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THE UNIVERSITY of NORTH CAROLINA at CHAPEL HILL

RE: SARS-Uganda Constructs March 20, 2017

Dear Fang and Erik,

We are considering the synthetic recovery of a chimeric bat SARS-like coronavirus. It contains the bat coronavirus SCH014 (group 2b) genome backbone, but will include the bat SARS-Uganda (PDF-2386) spike glycoprotein with and without mutation sets in the virus receptor binding domain designed to enhance interaction with the mouse ACE2 receptor. Neither of these viruses have caused disease or are likely capable of wide and uncontrollable spread in human populations, nor is the virulence of these isolates in humans known, as neither have caused human infections. We believe that potential concerns and oversight of this chimera remain focused on sections 2.1 and 2.2 of the P3C0 document.

2.1. A *potential pandemic pathogen* (PPP) is one that satisfies both of the following:

2.2.1. It is likely highly transmissible and likely capable of wide and uncontrollable spread in human populations, and

2.2.2. It is likely highly virulent and likely to cause significant morbidity and/or mortality in humans.

2.2. An *enhanced PPP* is a PPP resulting from the enhancement of a pathogen's transmissibility and/or virulence. Wild-type pathogens that are circulating in or have been recovered from nature are not enhanced PPPs, regardless of their pandemic potential.

We do not believe that this chimera satisfies either of the above for mentioned criteria as: 1) bat coronavirus SCH014 is a natural bat coronavirus that has not been demonstrated to be virulent, cause human mortality or morbidity, replicate in humans or shown to be highly transmissible or capable of wide and uncontrolled spread in human populations. Importantly, the SCH014 spike glycoprotein can use human ACE2 receptors for entry and grow on human primary airway cells, but has never been associated with human disease. The replicative capacity of the SARS-Uganda strain is unknown in human or primate cells, however, this strain has never been associated with human disease. Hence, S glycoprotein chimeras between these two strains are not likely to represent an increased threat over SCH014, even with mutations that enhance SARS-Uganda spike (PDF-2386) ACE2 receptor usage in mice, because SHC014 already replicates efficiently in the mouse. These recombinants have utility and are important for evaluating the breadth of current SARS-CoV vaccines, drugs and immunotherapeutics and will provide some information regarding the threat potential of the Uganda SARS like viruses. It would represent the most distant SARS like CoV strain yet available for evaluating the breadth of vaccines and immunotherapeutics.

Figure 1. S Glycoprotein Sequence Comparisons.

	Urbani	PDF-2386	HKU3	WIV1	SCH014
20150715\SARS\Urbani\Spike\AAP13441 (modified) extraction		77	69	96	85
PDF-2386/SARS-like/Uganda translation extraction	77		69	78	78
20150715\Bat\CoV\HKU3-1\Spike\AAY88866 extraction	69	69		70	68
20150715\WIV1\Spike\AGZ48831 extraction	96	78	70		86
20150715\SCH014\Spike\AGZ48806 extraction	85	78	68	86	

Figure 2. Comparison of SARS RBD ACE2 interaction residues across strains.

Strain	402	426	436	441	442	472	473	475	479	484	486	487	488	491
Urbani	T	R	Y	R	Y	L	N	Y	N	Y	T	T	G	Y
MA15	T	R	H	R	Y	L	N	Y	N	Y	T	T	G	Y
Rs3367	T	R	Y	R	S	F	N	Y	N	Y	T	N	G	Y
PDF-2386	T	N	*	R	L	L	G	Y	K	T	T	V	G	Y
RsSHC014	T	N	Y	R	W	P	N	Y	R	F	T	A	G	H
HKU3	T	A	*	R	S	*	N	V	K	N	N	V	G	Y
ACE2 interaction s	325				31*	79* 82*			34*	41 42 45		41 353*		37 353* 354

* indicates sequence differences between human and mouse ACE2 at this position

Background

SARS-CoV emerged from bats, likely underwent additional selection in civets and raccoon dogs before colonizing humans, leading to the 2002-2003 outbreak, which infected ~8,000 individuals and causing about 800 deaths. Several bat coronaviruses (e.g., SCH014, WIV-1) retain the capacity to use the human ACE2 receptor for docking and entry (PMC4801244, PMC4797993), but have not been shown to cause human disease. SARS-Uganda was sequenced from bats and is about 77% identical to SARS-CoV (**Fig 1**), it is equally distant from WIV1 (Rs3367) and RsSHC014). It has not been successfully cultured, but rather exists as

a sequence isolated from bats. Analyses of the receptor binding domain suggests that it will not be able to recognize the human or mouse ACE2 receptor for entry (**Fig 2**), although it does retain some contact interface recognition sites with human (Y491, L472) or mouse (Y491, L472, L442) ACE2 receptors. The sequence comparisons predict, however, that incorporation of 5 residues *436Y, L442W, K479R, T484F, V487A from SCH014 into PDF-2386 (PDF-2386-SHC-RBD) or the 8 known mouse adapting mutations (K411E, *436H, L472F, K411E, G473N, K479N, T484Y and V487T) into the PDF-2386 Spike (PDF-2386-MA) which are predicted to confer replication of the PDF-2386 strain in mice, and perhaps, primate cells.

Experimental Design.
The SARS Uganda PDF-2386 S glycoprotein gene will be purchased from commercial vendors with two small adaptive cassettes, which allow for rapid insertion of the SCH014 or mouse adapted residues into the PDF-2386 S gene. The entire SARS Uganda spike will first be dropped into SHC014 genetic backbone and recombinant viruses tested for their ability to replicate in vero cells and in mouse cells expressing the civet, mouse, human and bat ACE2 receptors. Then the Uganda spike RBD residues will be replaced with the five SHC014 contact interface sites (PDF-2386-SHC-RBD) or the 8 known mouse adaptive mutation set clusters (PDF-2386-MA) in the SHC014 genetic backbone. Following electroporation of full length transcripts into cells, any viable viruses will be recovered and then tested for their abililty to replicate efficiently in vero cells and in mouse cells expressing civet, mouse, human and bat ACE2 receptors. Viruses that can replicate efficiently in mouse cells expressing the mACE2 receptor will be evaluated for growth and pathogenic outcomes in young and then aged BALB/c mice. After demonstration of a spike formulation that allows for efficient replication in mice and mouse cells expressing the mouse ACE2 receptor, we will synthetically reconstruct full length SARS-Uganda viruses, with mouse ACE2 enhancing mutations.

Risk Management.
If any chimeras are capable of using the human ACE2 receptor for docking and entry, we will evaluate the ability of the viruses to replicate in primary human airway epithelial cells and vero cells, predicting that these chimeras will demonstrate significant attenuation in growth, as compared to wildtype SARS-CoV. Should the chimeras replicate >10X more efficiently than wildtype SARS-CoV, we will halt all experiments, inform the local IBC, the program officer at NIH and the NIH and participate in any discussions regarding the suitability of these experiments to move forward.

Please do not hesitate to contact me should you acquire additional information. We thank you for your consideration of this request.

Sincerely,

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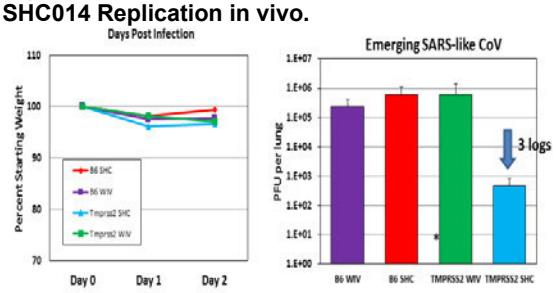
Introduction. CoVs recognize a variety of host receptors through one or both of its spike S1 domains (S1-NTD and S1-CTD), and are also regulated for cell entry by host proteases that cleave at the S1/S2 boundary. Understanding the receptor recognition and cell entry by CoVs has important implications for virology, medicine, and epidemics. Our studies have elucidated how CoVs explore different host receptors and host proteases to expand their host ranges, and how SARS-CoV and MERS-CoV transmit from animals to humans to cause epidemics. During the past year, the Baric laboratory has made considerable progress in two principle areas of Aims 1-3 of the grant; specifically, developing a molecular clone for porcine delta coronavirus and in designing and recovering synthetic bat SARS-like CoV.

Porcine epidemic diarrhea virus. Previously, our group developed a molecular clone for porcine epidemic diarrhea virus. Several S glycoprotein deletions mutants have been described in the literature, including TC-PC177. To investigate whether 197 aa-deletion was the determinant for the attenuation of TC-PC177, we generated a mutant (icPC22A-S1Δ197) bearing the 197 aa-deletion based on an infectious cDNA clone of PC22A strain (icPC22A). In collaboration with the Wang laboratory at Ohio State University, the icPC22A-S1Δ197 virus caused mild to moderate diarrhea and no mortality, whereas the icPC22A virus caused severe diarrhea and death in all piglets. Our data indicate that the deletion of this 197 aa-fragment in spike protein can attenuate a highly virulent PEDV, but may lose important epitopes for inducing robust protective immunity. A manuscript detailing the construct of the mutant and its pathogenic outcomes in swine has been drafted and will soon be submitted for review.

Porcine delta coronavirus reverse genetic platforms. In addition to porcine epidemic diarrhea virus, porcine delta coronavirus was first reported in the United States in February 2014, causing severe disease outbreaks of lethal disease in piglets in swine herds. *Deltacoronavirus* genus is new, having only been recently defined by genomic sequence analysis from both pig and avian isolates. Since 2009, avian deltacoronaviruses have been detected in a wide range of domestic and wild birds and then porcine deltacoronaviruses (PdCV) Hong Kong (HK) strains emerged suddenly in south east asia. To develop a PdCV molecular clone, we developed a collaboration with Dr. Linda Saif, who provided us with wildtype and tissue culture adapted strain OH-FD22. We have deep sequenced these isolates and have ordered a molecular clone from BioBasic. We anticipate having recombinant viruses within a few months of obtaining the reverse genetic platform. In parallel, we have dropped the PdCV S glycoprotein into Venezuelan equine encephalitis virus replicon particles (VRP-PdCV-S), and after VRP vaccination, we will obtain antisera against the S glycoprotein gene. The development of these two molecular clone reagent sets will be extremely valuable for downstream studies focusing on mechanisms of coronavirus entry.

Figure 1. TMPRSS2 KO Blocks SARS-like Bt CoV SHC014 Replication in vivo.



SARS-like Bat Coronaviruses. We have synthetically reconstructed recombinant bat SARS-like viruses encoding the WIV-1 and SHC014 spike, which are about 10-15% distinct from the SARS-CoV epidemic S glycoprotein. While these strains are not pathogenic in mice, infection of TMPRS2 KO mice revealed that SHC014 S, but not isogenic backbone viruses encoding the SARS or WIV-1 S glycoproteins, cannot replicate in the absence of TMPRS2 in vivo (Fig 1). These data suggest that virus emergence may have been associated with adaptive changes that allowed for epidemic SARS-CoV strains to use the TMPRSS2 to replicate efficiently in the human lung. We are currently in the process of studying the exact role of specific mutations in SHC014 TMPRSS2 protease usage, as well as evaluating tropism differences in the lungs of infected control and KO animals.

New SARS-Like Bat Coronaviruses. The SARS-like bat coronavirus, WIV16 was isolated from a single fecal sample of *Rhinolophus sinicus*, which was collected in Kunming, Yunnan Province, in July 2013. The full genomic sequence of SL-CoV WIV16 (GenBank accession number KT444582) was determined (PMC4810638). The overall nucleotide sequence of WIV16 has 96% identity (higher than that of any previously reported bat SL-CoVs) to human and civet SARS-CoVs, including 97% amino acid identity in the S glycoprotein gene. Thus, WIV16 bridges the divide between zoonotic and epidemic SARS like viruses, but may retain mutation profiles in S2 that prevent TMPRSS2 proteolytic cleavage and efficient replication in vivo. To address this question (previously GOF requested and approved to move forward), we inserted the WIV16 S

glycoprotein into the genome backbone of SARS-CoV and isolated recombinant viruses. Sequence analyses revealed the recombinant virus encoded with WIV16 S glycoprotein. In preliminary in vivo studies, the WIV16-S virus is attenuated as compared to the wildtype SARS-CoV MA strain. We are characterizing WIV16-S growth in Vero and primary human airway epithelial cells in vitro and for its ability to recognize the murine, human, civet and bat ACE2 receptors ectopically expressed in DBT cells for entry and to replicate in KO mice lacking the TMPRSS2, TMPRSS4 and TMPRSS2/4 genes.

PDF-2386 (SARS-Ubanda). We have recently identified a new bat SARS like virus from Uganda (SARS-Uganda) in collaboration with Simon Anthony at EcoHealth. Analyses of the receptor binding domain suggests that it will not be able to recognize the human or mouse ACE2 receptor for entry (**Fig 2**), although it does retain some contact interface recognition sites with human (Y491, L472) or mouse (Y491, L472, L442) ACE2 receptors. The sequence comparisons predict, however, that incorporation of 5 residues *436Y, L442W, K479R, T484F, V487A from SCH014 into PDF-2386 (PDF-2386-SHC-RBD) or the 8 known mouse adapting mutations (K411E, *436H, L472F, K411E, G473N, K479N, T484Y and V487T) into the PDF-2386 Spike (PDF-2386-MA) which are predicted to confer replication of the PDF-2386 strain in mice, and perhaps, primate cells. As we are proposing to introduce the PDF-2386 S glycoprotein, with and without mouse ACE2 binding residues, into the bat SARS-like SHC014 coronavirus, we believe these experiments are exempt because neither virus has been demonstrated to have pathogenic potential or be able to be transmitted in humans. Moreover, we are introducing SHC014 preferred and mouse adapted RBD residues into PDF-2386, hence, it is unlikely to be more dangerous than the original SHC014 strain. We have not started these experiments, as we are awaiting permission to proceed.

Strain	402	426	436	441	442	472	473	475	479	484	486	487	488	491
Urbani	T	R	Y	R	Y	L	N	Y	N	Y	T	T	G	Y
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RsSHC014	T	N	Y	R	W	P	N	Y	R	F	T	A	G	H
HKU3	T	A	*	R	S	*	N	V	K	N	N	V	G	Y
ACE2 interaction s	325				31*	79* 82*			34*	41 42 45		41 353*		37 353* 354

* indicates sequence differences between human and mouse ACE2 at this position

Figure 2. PDF-2386 (SARS-Uganda) Receptor Binding Domain Residues.