

genes of novel SARSr-CoV will be inserted into VEE 3526 replicon vectors (VRP3526-S), packaged and used to vaccinate mice (59). Polyclonal sera will be harvested and tested for ability to cross neutralize SARS-CoV, GD03, WIV-1, SHC014, WIV-16, other novel SARSr-CoV and HKU3-SRBD by PRNT50 assay (55, 60, 61). Using PRNT50 titers with sera (n=4 each) among these viruses, antigenic cartography (62) will allow comparison of antigenic vs. phylogenetic distance, identifying the transition at which SARSr-CoV strains escape SARS-CoV based vaccines, informing immunotherapeutic and vaccine design strategies (63-65).

**3.3.c Humanized mouse infection experiments:** Briefly, in BSL3, n=5 10- to 20-week old hACE2 transgenic mice will be intranasally inoculated with  $1 \times 10^4$  PFU of wildtype WIV-1 or chimeric bat SARSr-CoVs with different spike proteins, then monitored daily for weight loss, morbidity, and clinical signs of disease. Mice will be euthanized at 2, 4, and 6 dpi (endpoint of 14 dpi), organs harvested and virus quantified by SARS-CoV NP RT-PCR. After 7 days in formalin, tissues for histopathology will be removed from the BSL3 and stained with H&E, and for immunohistochemistry using polyclonal antibodies against the N protein. We will conduct limited evaluation of existing countermeasures using therapeutic monoclonal antibodies *in vitro* and *in vivo*. Existing SARSr-CoV mAbs will be diluted in DMEM starting at 1:20, and serial 2-fold dilutions mixed with an equal volume of 50 PFU of chimeric bat SARSr-CoVs with different spike proteins, then incubated on Vero E6 cells at 37°C for 1 h, then changed to a semi-solid medium (DMEM containing 1% methylcellulose and 2% FBS). Antibody neutralization titers will be evaluated by plaque quantification at 4-5dpi. hACE2 transgenic mice will be injected with SARS-CoV mAbs, and infected with chimeric bat SARSr-CoVs. Clinical signs and morbidity will be assessed and tissue pathology examined and compared with mice without treatment of mAbs to determine the therapeutic effect on SARSr-CoV infection, and protection of SARSr-CoV by wildtype SARS-S based vaccines assessed as described (56, 66). We will sequence full length genomes of high risk strains that are antigenically distinct and escape SARS cross neutralization, synthetically reconstruct a small subset (1-2) and evaluate the ability of nucleoside analogues to inhibit growth in HAE cultures and/or *in vivo* (55, 56).

**3.3.d HKU3 clade cellular receptor:** We will screen potential receptor molecules by pull-down analysis on membrane proteins interacting with the spike protein, initially using bat primary intestinal epithelial cell lines and lysates to extract protein, isolate membranes, and proteomically sequence intestinal proteins. The fusion protein of the HKU3 and HKU3r-CoV S proteins containing human Immunoglobulin Fc fragment will be eukaryotically expressed and purified. SARSr-CoV S will be incubated as bait protein with the membrane proteins extracted from *Rhinolophus sinicus* intestinal cells, to capture and precipitate membrane proteins that interact with the S protein. Mass Spectrometry will be performed to screen for the candidate receptor molecules and Co-Immunoprecipitation assay to confirm binding of the SARSr-CoV S protein to the candidate receptor. Alternatively, retroviruses pseudotyped with the SARSr-CoV S protein will be constructed and used to infect cells trans-expressing the candidate receptor molecule. Luciferase activity will be measured to test whether the S protein can bind to the receptor. If successful, this work will allow future research to clone and study human HKU3 receptor ortholog's ability to function as a receptor for other clade 2 strains and will allow better assessment of risk of clade 2 SARSr-CoV spillover to humans.

**3.4 Combined spatial risk 'hotspot' analyses:** We will use data from 3.3 to identify rank SARSr-CoV strains most likely to infect people and evade therapeutic and vaccine modalities. We will use bat survey and zoological data (44) to build species distribution models (67) and predict the distribution of bat species that harbor low, medium and high risk viral strains. Stacking these modeled distributions for the ~20-30 *Rhinolophus* and related species that occur in the region will allow estimates of SARSr-CoV diversity for a given locality. We will use machine learning models (boosted regression trees) and spatial 'hotspot' mapping approaches to identify the ecological, socio-economic and other correlates of SARSr-CoV diversity and spillover (from serosurveys) (21, 68, 69). We will include data from our human behavioral surveys and sampling to give a direct measure of where risk of spillover to people is likely to be highest in the region.

**Potential problems/alternative approaches:** We may not be able to glean further information about the **capacity of HKU3r-CoVs to infect human cells, or bind to human cell surface receptors**. If attempts at culture are unsuccessful, and efforts to identify the receptor too costly or time-consuming, we will cease this line of work. In that event, we will focus entirely on filling out the gaps in the 10-25% S protein sequence divergence from SARS-CoV, by working on a greater diversity of lineage 2 SARSr-CoVs.