Accepted Manuscript Posted Online
Virologica Sinica. **DOI: 10.1007/s12250-017-4124-2**
Received: 21 November 2017, Revised: 2 January 2018, Accepted: 8 January 2018

LETTER

Serological evidence of bat SARS-related coronavirus infection in humans, China

Running title: SARSr-CoV serological detection in human

Ning Wang[1,2], Shi-Yue Li[3], Xing-Lou Yang[1], Hui-Min Huang[3], Yu-Ji Zhang[1], Hua Guo[1,2], Chu-Ming Luo[1,2], Maureen Miller[4], Guangjian Zhu[4], Aleksei A. Chmura[4], Emily Hagan[4], Ji-Hua Zhou[5], Yun-Zhi Zhang[5,6], Lin-Fa Wang[7], Peter Daszak[4], Zheng-Li Shi[1]✉

1. CAS Key Laboratory of Special Pathogens and Biosafety, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China
2. University of Chinese Academy of Sciences, Beijing 100049, China
3. School of Health Sciences, Wuhan University, Wuhan 430071, China
4. EcoHealth Alliance, New York NY10001, USA
5. Yunnan Provincial Key Laboratory for Zoonosis Control and Prevention, Yunnan Institute of Endemic Diseases Control and Prevention, Dali 671000 China
6. School of Public Health, Dali University, Dali 671000, China
7. Programme in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore 169857, Singapore

✉Correspondence:
Zheng-Li Shi
CAS Key Laboratory of Special Pathogens and Biosafety, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China
Email: zlshi@wh.iov.cn

Please cite this article as: Ning Wang, Shi-Yue Li, Xing-Lou Yang, Hui-Min Huang, Yu-Ji Zhang, Hua Guo, Chu-Ming Luo, Maureen Miller, Guangjian Zhu, Aleksei A. Chmura, Emily Hagan, Ji-Hua Zhou, Yun-Zhi Zhang, Lin-Fa Wang, Peter Daszak, Zheng-Li Shi. Serological evidence of bat SARS-related coronavirus infection in humans, China. Virologica Sinica. DOI: 10.1007/s12250-017-4124-2.

ABSTRACT

In our previous works, we have reported genetically diverse SARS-related coronaviruses (SARSr-CoV) in a single bat cave, Yunnan province, China, and suggested that some SARSr-CoVs may have high potential to infect humans without the necessity for an intermediate host. In this report, we developed a specific ELISA based on the nucleocapsid protein of a SARSr-CoV strain and detected its antibody in humans who are highly exposed to bat populations. From 218 human serum samples, 6 were positive against the nucleocapsid protein by ELISA and further confirmed by Western blot. For the first time, we demonstrated the SARSr-CoV had spillover to humans, although did not cause clinical diseases.

KEYWORDS Bats, Coronavirus, SARS, SARS-related coronavirus, zoonoses, spillover events

Dear Editor,

Severe acute respiratory syndrome coronavirus (SARS-CoV) was the causative agent of the 2002–2003 SARS pandemic, which resulted in more than 8,000 human infections worldwide with an approximately 10% fatality rate (Ksiazek et al. 2003; Peiris et al. 2004). The virus infects both upper airway and alveolar epithelial cells, resulting in mild to severe lung injury in humans (Peiris et al. 2003).

During investigation into the SARS epidemic, epidemiological evidence of a zoonotic origin of SARS-CoV was identified (Xu et al. 2004). Isolation of SARS-related coronavirus (SARSr-CoVs) from masked palm civets and detection of virus infection in humans working at the wet market suggested that masked palm civets could serve as source of human infection (Guan et al. 2003). Subsequent work has identified genetically diverse SARSr-CoVs in Chinese horseshoe bats (*Rhinolophus sinicus*) in a county of Yunnan Province, China and provided strong evidence that bats are the natural reservoir of SARS-CoV (Ge et al. 2013; Li et al. 2005; Yang et al. 2016). Since then, diverse SARS-related coronaviruses (SARSr-CoVs) have been detected and reported in bats in different regions globally (Hu et al. 2015). Importantly, SARSr-CoVs which use the SARS-CoV receptor, angiotensin converting enzyme 2 (ACE2) have been isolated (Ge et al. 2013). These results indicate that some SARSr-CoVs may have high potential to infect human cells, without the necessity for an intermediate host. However, to date, no evidence of direct transmission of SARSr-CoVs from bats to people has been reported.

In this study, we performed serological surveillance on residents who live in close proximity to caves that are roost sites for bats carrying diverse SARSr-CoVs. In October 2015, we collected serum samples from 218 residents in four villages in Jinning County, Yunnan province, China (Figure 1A), located 1.1–6.0 km from two caves (Yanzi and Shitou). We have been conducting longitudinal molecular surveillance of bats for CoVs in these caves since 2011 and they are inhabited by large numbers of bats including *Rhinolophus spp*., a major reservoir of SARSr-CoVs. This region was not involved in the 2002–2003 SARS outbreaks and none of the subjects exhibited any evident respiratory illness during sampling. Among those sampled, 139 are female and 79 male, median age of 48 (range 12–80). Occupational data were available for 208 (95.4%) participants: 85.3% farmers and 8.7% students. Most (81.2%) kept or owned livestock or pet, and the majority (97.2%) had a history of exposure to or contact with livestock

or wild animals. Importantly, 20 (9.1%) participants have witnessed bats flying close to their houses, and one had handled a bat corpse. As a control, we also collected 240 serum samples from random blood donors in 2015 in Wuhan, Hubei Province more than 1,000 km away from Jinning (Figure 1A) and where inhabitants have a much lower likelihood of contact with bats. None of the donors had prior SARS infection or known contact with SARS patients.

His-tagged nucleocapsid protein (NP) of the following viruses were expressed and purified in *E.coli* for this study: SARSr-CoV Rp3; human coronaviruses (HCoVs) HKU1, OC43, 229E, NL63; Middle East Respiratory Syndrome Coronavirus (MERS-CoV); and Ebola virus (EBOV). In addition, the receptor binding domain (RBD) of the spike protein (S) was also produced in mammalian cells from SARS-CoV and bat SARSr-CoVs Rp3, WIV1, and SHC014 (Ge et al. 2013; Yang et al. 2016).

Polyclonal antibodies against each of the six NPs were prepared in rabbits as previously published (He et al. 2006). Cross-reactivity was evaluated with ELISA and Western blot (Supplementary Figure S1 – S2). No significant cross-reactivity was detected among NPs and their corresponding antibodies for Rp3, MERS-CoV, NL63, or 229E. Cross-reaction was detected between OC43 and HKU1 as reported previously (Lehmann et al. 2008).

The Rp3 NP was chosen to develop a SARSr-CoV specific ELISA for serosurveillance. Micro-titer plates were coated with 100 ng/well of recombinant Rp3 NP and incubated with human sera in duplicates at a dilution of 1:20, followed by detection with HRP labeled goat anti-human IgG antibodies (Proteintech, Wuhan, China) at a dilution of 1:20000. The 240 random serum samples collected in Wuhan and two SARS positive samples from Zhujiang Hospital, Southern Medical University (kindly provided by Prof. Xiaoyan Che) were used to set a cutoff value. We used the mean OD value of the 240 samples plus three standard deviations to set the cutoff value at 0.41. A total of 6 positive samples were detected by ELISA (Figure 1B). The specificity of these positive samples was confirmed by Western blot with recombinant Rp3 NP (Figure 1C) together with NP of NL63, MERS-CoV and EBOV. The degree of reactivity in Western blot correlated well with the ELISA OD readings, providing further confidence in the ELISA screening method. None of the sera reacted with NPs of either MERS-CoV or EBOV. On the other hand, all 10 human sera (9 from Jinning and 1 from Wuhan), regardless of their Rp3 NP reactivity, reacted strongly with the NL63 NP as expected due to

high prevalence of NL63 infection in humans worldwide (Abdul-Rasool and Fielding 2010).

We conducted a virus neutralization test for the six positive samples for the two SARSr-CoVs, WIV1 and WIV16 (Ge et al. 2013; Yang et al. 2016). None of them were able to neutralize either virus. These sera also failed to react in Western blot with any of the recombinant RBD proteins from SARS-CoV or the three bat SARSr-CoVs (Rp3, WIV1, and SHC014). We also performed the viral nucleic acid detection in the oral and fecal swab and blood cells, none of them were positive.

The demography and travel history of the 6 positive individuals (4 male, 2 female) are as follows. Two males (JN162, 45 yrs old, JN129, 51 yrs old) are from the Dafengkou village; two males (JN117, 49 yrs old, JN059, 57 yrs old) from the Lvxi village; and two females (JN053, JN041, both 55 yrs old), from the Tianjing village. In the 12 months prior to the sampling date, JN041 was the only one who travelled outside of Yunnan, to Shenzhen, a city 1400 km away from her home village (see Figure 1). JN053 and JN059 had travelled to another county 1.4 km away from their village. JN162 had travelled to Kunming, the capital of Yunnan, 63 km away. JN129 and JN117 had never left the village. It is worth to note that all of them have sighted bats flying in their villages.

Our study provides the first serological evidence of likely human infection by bat SARSr-CoVs or, potentially, related viruses. The lack of prior exposure to SARS patients by the people surveyed, their lack of prior travel to areas heavily affected by SARS during the outbreak, and the rapid decline of detectable antibodies to SARS-CoV in recovered patients within 2–3 years after infection strongly suggests that positive serology obtained in this study is not due to prior infection with SARS-CoV (Wu et al. 2007). The 2.7% seropositivity for the high risk group of residents living in close proximity to bat colonies suggests that spillover is a relatively rare event. During questioning, none of the 6 sero-positive subjects could recall any clinical symptoms in the past 12 months, suggesting that their bat SARSr-CoV infection either occurred before the time of sampling, or that infections was subclinical or caused only mild symptoms. Our previous work based on cellular and humanized mouse infection studies suggest that these viruses are less virulent than SARS-CoV (Ge et al. 2013; Menachery et al. 2016; Yang et al. 2016). Masked palm civets play a significant role as the intermediate host of SARS-CoV in the 2002–2003 outbreak (Guan et al. 2003). However, considering that these individuals have a

high chance of direct exposure to bat secretion in their villages, this study further support the notion that some bat SARSr-CoVs are able to directly infect humans without intermediate hosts as suggested by receptor entry and animal infection studies (Menachery et al. 2016).

The failure of these NP ELISA positive sera to either neutralize live virus or react with RBD proteins in Western blot could be explained by at least two hypotheses. First, the immune response to the bat SARSr-CoV S protein may be weaker than that to the NP protein or may wane more rapidly, especially in subclinical infections, and hence its antibody level is too low to be detected in our assay systems. Second, other bat SARSr-CoV variants may be circulating in bats of these villages that have highly divergent S proteins that have not yet been detected in our previous surveillance studies.

Coronaviruses are known to have a high mutation rate during replication and are prone to recombination if different viruses infect the same individual (Knipe et al. 2013). From our previous studies of bat SARSr-CoVs in the two caves near these villages, we have found genetically highly diverse bat SARSr-CoVs and evidence of frequent coinfection of two or more different SARSr-CoVs in the same bat (Ge et al. 2013). Our current study suggests that our surveillance is not exhaustive, as one would have expected, and further more extensive surveillance in this region is therefore warranted. It might also be prudent to combine serological surveillance with molecular surveillance of bats in future, despite the technological challenge.

ACKNOWLEDGMENTS

This study was jointly funded by the National Natural Science Foundation of China grant (81290341) to ZLS; the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (Award Number R01AI110964) to PD and ZLS, United States Agency for International Development (USAID) Emerging Pandemic Threats PREDICT project grant (Cooperative Agreement no. AID-OAA-A-14-00102) to PD; and Singapore NRF-CRP grant (NRF2012NRF-CRP001–056) and CD-PHRG grant (CDPHRG/0006/2014) to LFW.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare that they have no conflict of interest.

Animal and Human Rights Statement This study was approved by the Wuhan Institute of Virology Institutional Review Board (China) and by Hummingbird IRB (USA).

Supplementary figures are available on the websites of *Virologica Sinica*: www.virosin.org; link.springer.com/journal/12250.

REFERENCES

Abdul-Rasool S, Fielding BC (2010) Understanding Human Coronavirus HCoV-NL63. The open virology journal 4:76-84 doi:10.2174/1874357901004010076

Ge XY, Li JL, Yang XL, Chmura AA, Zhu GJ, Epstein JH, Mazet JK, Hu B, Zhang W, Peng C, Zhang YJ, Luo CM, Tan B, Wang N, Zhu Y, Crameri G, Zhang SY, Wang LF, Daszak P, Shi ZL (2013) Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. Nature 503:535-538 doi:10.1038/nature12711

Guan Y, Zheng BJ, He YQ, Liu XL, Zhuang ZX, Cheung CL, Luo SW, Li PH, Zhang LJ, Guan YJ, Butt KM, Wong KL, Chan KW, Lim W, Shortridge KF, Yuen KY, Peiris JSM, Poon LLM (2003) Isolation and characterization of viruses related to the SARS coronavirus from animals in Southern China. Science 302:276-278 doi:10.1126/science.1087139

He YX, Li JJ, Li WH, Lustigman S, Farzan M, Jiang SB (2006) Cross-neutralization of human and palm civet severe acute respiratory syndrome coronaviruses by antibodies targeting the receptor-binding domain of spike protein. J Immunol 176:6085-6092

Hu B, Ge XY, Wang LF, Shi ZL (2015) Bat origin of human coronaviruses. Virol J 12 doi:10.1186/s12985-015-0422-1

Knipe DM, Howley PM, Cohen JI, Griffin DE, Lamb RA, Martin MA, Racaniello VR, Roizman B (2013) Fields Virology 6th edition. Wolters Kluwer/Lippincott Williams and Wilkins; Volum 1,

Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, Tong SX, Urbani C, Comer JA, Lim W, Rollin PE, Dowell SF, Ling AE, Humphrey CD, Shieh WJ, Guarner J, Paddock CD, Rota P, Fields B, DeRisi J, Yang JY, Cox N, Hughes JM, LeDuc JW, Bellini WJ, Anderson LJ, Grp SW (2003) A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med 348:1953-1966 doi:10.1056/NEJMoa030781

Lehmann C, Wolf H, Xu JG, Zhao QB, Shao YM, Motz M, Lindner P (2008) A line immunoassay utilizing recombinant nucleocapsid proteins for detection of antibodies to human coronaviruses. Diagn Microbiol Infect Dis 61:40-48 doi:10.1016/j.diagmicrobio.2007.12.002

Li WD, Shi ZL, Yu M, Ren WZ, Smith C, Epstein JH, Wang HZ, Crameri G, Hu ZH, Zhang HJ, Zhang JH, McEachern J, Field H, Daszak P, Eaton BT, Zhang SY, Wang LF (2005) Bats are natural reservoirs of SARS-like coronaviruses. Science 310:676-679 doi:10.1126/science.1118391

Menachery VD, Yount BL, Sims AC, Debbink K, Agnihothram SS, Gralinski LE, Graham RL, Scobey T, Plante JA, Royal SR, Swanstrom J, Sheahan TP, Pickles RJ, Corti D, Randell SH, Lanzavecchia A, Marasco WA, Baric RS (2016) SARS-like WIV1-CoV poised for human emergence. Proc Natl Acad Sci U S A 113:3048-3053 doi:10.1073/pnas.1517719113

Peiris JSM, Guan Y, Yuen KY (2004) Severe acute respiratory syndrome. Nat Med 10:S88-S97 doi:10.1038/nm1143

Peiris JSM, Lai ST, Poon LLM, Guan Y, Yam LYC, Lim W, Nicholls J, Yee WKS, Yan WW, Cheung MT,

Cheng VCC, Chan KH, Tsang DNC, Yung RWH, Ng TK, Yuen KY, Grp SS (2003) Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet 361:1319-1325 doi:10.1016/s0140-6736(03)13077-2

Wu LP, Wang NC, Chang YH, Tian XY, Na DY, Zhang LY, Zheng L, Lan T, Wang LF, Liang GD (2007) Duration of antibody responses after severe acute respiratory syndrome. Emerging infectious diseases 13:1562-1564 doi:10.3201/eid1310.070576

Xu RH, He JF, Evans MR, Peng GW, Field HE, Yu DW, Lee CK, Luo HM, Lin WS, Lin P, Li LH, Liang WJ, Lin JY, Schnur A (2004) Epidemiologic clues to SARS origin in China. Emerg Infect Dis 10:1030-1037

Yang XL, Hu B, Wang B, Wang MN, Zhang Q, Zhang W, Wu LJ, Ge XY, Zhang YZ, Daszak P, Wang LF, Shi ZL (2016) Isolation and Characterization of a Novel Bat Coronavirus Closely Related to the Direct Progenitor of Severe Acute Respiratory Syndrome Coronavirus. J Virol 90:3253-3256 doi:10.1128/jvi.02582-15

TITLES AND LEGENDS TO FIGURES

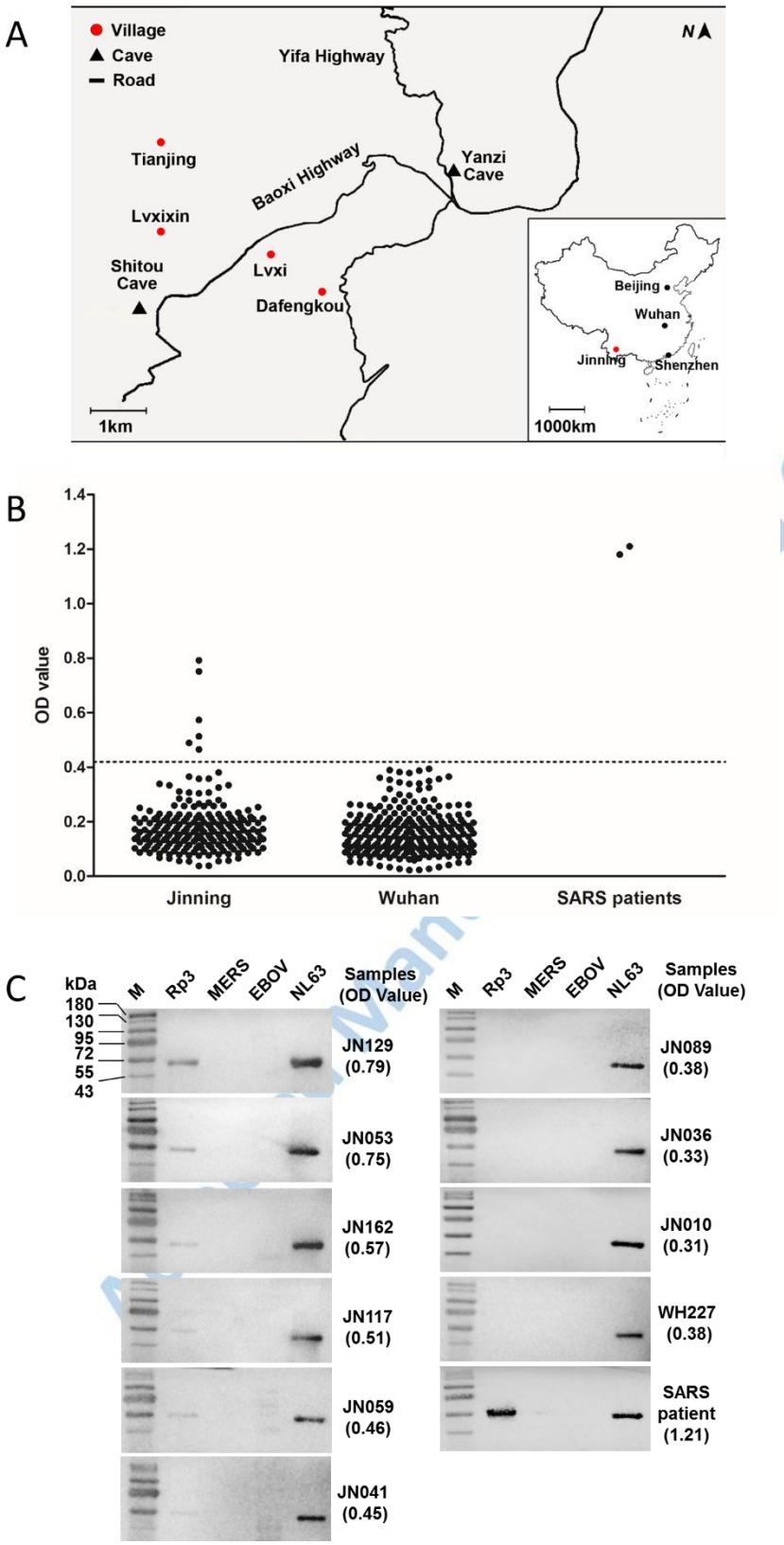
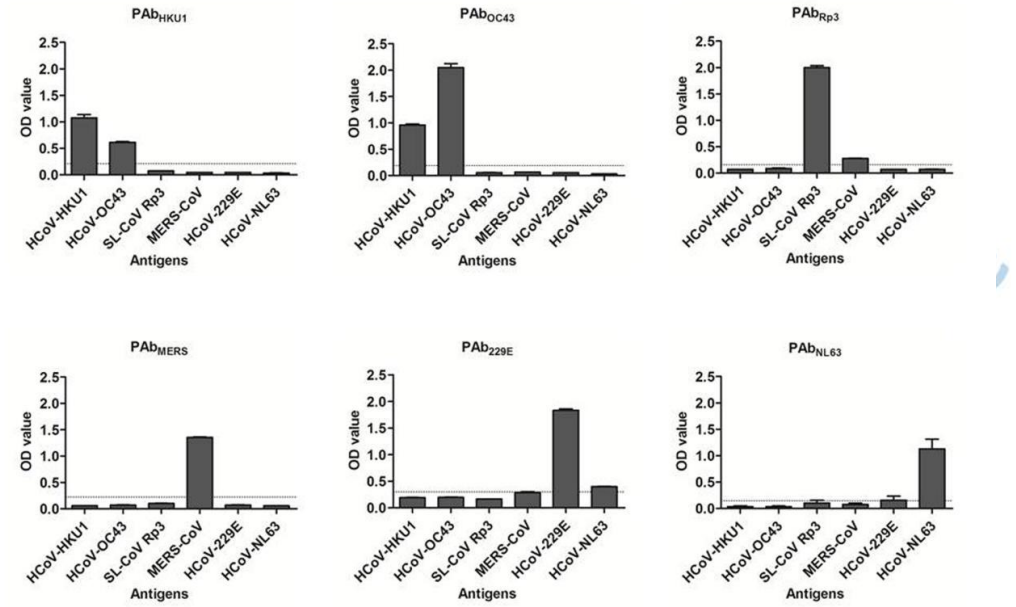


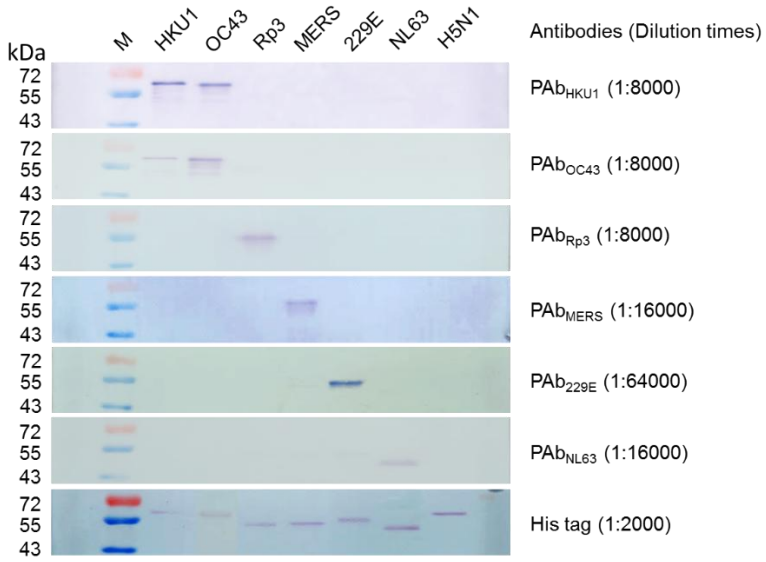
Figure 1. SARSr-CoV serosurveillance. Map of Xiyang town, Jinning County, Yunnan

Province, China. Shown here is the location of the 4 villages (Tianjing, Dafengkou, Lvxi, Lvxixin) around 2 bat caves (Yanzi Cave and Shitou Cave) chosen for this study (A). The map of China is also shown in the inset indicating the location of Wuhan, where the negative control sera were collected, in relation to Jinning, Shenzhen and the capital Beijing. Serological reactivity of serum samples with recombinant SARSr-CoV NP protein. (B) ELISA test. The dotted line represents the cutoff of the test. (C) Western blot analysis. Numbers on the left are molecular masses in kDa.

SUPPLEMENTARY MATERIALS



Supplementary Figure S1. Two-way cross-reaction ELISA testing between 6 coronavirus NPs and their corresponding rabbit polyclonal antibodies. The NP proteins (100 ng/well) were coated in 96-well micro-plate and tested with polyclonal antibody against NPs of SARS-related CoV Rp3 (PAb_{Rp3}), HCoV HKU1 (PAb_{HKU1}), HCoV OC43 (PAb_{OC43}), MERS-CoV (PAb_{MERS}), HCoV229E (PAb_{229E}) and HCoV NL63 (PAb_{NL63}), respectively. The serum was diluted at 1:16,000 or 1:64,000 (for PAb_{229E} and PAb_{NL63}). HRP labeled goat anti-rabbit IgG (1:20,000) was used as secondary antibody and detected with TMB substrate. The horizontal line in the diagram indicates cutoff value determined from negative rabbit sera collected before immunization.



Supplementary Figure S2. Two-way cross-reaction Western blotting between 6 coronavirus NPs and their corresponding rabbit polyclonal antibodies. The NP proteins (100 ng) were run on 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Roche Diagnostics GmbH, Mannheim, Germany). The membrane was incubated with the different rabbit sera at different dilutions indicated on the right (in brackets). Goat anti-rabbit IgG conjugated with AP (Proteintech, Wuhan, China) were used for detection at a dilution of 1:2000. Influenza virus H5N1 NP was used as negative control. Numbers at the left are molecular masses (in kilodaltons).