *Activating dampened bat-specific innate immune pathways which include DNA-STING-dependent and TLR-dependent pathways:* Our work showing that mutant bat STING or reconstitution of AIM2 and functional NLRP3 homologs restores antiviral functionality suggests these pathways are important in bat-viral coexistence and that the majority of the pathway is preserved. By identifying small molecules to directly activate pathways downstream of STING or TLR/RLRs, such as TBK1 activation, we will activate bat innate defense by interferons and promote viral clearance. We hypothesize that these small molecules we will be able to significantly reduce viral load in bats. *Validation in a bat-mouse model.* Various CoVs show efficient infection and replication inside the human host but exhibit defective entry and replication using mouse as a host due in part to differences in DPP3 and ACE2 receptors. We have shown efficient reconstitution of irradiated mice using bat bone marrow from multiple species, including *E. spelaea*. Fig. 9 shows the efficient reconstitution of bat PBMC's in the mouse, presence of circulating bat cells and generation of bat-specific antibodies in mice incapable of producing an antibody response. This 'batized' mouse model can be utilized for both circulating infection of SARS/MERS CoV (in the immune compartment only) and as a model for generating bat-specific antibodies against CoV proteins. Efficient validation of infection into bat cells will be used to validate the infectivity of the viruses and generation of bat antibodies will facilitate validation of the best proteins/peptide to elicit an effective immune response.

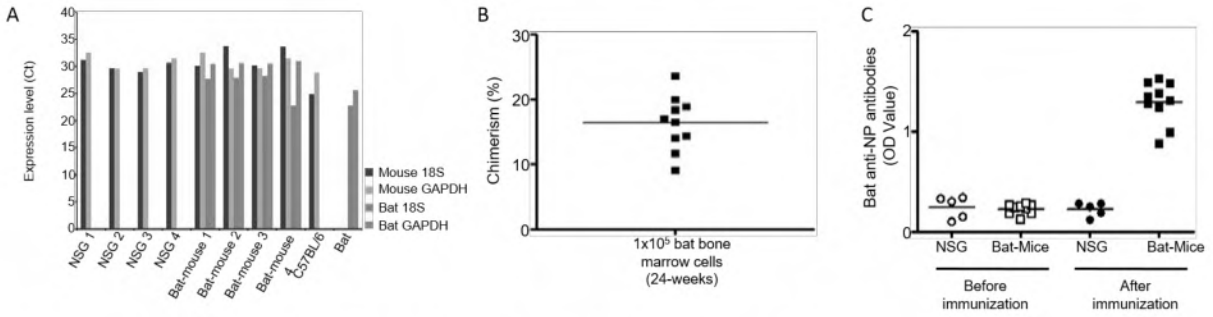

Fig. 9: A) Presence of bat-specific qPCR in reconstituted mice after 12 weeks. B) chimeric ratio of bat-mouse cells in circulation after 24 weeks. C) Specific antibody response to a KLH-tetanus antigen generated by bat-reconstituted mice.

Viral infection models in cave-nectar bat (Duke-NUS): To test and compare the efficacy of the immune modulating approaches above, we will use our cave-nectar bat (*Eonycteris spelaea*)

breeding colony infected with Melaka virus (family *Reoviridae*) which is known to infect this species[93,94]. We will also use two coronaviruses (SARSr-CoV WIV1 and MERS-CoV in ABSL3. Details of infection, housing, prior infection trials in the facility… Viral loads will be measured by qPCR, titration of produced virus, NGS transcriptomics and nanostring probes added to the immunoprofiling panel. Antibody responses will be measured by LIPS assay. This approach allows us to test our immune-boosting strategies, in a safe and controlled environment, prior to expanding to field-based evaluation. The analytical methods used for the *E. spelaea* colony will be replicated to analyze the experimental infection of *Rhinolophus* in a wild-cave scenario. Additionally, the versatility of the analysis should allow easy application to multiple species of bats

Targeted Immune Priming (led by Baric, UNC). We have developed novel group 2b SARSr-CoV chimeric S glycoproteins that encode neutralizing domains from phylogenetically distant strains (e.g. Urbani, HKU3, BtCoV 279), which differ by ~25%. The chimeric S programs efficient expression when introduced in the HKU3 backbone full length genome, and elicit protective immunity against multiple group 2b strains. We will develop robust expression systems for SARSr-CoV chimeric S using ectopic expression *in vitro*. Then, we will work with Dr. Ainslie (UNC-Pharmacy) who has developed **novel microparticle delivery systems and dry powders for aerosol release**, and which encapsidate recombinant proteins and adjuvants (innate immune agonists) that will be used for **parallel broadscale immune boosting strategies ± chimeric immungens.** Simultaneously, we will introduce chimeric and wildtype S in raccoon poxvirus (RCN), in collaboration with Dr. Rocke and confirm recombinant protein expression, first *in vitro* and then in the Duke-NUS bat colony, prior to any field trial. The goal of this aim is to develop a suite of reagents to remotely reduce exposure risk in high risk environmental settings.

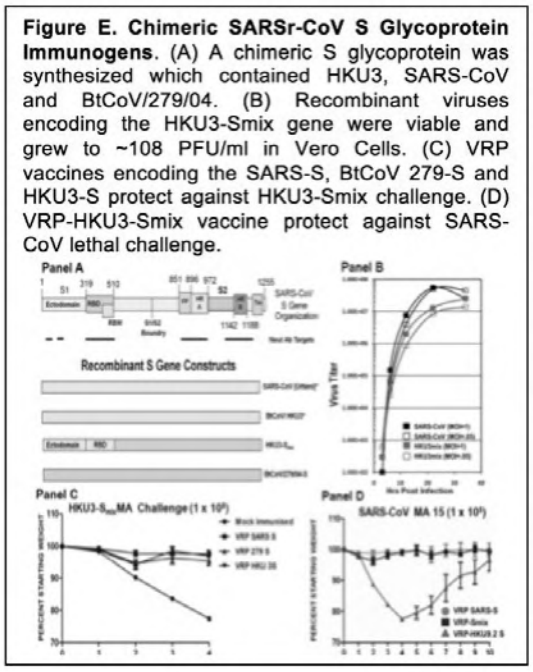

Figure E. Chimeric SARSr-CoV S Glycoprotein Immunogens. (A) A chimeric S glycoprotein was synthesized which contained HKU3, SARS-CoV and BtCoV/279/04. (B) Recombinant viruses encoding the HKU3-Smix gene were viable and grew to ~108 PFU/ml in Vero Cells. (C) VRP vaccines encoding the SARS-S, BtCoV 279-S and HKU3-S protect against HKU3-Smix challenge. (D) VRP-HKU3-Smix vaccine protect against SARS-CoV lethal challenge.

Chimeric SARSr-CoV S Immunogens: CoV evolve quickly by mutation and RNA recombination, the latter provides a strategy to rapidly exchange functional motifs within the S glycoprotein and generate viruses with novel properties in terms of host range and pathogenesis[30,95]. CoV also encode neutralizing epitopes in the amino terminal domain (NTD), RBD and S2 portion of the S glycoprotein[57,96,97], providing a strategy to build chimeric immunogens that induce broadly cross reactive neutralizing antibodies. Given the breadth of SARSr-CoV circulating in

natural settings, chimeric immunogens will be designed to increase the breadth of neutralizing epitopes across the group 2b phylogenetic subgroup[40]. Using synthetic genomes and structure guided design, we fused the NTD of HKU3 (1-319) with the SARS-CoV RBD (320-510) with the remaining BtCoV 279/04 S glycoprotein molecule (511-1255), introduced the chimeric S glycoprotein gene into the HKU3 genome backbone (25% different than SARS-CoV, clade 2 virus) and recovered viable viruses (HKU3-S_{mix}) that could replicate to titers of about 10^8 PFU/ml on Vero cells (Fig. 10). HKU3-Smix is fully neutralized by mAb that specifically target the SARS RBD (data not shown). In parallel, we inserted the $HKU3_{mix}$ S glycoprotein gene into VEE virus replicon vectors (VRP-$S_{chimera}$) and demonstrated that VRP vaccines protect against lethal SARS-CoV challenge and virus growth. In addition, VRP-S_{HKU3} and VRP-S_{279} both protect against $HKU3_{mix}$ challenge and growth *in vivo* (Fig. 9), demonstrating that neutralizing epitopes in the $HKU3_{mix}$ S glycoprotein are appropriately presented and provide broad cross protection against multiple SARSr-CoV strains. In addition to using these immunogens as a targeted broad-based boosting strategy in bats, we will also produce a chimeric SHC014/SARS-CoV/HKU3 S and a SCH014/SARS-CoV/WIV-1 S gene for more focused immune targeting on known high risk strains. In parallel, we will work with the Protein Expression Core at UNC (https://www.med.unc.edu/csb/pep) to produce codon optimized, stabilized and purified prefusion SARS-CoV glycoprotein ectodomains as published previously[17]. Purified recombinant protein will be used by Drs. Rocke and Ainslie for inclusion in delivery matrices (e.g. purified powders, dextran beads, gels – see below) with broadscale immune agonists (adjuvants-Dr. Wang) like poly IC, TLR4 and Sting agonists.

2nd Generation Chimeric S glycoprotein Design and Testing: We will also produce a chimeric SHC014 NTD/SARS-CoV-RBD/HKU3 S C terminal and generate recombinant HKU3 encoding the trimer spike (HKU3-S_{S014}), for more focused immune targeting on known high and low risk strains designated from our experimental and modeling analyses. A second construct will be synthesized with a SHC014 NTD domain, SARS-CoV RBD and WIV-1 C terminal domain (WIV-S_{S014}). After sequence variation, we will evaluate virus growth in Vero and HAE cultures and the ability of SARS RBD monoclonal antibodies (S227, S230, S109) to neutralize chimeric virus infectivity[89,96]. We will also evaluate *in vivo* pathogenesis in C57BL/6 mice and hACE2 transgenic mice. The recombinant HKU3-S_{S014} S genes will be introduced into VRP vectors and sent to Dr. Rocke for insertion into the raccoon poxvirus vaccine vector. Using established techniques, we will characterize S expression and then provide virus vectors to Prof. Wang for immune boosting trials at Duke-NUS, and ultimately if successful in the field (Prof. Shi). We will also synthesize human codon optimized the HKU3-S_{S014}, WIV-S_{S014} and HKU3-S_{mix} chimeric spikes for expression and purification by the UNC proteomics core, producing mg quantities for inclusion in nanoparticle and microparticle carriers in collaboration with Dr. Ainslie. We will produce enough material for *in vivo* testing in mice and in bats. Recombinant HKU3-S_{S014} and WIV-S_{S014} glycoprotein expression will be validated by Western blot and by vaccination of mice, allowing

us to determine if the recombinant protein elicits neutralizing antibodies that protect against lethal SARS-CoV, HKU3-S_{mix} and SHC014 challenge. In parallel, we will survey the RNAseq data for evidence of complex S glycoprotein gene RNA recombinants in the SARSr-CoV population genetic structure. If present, we will synthesize 2-3 interesting recombinant S genes, insert these genes into SHCO14 or HKU3 genome backbones and VRP and characterize the viability and replicative properties of these viruses in cell culture and in mice and the VRP for S glycoprotein expression and vaccine outcomes. We will produce immunogens and evaluate their ability to protect against infection.

Adjuvant and Immunogen Delivery Vehicles. Dr. Ainslie (UNC) and collaborators have developed the biodegradable polymer acetalated dextran (Ac-DEX) for the delivery of antigens and adjuvants in vaccine applications (Fig. 11). Ac-DEX has distinct advantages over other polymers for vaccine development: 1) synthesis is straightforward and scalable. An FDA-approved water soluble dextran polysaccharide is modified and rendered insoluble in water by a simple one-step modification of its hydroxyl groups with pendant acyclic or cyclic acetal groups[98-100]. Unlike other dextran based vaccine materials, our material is acid sensitive, which has been shown to greatly improve antigen presentation; 2) Ac-DEX microparticles (MPs) can passively target antigen-presenting cells (APCs) based on their size (5-8μm), being phagocytosed by DCs and traffic to the lymph node[101]. Furthermore, APCs have acidic phagosomes that can result in triggered intracellular release due to the acid-sensitivity of Ace-DEX; 3) Ac-DEX MPs and their hydrolytic byproducts are pH-neutral, biocompatible, and safe compared to other commonly used polyesters have acidic hydrolytic byproducts (e.g. lactic and glycolic acid, in the case of PLGA) that damage vaccine components such as protein antigens[102]. The complete hydrolysis of Ac-DEX results in particle breakdown with release of the metabolic side products. 4) Ac-DEX MPs are stable outside the cold-chain. MPs can be stored for at least 3 months at 45°C without any loss of integrity or encapsulated cargo bioactivity[103]. Other common formulations (e.g. liposomes[104], PLGA MPs[103], squalene emulsions [Fluad™ package insert]) have limited shelf-life that requires the cold-chain. Ac-DEX MPs can be aerosolized, or delivered in sprays or gels to bat populations, providing new modalities for zoonotic virus disease control in wildlife populations[98,105]. 5) We have previously encapsulated Poly (I:C)(1), resiquimod[101], and a STING agonist into our novel MPs[106].

As seen in Fig. 10, encapsulation of Poly (I:C) drastically enhances the activity of the TLR agonist. Additionally, encapsulation of adjuvants in MPs drastically enhances the activity of subunit vaccines. We have

Figure F. Particle Delivery Systems. Broadscale immune boosting strategies include (A) Dextran microparticles or Dry nanoparticle powders. (B) Macrophages cultured with either free poly (I:C) or poly (I:C) encapsulated into Ac-DEX MPs produce significant TNFα. (C) Comparison of (left) neutralizing titer and (right) viral load when ferrets are vaccinated with Ac-DEX MPs. Day 0, 28, and 56 (prime, boost, and challenge.)

displayed better efficacy than state-of-the-art FDA-approved inactivated flu virus (Fluarix) in a ferret model of influenza. The ferret model is the ideal animal model for influenza because of their relatively small size and they possess various clinical features associated with human influenza infection[107]. This formulation used HA with encapsulated STING agonist cyclic [G(3',5')pA(3',5')p](16)

Microparticle Performance Metrics in vitro and in Rodents and Bats: MPs are designed for aerosol delivery due to their relatively effective low aerodynamic diameter[108], their low density microporous nature which allows for efficient aerosol dispersal and deep penetration into the lung, or deposition on the skin for oral uptake by grooming. We will encapsulate Poly (I:C), resiquimod (TLR 7) or other innate immune agonists to enhance type I interferon production in in consultation with Prof Wang. Agonist laden particles will be made separately or in combination with recombinant SARS-CoV chimeric spike proteins, encapsulated into our aerodynamic MPs as well as nanoparticles.

Delivery system development (Rocke, NWHC). We have previously developed, tested and registered oral vaccines and delivery methods to manage disease in free-ranging wildlife including a sylvatic plague vaccine for prairie dogs[24], vaccines against bat rabies[25] and white-nose syndrome (unpubl. data). We have optimized vaccine delivery methods, uptake by the target species and safety in non-target hosts using biomarkers prior to deployment[109]. We will use a similar approach to develop, test and optimize delivery methods to *Rhinolophus* bats in SE Asia. While work on immune modulating agents progresses, we will concurrently develop and test mediums, routes, and methods of delivery to large colonies of bats. We will determine the most feasible and simple method of delivery that achieves high uptake by bats, is safe for humans as well as target and non-target species, and minimizes colony disturbance. Sticky edible gels or pastes that bats groom from themselves and each other have been used previously to deliver pharmaceuticals to bats orally and we are currently testing these for use in rabies vaccine delivery. These may also be useful for delivering immune modulators and recombinant SARSr-CoV spike proteins to *Rhinolophus* bats, but may need to be combined with viral vectors (like poxvirus or adenovirus) or nanoparticles/nanoemulsions that enhance uptake through mucous membranes or transdermally after topical application. *Poxvirus vectors:* Poxviruses are effective viral vectors for delivering vaccines to wildlife [24,110,111], and can replicate safely at high levels in bats after oronasal administration[26]. We have demonstrated proof-of-concept in bats. We tested modified vaccinia Ankara (MVA) and raccoon poxvirus (RCN) vectors for safety and replication in bats using *in vivo* biophotonic imaging[25]. RCN replicated to higher levels in bats than MVA, even via the oral route, and was found to be highly safe for bats (Fig. 12). We used raccoon poxvirus-vectored novel rabies glycoprotein (mosaic or MoG) and demonstrated protective efficacy in bats after oronasal and topical administration[25] (Fig. 13). We are currently developing vaccine delivery for vampire bats in several Latin

American countries, and vaccines for white-nose syndrome in bats, a devastating disease that has killed millions of insectivorous bats in North America.

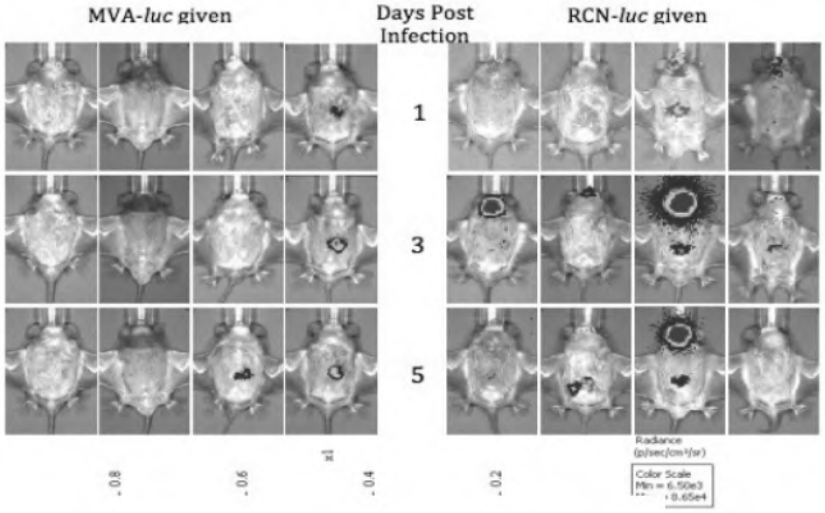

Fig. 12. Luminescence, indicative of viral replication of modified vaccinia Ankara (MVA) and raccoon poxvirus RCN) in the bat Tadarida brasiliensis *on 1, 3 and 5 dpi via the oronasal route.*

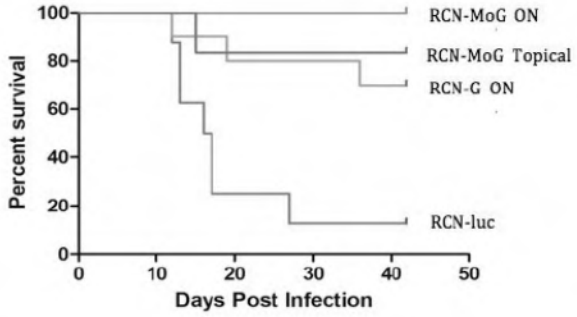

Figure 13. Vaccine efficacy and rabies challenge in Epstesicus fuscus immunized with raccoon poxvirus expressing a mosaic G protein (RCN-MoG) either oronasally (ON) or topically in comparison to RCN expressing typical G protein and RCN expressing luciferase (negative control).

Poxviruses are safe in a wide variety of wild and domestic animals, and allow for large inserts of foreign DNA. We have previously used a raccoon poxvirus vectored vaccine expressing plague antigens that was incorporated into a peanut-butter flavored bait matrix to manage plague caused by *Yersinia pestis* in prairie dogs. We incorporated the biomarker Rhodamine B (RB) into baits to assess uptake by target and non-target species [109,112] (Fig. 14). RB is visible under a UV microscope until the hair grows out (~50 days in prairie dogs). We have since conducted a large field trial (approved by USDA Center for Veterinary Biologics) that demonstrated vaccine efficacy in four species of prairie dogs in seven western states[24]. We used biomarker analysis to assess site- and individual host-specific factors that increased bait consumption including age, weight, and the availability of green vegetation.

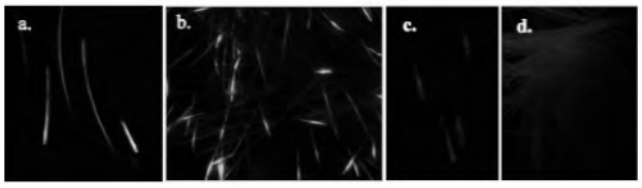

Fig. 14. Prairie dog hair and whisker samples under fluorescence microscope (excitation wavelength: 540 nm, emission wavelength: 625 nm) to determine uptake of baits containing Rhodamine B. a) 20 days after

bait distribution, b) 16 days after bait distribution, c) and d) controls (note natural dull fluorescence).

Transcutaneous delivery: In addition to viral vectors, we will also consider methods to achieve transcutaneous delivery of the immune boosting proteins without the use of live agents. Nanoparticles have been used to increase transcutaneous delivery efficiency[113]. However, the impermeable stratum corneum provides a difficult barrier to breach. Mechanical approaches have been used[113] but are somewhat unethical and impractical for wildlife. We are currently testing poly lactic-co-glycolic acid (PLGA) as a nanoparticle to encapsulate rabies glycoprotein as a method of transcutaneous delivery of vaccine to bats via dendritic cell uptake[114], as has been shown for delivery of TLR agonists and antigens simultaneously to mice[115]. This approach will be competitively trialed against ac-DEX to encapsulate and deliver SARSr-CoV glycoproteins, with and without adjuvants[116], e.g. Matrix M1 (Isconova, Sweden) which has been shown to significantly enhance the immune response in mice to SARS-CoV spike proteins[18]. For efficiency and to reduce costs, initial trials will be conducted in the USA with locally acquired insectivorous big brown bats (*Eptesicus fuscus*) which we have maintained and housed for several experiments at our facility previously[25,26]. We will treat bats via topical application with various test formulations that include the biomarker Rhodamine B (RB), co-house them with untreated bats, and monitor transfer between bats by collecting hair and whiskers for biomarker analysis.

Initial field trials: Bat are not attracted to baits, so delivery in the field is challenging. The high rates of self and mutual grooming observed in bats has previously been exploited to cull vampire bats using poisons like warfarin, applied topically to a small number of bats. Once released, contact and mutual grooming transfers the poison within the colony. We have conducted preliminary biomarker studies in vampire bats in both Mexico and Peru and also in insectivorous bats in Wisconsin. In Peru, we conducted trials with RB-labeled glycerin jelly. Based on capture-recapture data, we estimated a rate of transfer from 1.3 – 2.8 bats for every bat marked. We are analyzing factors associated with rates of transfer, e.g. sex and age of initially treated bats, time of day, to model the rate of vaccination and impact on rabies transmission with different rates of application, prior to actual deployment of vaccine in the field. More recently, we applied RB marked glycerin jelly to the entry of bat houses used by little brown bats (*Myotis lucifugus*). Of 29 bats trapped one week post-application, 59% were positive for biomarker indicating they had eaten the jelly. We will conduct initial trials with each of the delivery vehicles in caves in Wisconsin, targeting local US insectivorous bats. Within one week of application, bats will be trapped at the cave entrance using mist nets or Harp traps and hair will be collected to assess the rate of uptake via biomarker analysis. The bats will be released immediately afterward. The procedures will be tested at several different locations as it will likely take some manipulation to determine appropriate dosages for maximum uptake. After we have determined the most optimal approaches for mass delivery, we will then test

them on wild bats in our three cave sites in Yunnan Province. Again, biomarker will be used to assess rates of uptake and this data can then be used in modeling studies to help determine the optimal rates of application of immunomodulating agents. Biomarker studies can also be used to assess uptake by non-target species, an important consideration in evaluating safety. Fieldwork will be conducted in collaboration with Dr. Yunzhi Zhang (Yunnan CDC, Consultant at EcoHealth Alliance).

Innovative Aerosol Approach to Bat Inoculation: Once we have confirmed uptake in laboratory studies, we will then assess scalable delivery methods in local caves and hibernacula (using biomarker-labeled mediums but without immunomodulatory substances). In collaboration with Dr. Jerome Unidad of Palo Alto Research Center (PARC), we will develop an innovative aerosol platform technology unique to PARC into a field-deployable prototype for use in cave settings. The technology called Filament Extension Atomization (FEA) can spray fluids with a wide-range of viscosities ranging from 1mPa-s (the viscosity of saliva and most aqueous vaccine formulations) up to 600Pa-s (the viscosity of creams and gels for topical delivery) using a roll-to-roll misting process (https://www.parc.com/services/focus-area/amds/) that results in narrowly-dispersed droplets with tunable sizes from 5-500 microns. FEA technology is compatible with all the formulations of interest to project DEFUSE, including aqueous formulations intended for conventional spraying and the edible gels and creams intended for topical delivery with no limit on bioactive ingredient loading. FEA can then be a universal delivery platform for direct spraying onto bats with the formulation geared towards bio-efficacy.

We will subcontract to PARC to develop a field-deployable FEA prototype, potential form factors for which are shown in Fig. 15F, that can be used in cave settings. PARC will develop the prototype in close collaboration with USGS-NWHC and will conduct the initial trials with them on Wisconsin cave bats. After initial trials, PARC will develop the prototype to a form that will be used for the proof-of-concept demonstration at the test sites in the Kunming bat caves, Yunnan province, China. The field-deployable system will be motion-actuated, and on a timer so that bats will be targeted at fly-in and fly-out but diurnal flying non-target species (e.g. cave swiftlets) can be avoided.

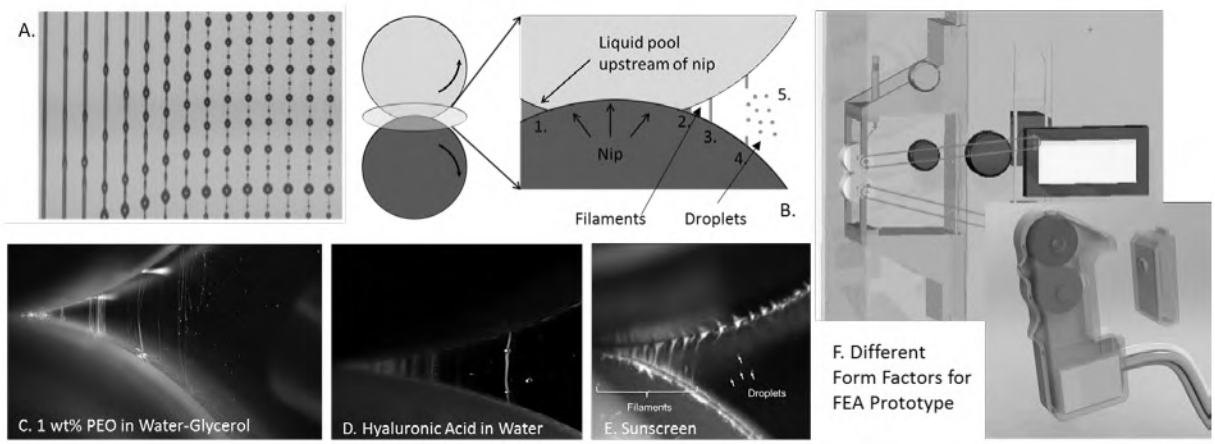

Fig. 15: PARC FEA Technology – A. Beads-on-a-string structures in viscoelastic fluids, B. Parallelization of filament formation and droplet break-up in an FEA roller system, C.-E. Images from high speed videos of representative fluids sprayed with FEA (Polyethylene Oxide in Water-Glycerol, Hyaluronic Acid and Sunscreen), F. Potential form factors of the field-deployable prototype for Project DEFUSE (benchtop, handheld)

Dynamic circulation modeling to optimize deployment strategy. To select amongst various options for immune boosting, priming, and targeting, and multiple delivery options and schedules, we will simulate deployment using a model of viral circulation in cave bat populations. The model will be fit to data from our three-cave test system but designed to be robust to be generalizable to other cases. We will simulate outcomes under a variety of different deployment scenarios to produce conservative estimate of necessary application under real-world conditions. *Fit stochastic viral circulation models to longitudinal sampling data:* We will use longitudinal viral prevalence, mark-recapture estimates of bat populations, radiotelemetry and infrared camera data collected during our field sampling to parameterize and construct models of bat population dynamics and viral circulation in our test caves. We will use a simple but robust stochastic SIR process model with immigration and emigration and flexible, nonlinear contact rates between bats[117]. This model structure can capture a wide range of viral dynamics from intermittent viral outbreaks to regular, endemic circulation with a relatively small number of parameters. We will fit these models to our sampling data using the **partially observable markov process (pomp)** framework[118], allowing estimates of the underlying latent dynamic disease transmission process, accounting for and separating the natural stochasticity of viral circulation and observation error in sampling. We will validate our models via temporal cross-validation: leaving out successive sections on longitudinal time-series from our model fitting to test the model, and by testing the results of a fit from two cave sites on data from a third. *Simulate circulation under a set of plausible deployment scenarios.* Using the top performing sets of immune boosting and targeted immune priming molecules from captive trials, and the delivery media and methods with the greatest uptake rates in cave

studies, we will use the stochastic SIR model to generate simulations of viral circulation under a series of treatment deployments in our focal study caves. These scenarios will cover a range of plausible intensities, frequencies, and combinations of suppression strategies. They will incorporate uncertainty in the efficacy of each of the treatment strategies. From these simulations, we will estimate the expected degree and time period of suppression of viral circulation and shedding and the uncertainty in this expectations. We will determine the optimal scenario for deployment in our focal study caves. *Test robustness of deployment strategies under broader conditions:* We will use our simulation models to determine best strategies for deployment under a variety of conditions covering likely environments. We anticipate the deployment is likely to occur under (a) highly varied species population and compositions, with uncertain estimates based on rough observations (b) varied uptake and efficacy of immune boosting and targeting molecules due to different environmental conditions, and (c) limited time or resources to deploy treatment. Thus, we will simulate deployment under many potential conditions to determine how optimal deployment differs according to condition, and determine deployment strategies which are conservative and robust to these uncertainties and limitations.

Proof-of-concept deployment of immune modulation molecules in test caves in Yunnan Province, China.

MANAGEMENT PLAN

- Provide a summary of expertise of the team, including any subcontractors, and key personnel who will be doing the work. Resumes count against the page count.

- Identify a principal investigator for the project.

- Provide a clear description of the team's organization

- Include an organization chart with the following information, as applicable:
  A) Programmatic relationship of team members
  B) Unique capabilities of team members
  C) Task responsibilities of team members
  D) Teaming strategy among the team members
  E) Key personnel with amount of effort to be expended by each during each year

- Provide a detailed plan for coordination including explicit guidelines for interaction among

collaborators/subcontractors of the proposed effort.

- Include risk management approaches.

- Describe any formal teaming agreements that are required to execute this program.

The lead institution for Project DEFUSE is EcoHealth Alliance, New York, an international research organization focused on emerging zoonotic diseases. The PI, Dr. Peter Daszak, has 25+ years' experience managing lab, field and modeling research projects on emerging zoonoses. Dr. Daszak will commit 3 months annually to oversee and coordinate all project activities, and lead modeling and analytic work for TA1. Dr. Billy Karesh has 40+ years' experience leading zoonotic and wildlife disease projects, and will commit 1 month annually to manage partnership activities and outreach. Dr. Jon Epstein, with 15 years' experience working emerging bat zoonoses will coordinate animal trials. Dr. Kevin Olival and Dr. Noam Ross will manage and conduct the modeling and analytical approaches for this project. Support staff include field surveillance teams, modeling analysts, and consultants based in Yunnan Province, China, to oversee field trials. The EHA team has worked extensively with all other collaborators: Prof. Wang (15+ years); Dr. Shi (15+ years); Prof. Baric (5+ years) and Dr. Rocke (15+ years). **Subcontracts: #1** to Prof. Ralph Baric, UNC, to oversee reverse engineering of SARSr-CoVs, BSL-3 humanized mouse experimental infections, design and testing of immune priming treatments based on recombinant spike proteins. Assisted by senior personnel Dr. Tim Sheahan, Dr. Amy Sims, and support staff; **#2** to Prof. Linfa Wang, Duke NUS, to oversee the immune boosting approach, captive bat experiments, and analyze immunological and virological responses to immune boosting treatments; **#3** to Dr. Zhengli Shi, Wuhan Institute of Virology, to conduct PCR testing, viral discovery and isolation from bat samples collected in China, spike protein binding assays, and some humanized mouse work, as well as experimental trials on *Rhinolophus* bats. Her team will include Dr. Peng Zhou and support staff; **#4** to Dr. Tonie Rocke, USGS National Wildlife Health Center, to refine delivery mechanisms for both immune boosting and immune priming treatments. With a research technician, Dr. Rocke will use a captive colony of bats at NWHC for initial trials, and oversee cave experiments in China; **#5** to Dr. Jerome Unidad, PARC, to develop their innovative aerosol platform into a field-deployable device for large-scale inoculation of the bats. Dr. Unidad will collaborate closely with Dr. Rocke in developing a field-deployable prototype for both initial trials and cave experiments in China.

Dr. Peter Daszak is President and Chief Scientist of EcoHealth Alliance, a US-based research organization focused on emerging zoonotic diseases. His >300 scientific papers include the first global map of EID hotspots[119,120], estimates of unknown viral diversity[121], predictive models of

virus-host relationships[3], and evidence of the bat origin of SARS-CoV[29] and other emerging viruses [122-125]. He is Chair of the NASEM Forum on Microbial Threats, and is a member of the Executive Committee and the EHA institutional lead for the $130 million USAID-EPT-PREDICT. He serves on the NRC Advisory Committee to the USGCRP, the DHS CEEZAD External Advisory Board, the WHO R&D Blueprint Pathogen Prioritization expert group, and has advised the Director for Medical Preparedness Policy on the White House National Security Staff on global health issues. Dr. Daszak won the 2000 CSIRO medal for collaborative research.

Prof. Ralph Baric is a UNC Lineberger Comprehensive Cancer Center member and Professor in the UNC-Chapel Hill Dept. of Epidemiology and Dept. of Microbiology & Immunology. His work focuses on coronaviruses as models to study the genetics of RNA virus transcription, replication, persistence, cross species transmission and pathogenesis. His group has developed a platform strategy to access the potential "pre-epidemic" risk associated with zoonotic virus cross species transmission potential and evaluation of countermeasure potential to control future outbreaks of disease (REFS).

Prof. Linfa Wang is Director, Programme in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore. His proven track record in the field includes identifying the bat origin of SARS-CoV, pioneering work on Henipaviruses and many more. His work has shifted from identifying the bat-origin of pathogens to understanding basic bat biology and the mechanisms by which they can endure sustained virus infection. He has received multiple awards including the 2014 Eureka Prize for Research in Infectious Diseases. He currently heads and administers a Singapore National Research Foundation grant on "Learning from bats" for $9.7M SGD. He is an advisory member of …. an Editor of multiple journals and current Editor-in-Chief for the Journal *Virology*.

Dr. Danielle Anderson is the Scientific Director of the Duke-NUS ABSL3 laboratory and is an expert in RNA virus replication. Dr Anderson has extensive experience in both molecular biology and animal models and will lead the animal studies. Dr Anderson has established Zika, Influenza and Reovirus non-human primate (NHP) models in Singapore, using different inoculation routes (such as mosquito inoculation), and has performed trials on over 30 NHPs.

Dr Aaron Irving is an experienced postdoctoral fellow in the field of innate immunity and viral sensing with expertise focusing on host-pathogen interactions and intrinsic immunity. He oversees multiple projects on bat immune activation within Prof. Linfa Wang's laboratory at Duke-NUS Medical School and has experience in *in vivo* animal infection models.

Prof. Zhengli Shi: Dr. Shi is the director of the Center for Emerging Infectious Diseases of the Wuhan Institute of Virology, Chinese Academy of Sciences. She got Ph.D training in Virology in Montpellier University II from 1996 to 2000, biosafety training at Australian Animal Health Laboratory in May 2006 and at Lyon P4 in October 2006. She is now in charge of the scientific

activity in BSL3 and BSL4 of the Institute. Her research focuses on viral pathogen discovery through traditional and high-throughput sequencing techniques. She has been studying the wildlife-borne viral pathogens, particularly bat-borne viruses since 2004. Her group has discovered diverse novel viruses/virus antibodies in bats, included SARS-like coronaviruses, adenoviruses, adeno-associated viruses, circoviruses, paramyxoviruses and filoviruses in China. One of her great contributions is to uncover genetically diverse SARS-like coronaviruses in bats with her international collaborators and provide unequivocal evidence that bats are natural reservoir of SARS-CoV by isolation of one strain that is closely related to the SARS-CoV in 2002-3. She has coauthored >100 publications on viral pathogen identification, diagnosis and epidemiology.

Dr. Tonie Rocke is a is a research scientist at the USGS National Wildlife Health Center, the only federal laboratory with the sole mission to manage disease in wild animals. Dr. Rocke's current research is focused on the ecology and management of diseases in wild mammals (e.g. plague, monkeypox, rabies and white-nose syndrome) with the overreaching goal of conservation of threatened and endangered species. She and other colleagues developed an oral recombinant plague vaccine for use in wild rodents. Dr. Rocke lead a large-scale field trial in 7 western states of the U.S. demonstrating that oral vaccination through consumption of vaccine-laden baits could prevent plague in wild prairie dogs, thus reducing the risk of disease for the endangered black-footed ferret, other animals, and possibly humans. Research is ongoing in Dr. Rocke's laboratory to develop a similar oral recombinant vaccine to manage rabies in vampire bats in Latin America and also white-nose syndrome in North American bats, a fungal disease that has killed millions of bats in the last few years in the U.S.

Dr. Jerome Unidad is a Member of Research Staff at the Hardware Systems Laboratory at PARC. His research interests revolve around novel fluid delivery systems (including aerosol delivery) for high viscosity fluids, polymers and biomacromolecules. At PARC, he is the technical lead in developing the FEA spray technology for consumer and biomedical applications, as well as additive manufacturing. He has a PhD in Chemical Engineering, specializing in polymer science and rheology, from the University of Naples "Federico II" in Naples, Italy and was a postdoctoral researcher at Forschungszentrum Juelich in Munich, Germany.

Dr. Peng Zhou is a

Dr. Xinglou Yang

Dr. Ben Hu

Dr. Kevin Olival is VP for Research at EcoHealth Alliance. His research over the last 15 years has focused on understanding the ecology and evolution of emerging zoonoses, with a focus on

developing analytical tools and modeling approaches to forecast and prioritize the discovery and surveillance of viral zoonoses. This includes a recent large scale analysis identifying host and viral predictors of spillover in mammals [REF, Nature]. He has led several international field teams to investigate bat-borne viruses globally. Dr. Olival is the Modeling and Analytics coordinator for the USAID PREDICT-2 project; co-PI on an NIH-NIAID project to investigate CoVs in China; and PI on recent DTRA-CBEP grant to characterize CoVs from bats in Western Asia.

Please follow the same format and create Bios for all other personnel with Ph.D and higher. Peter Daszak will then work out how much space we have and decide who to include…

CAPABILITIES

- Describe organizational experience in relevant subject area(s), existing intellectual property, specialized facilities, and any Government-furnished materials or information.

- Discuss any work in closely related research areas and previous accomplishments.

(The following information was taken from the 'Goals and Impact' section of the abstract we submitted).

The SARSr-CoV-bat system, and immune modulation focus: Our group's 15 yrs work on the SARSr-CoV – *Rhinolophus* bat system in China has identified and isolated SARSr-CoVs with remarkable sequence identity in the spike protein to SARS-CoV (e.g. SCH014 & WIV-1). We have shown they bind and replicate efficiently in primary human lung airway cells and that chimeras with SARSr-CoV spike proteins in a SARS-CoV backbone cause SARS-like illness in humanized mice, with clinical signs that are not reduced by SARS monoclonal therapy or vaccination. We have identified a single cave site in Yunnan Province where bat SARSr-CoVs contain all the genetic components of epidemic SARS-CoV (*7,8,9*). We have now shown that people living up to 6 kilometers from this cave have SARSr-CoV antibodies (3% seroprevalence in 200+ cohort), suggesting active spillover, and marking these viruses as **a clear-and-present danger of a new SARS-like pandemic**. Our work on bat immunology suggests that bats' unique flying ability has led to downregulated innate immune genes, and their ability to coexist with viruses such as SARSr-CoVs, henipa- and filoviruses that are lethal in many other mammals (*3*). We have identified bat-specific constitutively expressed bat interferon, a dampened STING-interferon production pathway (*4, 5*), and have identified a series of other innate immunity factors that are dampened in bats (*6*).

STATEMENT OF WORK

- Provide a detailed task breakdown, citing specific tasks and their connection to the interim milestones and program metrics.

- Each phase of the program (Phase I base and Phase II option) should be separately defined in the SOW and each task should be identified by TA (1 or 2).

NOTE: The SOW must not include proprietary information.

- For each task/subtask, provide:

    - A detailed description of the approach to be taken to accomplish each defined task/subtask.

    - Identification of the primary organization responsible for task execution (prime contractor, subcontractor(s), consultant(s), by name).

    - A measurable milestone, i.e., a deliverable, demonstration, or other event/activity that marks task completion. Include quantitative metrics.

    - A definition of all deliverables (e.g. data, reports, software) to be provided to the Government in support of the proposed tasks/subtasks.

Phase I:

TA-01 Task 1.1 Construct species distribution models to predict viral spillover risk in cave bats in South and Southeast Asia
Sub-task 1.1.1.;lkj;lkj;klj
Sub-task 1.1.2.;lj;lkj;lkj
Deliverables: models capable of .....

TA-01 Task 2.5: Field studies to collect tolerant reservoir species. (EcoHealth Alliance, William Karesh).

Sub-Task 2.5.1. Apply for and obtain IACUC approval and appropriate wildlife permits in Bangladesh for sample collection. Collection of blood and urogenital, oropharyngeal and rectal swab

specimens from targeted bat, rodent and non-human primate species from Bangladesh (n = 1000 specimens). Collection of wing-punch dermal tissue biopsies from bats (n = 300).

Sub-Task 2.5.2. Field work is to be conducted by a trained field team using ethical, nondestructive capture, restraint, and sample collection techniques (with IACUC and local government approval). Samples are to be preserved in RNA later (or other preservative) to maintain cellular integrity and frozen at the point of collection using a liquid nitrogen dry shipper and maintained in -80°C. All samples are to be shipped with appropriate government permission and export permits.

Deliverables: 1000 field specimens (whole blood, nasal/rectal swabs) collected from reservoir bats, rodents and non-human primates which have been obtained with all proper permits and permissions are appropriately shipped for further analysis.

TA1:

Task 1.1

Sub-task 1.1.1. Models to predict bat community in caves across S. and SE Asia.

Organization leading task: EcoHealth Alliance

Sub-task 1.1.2. Models to predict presence of viruses with zoonotic potential in bats across S. and SE Asia.

Progress Metrics:

- Joint species distribution model fit for Asian Bats
- Cave-level predictions of bat community composition
- Linear predictions of viral diversity in cave populations
- JSDM predictions of viral diversity in cave populations
- Prediction validations

Deliverable(s):

- Deployable spatial model software of bat community composition
- Deployable spatial model software of viral diversity in bat cave populations

Progress Metrics:

- Joint species distribution model fit for Asian Bats
- Cave-level predictions of bat community composition
- Linear predictions of viral diversity in cave populations
- JSDM predictions of viral diversity in cave populations
- Prediction validations

Deliverable(s):

- Deployable spatial model software of bat community composition
- Deployable spatial model software of viral diversity in bat cave populations

Subtask 1.1.3: Develop prototype app for the warfighter
Description and execution:
Preliminary Data:
Organization leading task: EcoHealth Alliance
Progress Metrics: Development of fully functional and user-friendly application. Use of application in the field.
Deliverables:

Task 1.2: Determining baseline risk of SARSr-CoV emergence in Yunnan, China
Subtask 1.2.1. Longitudinal sampling of bats to determine virus prevalence and diversity in Yunnan cave sites.
Subtask 1.2.2. Analyzing ability of CoVs to infect and emerge in people

***(TA1)* Subtask 5: Assay SARr-CoV quasispecies for spillover potential via assays for binding, cell entry, and pathogenesis in mouse models.**

Organization leading task: University of North Carolina

Progress Metrics: Not sure how to do this.

Deliverable(s):

1. *Methods to Produce Synthetic SARSr-CoV Virus Molecular Clones and Reverse Genetics.*
    a. ***Preliminary Data**:* Molecular Clones for SARSr-CoV WIV1, WIV16, SHC014 and HKU3-SRBD exist. We have demonstrated in the preliminary data that these reagents are already available.
    b. ***Target Goals**:* We will generate molecular constructs for 20+ chimeric SARSr-CoV encoding different S glycoprotein genes/yr
    c. **Target Goals***: We will generate 2-5 full length molecular clones of SARSr-CoV.*

2. *Methods of Recombinant virus Recovery and Characterization*
    a. **Preliminary Data***: Demonstrated recovery recombinant chimeric SARSr-CoV WIV1, WIV16, SHC014, HKU3-SRBD, including full length recombinant viruses of WIV1, WIV16, SHC014 and HKU3-SRBD.*
    b. *Target Goals:* We will isolate 20+ chimeric SARSr-CoV encoding novel S

glycoprotein genes

c. *Target Goals:* We will isolate 2-5 full length SARSr-CoV/year/
    i. **Key Deliverables for Program-wide Success***: These two key reagents position us for immediate testing of the antiviral effects of* **broadscale immune boosting molecules** +/- immunogens on virus growth *in vitro* and *in vivo*, and on virus levels in models of chronic SARS-CoV infection in mice.

3. *Virus Phenotyping: Receptor Interactions and In Vitro Growth.*
    a. ***Preliminary Data****: Cell lines encoding bat, human, civet and mouse ACE2 receptors exist and have been validated. We have demonstrated the use of primary human airway epithelial cultures to characterize SARSr-CoV pre-epidemic potential.*
    b. ***Target Goals****: We will characterize SARSr-CoV recombinant virus growth in Vero cells, nonpermissive cells encoding the civet, bat and human ACE2 receptors.*

4. *Virus Pathogenic Potential in Humans:*
    a. *Preliminary Data: We also have transgenic human ACE2 mouse models to compare the pathogenic potential of SARSr-CoV*
    b. *Target Goals: We will evaluate SARSr-CoV pathogenic outcomes in hACE2 transgenic mice.*

5. *Virus Antigenic Variation:*
    a. *Preliminary Data: We have robust panels of broadly cross reactive human monoclonal antibodies against SARS and related viruses and mouse models to evaluate protection against SARSr-CoV replication and pathogenesis.*
    b. *We will evaluate SARS-vaccine performance against a select subset of SARSr-CoV (10), chosen based on the overall percent of antigenic variation, coupled with distribution across the S glycoprotein structure.*

6. *Low Abundant High Consequence Sequence Variants:*
    a. *We will identify the presence of low abundant, high risk SARSr-CoV, based on deep sequencing data*

7. *Proteolytic Processing and Pre-epidemic Potential.*
    a. *We will evaluate the role of proteolytic cleavage site variation on SARSr-CoV cross species transmission and pathogenesis in vivo.*

(TA1) **Subtask 4: Build models to predict viral species spillover potential and evoluation**

Organization leading task: EcoHealth Alliance

Description and execution:

Progress Metrics:

- *Development of prior-based pathogenicity predictions and sequence testing guidance*
- *Model fits from initial rounds of viral characterization*
- *Model fits from secondary rounds of viral characterization*
- *Predictions of spillover probability of sequenced viral QS*
- *Deployable predictive model*

Deliverable(s):

- Fit models as reproducible, deployable software providing virus spillover potential predictions and uncertainties based on input of host species and viral sequence data
- Ranking of potential pathogenicity of virus QS from both Task X sampling and previous data.

***(TA2)* Task 5: Trial experimental approaches aimed towards 'Broadscale Immune Boosting' using experimental bat colonies**

TA2: Develop scalable approaches that target and suppress the animal virus in its reservoir(s) and/or vector(s), to reduce the likelihood of virus transmission into humans.

Organization leading task: Wuhan Institute of Virology, Duke-NUS

***(TA2)* Task 6: Trial experimental approaches aimed towards 'Immune Targeting' using experimental bat colonies**

Organization leading task: University of North Carolina

Progress Metrics:

Deliverable(s):

1. ***Chimeric S-Glycoprotein Antigen Design, Recovery and Phenotyping for Immune Boosting.***
    a. **Preliminary Data***: Demonstrated recovery recombinant chimeric HKU3-*S_{mix}*, demonstrating preservation of entry functions in the chimeric spike. Neutralizing*

epitopes and in vivo pathogenesis phenotypes were also preserved. Chimeric Spikes are biologically functional.

b. ***Target Goals****:* We will isolate chimeric HKU3-S_{S014} S and WIV-S_{S014} genes, chimeric viruses and express the S glycoprotein from VRP and raccoon poxvirus expression vectors.

c. ***Target Goals:*** We will synthesize 2-3 chimeric S glycoproteins, recover recombinant viruses derived from natural recombinants in the population genetic structure of SARSr-CoV. We will also characterized recombinant protein expression from VRP and raccoon poxviruses.

d. ***Target Goals****:* We produce sufficient recombinant HKU3-S_{S014}, WIV-S_{S014} and HKU3-S_{mix} S glycoproteins for inclusion in nanoparticle and microparticle delivery vehicles.

   i. **Key Deliverables for Program-wide Success***: These two key reagents position us for immediate testing of the antiviral effects of* **broadscale immune boosting molecules** +/- immunogens.

2. ***Virus Phenotyping: Receptor Interactions and Growth in vitro and in vivo****.*

   a. ***Preliminary Data****: We have well developed metrics for evaluating chimeric S glycoprotein function in the context of whole virus, neutralization phenotypes and expression as recombinant proteins vaccines for testing in mice.*

   b. ***Target Goals****: Demonstrate chimeric S function in the context of virus infection in Vero and HAE cells and susceptibility to neutralizing antibodies targeted the SARS RBD.*

   c. ***Target Goals****: Evaluate chimeric virus pathogenesis in hACE2 transgenic mice and the ability of VRP vaccines encoding chimeric spikes to elicit protective immunity against lethal SARS-CoV, HKU3-S_{mix} and SCH014 challenge.*

3. ***Production of Agonist (TLR4, dsRNA, Sting) and Chimeric S glycoprotein Nanoparticle and Microparticle Suspensions for in vivo studies***

   a. ***Preliminary Data****: Robust preliminary data exists on the production and immunogenicity of nanoparticle and microparticle delivery systems.*

   b. ***Target Goals****: Produce nanoparticle and microparticle delivery systems encoding agonists, coupled with in vitro testing in vitro in bat and in other reporter cells, mice and bats.*

   c. ***Target Goals****: Inclusion of chimeric recombinant proteins and agonists in nanoparticle and microparticle delivery vehicles, coupled with testing in vitro and in vivo in mice and bats.*

   d. ***Target Goals***: Perform *in vivo* testing in collaboration with Dr. Shi and Dr. Wang.

Task 7: Develop and assess delivery methods to bats for immune boosting and priming molecules

Organization leading task: USGS National Wildlife Health Center
Participating organizations: Palo Alto Research Center (PARC)

Progress Metrics: Still not sure what format you want this in?

Deliverable(s):

1. Poxvirus construct expressing optimal SARS/CoV spike protein for immunizing bats

a. Genetically insert SARS/CoV spike proteins into raccoon poxvirus and confirm antigen expression

b. Conduct laboratory studies to confirm serologic conversion, first in mice (UNC) and then in bats (NWHC)

c. Master seed production of viral stocks for use in later field trials

2. Mediums/vehicles and methods to deliver immunomodulatory agents to bats.

a/ Determine appropriate medium (e.g. glycerin jelly or other viscous substance) for delivering virally vectored vaccines and nanoparticles to bats

b. Assess minimum dosage required for adequate uptake by bats after topical application

c. Determine appropriate delivery methods to apply appropriate dosages in conjunction with PARC, first in laboratory settings, and then in local field sites

3. Prototype system for automatic, mass delivery of immunomodulatory substances to bats (in collaboration with PARC)

a. Conduct biomarker studies to validate application methods in bats, first in local field sites and then at sites in China

b.. Conduct field trial in China with prototype delivery method using selected immunomodulatory substances deemed most useful for bats

4. Data on uptake in insectivorous bats.

a. Provide data on biomarker uptake in insectivorous bats for use in modeling studies

5. Annual reports, manuscripts, presentations.

SCHEDULE AND MILESTONES

- Provide a detailed schedule showing tasks (task name, duration, work breakdown structure element as applicable, performing organization), milestones, and the interrelationships among tasks.

  NOTE: Task structure must be consistent with that in the SOW.

- Measurable milestones should be clearly articulated and defined in time relative to the start of the project.

PREEMPT TRANSITION PLAN

- Indicate the types of partners (e.g. government, private industry, non-profit)

- Submit a timeline with incremental milestones toward successful engagement.
  NOTE: begin transition activities during the early stages of the program (Phase I).

- Describe any potential DARPA roles.

Project DEFUSE partners come from academic, government, private industry, private non-profit institutions and will develop a coherent transition plan for research findings, data and any technology developed in this work.

PARC as a private industry partner (large business) is a fully-owned subsidiary of Xerox Corporation and is committed to commercializing the FEA technology through IP licensing for different applications spaces to different commercial partners. In the context of project DEFUSE, PARC has been and will continue to engage potential licensees (OEMs) in the biotechnology and biomedical fields for eventual transitioning of targeted delivery technology that might result in the project. PARC already has existing networks of business relations in the biotechnology and biomedical space, both large companies (Fortune 500, Fortune 1000) and small businesses and start-ups who could be transition partners for FEA as a wide-scale, large-area drug delivery device. In addition, in collaboration with our extended network of DEFUSE partners and with DARPA, we will further identify existing government needs for our delivery technology, particularly in wildlife health management (in collaboration with EHA and USGS-NWHC) as well as in suppression of emerging threats (in collaboration with government agencies such as the CDC). PARC will leverage this knowledge in developing a needs-based commercialization plan with potential partners.

PREEMPT RISK MITIGATION PLAN

- Provide the following:
    - An assessment of potential risks to public health, agriculture, plants, animals, the environment, and national security.
    - Guidelines the proposer will follow to ensure maximal biosafety and biosecurity.
    - A communication plan that addresses content, timing, and the extent of distribution of potentially sensitive dual-use information. The plan must also address how input from DARPA, other government, and community stakeholders will be taken into account in decisions regarding communication and publication of potentially sensitive dual-use information.

ETHICAL, LEGAL, SOCIETAL IMPLICATIONS

- Address potential ethical, legal, and societal implications of the proposed technology.

BIBLIOGRAPHY

A) Brief Bibliography (no page limit indicated – can be published/unpublished)
This and next part don't count toward 36 page limit

RELEVANT PAPERS

B) Up to 3 relevant papers attached (optional)
Propose:

- Ge et al. Nature
- Menacherry et al.
- Zhou et al. SADS-CoV

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