

1. Vertebrate Animals Section

Are vertebrate animals euthanized?

If "Yes" to euthanasia

Is the

Yes No

If "No" to AVMA guidelines, describe method and provide

.....

2. *Program Income Section

*Is

Yes No

If you checked "yes" above, indicating that program income is derived from source(s),

Introduction

1. Introduction to Application
(for Resubmission and Revision applications)

Research Plan Section

2. Specific Aims

NIAID_COV_2019_SPECIFIC_AIMS_Final.pdf

3. Research Strategy

NIAID_COV_2019_RESEARCH_STRATEGY_Final.pdf

4. Progress Report

Other Research Plan Section

5. Vertebrate Animals

NIAID_COV_2019_VERTEBRATE_ANIMALS_Final.pdf

6. Select Agent Research

NIAID_COV_2019_SELECT_AGENT

7. Multiple PD/PI Leadership Plan

8. Consortium/Consortium

NIAID_COV_2019_CONSORTIUM

9. Budget of Support

NIAID_COV_2019_LOS_Final.pdf

10. Resource Sharing Plan(s)

NIAID_COV_2019_RESOURCE_SHARING_PLAN_Final.pdf

11. Anticipation of Technology Transfer

Appendix

Appendix

SPECIFIC AIMS

Zoonotic coronaviruses are a significant threat to global health, as demonstrated with the emergence of Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) in 2002. Middle East Respiratory Syndrome (MERS) and our group as bat species, and since then we have sequenced dozens of novel SARS-related CoV (SARSr-CoV) strains. Our previous R01 work demonstrates that bats in southern China harbor an extraordinary diversity of SARSr-CoVs, many of which are able to use human ACE2 to enter into human cells and infect humanized mouse models to cause SARS-like illness, and evade available therapies or vaccines.

We found that people living close to bat habitats are the primary groups for spillover. At one of these sites, we found evidence of human exposure among people living near a SARSr-CoV and of public health impacts. For some

and sites that harbor CoV strains with high potential for spillover? Which human behaviors do pose a risk of bat SARSr-CoV exposure? What are the risk factors for spillover? Does human exposure to SARSr-CoV cause illness like or other illness? Can we characterize viral strain diversity, bat traits and human behaviors to predict potential future CoV spillover? The proposed work in this renewal R01 will build on our findings to address these issues by conducting: 1) focused sampling of bats in southern China to identify hotspots of high risk SARSr-CoV predicted risks for spillover; 2) community-based, and clinic-based surveillance of people to identify spillover, and assess human risk factors and evidence of illness.

viral characterization and analyze epidemiological data to identify hotspots of future CoV spillover risk. This work will follow 3 specific aims:

Aim 1: Characterize the diversity and distribution of SARSr-CoVs in southern China. We will conduct targeted sampling at sites where we predict high risk SARSr-CoV strains exist. Bat sampling will be targeted geographically and by host species to test for evolutionary diversity of SARSr-CoVs and to assess the risk of spillover to people in Aim 3.

Aim 2: Assess human exposure to SARSr-CoVs and potential public health consequences. We will conduct focused, targeted human surveys and sampling to identify key risk factors for SARSr-CoV spillover and evidence of illness. To maximize our opportunity capturing human exposure to bat CoVs, we will conduct surveillance of people living in close proximity to bat habitats with high SARSr-CoV seropositive status. We will also conduct clinic-based syndromic surveillance close to bat habitats for people with SARS-like illness and severe acute respiratory illness, assess the risk of spillover, and sample for PCR and serological evidence of SARSr-CoV infection. We will conduct follow-up sampling to capture patients who had not yet sought medical attention.

Aim 3: *In vitro* and *in vivo* characterization of SARSr-CoVs. We will perform phylogenetic analyses to identify the regions and viruses of public health concern. We will test the propensity of novel SARSr-CoVs to infect people *in vitro* using primary human airway epithelial cells, and *in vivo* using the transgenic hACE2 mouse model. We will also test the ability of SARSr-CoVs to infect human cells, and to evade mAb therapeutics and vaccines. We will then map the geographic distribution of their bat hosts and other ecological risk factors to identify the key hotspots of risk for future spillover.

Conclusions: Our SARSr-CoV program will identify key risk factors contributing to CoV emergence while providing insights into the mechanisms of zoonotic pandemics. This includes providing a framework for future SARSr-CoV pandemic preparedness and health preparedness.

previous work also suggests that SARSr-CoVs with S proteins that are ~10% divergent from SARS-CoV resist neutralization by therapeutic mAbs and escape SARS-CoV vaccines (17, 18, 23), suggesting that . However, viruses with 10-25% divergence in S proteins may bind to human cell receptors, but completely evade therapeutic and vaccine effects, and could therefore be a higher risk for public health. We will sequence the S proteins of novel SARSr-CoV for experimental work in Aim 3 to test

1.2 General Approach: We will use sequence data from our previous study (2019) to pinpoint sites and host species in Guizhou and Guangdong, China, where we discovered SARSr-CoVs and with competent bat species (Y1). We will target at least 5,000 individual bats over 5 years from 15 sites in *Rhinolophus* bats, which will allow us to almost fully characterize the expected natural diversity of SARSr- and other p-CoVs in the region. Bats will be captured, sampled, and released at the site. Specimens will be transported in liquid N₂ to Wuhan Inst. Virology (WIV) for PCR screening, and positive samples selected for further molecular characterization. WIV will lead the testing and viral sequencing and data analysis for this Aim; and WIV will lead the testing and viral sequencing.

1.3 Sampling and testing of bats: 1.3.a Site selection We will use viral trait modeling, phylogeographic analyses of RdRp and S Protein sequences, and geographic and host species-based viral discovery curve analyses to identify SARSr-CoV diversity hotspots. We will sample at 8 new sites in Yunnan, Guizhou, and Guangdong, China, which humans likely have contact with bats. In Yunnan, we will sample caves close to, but distant from, the sites. This will provide adequate coverage of lineage 1 and 2 SARSr-CoVs, including a hot source of new SARSr-CoVs, which have unknown potential for zoonotic spillover. Sampling will begin to use survey data from our previous study and possible 10 under-sampled *Rhinolophus* spp. In Guangdong, we previously found PCR-positive related bat genera (including *Hipposideros* spp. and *Acelliscus* spp.) we previously found PCR-positive SARSr-CoVs (Table 1). We will sample at least 500 bats from these 4 provinces with ~5-12% prevalence of SARSr-CoV. This will result in us 425 (±175) positive individual bats and ~125 novel strains.

1.3.b CoV screening: Total RNA will be extracted from bat samples using the Viral RNA Kit (Qiagen). RNA will be stored at -80°C. A RT-PCR kit will be used to detect the presence of CoV sequences using primers that target a 440-nt fragment in the dependent RNA polymerase gene (RdRp) of all known α -CoV (149). PCR products will be sequenced with an ABI Sanger sequencer. We will also identify and sequence any interesting novel CoVs, using Vero E6 cells and bat primary cell culture.

1.3.c Sequencing S proteins: For all novel SARSr-CoV strains, we will sequence the complete S gene by amplifying overlapping fragments using degenerate primers as shown previously. Full-length genomes of selected SARSr-CoV strains (representative across subclasses) will be sequenced via high throughput sequencing method followed by genome walking. The sequencing libraries will be constructed using NEBNext Ultra II DNA Library Prep Kit for Illumina. Sanger PCR will be performed to fill gaps in the genome. The full length S gene sequences, including the amount of variation in the S receptor binding residues that bind the ACE2 receptor, will be used to select strains for Aim 3 experiments.

1.3.d Host ACE2 receptors: We will sequence host ACE2 receptors of different bat species or different populations from a single site as well as *Rhinolophus* spp. We will evaluate the relative importance of high risk SARSr-CoVs across to bat and human orthologous ACE2 contact interface residues that engage the SARSr-CoV S protein as a potential indicator of SARSr-CoV cross species transmission potential.

1.4 Analyses: 1.4.a Bat-CoV evolution and phylogenetic analyses: We will use SnpSites and full genome sequence (when available) data to reconstruct the evolutionary history of the novel bat SARSr-CoV lineages.

reconstruct β -CoV evolution using phylogenetic methods. Phylogenetic models will be used to predict spatial distribution of SARS-CoV-2 positive species, including sampling, and overlap with people. We will use our viral discovery/accumulation curve approach (Fig. 4) to monitor progress toward a discovery of 70% of predicted diversity of SARS-CoV-2 (image 1 & 2) within 100 public locations, halting sampling when this target is reached, and reusing resources for work

Which Viral strain prioritizations? We will prioritize 100-200 SARS-CoV-2 strains we will down-select to prioritize for further characterization based on SARS-CoV-2 RBDs are: i) different from SHC014, WIV1, SARS-CoV with diversity ranges of 10-25%; ii) have virus S RBD that could use human/bat receptors; iii) recombinant chimeric spikes indicative of a recombination event and ns1 amino acid R522 receptors that might select for spike RBDs that can use ACE2 receptors for entry (15/18 conserved residues in human/bat ACE2 molecules that bind SARS-CoV S RBD domains are likely more efficient receptors than 3/18 conserved sites). Using structural models based on the SARS-CoV-2 glycoprotein, the extent and location of antigenic variation will be annotated onto the structure, identifying the locations of highly evolving and conserved sites.

1.5 Potential problems/alternatives
selection We have a

the start of this project. Due to the abundance of distinct caves in the region, we will rapidly identify a suitable, complementary cave with similar bat species composition. **may identify β -CoVs in our sample bases due to seasonal viral shedding.** The same regions we have selected are not seasonal in SARS-CoV shedding. Nonetheless, for this we will conduct sampling every on quarterly basis within each province.

Aim 2: Community and clinic-based surveillance to capture SARS-CoV-2 spillover, routes of exposure and potential public health consequences

2.1 Rationale: Our previous R01 study identified serological evidence of exposure to SARS-CoV-2 in rural communities in S. China (Table 2, Fig. 10) (19). However, the low prevalence (1.5% at 10 sites) suggests we need a larger sample size, and a focused, targeted study to maximize the likelihood of identifying seropositive cases. In Aim 2, we will use combined biological-behavioral risk surveillance in targeted populations within the community and clinical settings to 1) identify risk factors of exposure to and potential health impacts of SARS-CoV-2, and 2) assess potential health impacts of SARS-CoV-2 spillover events and their potential for the emergence of SARS-like diseases. It will also support the development of risk mitigation strategies by public health authorities.

2.2 General Approach/Innovation: we will use a dual study design to gain in-depth understanding of exposure and risk factors for SARS-CoV-2 spillover (Fig. 13). We will conduct community-based surveillance with more focused questionnaires and biological sampling to determine the seroprevalence of SARS-CoV-2 in at-risk human populations, and to identify risk factors for SARS-CoV-2 spillover in these communities. We will conduct clinic-based syndromic surveillance at 10 sites within their catchment. This will include follow-up sampling of individuals with acute respiratory illness (ARI) and SARS-CoV-2 seroconversion.

SARS-CoV-2 replication. Both community-based and clinic-based syndromic surveillance programs are case-control studies designed with the sample sizes necessary to statistically identify risk factors and health impacts for SARS-CoV-2 spillover, linked to

2.3 Target Population & sample size: We will target sites in the same four provinces, and close to those for the S protein sequence divergence of 5-25% and C) high levels of human-bat interactions. Community-based surveillance will be conducted at 2 sites in each of the 4 provinces, a total of 8 sites. From our previous work we anticipate that 10-30% of the community population will have

Aim 3: *In vitro* and *in vivo* characterization of SARSr-CoV spillover risk, coupled with spatial and phylogenetic analyses to identify the regions and viruses of public health concern

3.1 Rationale and justification: In Aim 1 we aim to expand the known diversity of SARSr-CoV strains, targeting 10-25% S protein divergence from the WIV1 backbone. This will be important for understanding the therapeutic and vaccine efficacy. In Aim 3, we will further characterize the zoonotic potential of a group of these novel bat SARSr-CoVs using molecular biology technology, including receptor binding and analysis of HKU3r-CoV receptor binding to test whether they meet the 10-50% divergence threshold to predict spillover potential (18, 55). We will conduct *in vitro* and *in vivo* infection experiments, coupled with bat host distribution, viral diversity and phylogenetic analyses to assess spillover risk of SARSr-CoVs in different bat species across southern China. This will enable us to identify high risk bat species for targeted surveillance to prevent the emergence of a novel SARSr-CoV.

3.2 General approach: We will use S protein sequences with 25% S protein divergence we predict as high public health potential and construct chimeric SARSr-CoVs using the WIV1 backbone and these S genes. We will assess infection of non-permissive cells expressing human, bat and civet ACE2 receptors, Vero cells, primary human airway epithelial cells (which have not previously been shown to be permissive for SARSr-CoV infection, but may use intestinal epithelium in nature). We will conduct experimental infections in ACE2 transgenic mice to assess pathogenicity and clinical signs (18). Finally, using a panel of monoclonal antibodies that neutralize SARSr-CoVs with different S protein sequences to evade therapeutic monoclonal antibodies with high public health potential, we will also conduct limited experiments to analyze HKU3r-CoV receptor binding. Using these results, and data from Aims 1 and 2, we will use spatial modeling techniques to identify geographic hotspots in southern China where bat species that harbor high risk SARSr-CoVs inhabit, where communities that have high exposure to bats exist, where there is no evidence of spillover, and where underlying demographic or environmental trends suggest high risk of future emergence.

3.3 Virus characterization. 3.3.a Construction of chimeric SARSr-CoV viruses: The S gene of novel bat SARSr-CoVs and the SARSr-CoV WIV1 genome backbone using the reverse genetic system developed in our previous R01 (24). The correct infection-competent clones will be screened by BAC DNA digestion with appropriate restriction enzymes and verified by sequence analysis. Our research group is well versed in coronavirus reverse genetics.

3.3.b Cell entry analysis: HeLa cells expressing human ACE2 are cultured on 6-well plates at 37°C and incubated with the chimeric bat SARSr-CoVs with different spike proteins at a multiplicity of infection (MOI) = 1.0 for 1h. The inoculum is removed and the cells are washed 3 times with PBS and supplemented with medium. HeLa cells without ACE2 are used as negative control. Two-four hours after infection, cells are washed with PBS and fixed with 4% formaldehyde in PBS (pH 7.4) at 4°C for 1h. ACE2 expression is detected by goat anti-human ACE2 immunoglobulin followed by FITC-labelled donkey anti-goat immunoglobulin. Virus replication is detected by using rabbit antibody against the nucleocapsid protein of bat SARSr-CoV followed by Cy3-conjugated mouse anti-rabbit IgG. The cells are then washed and analyzed by flow cytometry. This is conducted to determine the viral titers and growth kinetics in the infected cells.

3.3.c Primary cell infection: Primary cell cultures from the lungs of bat transplant recipients represent highly diverse and unique cell types containing ciliated and non-ciliated epithelial and goblet cells, grown on an air-liquid interface for several weeks prior to use (18, 55, 56). We will prepare HAE cultures from three different patient codes, triplicate in collaboration with the CDC. The HAE cultures will be inoculated with chimeric bat SARSr-CoVs to assess efficient replication. At 70-80% confluence, cells will be fixed for immunofluorescent staining using antisera to SARSr-CoV conserved nucleocapsid protein (N) (57, 58). SARSr-CoVs that differ significantly in S protein sequence (11-12) will be tested for their ability to infect *in vitro*, with a panel of SARS-CoV cross reactive human mAbs S227:14, S230:14, S230:15, S230:16, S230:17, S230:18, S230:19, S230:20, S230:21, S230:22, S230:23, S230:24, S230:25, S230:26, S230:27, S230:28, S230:29, S230:30, S230:31, S230:32, S230:33, S230:34, S230:35, S230:36, S230:37, S230:38, S230:39, S230:40, S230:41, S230:42, S230:43, S230:44, S230:45, S230:46, S230:47, S230:48, S230:49, S230:50, S230:51, S230:52, S230:53, S230:54, S230:55, S230:56, S230:57, S230:58, S230:59, S230:60, S230:61, S230:62, S230:63, S230:64, S230:65, S230:66, S230:67, S230:68, S230:69, S230:70, S230:71, S230:72, S230:73, S230:74, S230:75, S230:76, S230:77, S230:78, S230:79, S230:80, S230:81, S230:82, S230:83, S230:84, S230:85, S230:86, S230:87, S230:88, S230:89, S230:90, S230:91, S230:92, S230:93, S230:94, S230:95, S230:96, S230:97, S230:98, S230:99, S230:100.

Progress report publication list: R01 AI110964, Daszak PI, Project Period: 06/01/2014 - 05/31/2019

The following are peer-reviewed papers published from work funded by this IAID R01 during the project period (1-18). Other manuscripts on behavioral risk, phylogenetic and viral risk characterization are in prep and awaiting submission prior to the end date of the grant.

1. Hu et al., Detection of diverse novel astroviruses from small mammals in China. *Journal of General Virology* **95**, 2442-2449 (2014). PMID: 25031967
2. B. Hu, X. Y. Ge, F. Wang, J. Li, B. Liu, Origin of human coronaviruses. *Virology journal* **12**, (2015). PMID: 26529940
3. V. D. Menachery et al., A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence. *Nature Medicine*, **21**, 1508-1513 (2015). PMID: 26532008
4. J. N. Mandl et al., Reservoir Hosts of SARS-like Coronaviruses. *Journal of Virology* **89**, 2202-2207 (2015). PMID: 25533784
5. M. N. Wang et al., Longitudinal surveillance of SARS-like coronaviruses in bats by quantitative PCR. *Virologica Sinica* **31**, 70-83 (2010). PMID: 21920713
6. L. Brierley, M. J. Vonhof, K. J. Olival, P. Daszak, K. E. Jones, Quantifying Global Drivers of Zoonotic Bat Viruses: A Process-Based Approach. *Journal of Biogeography*, **43**, 103-114 (2016). PMID: 26807755
7. X. M. Ge et al., Coexistence of multiple coronaviruses in several bat colonies in an abandoned mineshaft. *Virologica Sinica* **31**, 1-10 (2016). PMID: 26920100
8. P. Zeng et al., Accessory Protein, ORFX, in a Novel Coronavirus Closely Related to the Direct Progenitor of Severe Acute Respiratory Syndrome Coronavirus. *Journal of virology* **90**, 6573-6582 (2016). PMID: 27170748
9. X. L. Yang et al., Isolation and Characterization of a Novel Coronavirus Closely Related to the Direct Progenitor of Severe Acute Respiratory Syndrome Coronavirus. *Journal of virology* **90**, 3255-3256 (2016). PMID: 26713272
10. K. J. Olival et al., Host and viral traits predict zoonotic spillover from mammals. *PLoS Pathogens* **13**, e1005312 (2017). PMID: 28636390
11. L. P. Zeng et al., Cross-neutralization of SARS-like coronaviruses. *Science* **355**, 144-146 (2016). PMID: 27134417
12. X. L. Yang et al., Genetically Diverse Filoviruses in *Rousettus* and *Eonycteris* spp. Bats, China, 2012 and 2015. *Emerging Infectious Diseases* **23**, 482-486 (2017). PMID: 28221123
13. B. Hu et al., Discovery of a rich gene pool for bat SARS-related coronaviruses provides evidence for the origin of SARS coronavirus. *PLoS pathogens* **13**, (2017). PMID: 29190287
14. N. Wang et al., Serological Evidence of SARS-related Coronavirus Infection in Humans, China. *Virologica Sinica* (2018). PMID: 29669833
15. C. M. Luo et al., Discovery of Novel Bat Coronaviruses in Guangdong, China, Closely Related to Severe Middle East Respiratory Syndrome Coronavirus. *Journal of Virology* **92**, (2018). PMID: 29669833
16. Y. Li et al., Longitudinal Surveillance of Bat Coronavirus in Guangdong, China, 2009-2016. *Virologica Sinica* **33**, 8 (2018). PMID: 29669833
17. Z. Wu et al., The Role of Bats in the Origin of Emerging Infectious Diseases. *Journal of Virology* **91**, 1-10 (2017). PMID: 28221123
18. P. Zhou et al., Fatal outcome associated with a novel coronavirus identified in a patient with atypical pneumonia in China. *Nature* **556**, 255-258 (2018). PMID: 29981333

PHS Human Subjects and Institutional Review Board

OMB Number: 0920-0045

Expiration Date: 03/31/2020

Are you a Human Subjects Investigator? Yes No

Is the Direct Exemption from Federal Review Applicable? 1 2 3 4

Exemption 1 2 3 4

Other Requested Information

Human Subject

Study#	Study title	Clinical Trial?
1	Understanding the surveillance to capture the 5r-CoV spillover routes of exposure	

Section 1. Basic Information (Study

Expiration Date: 03/31/2020

1.1. Study Title *

Understanding the Risk of Bat Coronavirus Emergence
SARSr-CoV s routes of e

1.2. Is this study exempt from Federal Regulations *

1.3. Ex

1.4. Clinical Trial Characteristics *

1.4.a. Does the study have a control group? Yes No

1.4.b. Are participants prospectively assigned to an intervention? Yes No

1.4.c. Is the study designed to evaluate a specific effect of the intervention on participants? Yes No

1.4.d. Is the effect that is being evaluated behavioral or outcome? Yes No

1.5. Provide the ClinicalTrials.gov Identifier (e.g. NCT87654)